plant material may be regenerated into a plant, or plantlet, before subjecting the derived plant, or plantlet, to selection or screening for the marker gene traits. Procedures for regenerating plants from plant cells, tissues or organs, either before or after selecting or screening for marker gene(s), are well known to those skilled in the art.

A transformed plant cell, callus, tissue or plant may be identified and isolated by selecting or screening the engineered plant material for traits encoded by the marker genes present on the transforming DNA. For instance, selection may be performed by growing the engineered plant material on media containing inhibitory amounts of the antibiotic or herbicide to which the transforming marker gene construct confers resistance. Further, transformed plants and plant cells may also be identified by screening for the activities of any visible marker genes (*e.g.*, the ß-glucuronidase, luciferase, B or C1 genes) that may be present on the recombinant nucleic acid constructs of the present invention. Such selection and screening methodologies are well known to those skilled in the art.

Physical and biochemical methods may also be used to identify a plant or plant cell transformant containing the gene constructs of the present invention. These methods include but are not limited to: 1) Southern analysis or PCR amplification for detecting and determining the structure of the recombinant DNA insert; 2) northern blot, S-1 RNase protection, primer-extension or reverse transcriptase-PCR amplification for detecting and examining RNA transcripts of the gene constructs; 3) enzymatic assays for detecting enzyme or ribozyme activity, where such gene products are encoded by the gene construct; 4) protein gel electrophoresis, western blot techniques, immunoprecipitation, or enzyme-linked immunoassays, where the gene construct products are proteins; 5) biochemical measurements of compounds produced as a consequence of the expression of the introduced gene constructs. Additional techniques, such as *in situ* hybridization, enzyme staining, and immunostaining, may also be used to detect the presence or expression of the recombinant construct in specific plant organs and tissues. The methods for doing all these assays are well known to those skilled in the art.

5.1.1. PLANTS TRANSFORMED TO EXPRESS OXYGEN-BINDING PROTEINS

In accordance with the present invention, a plant that expresses a recombinant oxygen-binding protein gene may be engineered by transforming a plant cell with a gene construct comprising a plant promoter operably associated with a sequence encoding oxygen-binding protein. (Operably associated is used herein to mean that transcription controlled by the "associated" promoter would produce a functional messenger RNA, whose translation would produce the enzyme.) The plant promoter may be constitutive or inducible. Useful constitutive promoters include, but are not limited to, the CaMV 35S promoter, the T-DNA mannopine synthetase promoter, and their various derivatives. Useful inducible promoters include but are not limited to the promoters of ribulose bisphosphate carboxylase (RUBISCO) genes, chlorophyll a/b binding protein (CAB) genes, heat shock genes, the defense responsive gene (*e.g.*, phenylalanine ammonia lyase genes), wound induced genes (*e.g.*, hydroxyproline rich cell wall protein genes), chemically-inducible genes (*e.g.*, nitrate reductase genes, gluconase genes, chitinase genes, PR-1 genes *etc.*), dark-inducible genes (*e.g.*, asparagine synthetase gene (Coruzzi and Tsai, U.S. Patent 5,256,558, October 26, 1993, Gene Encoding Plant Asparagine Synthetase) to name just a few.

In yet another embodiment of the present invention, it may be advantageous to transform a plant with a gene construct operably linking a modified or artificial promoter to a sequence encoding oxygen-binding protein. Typically, such promoters, constructed by recombining structural elements of different promoters, have unique expression patterns and/or levels not found in natural promoters. *See, e.g.* Salina et al., 1992, Plant Cell 4:1485-1493, for examples of artificial promoters constructed from combining cis-regulatory elements with a promoter core.

In a preferred embodiment of the present invention, the associated promoter is a strong and/or embryo-specific plant promoter such that the oxygen-binding protein is overexpressed in the transgenic plant. Additionally, particular promoters may be used to direct tissue specific expression of the oxygen-binding protein. For example, root-specific expression of an oxygen-binding protein may be engineered.

Further, recombinant expression of an oxygen-binding protein may be directed to specific cellular compartments. One of skill in the art may direct expression of the oxygen-binding protein intracellularly, to the chloroplasts, or to the mitochondria, to give just a few examples.

In yet another preferred embodiment of the present invention, the overexpression of oxygen-binding protein in plants may be engineered by increasing the copy number of the oxygen-binding protein gene. One approach to producing such transgenic plants is to transform with nucleic acid constructs that contain multiple copies of a plant expression construct directing the expression of an oxygen-binding protein gene (*i.e.*, operatively linked to a plant promoter). Another approach is to repeatedly transform successive generations of a plant line with one or more copies of the plant expression construct. Yet another approach is to place an oxygen-binding protein expression construct in a nucleic acid construct containing an amplification-selectable marker (ASM) gene such as the glutamine synthetase or dihydrofolate reductase gene. Cells transformed with such constructs are subjected to culturing regimes that select cell lines with increased copies of the oxygen-binding protein gene. *See* Donn et al., 1984, J. Mol. Appl. Genet. 2:549-562, for a selection protocol used to isolate of a plant cell line containing amplified copies of the GS gene. Because the desired gene is closely linked to the ASM, cell lines that amplified the ASM gene are also likely to have amplified the desired oxygen-binding protein gene. Cell lines with amplified copies of the oxygen-binding protein gene can then be regenerated into transgenic plants.

5.1.2. SCREENING OF TRANSFORMED PLANTS FOR THOSE HAVING DESIRED ALTERED TRAITS

It will be recognized by those skilled in the art that in order to obtain transgenic plants having the desired engineered traits, screening of transformed plants (i.e., those having an gene construct of the invention) having those traits may be required. For example, where the plants have been engineered for overexpression of an oxygen-binding protein gene, transformed plants are examined for those expressing the oxygen-binding protein gene at the desired level and in the desired tissues and developmental stages. The plants exhibiting the

desired physiological changes, *e.g.*, oxygen-binding protein expression, may then be subsequently screened for those plants that have the desired phenotypic changes at the plant level (e.g., increased chlorophyll, heme, amino acid, or alkaloid biosynthesis, increased fatty acid desaturation, or increased levels of oxmoprotectants such as glycine betaine which requires oxygen for its biosynthesis). The same principle applies to obtaining transgenic plants having tissue-specific expression of a heterologous gene by the use of a tissue specific promoter driven expression construct.

Alternatively, the transformed plants may be directly screened for those exhibiting the desired structural changes. In one embodiment, such screening may be for the color of the transformed plants. In other embodiments, the screening of the transformed plants may be for improved agronomic characteristics (e.g., faster growth, greater vegetative or reproductive yields, or improved protein contents, etc.), as compared to unengineered progenitor plants, when cultivated under various growth conditions (e.g., soils or media containing different amount of nutrients, water content, and/or salt content).

According to the present invention, plants engineered with oxygen-binding protein expression constructs, particularly *Vitreoscilla* hemoglobin, may exhibit improved vigorous growth characteristics, increased levels of chlorophyll, and of secondary plant metabolites, particularly alkaloids.

Engineered plants and plant lines possessing such improved agronomic characteristics may be identified by examining any of following parameters: 1) the rate of growth, measured in terms of rate of increase in fresh or dry weight, or rate of increase in height; 2) vegetative yield of the mature plant, in terms of fresh or dry weight; 3) the rate of germination; 4) the level of chlorophyll content; 5) the level of a secondary metabolite of interest, such as nicotine, scopolamine, hyoscyamine; and 6) the color of the plant. The procedures and methods for examining these parameters are well known to those skilled in the art.

According to the present invention, a desired plant is one that exhibits improvement over the control plant (*i.e.*, progenitor plant) in one or more of the aforementioned parameters. In an embodiment, a desired plant is one that shows at least 5% increase over the control plant in at least one parameter. In a preferred embodiment, a desired plant is one that shows at least 20% increase over the

control plant in at least one parameter. Most preferred is a plant that shows at least 50% increase in at least one parameter.

6. EXAMPLE: EXPRESSION OF VITREOSCILLA HEMOGLOBIN IN TOBACCO

An extensively investigated plant alkaloid is nicotine, the principal alkaloid found in tobacco (*Nicotiana tabaccum*). The biosynthetic pathways for the tobacco alkaloids have become accepted but regulation of this pathway is not yet fully understood, and all of its enzymes are not characterized (Bush, L.P., 1981, Recent Adv. Tob. Sci. 7:75-106). However, the activity of the final enzyme in nicotine biosynthesis, nicotine synthase, which catalyzes the formation of (s)-nicotine from nicotinic acid and N-methyl-Δ'-pyrrolinium chloride, has proven to be oxygen-dependent (Figure 1) (Friesen, J.B. and Leete, E., 1990, Tetrahedron Lett. 31:6295-6298), and the competing pathway from nicotinic acid and N-methyl-Δ'-pyrrolinium chloride to anabasine is not, so far as is known, stimulated by oxygen. Thus, this system was chosen as a first experiment to investigate the effects of increasing oxygen availability by providing oxygen-binding proteins to the plant. In this example, tobacco was transformed with a VHb expression vector via *Agrobacterium* mediated gene transfer. Subsequent assays were performed to determine: (i) if the VHb gene was correctly transcribed and translated in tobacco, (ii) the response of nicotine and anabasine accumulation to VHb expression, and, based on other features observed for the VHb-expressing transgenic plants, (iii) chlorophyll content and growth rate of VHb containing plants.

6.1. Experimental protocol

Recombinant DNA techniques and bacterial strains

Plasmid procedures were performed according to the methods described in Sambrook, J. *et al.*, 1990, Molecular cloning. A laboratory manual, Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, and PCR was performed using standard protocols (Saiki, *supra*). *Escherichia coli* strain TGI (F'*traD36lacI9Δ[lacZ]M15proA+B+/supE[hsdM-mcrB]5[r_K-m_K-McrB-]thi[lac-proAB][lac-pro]*) was cultivated and transformed, using standard techniques (Sambrook, J., *supra*). Restriction enzymes were used according to the suppliers'

recommendations (GIBCO BRL, Labdesign). *Agrobacterium tumefaciens* strain LBA 4404 (Walden, R. *et al.*, 1990, MMCB 1:175-194) was cultivated in LB medium (tryptone 10 g/l, yeast extract 5g/l, NaCl 10 g/l) and transformed with the aid of *E.coli* HB 101, harboring conjugative plasmid pRK 2013 (Figurski, D.H. and Helinski, D.R., 1979, Proc. Natl. Acad. Sci. U.S.A. 76:1648-1652). Extraction of genomic tobacco DNA for PCR analysis was performed as described by Edwards, K. *et al.*, 1991, Nucleic Acids Res. 19:1349).

DNA-constructions

PCR was used to amplify the VHb gene from plasmid pINT1 (Khosla, C. and Bailey J.E., 1989, J. Mol. Biol. 210:79-90). The 5' primer H1 (with sequence 5'- GCA AAC AGG ATC CCG CGT CTA GAG G 3') was complementary to a region upstream of the transcription initiation codon containing several restriction enzyme sites, the 3' primer H2 (with sequence 5'- GCA GAT TTG TAC GCT CAA GCG GTT GAA TGA GCT CCC AAA G - 3') was complementary to the coding region of the VHb gene upstream of the stop codon; primers were synthesized at the Biomolecular Unit, Lund University. The stop codon was also altered, using PCR mediated mutagenesis, from TAA to TGA, which is a more frequently used stop codon in tobacco. The amplified PCR fragment was inserted into pUC19 which was subsequently transformed into *E. coli*. Bacteria expressing the VHb gene exhibit a red tint when pelleted. One of the best expressing *E. coli* clones, pUH4, was chosen for further experiments. To verify that the cloned PCR fragment of the VHb gene expressed a functional protein, difference spectrum analysis was used (Webster, D.A. and Liu, C.Y., 1974, J. Biol. Chem. 249:4257-4260). Analysis of the CO spectrum of recombinant *E. coli* harboring pUH4 revealed a pronounced peak at 419 nm which is characteristic for the VHb activity (Lilius, *supra*). The VHb gene fragment was cleaved out with *XbaI* and *SacI* and ligated into plasmid pBin121:2 (Jefferson, R.A., 1987, Plant. Mol. Biol. Rep. 5:387-405) giving pBH4 (Figure 2).

Plant material

Nicotiana tabaccum SR. plants were exposed to 16 h light at 25°C and were either kept in soil or in sterile jars on MS-media (Murashige, T. and Skoog, F., 1962, Physiol. Plant 15:473-497). The tobacco plants were transformed by *Agrobacterium*-mediated gene transfer as described by Rogers, S.G. and Fraley,

R.T., 1986, Methods Enzymol. 118:627-684. Stable heterozygous transformants of the F_1 generation were used in all plant experiments.

Transcription analysis using RT-PCR

Polyadenylated mRNA was isolated from 100 mg leaf material using oligo(dT)-cellulose as in the Pharmacia Quick Prep™ manual. The VHb gene transcript was amplified by *rTth DNA polymerase* and the primers according to the instructions given by Perkin Elmer.

Western immunoblot analysis

Protein extracts of two transgenic tobacco lines and a wild-type control were prepared as follows: One gram leaf and stem material was homogenized in 0.1 M sodium phosphate, 5 mM DTT and 5 mM EDTA buffer at pH 7.5. The homogenates were centrifuged in 1.5 mL test tubes at 12,000 rpm for 15 min; the protein-containing supernatants were decanted and used for further studies. The proteins were separated on 15 % SDS-PAGE gels and blotted onto Immobilon P nylon membranes supplied by Millipore. The electrophoreses and blotting were performed as described by Laemmli (Laemmli, U.K., 1970, Nature 277:680-685) and Sambrook, *supra*, respectively. The VHb monomers were detected by VHb antiserum generated in rabbit, and visualized by peroxidase conjugated swine anti-rabbit immunoglobulins (Dako).

Alkaloid analysis

Transgenic tobacco and wild-type plants were analyzed for their nicotine and anabasine levels. The plants were grown for 30 days on MS medium, then harvested and homogenized in approximately 40 mL 0.1 M Tris-HCl buffer pH 9.0, centrifuged and stored at -70°C. The nicotine and anabasine concentrations were determined using capillary column gas chromatography as described by Curvall *et al.*, 1982, J. Chrom. 232:283-293.

Chlorophyll assay

Leaf material from three-week old seedlings was homogenized in 1.5 ml tubes and the chlorophyll was extracted with ice-cold 80% acetone. The debris was removed and the supernatants were collected. The absorbance of the chlorophyll extracts were monitored at 645 and 633 nm. The absorbencies were normalized to the amount of plant material used in each extraction. The amounts

of chlorophyll-A and B were calculated as described by Arnon, D.I., 1949, Plant Physiol. 24:1-15.

6.2. Results

Preparation of Transgenic Tobacco

A vector was constructed for expression of the VHb gene in plants (Figure 2). Genomic DNA from several tobacco transformants was purified and analyzed by PCR to determine if the VHb gene was inserted into the genome. Approximately 90% of the analyzed plants were positive. PolyA+ mRNA was isolated from plant leaves of two PCR-positive transgenic plant lines, He3 and He4, and a wild-type control line. Two polynucleotide primers, H1 and H2 (see Experimental protocol), were used in a reverse transcriptase PCR reaction to amplify and detect the mRNA transcript of the VHb gene. Both transgenic tobacco lines exhibited a full-length VHb mRNA transcript which corresponded well to the theoretical size of 460 base pairs.

Expression of VHb protein in tobacco could easily be detected by Western immunoblot analysis on leaf and stem protein extracts. A 15.7 kDa protein that cross-reacted with a VHb antiserum was present in both transgenic tobacco lines but absent in the wild-type control. The level of VHb expression using the 35S CaMV promoter of pBi121:2 is approximately 0.1 % of total protein. However, the presence of several cytochromes in tobacco precluded the use of the CO binding assay for VHb activity (Saiki, *et al.*, 1988, Science 239:487-491).

Improved overall growth in transgenic tobacco

Transgenic tobacco seeds of two transgenic lines, He3 and He4, from the F1 generation and a wild-type control were germinated in soil and their development was followed visually and by harvesting plants at regular intervals. Germination was markedly accelerated in plants transformed with VHb-expression constructs, particularly in the transgenic line He4, over that in non-transformed plants. Analysis showed that the germination time for the transformed plants was approximately 3-4 days compared to 6-8 days for wild-type tobacco. Additionally, the growth rates and final yields were higher in the transgenic plants (Figure 3). For instance, the dried weights were enhanced by 80 to 100% after 35 days of

growth. Furthermore, the period between germination and flowering of the transgenic plants was reduced by 3-5 days compared to wild-type.

Alkaloid analysis of the VHb-expressing transgenic tobacco Extracts of two VHb-expressing transgenic tobacco plant lines, He3 and He4, and a wild-type control were analyzed for nicotine and anabasine content by gas chromatography (Figure 4). Compared with the wild-type, the transgenic plaints (He3) were holding on average 34% higher ratio of nicotine corresponding to 108 μg/g dry weight. At the same time, the anabasine levels decreased substantially in the VHb-expressing transgenic plants. The highest yielding individual transgenic tobacco plant had 130 μg/g dry weight of nicotine which is very similar to the highest nicotine content in tobacco given in the literature (Sheen, S.J., 1988, J. Food Sci. 53:1572-1573), 140 μg/g dry weight.

Chlorophyll content of VHb-expressing transgenic tobacco

Visual observation of VHb-expressing tobacco transgenes indicated that many of these plants appeared more green than wild-type control plants. As depicted in Figure 1B, several steps in chlorophyll and heme biosynthesis are oxygen dependent. Therefore, chlorophyll and growth rate assays were conducted on several Fl generation plants obtained from wild-type tobacco and from the VHb-expressing clones designated He3 and He4. Assays of chlorophyll content showed small but significant increases in chlorophyll content particularly in the He4 plants (Figure 5). These plants also grew about 20-40% faster than the wild-type as determined by height. Differences in growth rate between transgenic and wild-type plants were not observed when these plants were grown in an atmosphere with reduced oxygen.

6.3. Discussion

Engineering VHb expression in tobacco plants influenced nicotine (and anabasine) production, but indications of more global effects of VHb expression began with the observation that young transgenic plants exhibit a darker green shade of color than do wild-type plants. Subsequent measurements of chlorophyll levels revealed 10-20% increased levels in the He3 plants which also corresponded 20-40% increased growth rates as determined by plant heights. Cloning of VHb in tobacco affected the He3 and He4 plants somewhat differently. Enhanced levels of

nicotine and chlorophyll were associated with He3 and He4 plants, respectively. Such differences among clones are common, particularly when using integrating vectors.

One hypothesis to explain this dramatic shift in nicotine and anabasine production in VHb-expressing transgenic tobacco plants, is that the presence of VHb increases the oxygen availability, which in turn significantly affects distribution of secondary metabolite production in the whole tobacco plant. Alternatively, these shifts in secondary metabolite production in VHb-expressing transgenes may result from other metabolic effects of VHb such as an increase in the ATP synthesis rate and/or membrane energy. Availability of more energy in the cell might lead to the improvement of many metabolic functions including increased nicotine production.

7. EXAMPLE: EXPRESSION OF VITREOSCILLA HEMOGLOBIN IN DATURA

Hyoscyamine and scopolamine are tropane alkaloids produced by plants; hyoscyamine is the levorotatory component of racemic atropine. Atropine, hyoscyamine, and scopolamine affect the parasympathetic nervous system and exhibit a wide range of pharmaceutical activity. In medicine they are used to relieve Parkinson-ism, dilate pupils, increase the heart rate, counteract toxic agents, and reduce secretions such as sweat. Scopolamine produces fewer undesirable side effects than hyoscyamine and is the preferred substance for treatment of motion sickness and production of derivative drugs for gastric disorders. Therefore, there has been a long-standing interest in raising the scopolamine level of producing plants. *Datura innoxia* a medicinal plant grown in many tropical and sub-tropical countries is one of the principal sources of tropane alkaloids. The biosynthesis of these alkaloids involves several oxygen-requiring steps.

7.1. Experimental Protocol

Recombinant DNA techniques, Transcription analysis and Western immunoblot analysis were performed as above for tobacco. The same transformation vector which was used for tobacco was also used for *Datura*.

Plant material

Plants of *Datura innoxia* Mill were exposed to 16 h light at 24°C and were kept in sterile jars on basal medium (BM) containing Murashige and Skoog's macro salts, and Nitsch and Nitsch micro salts and vitamins (Sangwan *et al.*, 1991, Plant Cell Rep. 10:90-93). The plants were transformed by *Agrobacterium*-mediated gene transfer using the leaf disc method as described by Sangwan *et al.* (1991). Transformants of the F_0 generation were used in this experiment.

Alkaloid analysis

Transgenic *Datura* and wild-type plants were analyzed for their scopolamine and hyoscyamine levels. The plants were grown in soil until they were budding, then the leaves were harvested and dried. The scopolamine and hyoscyamine levels were determined using HPLC as described by Gontier *et al.*, 1994, Agro-Food-Ind. Hi-Tech 5, 26-28.

7.2. Results

Several transgenic plants were positive for the VHb gene as determined by PCR of genomic DNA. PolyA$^+$ mRNA was isolated from plant leaves of six PCR-positive transgenic plants and two wild-type plants. All six transgenes exhibited a full-length VHb transcript. Preliminary results showed that the *Datura* plants transformed with the VHb expression construct also exhibited increased growth rates as compared to the wild-type plants.

Extracts from the six transgenic and two wild-type *Datura* plants were analyzed for scopolamine and hyoscyamine content by HPLC. Results are presented in the following table.

TABLE I

Transgenic plant No.	Production of Scopolamine mg/100 g D.W.	Production of Hyoscyamine mg/100 g D.W.
1	115	47
2	148	35
3	246	20
4	86	28
5	118	17
6	70	10
Control A	26	7.1
Control B	55	8.0

7.3. Discussion

Similarly to the results in tobacco, engineering *Datura* plants to express a VHb gene also resulted in transformed plants which both grew faster and contained altered levels of desired secondary metabolites. These results show that the expression of oxygen-binding proteins in whole plants, particularly proteins which reversibly bind oxygen via a heme group such as VHb, is generally applicable to obtain improved transformed plants.

8. EXAMPLE: PHENOTYPIC SCREEN FOR VITREOSCILLA HEMOGLOBIN MUTANTS

In order to create a library of randomly mutated VHbs, we used an earlier construct, pINT1 which carries the VHb gene as a template (Khosla and Bailey, 1989, *supra.*). The VHb sequence used as a template encoded the wild-type VHb protein with an additional 8 amino acid extension - MTMITPSF - at the amino-terminal end of the protein. However, all numbering conventions given herein correspond to the wild-type amino acid sequence positions as described in U.S. Patent No. 5,049,493 *supra.*. The VHb gene was amplified by low fidelity PCR with an estimated error rate of 2-4% (Leung *et al.*, 1989, Technique, 1, 11-15) using primers H1 and H2.

8.1. Experimental Protocol

PCR

Low fidelity PCR was performed with 1 mM dCTP, dGTP and dTTP, 0.2 mM dATP, 0.45 mM of each primer, 0.1 ng template, 1% 1 M mercaptoethanol, 10% DMSO, 0.5 mM $MnCl_2$ and 10 U Taq DNA polymerase. A temperature profile was employed of 2 min. at 95°C, hold at 70°C while the Taq DNA polymerase was added, 30 cycles of 15 sec. at 92°C, 30 sec. at 50°C, 1 min. at 72°C and an additional 5 cycles of 15 sec. at 92°C, 5 min. at 72°C. The amplified PCR fragments were inserted into pUC19 which was subsequently transformed into *E coli.* Sequencing of the mutated genes was performed on an automatic sequencer with the terminator chemistry.

Screening the library for colonies carrying mutated VHb

The plasmid library was transformed into *E. coli.* TG1 and selected on LB agar (Sambrook *et al.*) by visually assessing the color of the colonies. Expression of wild-type VHb results in a red colored colony. The red color phenotype is indicative of a VHb protein capable of binding oxygenated heme. Approximately 10,000 colonies were identified ranging in color from white to deep red.

Western immunoblot analysis

Overnight cultures were pelleted, resuspended in a sodium phosphate buffer (50 mM, pH 7.0) and sonicated to obtain protein extracts. The proteins were separated on 15% SDS-PAGE gels and blotted onto Immobilon P nylon membranes supplied by Millipore. The electrophoresis and blotting were performed as described by Laemmli (1970) and Sambrook *et al.* (1989), respectively. The VHb mutants were detected by VHb antiserum generated in rabbit, and visualized by peroxidase conjugated swine anti-rabbit immunoglobulins (Dako).

8.2. Results

Randomly picked colonies, which exhibited the correct size VHb (15.7 kDa) according to Western blot, were sequenced. The following altered VHb amino acid sequences were identified.

TABLE II.

ALTERED VHB PROTEINS OBTAINED THROUGH PCR MUTAGENESIS

ISOLATE	AMINO ACID ALTERATION
A	$Ile^{129}Thr$
B	$His^{36}Arg$
C	$Lys^{79}Asn$
D	$Phe^{33}Tyr$, $Gln^{53}Arg$
E	$Lys^{124}Glu$, $Phe^{133}Leu$, $Ile^{134}Thr$
F	$Gly^{21}Asp$, $Val^{136}Glu$, $Ala^{138}Thr$
G	$Leu^{51}Ser$, $Val^{83}Ala$, $Tyr^{126}His$
H	$Lys^{11}Glu$, $Lys^{107}Glu$, $Lys^{124}Stop$

8.3. Discussion

The PCR mutagenesis technique allowed the phenotypic selection of VHb mutants which retain at least some functional activity of the VHb, as evidenced by their ability to bind oxygenated heme. Although these mutants were generated in a VHb protein with an 8 amino acid amino-terminal extension, these mutants will be similarly effective in a VHb protein with the wild-type amino-terminus. Further, this approach will enable the easy selection and generation of any altered hemoglobin molecule through mutagenesis of the coding sequence, followed by selection for clones which encode heme containing proteins which retain functional activity.

9. EXAMPLE: TRANSFORMATION OF RICE WITH A VITREOSCILLA HEMOGLOBIN EXPRESSION CONSTRUCT

This example illustrates the transformation of rice using particle bombardment with a polynucleotide that directs the expression of Vitreoscilla hemoglobin in plant cells.

9.1 Experimental Protocol

Plasmid constructions

pJFK2 was constructed as a derivative of pUC that contains the *vhb* gene under the regulation of the CaMV 35S promoter and a nos polyadenylation

sequence. Additionally, the stop codon of the *vhb* gene was altered using PCR mediated mutagenesis from TAA to TGA (a more frequently used stop codon in plants). For the selection of transgenic rice tissue, the *aph*IV gene from *E.coli* which confers resistance to Hygromycin was included in the transformation construct. This marker gene was under the control of the CaMV 35S promoter and the CaMV polyadenylation sequence. A map of pJFK is shown in Figure 6.

The pJFK2 plasmid was digested with I-SceI, and the larger fragment containing *vhb* gene and hygromycin resistance gene (and lacking the ampicillin resistance gene) was isolated for use in posterior bombardment of plant embryos.

Rice transformation

Immature *Oryza sativa* embryos of the Japonica variety Taipei 309 were aseptically isolated 10-14 days after pollination from green-house-plants and plated scutulum site up on solid MS medium (Murashige and Skoog, 1962) containing 3% sucrose, 2 mg l^{-1} 2,4-D and 50 mg l^{-1} cefotaxime (MS1). After 4-6 days (28°C, darkness) embryos were transferred to solid MS medium containing 10% sucrose, 2 mg l^{-1} 2,4-D and 50 mg l^{-1} cefotaxime (MS2) and subjected within 1 hour to microprojectile bombardment with a particle inflow gun. The DNA fragment containing *vhb* and *aph* IV genes (5μg) was precipitated on 1-3 μm gold particles (Aldrich) as described by Vain et al., 1993, Plant Cell Rep. 12:84-88. Gold particles (400 μg per bombardment) were accelerated to the target with a particle inflow gun (Finer et al., 1992, Plant Cell Rep. 11:323-328) at a pressure of 6 bar. Embryos were placed 16 cm below the syringe filter. Twenty-four hours post-bombardment embryos were subjected to selection on a solid selection media (MS1 medium containing 20 mg l^{-1} hygromycin B) and incubated at 28°C in the dark.

After 1 week embryos were transferred to a liquid selection media, R2 medium (Ohira et al., 1973, Plant Cell Physiol. 14:113-121) supplemented with: 3% sucrose, 1 mg l^{-1} thiamine, 1 mg l^{-1} 2,4-D, 50 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B. The embryos were incubated with shaking at 28°C in the dark and subcultured weekly. Developing calli were isolated 3 to 6 weeks later, and transferred to a callus increasing media (R2 medium supplemented with: 6% sucrose, MS vitamins, 100 mg l^{-1} inositol, 2 mg l^{-1} 2,4-D, 50 mg l^{-1} cefotaxime and 20 mg l^{-1} hygromycin B). The calli were incubated in this media at 28°C in the dark and subcultured weekly.

Resistant calli were transferred to solid R2 regeneration medium supplemented with 2% sucrose, 3% sorbitol, 20 mg l^{-1} Hygromycin B, 1 mg l^{-1} of zeatin, 0.5 mg l^{-1} IAA, MS vitamins and 0.65% agarose. The callus tissue was maintained at 28°C with 12 h of light in order to enhance shoot formation. The calli were then subcultured every 3 weeks until shoots had reached a length of 2-3 cm. They were transferred to half-strength MS rooting medium without hormones, supplemented with 1.5% sucrose and 0.3% gelride®. After 2-4 weeks of cultivation, plantlets were transferred directly to the greenhouse and planted in soil. Plantlets were grown in 7 liter aquaculture pots with fertilizer enriched earth, 3 plants per pot, (day: 12 h, 28°C, 80% humidity; night: 12h, 21°C, 60% humidity) until they flowered and set seeds.

Analysis of transgenic rice plants

To check for presence of the transgene, complexity of insertion(s) and number of copies present, Southern blot analysis was performed as described previously (Burkhardt et al., 1997). A PCR-amplified, DIG-labeled (Boehringer), 300-bp fragment of the coding region of *vhb* was used as a probe.

Transgene expression is confirmed using western immunoblots as described above and in the literature (Wünn et al., 1996, Bio/Technology 14:171-176).

9.2 Results

Transformed plants were analyzed by Southern blot and were found to carry the transgene. Western blot is performed and demonstrates that the transformed plants express the *vhb* transgene. Seeds from the transgenic R0 plants are collected and R1 plants are grown. Growth of the R1 generation plants, as compared to control plants without the transgene, is more rapid.

Although the invention is described in detail with reference to specific embodiments thereof, it will be understood that variations which are functionally equivalent are within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings such modifications are intended to fall within the scope of the appended claims. Various publications are cited herein, each of the disclosures of which is incorporated by reference in its entirety.

WHAT IS CLAIMED IS:

1. A plant which has been genetically engineered by transformation with a polynucleotide encoding a globin protein.

2. The plant of claim 1, wherein the globin protein is selected from the group consisting of *Vitreoscilla* hemoglobin, horse heart myoglobin, E. coli hemoprotein, B. subtilis hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin, or their functional equivalents.

3. The plant of claim 1 or 2, wherein the polynucleotide encoding the globin protein is operably linked to a plant promoter.

4. The plant of claim 3, wherein the plant promoter is a strong, constitutively expressed plant promoter.

5. The plant of claim 4, wherein said strong, constitutively expressed plant promoter is a CaMV 35S promoter.

6. The plant of claim 3, wherein the plant promoter is a tissue specific plant promoter.

7. The plant of any one of claims 1-6, wherein the polynucleotide is introduced into a plant cell via microprojectile bombardment.

8. The plant of any one of claims 1-6, wherein the polynucleotide is introduced into a plant cell on a Ti plasmid.

9. The plant of any one of claims 1-8, wherein the plant is a dicotyledon.

10. The plant of claim 9, wherein the plant is from the genus *Nicotiana*.

11. The plant of claim 10, wherein the plant is *Nicotiana tabaccum*.

12. The plant of claim 9, wherein the plant is from the genus *Datura*.

13. The plant of any one of claims 1-8, wherein the plant is a monocotyledon.

14. The plant of claim 13, wherein the plant is from the genus *Oryza*.

15. A method of producing a plant with an improved agronomic or medicinal characteristic comprising transforming the plant with a polynucleotide encoding a globin protein, wherein the transformed plant exhibits an increased rate of growth, an increase in vegetative yield of the mature plant, an increased rate of germination, and/or an increased chlorophyll content, relative to a non-transformed plant.

16. The method of claim 15, wherein the globin protein is selected from the group consisting of *Vitreoscilla* hemoglobin, horse heart myoglobin, E. coli hemoprotein, B. subtilis hemoprotein, yeast flavohemoglobin, soybean leghemoglobin, lupin leghemoglobin, and sperm whale myoglobin, or their functional equivalents.

17. The method of claim 15 or 16, wherein the polynucleotide encoding the globin protein is operably linked to a plant promoter.

18. The method of claim 17, wherein the plant promoter is a strong, constitutively expressed plant promoter.

19. The method of claim 18, wherein the strong, constitutively expressed plant promoter is a CaMV 35S promoter.

20. The method of claim 17, wherein the plant promoter is a tissue specific plant promoter.

21. The method of any one of claims 15-20, wherein the polynucleotide is introduced into a plant cell via microprojectile bombardment.

22. The method of any one of claims 15-20, wherein the polynucleotide is transformed into a plant cell on a Ti plasmid.

23. The method of any one of claims 15-22, wherein the plant is a dicotyledon.

24. The method of claim 23, wherein the plant is from the genus *Nicotiana*.

25. The method of claim 24, wherein the plant is *Nicotiana tabaccum*.

26. The method of claim 25, wherein the plant further exhibits an elevated level of nicotine.

27. The method of claim 23, wherein the plant is from the genus *Datura*.

28. The method of claim 27, wherein the plant further exhibits an elevated level of scopolamine and/or hyoscyamine.

29. The method of claim 17-22, wherein the plant is a monocotyledon.

30. The plant of claim 29, wherein the plant is from the genus *Oryza*.

31. A plant produced by the method of claim 17.

32. A method of altering the distribution of secondary metabolites in a plant, comprising transforming a target plant or target plant cell with a polynucleotide encoding a globin protein operably linked to a plant promoter.

33. The method of claim 32, wherein the plant is from the genus *Nicotiana* and the distribution of nicotine is altered relative to anabasine.

34. The method of claim 32, wherein the plant is from the genus *Datura* and the distribution of scopolamine and/or hyoscyamine is altered.

35. A mutant *Vitreoscilla* hemoglobin protein, wherein the mutant *Vitreoscilla* hemoglobin protein differs from the wild type *Vitreoscilla* hemoglobin protein by having an amino acid substitution selected from the group consisting of: $Ile^{129}Thr$; $His^{36}Arg$; $Lys^{79}Asn$; $Phe^{33}Tyr$, $Gln^{53}Arg$; $Lys^{124}Glu$, $Phe^{133}Leu$, $Ile^{134}Thr$; $Gly^{21}Asp$, $Val^{136}Glu$, $Ala^{138}Thr$; $Leu^{51}Ser$, $Val^{83}Ala$, $Tyr^{126}His$; and $Lys^{11}Glu$, $Lys^{107}Glu$, $Lys^{124}Stop$.

36. The mutant *Vitreoscilla* hemoglobin protein of Claim 35 which further comprises an amino-terminal amino acid extension having the sequence MetThrMetIleThrProSerPhe.

37. A polynucleotide that encodes the mutant *Vitreoscilla* hemoglobin protein of Claim 35 or 36.

38. A host cell that contains the polynucleotide of Claim 37.

39. The host cell of Claim 38, wherein the host cell is selected from the group consisting of a plant cell, a bacterial cell, an animal cell, a fungal cell and an algae cell.

40. A method of generating a polynucleotide encoding mutant *Vitreoscilla* hemoglobin protein comprising:

amplifying a polynucleotide that encodes *Vitreoscilla* hemoglobin protein using a low fidelity polymerase chain reaction;

transforming the amplified polynucleotides into bacteria; and

selecting bacteria that contain a polynucleotide sequence encoding a mutant *Vitreoscilla* hemoglobin protein.

41. The method of Claim 40, wherein said selecting step comprises assaying the color of the bacterial cells.

42. A polynucleotide that encodes a mutant *Vitreoscilla* hemoglobin protein isolated by the method of Claim 40 or Claim 41.

43. A mutant *Vitreoscilla* hemoglobin protein encoded by the polynucleotide of Claim 42.

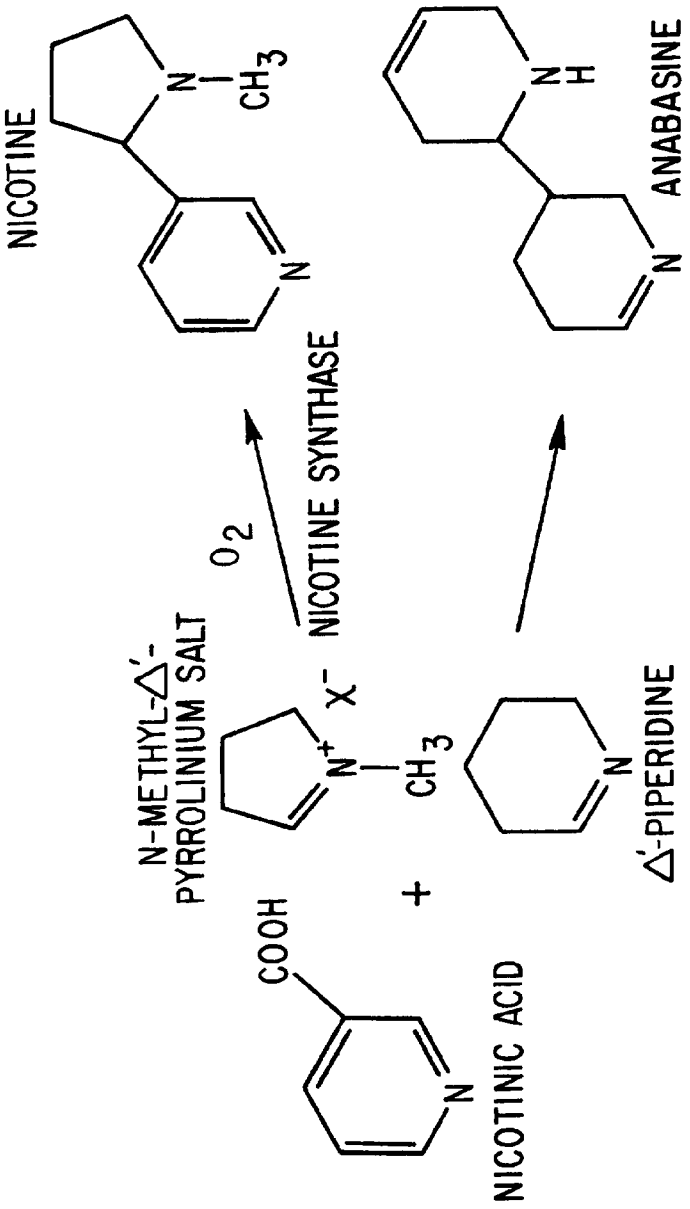

NICOTINE
N
CH3
N
ANABASINE
NH
N
O2
NICOTINE SYNTHASE
N-METHYL-Δ'-
PYRROLINIUM SALT
N+
X-
CH3
N
Δ'-PIPERIDINE
+
COOH
N
NICOTINIC ACID

FIG. 1A

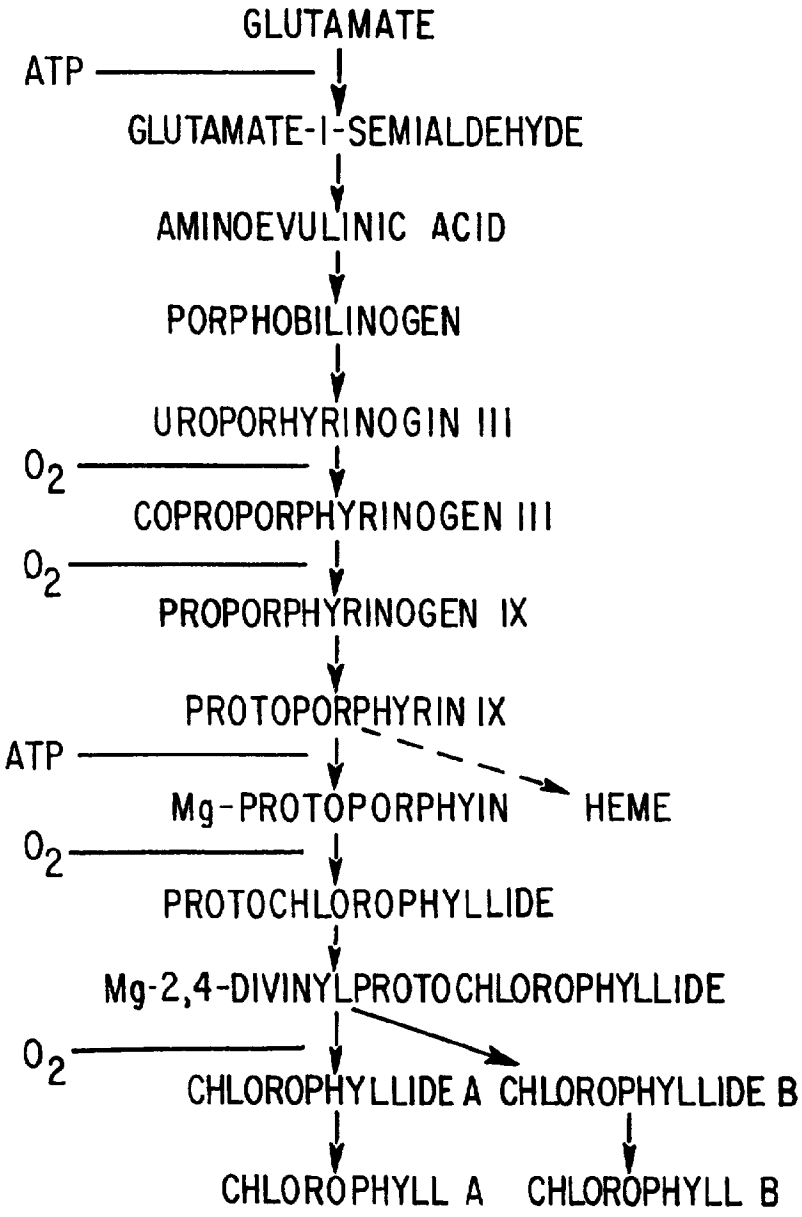

GLUTAMATE
ATP
GLUTAMATE-1-SEMIALDEHYDE
AMINOEVULINIC ACID
PORPHOBILINOGEN
UROPORHYRINOGIN III
O2
COPROPORPHYRINOGEN III
O2
PROPORPHYRINOGEN IX
PROTOPORPHYRIN IX
ATP
HEME
Mg-PROTOPORPHYIN
O2
PROTOCHLOROPHYLLIDE
Mg-2,4-DIVINYLPROTOCHLOROPHYLLIDE
O2
CHLOROPHYLLIDE A
CHLOROPHYLLIDE B
CHLOROPHYLL A
CHLOROPHYLL B

FIG. 1B

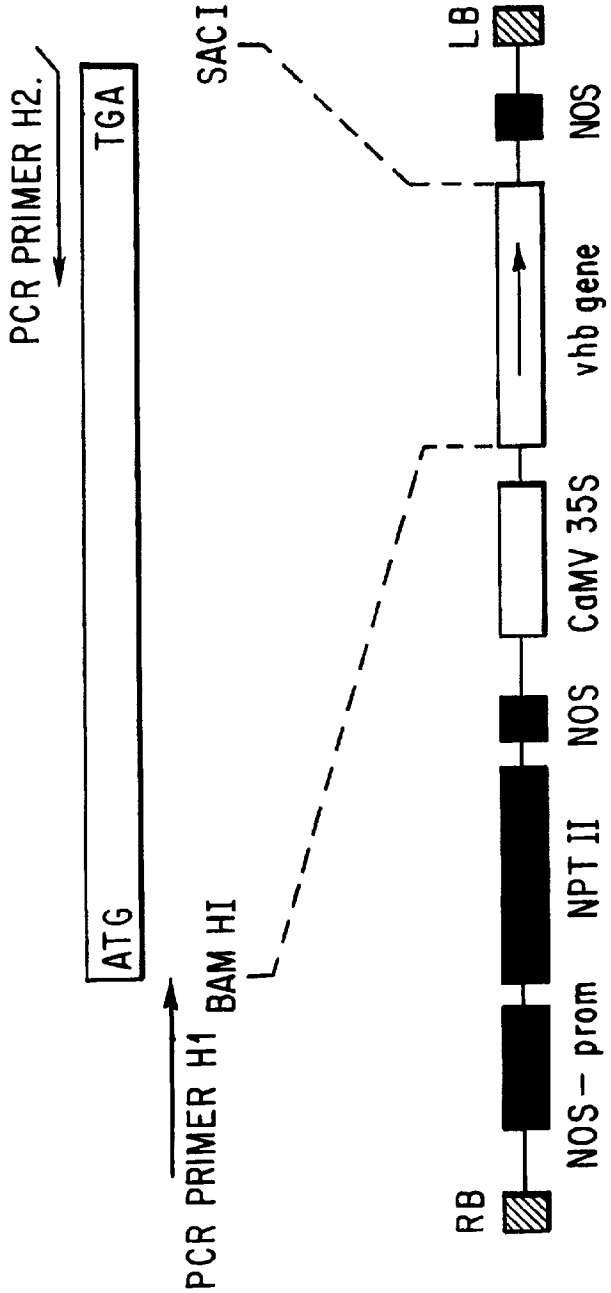

PCR PRIMER H2.
TGA
ATG
SACI
BAM HI
PCR PRIMER H1
LB
NOS
vhb gene
CaMV 35S
NOS
NPT II
NOS- prom
RB

FIG. 2

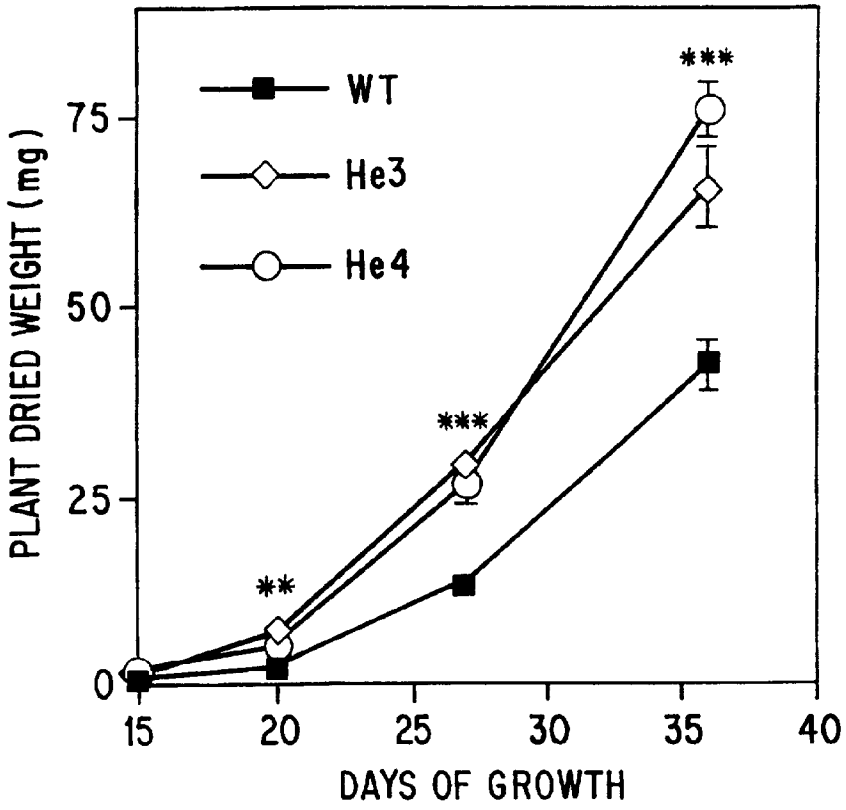

WT
He3
He4
PLANT DRIED WEIGHT (mg)
75
50
25
0
**
***
***
15
20
25
30
35
40
DAYS OF GROWTH

FIG. 3

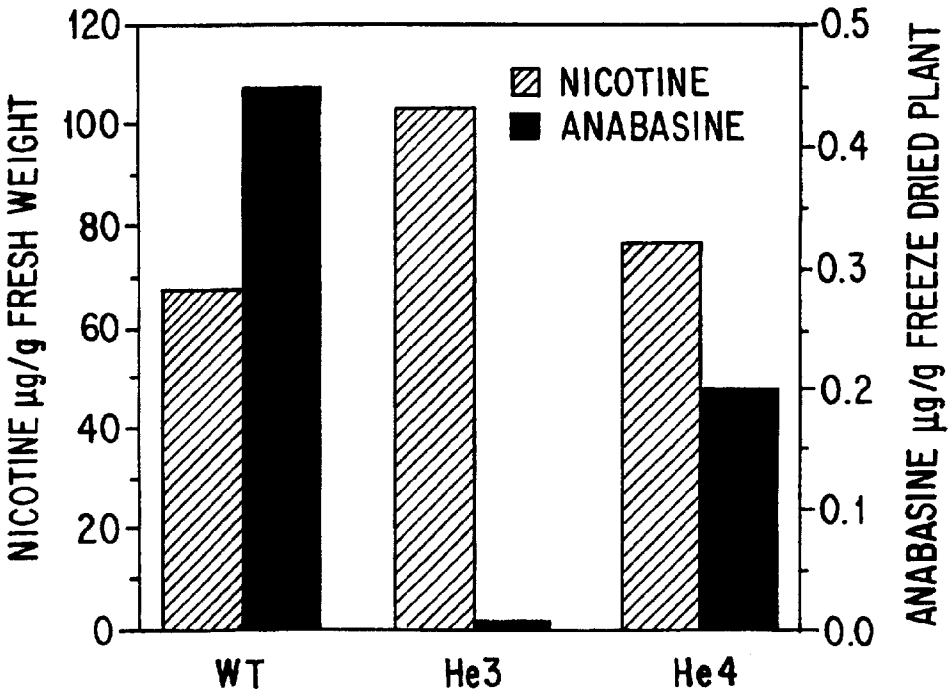

NICOTINE
ANABASINE
NICOTINE µg/g FRESH WEIGHT
ANABASINE µg/g FREEZE DRIED PLANT
120
100
80
60
40
20
0
0.5
0.4
0.3
0.2
0.1
0.0
WT
He3
He4

FIG. 4

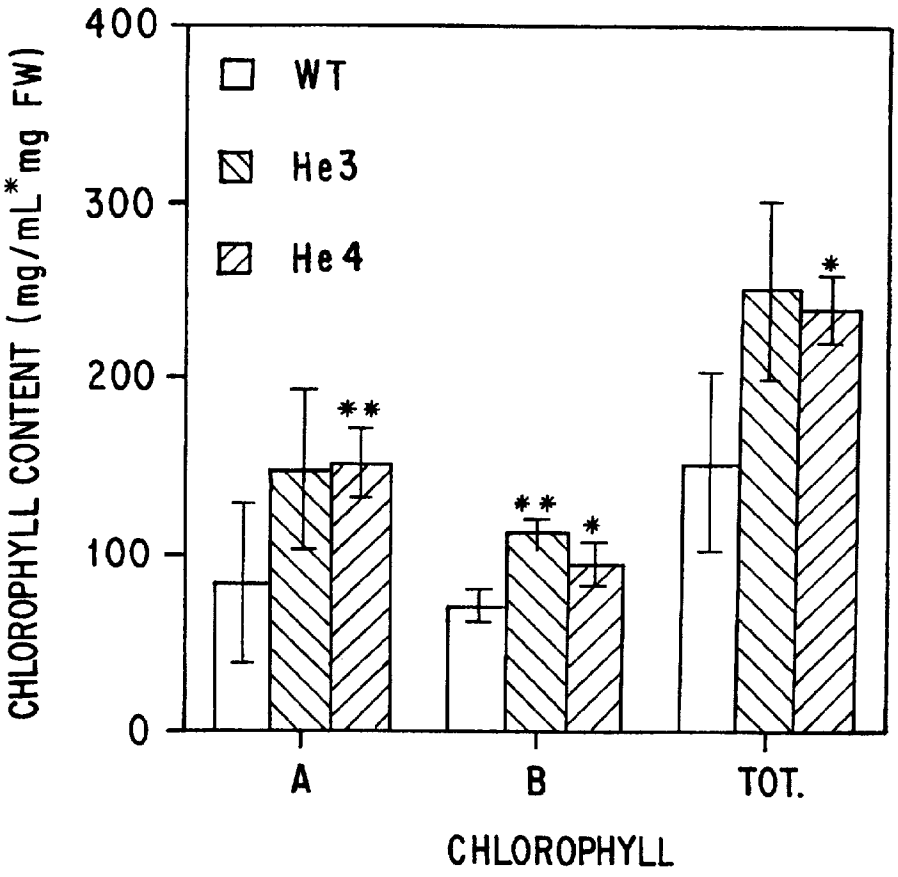

400
300
200
100
0
CHLOROPHYLL CONTENT (mg/mL*mg FW)
WT
He3
He4
**
**
*
*
A
B
TOT.
CHLOROPHYLL

FIG. 5

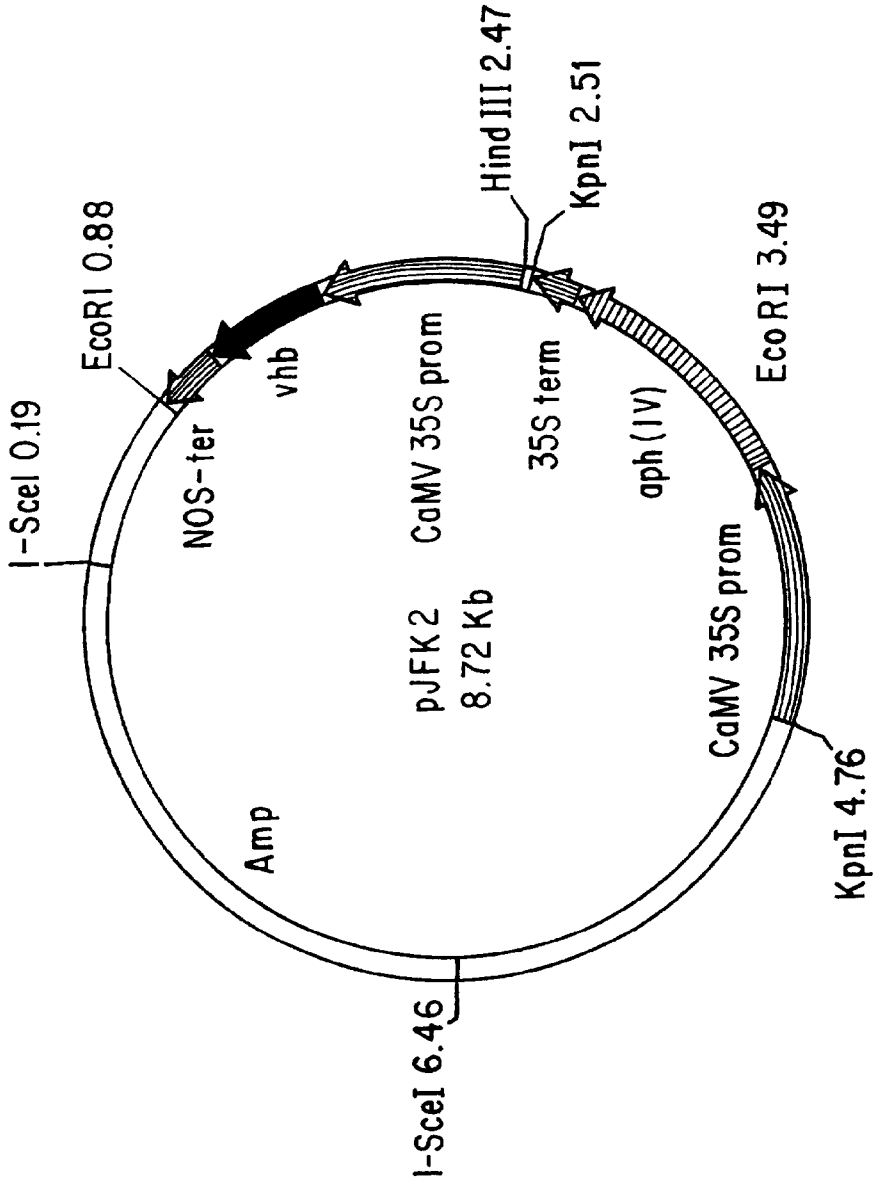

I-SceI 0.19
EcoRI 0.88
NOS-ter
vhb
CaMV 35S prom
35S term
HindIII 2.47
KpnI 2.51
aph(IV)
EcoRI 3.49
CaMV 35S prom
KpnI 4.76
I-SceI 6.46
Amp
pJFK2
8.72 Kb

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17246

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A01H 5/00; C12N 5/14, 15/31, 15/82
US CL :435/172.3, 320.1, 419; 536/23.7; 800/205, DIG40, DIG43, DIG57

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1, 419; 536/23.7; 800/205, DIG40, DIG43, DIG57

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, BIOSIS, EMBASE, CA, WPIDS
oygen binding, hemoglobin, plant, transgenic, transformation

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE ZOETEN et al. The Expression, Localization, and Effect of a Human Interferon in Plants. Virology. 1989, Vol. 172, pages 213-222, see entire article.	1-6, 1-12, 14-20, 24-28, 31-34
Y	KHOSLA et al. Heterologous Expression of a Bacterial Haemoglobin Improves the Growth Properties of Recombinant Escherichia coli. Nature. 18 February 1988, Vol. 331, pages 633-635, see entire article.	1-6, 10-12, 14-20, 24-28, 31-34
Y	BOGUSZ et al. Nonlegume Hemoglobin Genes Retain Organ-Specific Expression in Heterologous Transgenic Plants. The Plant Cell. July 1990, Vol. 2, pages 633-641, see abstract.	1-6, 10-12, 14-20, 24-28, 31-34

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 28 OCTOBER 1997	Date of mailing of the international search report 12 DEC 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer AMY NELSON Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17246

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 7, 8, 9, 13, 21-23, 29-30
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-6, 10-12, 14-20, 24-28, 31-34

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/17246

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s)1-6, 10-12, 14-20, 24-28, 31-34, drawn to method of producing transgenic plants comprising a globin gene, and transgenic plants thereby obtained.
Group II, claim(s) 35-36 and 43, drawn to mutant hemoglobin protein.
Group III, claim(s) 37-42, drawn to method of making mutant hemoglobin gene and mutant hemoglobin gene thereby obtained.

The inventions listed as Groups I, II and III do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is drawn toward wild type globin genes and Group III is drawn toward mutant globin genes and hence they lack a special technical feature.

Both Group III encompasses many different mutant genes and hence the mutant genes of Group III are not linked by a special technical feature to the proteins of Group II.

DNA and proteins have completely different structures, compositions and functions, and mutant DNA molecules and proteins differ structurally and functionally from wild type DNA and proteins. Hence, the inventions of Group I, II and III are not so linked as to form a single inventive concept under PCT Rule 13.1.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

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PCT

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/22829 A1

(54) Title: PROCESS FOR PREPARING SOY PROTEIN EXTENDED FRESH CHEESE

(57) Abstract: The present invention provides a process for the production of fresh cheese comprising the following steps: (a) mixing milk and isolated soy protein, (b) heating the mixture resulting from (a) to a temperature in the range of from about 70 °C to about 100 °C, (c) adding food grade acid to the mixture resulting from (b), and (d) separating the whey and the curd in the mixture resulting from (c).

PROCESS FOR PREPARING SOY PROTEIN EXTENDED FRESH CHEESE

Background of the Invention

Field of the Invention

The present invention relates, in general, to a process for the production of fresh cheese. The present invention further relates to a fresh cheese.

Background Information

The art of cheese making has been practiced for a number of years. The need to increase the amount of cheese from a given amount of milk is an economic necessity when the milk supply is limited, but the demand for such product is high. Soy protein has been used as an extender in cheeses with limited success. The patent literature is rich with patents which proclaim the ability to solve the various problems associated with using soy protein in a milk based fresh cheese. To date, none of the patents have described methods or products which have been commercial successes due to either the difficulty in incorporating the soy protein isolate into the finished cheese or because of the quality issues with the finished product.

Summary of the Invention

The present invention provides a process for the production of fresh cheese comprising the following steps (a) mixing milk and isolated soy protein; (b) heating the mixture resulting from (a) to a temperature in the range of from about 70°C to about 100°C; (c) adding food grade acid to the mixture resulting from (b); and (d) separating the whey and the curd in the mixture resulting from (c).

Detailed Description of the Invention

This invention relates to a process for the production of fresh cheese comprising (a) mixing milk and isolated soy protein; (b) heating the mixture resulting from (a) to a temperature in the range of from about 70°C to about 100°C; (c) adding food grade acid to the mixture resulting from (b); and (d) separating the whey and the curd in the mixture resulting from (c).

The milk used in the present invention can be present in amounts of from about 80 % to about 100 %, and preferable from about 90 % to about 99.9%. The milk can comprise of from about 0% to about 5% fat. It can be desirable to use milk with a low fat content and optionally add fat from other sources. As such, the fat contained in the milk can originate from at least one of the compounds of the group comprising milkfat or vegetable oil.

The invention provides the above and below described methods wherein said soy protein is present in amounts of from about 10% to 100% by weight, and preferably from about 15% to about 50%. By "percent" it is meant weight percent based on the calculated amount of casein solids in the milk. The isolated soy protein used in the present invention can be prepared by pH adjusting soy curd prior to spray drying or by blending different isolated soy proteins to obtain the desired pH. The pH of the isolated soy protein(s) can be of from about 3 to about 7, and preferably of from about 4.5 to about 6.5. The isolated soy protein can be less or greater than ambient temperature before addition to the milk. Preferably, the soy protein is at a temperature of about 0°C to about 60°C, and even more preferably at a temperature of from about 30°C to about 40°C.

After mixing the milk and isolated soy protein and heating this mixture to a temperature in the range of from about 70°C to about 100°C, a food grade acid is added to the mixture to coagulate the mixture. In this way, the casein matrix encloses discrete agglomerates of soy protein isolate and provides a cheese with the desired texture and flavor. The food grade acid used in the present invention can be selected from the group comprising organic and non-organic acids used alone or in combination. Preferably the organic and non-organic acids are selected from the group comprising acetic acid, malic acid, citric acid, hydrochloric acid, phosphoric acid and lactic acid. Other such acids will

be apparent to one of skill in the art. The invention provides the process of making the above and below described cheeses wherein said acid is present in amounts of from about 0.05% to about 5.0% and preferably from about 0.1% to about 2.5%.

In one embodiment, the present invention provides a method of making a cheese analog by adding at least one compound to a mixture of soy protein and milk, wherein said compound is selected from the group comprising a whitening agent.

Often it is desirable to produce a cheese with a white appearance. As such, the present invention provides for the optional addition of a whitening agent in amounts of from about 0.005% to about 3% and preferably from about 0.01% to about 1.5%. The whitening agent must be chemically inert and harmless to the animal or human who ingests the agent, but, of course, also must make the appearance of the cheese white. The invention provides the herein described methods wherein said whitening agent can be selected from the group comprising titanium dioxide, titanium dioxide blend and titanium dioxide dispersions.

A mixture comprising curds and whey results after step (c), above. The whey can be separated from the remainder of the above and below described mixtures by at least one of the methods selected from the group comprising draining, straining and filtering. Preferably, said separation results in elimination of from about 20% to 99% and preferably from about 30% to about 90% of the whey.

After separating a percentage of the whey from the remainder of the above and below described mixtures, the mixture can optionally be placed in molds. The mixture in the molds can also optionally be pressed to remove more moisture from the cheese analog.

The taste of the cheese analog of the present invention can be enhanced by the addition of flavoring agents to the above and below described mixtures. The flavoring agents can be selected from the group comprising sodium chloride and natural and artificial flavors.

The present invention is described in further detail in the following non-limiting examples.

Examples

Example 1

A control fresh cheese was made without the use of soy protein isolate. The quantity of 3000 grams of whole milk was heated to 82°C and held for 15 minutes under agitation. Diluted Lactic acid 8.8% (w/w) was added to the hot milk. The whey was then drained though a cheesecloth and the curd salted with 10 grams of sodium chloride, then placed in a mold for pressing. Of the 105 grams of protein in the milk, 90.09 grams were in 429.17 grams of cheese and 14.7 grams of protein in the recovered whey. The product had a good texture with a clean milky flavor.

Example 2

A fresh cheese was made with the use of soy protein isolate. To the quantity of 3000 grams of whole milk, 28 grams of a spray dried 5.4 pH soy protein isolate was added. The mixture was heated to 82°C and held for 15 minutes under agitation. Diluted Lactic acid 8.8% (w/w) was added to the hot milk. The whey was then drained though a cheese-cloth and the curd salted with 10 grams of sodium chloride, then placed in a mold for pressing. Of the 129.64 grams of protein in the milk and soy protein isolate, 116.74 grams were in 589.6 grams of cheese and 12.8 grams of protein in the recovered whey. The product had a good texture with a clean milky flavor.

Example 3

A fresh cheese was made with the use of soy protein isolate. To the quantity of 3000 grams of whole milk, 28 grams of a blended soy protein isolated at pH of 5.4 was added. The mixture was heated to 82°C and held for 15 minutes under agitation. Diluted Lactic acid 8.8% (W/w) was added to the hot milk. The whey was then drained though a cheesecloth and the curd salted with 10 grams of sodium chloride, then placed in a mold

for pressing. Of the 129.64 grams of protein in the milk and soy protein isolate, 117.36 grams were in 553.6 grams of cheese and 11.6 grams of protein in the recovered whey. The product had a good texture with a clean milky flavor.

Example 4

A fresh cheese was made with the use of soy protein isolate. To the quantity of 3000 grams of whole milk, 42 grams of a pH 5.4 spray dried soy protein isolated was added. The mixture was heated to 82°C and held for 15 minutes under agitation. Diluted Lactic acid 8.8% (w/w) was added to the hot milk. The whey was then drained though cheesecloth and the curd salted with I 0 grams of sodium chloride, then placed in a mold for pressing. Of the 142 grams of protein in the milk and soy protein isolate, 125.43 grams were in 620 grams of cheese and 16.5 grams of protein in the recovered whey. The product had a good texture and a milky flavor with a slight whey flavor.

Example 5

A fresh cheese was made with the use of soy protein isolate. To the quantity of 3000 grams of whole milk, 83 grams of a pH 5.4 spray dried soy protein isolate was added. The mixture was heated to 82°C and held for 15 minutes under agitation. Diluted Lactic acid 8.8% (w/w) was added to the hot milk. The whey was then drained though cheesecloth and the curd salted with 10 grams of sodium chloride, then placed in a mold for pressing. Of the 178 grams of protein in the milk and soy protein isolate, 156.13 grams were in 680 grams of cheese and 21.9 grams of protein were in the recovered whey. The product had a good texture and a milky flavor with a slight soy flavor.

Example 6

A fresh cheese was made with the use of soy protein isolate. To the quantity of 3000 grams of whole milk, 28 grams of a pH 4.5 spray dried soy protein isolate was added. The mixture was heated to 82°C and held for 15 minutes under agitation. Diluted

Lactic acid 8.8% (W/w) was added to the hot milk. The whey was then drained though cheesecloth and the curd salted with 10 grams of sodium chloride, then placed in a mold for pressing. Of the 129.64 grams of protein in the milk and soy protein isolate, 109 grams were in 560 grams of cheese and 20.6 grams of protein were in the recovered whey. The product had a good texture and a milky flavor with a slight acid flavor.

Example 7

A fresh cheese was made with the use of soy protein isolate. To the quantity of 3000 grams of whole milk, 28 grams of a pH 5.0 spray dried soy protein isolate was added. The mixture was heated to 82 °C and held for 15 minutes under agitation. Diluted Lactic acid 8.8% (w/w) was added to the hot milk. The whey was then drained though cheesecloth and the curd salted with 10 grams of sodium chloride, then placed in a mold for pressing. Of the 129.64 grams of protein in the milk and soy protein isolate, 116.3 grams were in 580 grams of cheese and 13.3 grams of protein were in the recovered whey. The product had a good texture and a milky flavor with a very slight acid flavor.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

What Is Claimed Is:

1. A process for the production of fresh cheese comprising:
   a. mixing milk and isolated soy protein;
   b. heating the mixture resulting from (a) to a temperature between about 70°C and about 100°C;
   c. adding food grade acid to the mixture resulting from (b); and
   d. separating the whey from the mixture resulting from (c).

2. The process of claim 1, wherein said milk is present in amounts of from about 80% to about 100%.

3. The process of claim 1, wherein said milk comprises 0% to 5% fat.

4. The process of claim 3, wherein said fat is selected from the group comprising milkfat and vegetable oil.

5. The process of claim 1, wherein said isolated soy protein is present in amounts of from about 10 to about 100%.

6. The process of claim 1, wherein said isolated soy protein has a pH of from about 3 to about 7.

7. The process of claim 6, wherein said pH of the isolated soy protein is adjusted by a method comprising spray drying or blending different pH soy protein isolate products to obtain the desired pH range.

8. The process of claim 1, wherein said isolated soy protein is added to the milk under agitation at a temperature from about 0°C to about 60°C.

9. The process of claim 1, wherein said food grade acid is selected from the group comprising organic acids and non-organic acids.

10. The process of claim 1, wherein said food grade acid is present in amounts from about 0.05% to about 5%.

11. The process of claim 1, wherein following step (a), a whitening agent is added to the mixture.

12. The process of claim 11, wherein said whitening agent is selected from the group comprising titanium dioxide, titanium dioxide blends and titanium dioxide dispersions.

13. The process of claim 11, wherein said whitening agent is present in amounts from about 0.005% to about 3%.

14. The process of claim 1, wherein said mixture of step (b) is held at said temperature for about 1 minute to about 60 minutes.

15. The process of claim 1, wherein said whey is separated from curd by at least one of the methods selected from the group comprising draining, straining, and filtering.

16. The process of claim 1, wherein following any of steps (a) through (d), salt is added to produce a cheese with an acceptable flavor.

17. The process of claim 1, further comprising placing curds into molds and pressing said curds at a pressure, temperature and length of time to produce the desired consistency of the finished fresh cheese.

18. A composition prepared according to the method of claim 1.

INTERNATIONAL SEARCH REPORT

Intern ial Application No
PCT/US 00/24871

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23C19/055 A23C19/05 A23C19/045 A23C19/076

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, FSTA, WPI Data, PAJ, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199404 Derwent Publications Ltd., London, GB; Class D13, AN 1994-032781 XP002154258 & SU 1 785 421 A (BELO MEAT DAIRY IND CONS TECHN INST), 30 December 1992 (1992-12-30)	1-10, 14-18
Y	abstract	11-13
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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Date of the actual completion of the international search	Date of mailing of the international search report
14 December 2000	05/02/2001

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INTERNATIONAL SEARCH REPORT

Intern ial Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANFURT/MAIN, DE; SMIETANA Z: "Study of guided modification of milk proteins for processing purposes." Database accession no. 80-2-11-p1849 XP002154257 abstract & ZESZYTY NAUKOWE AKADEMII ROLNICZO TECHNICZNEJ W OLSZTYNIE, TECHNOLOGIA ZYWNOSCI 1979 INST. INZYNIERII & BIOTECH. ZYWNOSCI, ART, OLSZTYN, POLAND, no. 14, 1979, pages 123-184, ---	1-4, 6-10, 15-18
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A	P.F. FOX: "Cheese: Chemistry, Physics and Microbiology" 1993 , CHAPMAN & HALL , GB XP002154413 page 396, line 4 - line 7 ---	1
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A	EP 0 815 736 A (UNIV CALIFORNIA POLYTECHNIC) 7 January 1998 (1998-01-07) claim 3 -----	1

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Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr nal Application No
PCT/US 00/24871

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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US 5676984 A	14-10-1997	US 5470593 A	28-11-1995
EP 0815736 A	07-01-1998	US 5766657 A	16-06-1998
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		AU 2616097 A	08-01-1998
		CA 2207881 A	21-12-1997
		NZ 328129 A	26-08-1998

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(54) Title: METHOD FOR THE USE OF SOY PROTEIN IN THE PRODUCTION OF FRESH AND RIPENED CHEESE

(57) Abstract: The invention provides a method for making a fresh, unriped cheese analog comprising, (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting the product resulting from (b); (d) agitating the product resulting from (c) for about 40 to about 60 minutes at a temperature selected from the range of about 30°C to about 50°C; (e) separating about 50 to about 80% of whey from product resulting from (d); and (f) placing product resulting from (d) in molds and placing molds at a temperature of at least about 28°C for about 2 to about 8 hours. The invention also relates to a method for making a hard, ripened cheese or a cheese analog comprising (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting product resulting from (b); (d) agitating the product resulting from (c) for about 40 to about 60 minutes at a temperature of from about 30°C to about 70°C; (e) separating from (f) in molds and placing the molds at a temperature appropriate for the desired type of cheese or cheese analog, for a length of time necessary to begin the ripening process.

METHOD FOR THE USE OF SOY PROTEIN IN THE PRODUCTION OF FRESH AND RIPENED CHEESE

Background of the Invention

Field of the Invention

The present invention relates, in general, to fresh, unripened cheese analog and a method for making a fresh, unripened cheese analog by incorporating isolated soy protein. The invention also relates to hard, ripened cheeses or cheese analogs and methods for making hard, ripened cheeses or cheese analogs by incorporating isolated soy protein.

Background Information

While the art of cheesemaking has been practiced for centuries, the cheesemaker is always looking for ways to improve the finished cheese yield obtained from the starting quantity of milk. This becomes very important in areas of the world where the milk supply is limited. Attempts have been made over the years to incorporate soy protein as an economic replacement for milk proteins. Problems can be encountered due to differences between milk and soy proteins in molecular and functional properties which can affect the quality of the finished cheese.

Therefore, a method is provided which incorporates isolated soy protein into a coagulated milk matrix.

Summary of the Invention

The invention provides a method for making a fresh, unripened cheese analog comprising, (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting the product resulting from (b); (d) agitating the product resulting from (c) for about 40 to about 60 minutes at a temperature selected from the range of about 30°C to about 50°C; (e) separating about 50 to about 80% of whey from product resulting from (d); and (f) placing product resulting from (d) in molds and placing molds at a temperature of at least about 28°C for about 2 to about 8 hours.

The invention also relates to a method for making a hard, ripened cheese or cheese analog comprising (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting product resulting from (b); (d) agitating the product resulting from (c) for about 40 to about 60 minutes at a temperature of from about 30°C to about 70°C; (e) separating about 50 to about 80% of whey from product resulting from (d); (f) processing the product resulting from (e) to produce the desired type of cheese or cheese analog; and (g) placing product resulting from (f) in molds and placing the molds at a temperature appropriate for the desired type of cheese or cheese analog, for a length of time necessary to begin the ripening process.

Detailed Description of the Invention

The invention provides a method for making a fresh, unripened cheese and cheese analog comprising, (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting the product resulting from (b); (d) agitating the product resulting from (c) for about 40 to about 60 minutes at a temperature selected from the range of about 30°C to about 50°C; (e) separating about 50 to about 80% of whey from product resulting from (d); and (f) placing product resulting from (d) in molds and placing molds at a temperature of at least about 28°C for about 2 to about 8 hours.

The invention also relates to a method for making a hard, ripened cheese or cheese analog comprising (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting product resulting from (b); (d) agitating the product resulting from (c) for about 40 to about 60 minutes at a temperature of from about 30°C to about 70°C; (e) separating about 50 to about 80% of whey from product resulting from (d); (f) processing the product resulting from (e) to produce the desired type of cheese or cheese analog; and (g) placing product resulting from (f) in molds and placing the molds at a temperature appropriate for the desired type of cheese or cheese analog, for a length of time necessary to begin the ripening process.

The milk used in the present invention may be present in amounts of from about 95% to about 99.99%, and preferably from about 98% to about 99.99%. The milk may comprise of from about 0% to about 10% fat and preferably from about 0.5% to about 5% fat. It may be desirable to use milk with a low fat content and optionally add fat from other sources. As such, the fat contained in the milk may originate from at least one of the compounds of the group comprising milkfat or vegetable oil.

The invention provides the above and below described methods wherein said soy protein is present in amounts of from about 10% to 100% by weight, and preferably from about 15% to about 50%. By "percent" it is meant weight percent based on the calculated amount of casein solids in the milk. The isolated soy protein used in the present invention may be prepared by pH adjusting soy curd prior to spray drying or by blending different isolated soy proteins to obtain the desired pH. The pH of the isolated soy protein(s) may be of from about 3 to about 7, and preferably of from about 4.5 to about 6.0.

In one embodiment, the present invention provides a method of making a fresh, unripened cheese analog by adding at least one compound to a mixture of isolated soy protein and milk, wherein said compound is selected from the group comprising transglutaminase, starch, a whitening agent, hydrocolloid gum, calcium chloride, and a coagulating enzyme.

In another embodiment, the present invention provides a method of making a hard, ripened cheese or cheese analog by adding at least one compound to a mixture of isolated soy protein and milk, wherein said compound is selected from the group comprising transglutaminase, starch, a whitening agent, hydrocolloid gum, calcium chloride, and a coagulating enzyme.

Transglutaminase is an enzyme which crosslinks proteins and which aids in crosslinking the isolated soy protein and milk proteins. The transglutaminase catalyzed crosslinks serve to compatibilize the soy and milk proteins. These crosslinks help in improving the functionality of the different proteins and therefore improving the texture of the finished product. Transglutaminase is preferably present in amounts from about 0% to about 1.0%, and preferably from about 0% to about 0.2%.

As a binder or filler in the cheese, generally any starch which is at least partially soluble in water at the described temperatures can be employed in the cheese or cheese analog making method of the present invention. Corn and potato starches are types of binder that may be used. The preferred starch should be pregelatinized by mixing with water and heating to its gelatinization temperature prior to addition to the milk. The gelatinized starch may optionally be added in amounts of from about 0% to about 20%, and preferably from about 0% to about 10%.

Often it is desirable to produce a cheese analog with a white appearance. As such, the present invention provides for the optional addition of a whitening agent in amounts of from about 0% to about 0.5%, preferably from about 0% to about 0.1%. The whitening agent must be chemically inert and harmless to the animal or human who ingests the agent, but, of course also must make the appearance of the cheese white. The invention provides the herein described methods wherein said whitening agent may be selected from the group comprising titanium dioxide, benzoyl peroxide, or green and blue food colorings.

A hydrocolloid gum may be optionally added as a stabilizer to improve the yield of the cheese, and may be added in amounts of from about 0% to about 2%,

and preferably from about 0% to about 0.8%. The hydrocolloid gum can be selected from the group comprising carrageenan, carob bean, guar and xanthan.

The invention provides a method for producing a soft, sliceable, unripened cheese-like product. Firming agents may optionally be added to firm the curd. Preferably, calcium chloride is added to produce a firm cheese curd and can be added in amounts of from about 0% to about 0.5%, preferably from about 0% to about 0.2%.

The invention also provides a method for producing a hard, sliceable, meltable, ripened cheese or cheese-like product. Firming agents may optionally be added to firm the curd. Preferably, calcium chloride is added to produce a firm cheese curd and can be added in amounts of from about 0% to about 0.5%, preferably from about 0% to about 0.2%.

Following step (a), above, a coagulating enzyme may be used to speed the coagulation process, and may be added in catalytic amounts. The coagulating enzyme may be selected from the group comprising rennet and its synthetic equivalents. Preferred rennet preparations can be selected from the group comprising AmericanPure, (SBI-Systems Bio-Industries, calf rennet purified via ion-exchange), Chy-Max (Pfizer, fermentation - using *Escherichia coli* K-12), Chymogen (Chris Hansen, fermentation - using *Aspergillus niger* var. *awamori*), ChymoStar (Rhône-Poulenc, fermentation - using *Aspergillus niger* var. *awamori*), Maxiren (Gist-Brocades, Fermentation - using *Kluyveromyces marxianus* var. *lactis*), Novoren (Marzyme GM) (Novo Nordisk, Fermentation - using *Aspergillus oryzae*). Kosikowski, F.V. and Mistry, V.V. (1997), "Cheese and Fermented Milk Foods," 3rd edn., F.V. Kosikowski, L.L.C., Westport, Conn., pp. 395 and 698.

Following step (a), above, a bacterial culture may optionally be added to the resulting mixture. The bacterial cultures can be added to the mixture to produce a desired flavor of cheese or to aid in acid development. Furthermore, these bacterial cultures may be added in lieu of, or in addition to, a coagulating agent. The types of bacterial cultures to be added to the mixture is obvious to one skilled in the art of making cheese. Examples of bacterial cultures that may be

added to the mixture include, but are not limited to, *Lactobacillus Bulgaris, Lactobacillus Lactus, Lactobacillus Helveticus, Streptococcus Thermophilus, Streptococcus Cremoris, Streptococcus Diacetilactis, Streptococcus Lactus, Streptococcus Durans, Leuconostoc Faecalis, Leuconostoc Citrovorum, Leuconostoc Dextranicum.* The bacterial cultures may be added individually, or as mixtures of bacterial cultures that contain 2 or more different species of bacteria. Appropriate mixtures of bacterial cultures are readily apparent to one skilled in the art.

A mixture comprising curds and whey results after step (d), above. The whey may be separated from the remainder of the above and below described mixtures by at least one of the methods selected from the group comprising draining, straining and filtering. Preferably, said separating results in elimination of from about 50% to 80% of the whey, and even more preferably, results in elimination of from about 60 to 70% of the whey.

After separating a percentage of the whey from the rest of the above described mixtures, the mixture is further processed to produce the appropriate type of cheese or cheese analog. The processing required to produce various types o cheeses or cheese analogs at this stage in the method is obvious to one skilled in the art of cheese-making. The mixture is also salted to improve flavor and texture. The salting agent can be selected from the group comprising sodium chloride. The salted, processed mixture is poured into molds to shape the cheese. The mixture in the molds may also optionally be externally pressed to remove moisture from the cheese or cheese analog. The resulting cheese composition after comprises from about 2% to about 17% soy isolate.

The present invention is described in further detail in the following non-limiting examples.

Examples

Example 1

Isolated soy protein was prepared by pH adjusting soy curd prior to spray drying. The pH of the isolated soy protein ranged from between 4.5 to 6.0. 2650 grams of milk were placed in a vat. Twenty eight grams of isolated soy protein was incorporated into 300 grams of milk with high shear agitation or the use of a homogenizer. The isolated soy protein/milk mixture was added when the milk in the vat reached a temperature of 32°C. Six grams of calcium chloride was dissolved in water then added to the mixture in the vat, followed by the synthetic rennet. The milk/soy protein matrix was then allowed to coagulate for a period of 40 minutes. The curd was then cut using cheese knives and allowed to "heal" for 5 minutes. The cut curds were then agitated occasionally for about 40 minutes and allowed to cook at a temperature of 37°C. Approximately 60-70% of the whey was drained and 3.5% salt was incorporated, based on the weight of the remaining whey and curds. The salted whey/curd mixture was poured into molds and allowed to set for 4 hours, without external pressing, at a minimum temperature of 28 °C. After four hours, the molds were placed in refrigerated storage overnight. The finished cheese was removed from the mold and packaged. The weight of the finished cheese product was increased by 3 to 12.5% compared to the product produced without the addition of isolated soy protein.

Example 2

Isolated soy protein was prepared by blending different isolated soy proteins to obtain the desired pH. The pH of the isolated soy protein ranged from 4.5 to 6.0. 2650 grams of milk were placed in a vat. Twenty-eight grams of an isolated soy protein blend and 6 grams of a 50% titanium dioxide dispersion were incorporated into 300 grams of milk with high shear agitation or the use of a homogenizer. The isolated soy protein/milk/titanium dioxide mixture was added

when the milk in the vat reached a temperature of 32°C. Six grams of calcium chloride was dissolved in water then added to the mixture in the vat, followed by the synthetic rennet. The milk/soy protein matrix was then allowed to coagulate for a period of 40 minutes. The curd was then cut using cheese knives and allowed to "heal" for 5 minutes. The cut curds were then agitated occasionally for about 40 minutes and allowed to cook at a temperature of 37°C. Approximately 60-70% of the whey was drained and 3.5% salt was incorporated, based on the weight of the remaining whey and curds. The salted whey/curd mixture was poured into molds and allowed to set for 4 hours, without external pressing, at a minimum temperature of 28 °C. After four hours, the molds were placed in refrigerated storage overnight. The finished cheese was removed from the mold and packaged. The weight of the finished cheese product was increased by 9 to 14% compared to the product produced without the addition of isolated soy protein.

Example 3

Isolated soy protein was prepared by either adjusting the soy curd prior to spray drying or by blending different isolated soy proteins to obtain the desired pH. The pH of the isolated soy protein or isolated soy protein blends ranged from 4.5 to 6.0. 2650 grams of milk were placed in a vat. Twenty-eight grams of the isolated soy protein or isolated soy protein blend; 6 grams of a 50% titanium dioxide dispersion; and 1 activity unit of transglutaminase were incorporated into 300 grams of milk with high shear agitation or the use of a homogenizer. The isolated soy protein/milk/titanium dioxide/transglutaminase mixture was added when the milk in the vat reached a temperature of 32°C. Six grams of calcium chloride was dissolved in water then added to the mixture in the vat, followed by the synthetic rennet. The milk/soy protein matrix was then allowed to coagulate for a period of 40 minutes. The curd was then cut using cheese knives and allowed to "heal" for 5 minutes. The cut curds were then agitated occasionally for about 40 minutes and allowed to cook at a temperature of 37°C. Approximately

60-70% of the whey was drained and 3.5% salt was incorporated, based on the weight of the remaining whey and curds. The salted whey/curd mixture was poured into molds and allowed to set for 4 hours, without external pressing, at a minimum temperature of 28 °C. After four hours, the molds were placed in refrigerated storage overnight. The finished cheese was removed from the mold and packaged. The weight of the finished cheese product was increased by 16.5 to 23% compared to the product produced without the addition of transglutaminase and isolated soy protein.

Example 4

A Manchego cheese was made using the following procedure. Isoalted soy protein was prepared by pH adjusting soy curd prior to spray drying. The pH of the isolate soy protein ranged between 4.5 to 6.0. 2.3 pound of the isolated soy protein was added to 500 pound of whole milk and then homogenized at 2000 psig and pasteurized at 72.2°C for 16 seconds. 6 grams of Rhodia EZAL MA 011 starter was added along with 2.5 ml of Annatto color. After 40 minutes 88 ml of Calcium chloride was added along with 17 ml of double strength Chymax synthetic rennet. The milk/soy matrix was then allowed to coagulate for a period of 30 minutes. The curd was then cut using cheese knives and allowed to "heal" for 5 minutes. The cut curds were then agitated occasionally for the next 10 minutes and then 50% of the whey was removed. After 10 minutes, the same amount of 54.4°C water was added slowly. The curds were then heated to 38.9°C for 20 minutes. The whey was then drained. The curds were then gathered and formed into blocks. These blocks were then turned for the next 15 minutes to drain more whey. The product was then cut, placed into hoops and pressed overnight. The product was then placed in a brine solution for 40 hours and then placed at 4°C for ripening.

The resulting cheese was 20% more than a control made without isolated soy protein. The texture and melt were similar to the control.

* * * * *

All publications mentioned hereinabove are hereby incorporated in their entirety by reference.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be appreciated by one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention and appended claims.

What Is Claimed Is:

1. A method for making a fresh, unripened cheese analog comprising

(a) mixing isolated soy protein and milk;

(b) adding a coagulating agent mixture from (a);

(c) cutting product resulting from (b);

(d) agitating the product resulting from (c) for about 0 to about 90 minutes at a temperature of from about 30°C to about 50°C;

(e) separating about 50 to about 80% of whey from product resulting from (d) and salting; and

(f) placing product resulting from (e) in molds and placing molds at a temperature of at least about 28°C for about 2 to about 8 hours.

2. The method according to claim 1, wherein a bacterial culture is added to said mixture from (a), instead of said coagulating agent.

3. The method of claim 1, wherein a bacterial culture is added to said mixture from (a), in addition to said coagulating agent.

4. The method of claim 1, wherein said milk is present in amounts of from about 95% to about 99%.

5. The method of claim 1, wherein said milk from step (a) comprises 0% to 10% fat.

6. The method of claim 5, wherein said fat comprises milk fat or vegetable oil.

7. The method of claim 1, wherein said soy protein is present in amounts from about 10% to about 100% by weight.

8. The method of claim 1, wherein said isolated soy protein from step (a) has a pH of from about 3 to about 7.

9. The method of claim 1, wherein following step (a), at least one compound selected from the group comprising transglutaminase, starch, whitening agent, hydrocolloid gum, and a firming agent is added to the mixture.

10. The method of claim 9, wherein said transglutaminase is present in amounts from about 0% to about 1%.

11. The method of claim 9, wherein said starch is present in amounts of from about 0% to about 20%.

12. The method of claim 9, wherein said whitening agent is selected from the group comprising titanium dioxide, benzoyl peroxide, green food coloring and blue food coloring.

13. The method of claim 9, wherein said whitening agent is present in amounts of from about 0% to about 0.5%.

14. The method of claim 9, wherein said hydrocolloid gum is selected from the group comprising carrageenan, carob bean, guar gum and xanthan gum.

15. The method of claim 9, wherein said hydrocolloid gum is present in amounts from about 0% to about 2%.

16. The method of claim 9, wherein said firming agent is calcium chloride.

17. The method of claim 9, wherein said firming agent is present in amounts of from about 0% to about 0.5%.

18. The method of claim 1, wherein said coagulating agent comprises rennet or a synthetic rennet equivalent.

19. The method of claim 1, wherein about 2 to about 10% salt is added to said product following step (d).

20. The method of claim 1, wherein during step (f), the mixture is subjected to external pressure.

21. A composition prepared according to the method of claim 1.

22. A method for making a hard, ripened cheese or cheese analog comprising

(a) mixing isolated soy protein and milk;

(b) adding a coagulating agent to said mixture from (a);

(c) cutting product resulting from (b);

(d) agitating the product resulting from (c) for about 0 to about 90 minutes at a temperature of from about 30°C to about 70°C;

(e) separating about 50 to about 80% of whey from product resulting from (d);

(f) processing the product resulting from (e) to produce the desired type of cheese analog; and

(g) placing product resulting from (f) in molds and placing said molds at a temperature appropriate for said desired type of cheese analog, for a length of time necessary to begin the ripening process.

23. The method according to claim 22, wherein a bacterial culture is added to said mixture from (a), instead of said coagulating agent.

24. The method of claim 22, wherein a bacterial culture is added to said mixture from (a), in addition to said coagulating agent.

25. The method of claim 22, wherein said milk is present in amounts of from about 95% to about 99.99%.

26. The method of claim 22, wherein said milk from step (a) comprises 0% to 10% fat.

27. The method of claim 26, wherein said fat comprises milk fat or vegetable oil.

28. The method of claim 22, wherein said soy protein is present in amounts from about 10% to about 100% by weight of the casein present in the finished said cheese analog.

29. The method of claim 22, wherein said isolated soy protein from step (a) has a pH of from about 3 to about 7.

30. The method of claim 22, wherein following step (a), at least one compound selected from the group comprising transglutaminase, starch, whitening agent, hydrocolloid gum, and a firming agent is added to the mixture.

31. The method of claim 30, wherein said transglutaminase is present in amounts from about 0% to about 1%.

32. The method of claim 30, wherein said starch is present in amounts of from about 0% to about 20%.

33. The method of claim 30, wherein said whitening agent is selected from the group comprising titanium dioxide, benzoyl peroxide, green food coloring and blue food coloring.

34. The method of claim 30, wherein said whitening agent is present in amounts of from about 0% to about 0.5%.

35. The method of claim 30, wherein said hydrocolloid gum is selected from the group comprising carrageenan, carob bean, guar gum and xanthan gum.

36. The method of claim 30, wherein said hydrocolloid gum is present in amounts from about 0% to about 2%.

37. The method of claim 30, wherein said firming agent is calcium chloride.

38. The method of claim 30, wherein said firming agent is present in amounts of from about 0% to about 0.5%.

39. The method of claim 22, wherein said coagulating agent comprises rennet or a synthetic rennet equivalent.

40. The method of claim 22, wherein about 2 to about 10% salt is added to said product following step (d).

41. The method of claim 22, wherein during step (f), the mixture is subjected to external pressure.

42. A composition prepared according to the method of claim 22.

INTERNATIONAL SEARCH REPORT

Int. ational Application No
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A. CLASSIFICATION OF SUBJECT MATTER
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B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
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Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
FSTA, EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE FSTA 'Online! INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANFURT/MAIN, DE; ALEKSEEV N G ET AL: "Method for producing cheese." Database accession no. 87-1-02-v0159 XP002156451	1,3-11, 13, 15-22, 24-32, 34,36-42
Y	abstract & USSR PATENT SU 1 205 870 A 1986, --- -/--	2,10-14, 23,31-35

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Koch, J

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INTERNATIONAL SEARCH REPORT

Int. ...tional Application No
PCT/US 00/26558

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 089 777 A (NEW ZEALAND DAIRY BOARD) 28 September 1983 (1983-09-28) page 2, line 27-35; claim 8; example 1 page 3, line 23-26 page 2, line 18,19	1,3,5,6, 8-11,13, 15, 17-22, 24,26, 27, 29-32, 34,36, 38-42
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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/US 00/26558

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		US 5681598 A	28-10-1997
US 5676984 A	14-10-1997	US 5470593 A	28-11-1995

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(54) Title: METHOD FOR THE USE OF SOY PROTEIN IN THE PRODUCTION OF FRESH AND RIPENED CHEESE

(57) Abstract: The invention provides a method for making a fresh, unripened cheese analog comprising (a) mixing isolated soy protein and milk; (b) adding a coagulating agent mixture, and/or bacterial cultures to said mixture from (a); (c) cutting product resulting from (b); (d) agitating the product resulting from (c) for about 0 to about 90 minutes at temperature of from about 30°C to about 50°C; (e) separating about 50 to about 80% of whey from product resulting from (d) and salting; and (f) placing product resulting from (e) in molds and placing molds at temperature of at least about 28°C for about 2 to about 8 hours. The invention also relates to a method for making a hard, ripened cheese or a cheese analog comprising (a) mixing isolated soy protein and milk; (b) adding a coagulating agent, and/or bacterial cultures to said mixture from (a); (c) cutting product resulting from (b); (d) agitating the product resulting from (c) for about 0 to about 90 minutes at a temperature of from about 30°C to about 70°C; (e) separating about 50 to about 80% of whey from product resulting from (d); (f) processing the product resulting from (e) to produce the desired type of cheese analog; and (g) placing product resulting from (f) in molds and placing said molds at a temperature appropriate for said desired type of cheese or cheese analog, for a length of time necessary to begin ripening process.

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(54) Title: PERSONAL CARE COMPOSITION CONTAINING LEGHEMOGLOBIN

(57) Abstract: A personal care composition comprising leghemoglobin and at least one preservative selected from the group consisting of alcohols, glycols, parabens, hydantoins, quaternary nitrogen-containing compounds, isothiazolinones, aldehyde-releasing agents, and halogenated compounds. Preferably, the leghemoglobin is a nitrogen fixation root nodule extract providing a leghemoglobin concentration in the composition of between 0.0001% and about 10% based upon the total weight of the composition. Also disclosed is a method for preparing the personal care composition.

WO 03/070172 A2

PERSONAL CARE COMPOSITION CONTAINING LEGHEMOGLOBIN

FIELD OF THE INVENTION

This invention relates generally to personal care compositions, and more specifically to such compositions containing leghemoglobin. The leghemoglobin is suitably extracted from nitrogen fixation root nodules. The leghemoglobin provides advantageous properties to the personal care composition during use by facilitating the control of reactive oxygen and nitric oxide free radicals on the skin.

BACKGROUND OF THE INVENTION

Personal care compositions encompass a wide variety of applications, including soaps, shampoos, skin care medicaments, cosmetics, as well as therapeutic and homeopathic skin care formulations. The use of plant-derived raw materials in personal care compositions is well established. By way of illustration, PCT patent publication WO 9913855 discloses the use of a combination of algae extract and exopolysaccharides for cosmetic and dermapharmaceutical applications.

Consideration of the use of plant-derived materials in personal care has become especially important in view of the recent discovery that certain animal-borne diseases, such as Bovine Spongiform Encephalopathy (BSE), are spread to humans through contact with infected meat and meat by-products. The sensitivity of the personal care industry to these issues is so great that even the sale of lanolin, a by-product derived from the shearing of sheep wool, has suffered because it is considered to be an animal-derived product.

The use of human-derived ingredients in personal care compositions is also frowned upon for the same reasons as suggested above for animal-derived products. Human-derived products can have even greater concerns than animal-derived constituents

Case No.: IPR2023-00209
U.S. Patent No. 10,039,306

because pathogens borne in the human-derived cosmetic ingredients are even more likely to have a detrimental effect on human health. It has been suggested, for example, in US 6,160,021 that human derived blood constituents, such as hemoglobin, when administered topically can influence the production of melanin in the skin providing a method of skin lightening.

As a consequence of these market pressures, personal care composition manufacturers are relying more heavily on plant-derived raw materials, such as plant proteins obtained from corn, soy and wheat replacing animal proteins. In addition, the cosmetic and personal care industries look closely to the health food and herbal supplement industries for trends in product development. Such trends are summarized, for example, in a recent article in *HAPPI* **38** (2001) 81. This article summarizes many of the pressures that face global cosmetic raw material manufactures and the recent trends towards 'natural' based products. Good examples of this are the recent trends of aromatherapy and natural extracts, particularly plant extracts, as useful ingredients for providing functionality and support the labeling claims as to such 'natural' based personal care products.

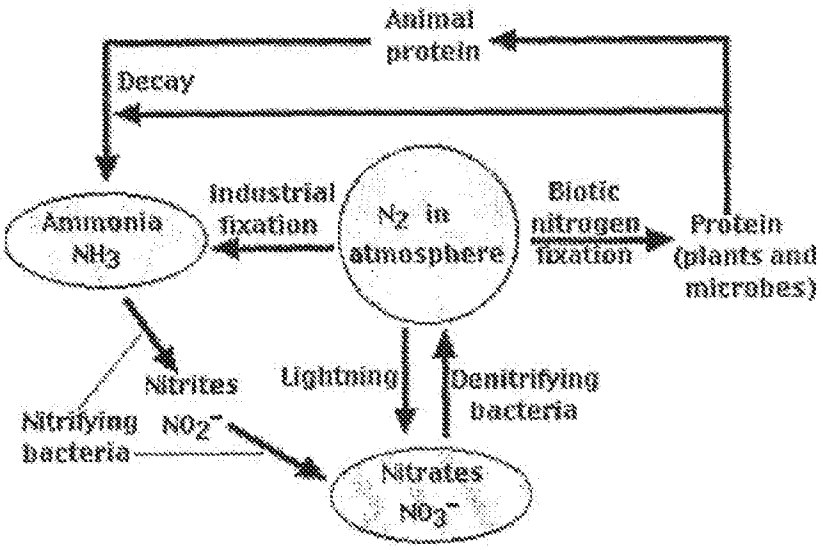
Several plants have enjoyed a healthy growth in personal care compositions because of their purported benefits to humans. For example, Aloe Vera is a well-known plant that provides an extract that is known to help aid wound healing. Gingo biloba and grapes are known to contain polyphenols that are purported to provide anti-oxidative effects to human skin, ameliorating the harmful effects of UV damage on the skin. Soybean extracts are extremely popular due to the presence of soy isoflavones that are suggested to contain plant phytoestrogens, compounds that mimic human estrogen and help ameliorate the effects of aging, particularly in post-menopausal women. Products such as extracts from *Asafaetida* and Yellow dock are suggested, for example, to minimize

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excess melanin formation that is responsible for the appearance of age spots on the skin. Recently, US Patents 5,371,089 and 5,602,139 disclose that plant growth regulators known as cytokinins appear to extend the longevity of human fibroblasts, the cells from which human keratinocytes arise. Keratinocytes become the protective cells that comprise the stratum corneum and make human life possible on this planet. Certain bacterial and yeast extracts are known to provide useful cosmetic, therapeutic or homeopathic effects, in particular, yeast such as *Saccharomyces cerevisiae*. In addition, some root extracts, for example, ginseng, have carried a historical trend for health and energy and are seen to appear in personal care products purporting such claims.

A major component of the atmosphere surrounding the earth is nitrogen gas, N_2. Both plants and animals are bathed in nitrogen gas from the air and yet, because of the extraordinary chemical stability of this molecule, nitrogen gas is essentially chemically inert to both plants and animals. However, molecular nitrogen is a key component of all of the amino acids, vitamins, proteins, enzymes, and cells of both Kingdoms. Without a means for converting nitrogen gas into useful nitrogen components such as ammonia, life as we know it on this planet would not exist. The conversion of nitrogen gas into useful nitrogen substrates occurs through natural and industrial means and comprises what is known as the nitrogen cycle, Figure 1.

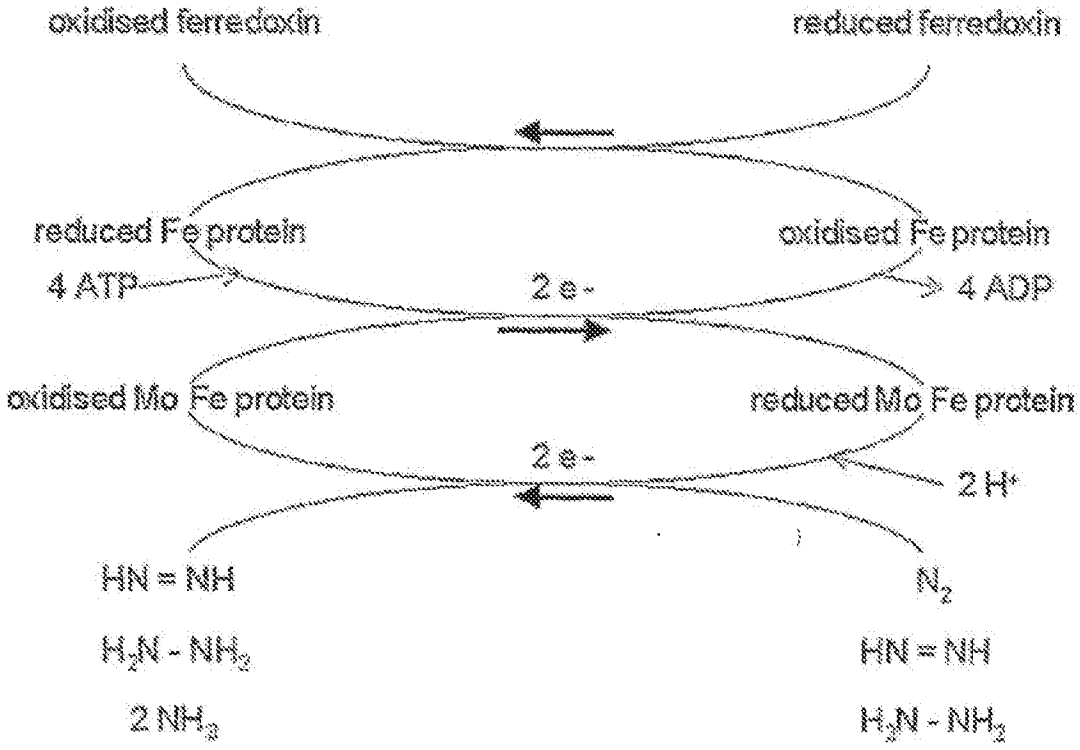
Figure 1. The Nitrogen Cycle



Fortunately, while plants and animals are incapable of chemically converting nitrogen into useful nitrogen-containing components, certain bacteria, especially those of the blue-green Cyanobacteria Kingdom, such as, for example, *Rhizobium*, have evolved a mechanism to convert nitrogen into more useful ammonia. The process of conversion is known as 'Nitrogen Fixation' and relies on the presence in these organisms of a key enzyme known as *nitrogenase*.

Nitrogen fixation is a complex enzymatic process illustrated in Figure 2 below.

Figure 2. Nitrogen Fixation.



The general enzymatic reaction to convert nitrogen into ammonia can be summarized as:

$$N_2 + 8H+ + 8e^- + 16\ ATP = 2NH_3 + H_2 + 16ADP + 16\ Pi$$

The process is intimately driven by conversion of adenosine triphosphate, ATP, into adenosine diphosphate through reduction of nitrogen to ammonia by *nitrogenase* enzyme. Because the reaction is a reduction reaction, it is very sensitive to the presence of oxygen.

Some free-living organisms, such as lichen, are capable of fixing nitrogen but typically on a very small scale. Interestingly, *Rhizobium* bacteria, however, will not convert nitrogen into ammonia when they exist alone. The bacteria are generally unable to keep the oxygen away that is detrimental to the overall nitrogen fixation reaction. In order to overcome this problem, the bacteria have evolved an unusual associative relationship with many green plants. The bacteria, which can be found readily in the soil, will infect the roots of various plants, in particular, the roots of legumes, for example. The bacteria

are attracted to the roots by the excretion of various flavonoids such as luteolin and hesperetin, such flavonoids being referenced, for example, in Begum, A. A. et. al., *J. Exper. Bot.* 2001; 52, 1537-1543.

Once within the roots of the plants, the bacteria force plants to build small, oxygen deficient homes known scientifically as symbiosomes and more commonly as 'root nodules' in which the bacteria can live and carry out the nitrogen fixation reaction. The plant, however, does not suffer from the infection, but instead benefits from the bacterial conversion of nitrogen into ammonia by using the ammonia for its own life processes. This relationship between the blue-green bacteria and the green plant is symbiotic. That is, both organisms benefit from the relationship. The seeds of many commercial crops that provide safe havens for nitrogen fixing bacteria are actually sold commercially with the fixing bacteria mixed with the seeds prior to planting. Such combinations can be found, for example, at commercial websites such as that provided for Agrobiologicals at http://www.agrobiologicals.com/glossary/Cont6.htm.

The bacteria build the root nodule by infecting the root hairs of the green plant and switching on the plant growth regulators that cause a rapid growth of plant cells around the infected site. The bacteria control the growth of the nodule by taking over the localized growth mechanisms of the root by using various plant growth regulators such as, for example, cytokinins as well as important complimentary plant growth regulators such as auxins, such plant growth regulators being referenced, for example in Schultze, M. et. al., *Anna. Rev. Genet.* 1998; 32, 33-57 incorporated in its entirety into the body of this invention. In addition, the bacteria begin expanding in size and shedding their cells walls, becoming bacteroids that begin forming a biofilm-like lining around themselves made from excreted lipopolysaccharides, exopolysaccharides and capsular polysaccharides that further prevents the infiltration of oxygen into the nodule. Such polysaccharides are

referenced, for example in Rodriquez-Carvajal, M. A. et. al., *Biochem. J.* 2001; 357, 505-511. Because the process of nitrogen fixation is so sensitive to the presence of oxygen and reactive oxygen radicals, the root nodule bacteria also accumulate and produce a variety of anti-oxidants including but not limited to, for example, peroxidases, superoxide dismutases, glutathiones, catalases, oxidases and other protective enzymes and molecules as might be found described in, for example, Iturbe-Ormaetxe, I. et. al., *Mol. Plant Microbe Interact.* 2001; 14, 1189-1196.

As a further defense mechanism, the bacteria begin to produce a protein known as 'leghemoglobin' that behaves much like human hemoglobin. The human body has four important globin molecules (distinguished by the presence of a heme-based porphyrin ring at the active site of the protein). These include myoglobin, neuroglobin, hemoglobin and a recently discovered fourth globin called histoglobin. Globin proteins are distributed throughout the human body where they capture and control oxygen reactive molecules such as O_2, CO_2, and NO (nitric oxide). In the symbiosomes, oxygen will bind to the leghemoglobin and is removed, through an oxygen transport mechanism, thus further improving the anaerobic conditions the bacteria require for the nitrogen fixation reaction to occur.

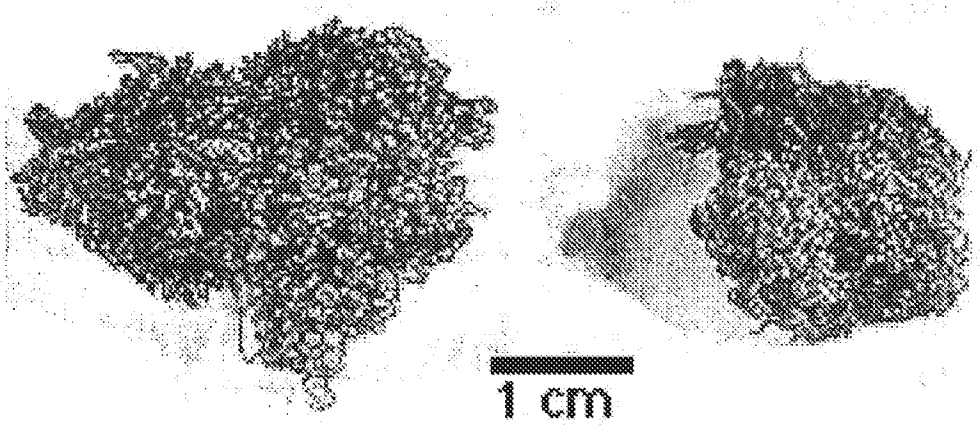
The interior of the nitrogen fixation nodule, therefore, is a complex mixture of plant growth regulators, amino acids, vitamins, proteins, polysaccharides, minerals and enzymes all which are either helping the plant grow, protecting or nourishing the bacteria. The composition of most of these root nodule extracts is also regionally controlled due to the soil and climates in which the individual plants are grown. However, regardless of the source of the plant, the climate or other factors, active root nodules will always contain leghemoglobin and nitrogenease.

In addition to binding oxygen, it has recently been established that globin-type proteins, such as hemoglobin, myoglobin and leghemoglobin, will bind nitric oxide (see for example, Hargrove, M et al., *J. Mol. Biol.* 1997; 266: 1032-1042). Nitric oxide has been found to be a potent controller of human blood flow (see, for example, Stix, G. *Scientific American* 2001, November). It has also become well established that an enzyme called nitric oxide synthase controls the presence of nitric oxide in the skin. Nitric oxide has been found to be part of the biochemical cascade that occurs in skin when the skin becomes irritated (see, for example, Bruch-Gerharz, D., et. al., *J. Invest. Dermatol.* 1998; 110: 1-7). In particular, the presence of nitric oxide free radicals in the skin has been demonstrated, along with the free radicals derived from oxygen such as, for example, hydrogen peroxide, to have detrimental effects on the health of the skin (see, for example, Herrling, T. et. al., *SOFW J.* 2000; 126: 20-26). Accordingly, treatments to reduce nitric oxide levels on the skin have been disclosed in the literature. Illustratively, U.S. Patent 6,160,021 discloses, at column 3, lines 28-45 thereof, a skin-treatment method for decreasing the level of nitric oxide by administering to the skin, either topically or subcutaneously, an inhibitor of NO synthase or an NO scavenger, such as a heme compound, such as hemoglobin.

In addition, it has recently been recognized that nitric oxide radicals appear to also be responsible for the initiation of the cascade that causes melanogenesis in human melanocytes. It has been suggested, for example, that human-derived hemoglobin can control the production of tyrosinase in fibroblasts as suggest in, for example, Romero-Graillet, C. et al., *J Clin Invest.*, 1997; 99: 635-642. Therefore, the control and modulation of oxygen and nitric oxide free radicals in the skin remains an area of considerable commercial and academic interest and research.

Plants can be grown and removed from the soil to expose the root nodules, Figure 3. Interestingly, the contents of the nitrogen fixation nodules can be extract using various

Figure 3. Nitrogen Fixation Nodules from the roots of Australian Pine.



methods of extraction well known to those skilled in the art. Such examples may comprise simple aqueous extractions as, for example, discussed on the Reed College website under "Nitrogen Fixation. Part II. Physiology and Anatomy of Nitrogen Fixation" found at http://web.reed.edu/academic/department/biology/nitrogen/Nfix2.html, or they may be more complex extractions as described, for example, in Mendonca, EHM. et. al., *Phytochemistry* 1999; 50: 313-316. In addition, the extractions may involve the use of super critical carbon dioxide extraction, methods of which are known to those skilled in the art. These extractions will comprise a mixture of the plant growth regulators, amino acids, vitamins, proteins, polysaccharides, minerals and enzymes that comprise the root nodules.

BRIEF SUMMARY OF THE INVENTION

In one aspect, the present invention relates to a personal care composition comprising leghemoglobin and at least one preservative selected from the group consisting of alcohols, glycols, parabens, quaternary nitrogen-containing compounds, isothiazolinones, aldehyde-releasing agents, antioxidants and halogenated compounds, such preservatives being found listed, for example, in Steinberg, D.C. *Cosmet. Toilet.* 1997; 112, 57-65. Preferably, the leghemoglobin is a nitrogen fixation root nodule extract providing a leghemoglobin concentration in the composition of between 0.0001% and about 10% based upon the total weight of the composition.

In another aspect, the present invention relates to a method for preparing a personal care composition comprising the steps of:

(a) extracting the leghemoglobin from root nodules infected with bacteria selected from the group consisting of Cyanobacteria, Anaerobic bacteria, Purple sulfur bacteria, Purple non-sulfur bacteria, Green sulfur bacteria, legumes bacteria, and combinations thereof, in order to provide a leghemoglobin extract, and

(b) combining the leghemoglobin extract with a preservative to provide the personal care composition.

In yet another aspect, the present invention relates to a method for binding free radicals on the skin that comprises contacting the skin with leghemoglobin, thereby causing said leghemoglobin to act as a scavenger for the free radicals.

These and other aspects will become apparent upon reading the following detailed description of the invention.

DETAILED DESCRIPTION OF THE INVENTION

It has now been now surprisingly found that the extracts of roots nodules from various plants, for example, perennial legumes such as peas, beans, and soybeans, annual legumes such as clover and alfalfa, and woody legumeous plants such as Australian Pine, contain leghemoglobin, as well as many other useful components, that provide advantages when incorporated into personal care compositions. The other useful components of the extracts comprise, for example, plant growth regulators, amino acids, vitamins, proteins, polysaccharides, minerals and enzymes. Additionally, these extracts can function as part of a growth media for other organisms such as, for example, yeast such as *Saccharomyces cerevisiae*, providing key growth factors to these organisms that can then be further extracted for use in personal care compositions. Moreover, one or more components of these extracts is suitably encapsulated within, for example, liposomes, niaosomes, sub-micron emulsions, polymeric encapsulants, gels, creams or lotions, to provide a time-release property to the extract components. Moreover, some of the extract components, notably leghemoglobin, bind to oxygen and nitric oxide in the skin, effectively controlling the presence of these molecules, and minimizing the formation of free radicals.

Without wishing to be bound by any particular theory, the present inventor postulates that the presence of leghemoglobin, topically applied to the skin, will bind to oxygen and nitric oxide present in the skin, effectively removing these molecules from their normal radical breakdown cycles and thus, effectively eliminating the detrimental effects of their free radicals on the skin. Without being further bound by theory, the inventor suggests that leghemoglobin can remove nitric oxide from binding with other critical heme-based enzymes such as, for example, guanylyl cyclase, the enzyme responsible for upregulating the production of cyclic-GMP, in a competitive fashion

effectively slowing significant biochemical pathways in human cells dependent on the upregulation of c-GMP.

The nitrogen fixation root nodule extracts of the present invention can be obtained from any number of microorganisms as summarized, for example, in a table provided on The University of Edinburgh's website under the heading "The Microbial World. The Nitrogen Cycle and Nitrogen Fixation" found on their website located at http://helios.bto.ed.ac.uk/bto/microbes/nitrogen.htm, and incorporated herein by reference in its entirety. Illustrative origins include, for example, aerobic bacteria such as those from the Genus' *Azotobacter, Beijerinckia, Klebsiella* and other Cyanobacteria, anaerobic bacteria such as, for example, organisms from the Genus' *Clostridium, Desulfovibrio,* Purple sulfur bacteria, Purple non-sulfur bacteria and Green sulfur bacteria, bacteria that inhabit legumes such as, for example, bacteria from the Genus *Rhizobium* and *Brachyorhizobium* and bacteria that infect other types of plants such as, for example, bacteria from the Genus *Frankia* and *Azospirillum*. Especially preferred are the nitrogen fixation root nodule extracts from the *Rhizobium* and *Brachyorhizomium* bacteria that infect annual and perennial legumes, in particular soybean root nodule extracts.

Plants from which the root nodules might be taken can include a variety of species found around the planet. Such plant species can include, but are not limited to, those listed in the article by Lindermann, W.C. and Glover, C. R., entitled "Nitrogen Fixation by Legumes" found at http://iubio.bio.indiana.edu/R35541-209718-/news/bionet/biology/n2-fixation/9702.newsm. Such plants might include, for example, perennial legumes such as alfalfa, sweetclover, vetches and true clover or annual legumes such as, for example, beans, faba beans, peas, cowpeas, peanuts and soybeans. Certain legumeous woody plants are known to carry nitrogen fixation bacterial infections such as, but not limited to, Australian pine. Especially preferred are the root nodules found on soybeans.

The personal care composition of the present invention suitably contains leghemoglobin and at least one preservative selected from the group consisting of alcohols, glycols, parabens, quaternary nitrogen-containing compounds, isothiazolinones, aldehyde-releasing agents, antioxidants and halogenated compounds. Illustrative alcohols include phenoxyethanol, isopropyl alcohol, and benzyl alcohol; illustrative glycols include propylene, butylene, and pentylene glycol; illustrative parabens (also known as parahydroxybenzoic acids) include methyl, propyl and butyl-parabens; illustrative quaternary nitrogen-containing compounds include, benzalkonium chloride, and Quaternium 15; illustrative isothiazolinones include methylisothiazolinone and methylchloroisothiazolinone; illustrative aldehyde-releasing agents include DMDM hydantoin, imidazolidinyl urea and diazolidinyl urea; illustrative antioxidants include butylated hydroxytoluen and tocopherol, and illustrative halogenated compounds include triclosan, and chlorohexidine digluconate.

Additionally, the personal care composition can optionally contain other functional ingredients such as, for example, water, surfactants, emulsifiers, conditioners, emollients, waxes, oils, polymers, thickeners, fixatives, colorants, humectants, moisturizers, stabilizers, diluents, solvents, fragrances and the like, as well as active ingredients such as, for example, botanicals, neutraceuticals, cosmeceuticals, therapeutics, pharmaceutics, antifungals, antimicrobials, steroidal hormones, antidandruff agents, anti-acne components, sunscreens, preservatives and the like. The concentration of the root extract component or components in the cosmetic composition is suitably in the range of 0.0001% to 99% by weight, preferably between 0.1% and 10%, based upon the total weight of the cosmetic composition. The composition of the present invention can be used in various types of cosmetic formulations including, but not limited to, lotions, ointments, creams, sprays, spritzes, aqueous or aqueous alcoholic mixtures, gels, mousses, patches, pads,

masks, moistened cloths and wipes, solid sticks, clear sticks, lipsticks, aerosol creams, anhydrous powders, talcs, tonics, oils, emulsions, and bath salts.

The root nodule extracts can be obtained from the roots by a variety of extraction methods known to those skilled in the art. Such extracts may involve the use of water, water miscible organic solvents such, for example, acetone, butylenes glcyol or ethanol, or organic solvents such as, but not limited to, hexane or methyl ethyl ketone. The extraction may also involve the use of supercritical fluid extraction, such as, but not limited to, super critical carbon dioxide extraction. The root nodules may be found to occur in a moistened state or a dried state, such conditions influencing the final composition of the extract, but not the method of extraction. The root nodule extraction may be done alone, or in the presence of other plant components such as leaves, stems, flowers, seeds and roots. The presence of these components can modify the resulting extraction composition. The extraction can be conducted at normal atmospheric pressure or under greater or lesser pressures depending on the method of extraction. The extraction can be conducted at temperatures between –30 °C and 200 °C, more preferably between 5 °C and 100 °C, most preferably between 25 °C and 45 °C, such extraction solvents, pressures and temperatures being suitably selected so as to not denature the leghemoglobin proteins found in the root nodule extracts.

The extracts of the present invention may be further purified by any number of means known to those skilled in the art including, but not limited to, chromatography, steam distillation, solvent extraction, centrifugation, decantation, filtration, or carbon treatment. The extracts of the present invention may be further concentrated by any means known to those skilled in the art including, but not limited to, evaporation, spray-drying, lyophylization, steam distillation or belt or drum drying.

The following Examples are intended to illustrate the art of the present invention and are not intended to limit the scope of the claims below.

EXAMPLE 1

Two kilograms of freshly pulled soybean (*Glycine max*) plants including the roots were washed with a gentle stream of deionized water to remove residual soil. The nitrogen fixation nodules, which were readily apparent on well-infected roots, were easily removed by hand and were collected. To approximately 100 grams of isolated nodules was added 200 mls of deionized water and the entire mixture was macerated in a Waring blender at high shear. The resulting dark heterogeneous lysate was filtered through cheesecloth to remove large pieces of undissolved material. The resulting hazy, dark solution was further purified by filtration through fine filter paper and by carbon and sterile filtration that removed the organisms responsible for a strong earthy odor in the extract.

The level of leghemoglobin in this particular extract was found to be approximately 0.3 mg/ml as determined by the following analysis method. To a 10 ml sample of the extract was added 1 ml of 0.1M sodium dithionite in 0.1 M Potassium Phosphate Buffer Solution (PBS) at pH 7.0. This sample was treated with gaseous carbon monoxide (CO) by gently bubbling a stream of the gas into the sample for 20 minutes. This procedure permanently binds carbon monoxide to the heme iron of the leghemoglobin providing a leghemoglobin–CO complex (referred to as Lba-CO) that has an extinction coefficient of 200 $mM^{-1}cm^{-1}$ at 416 nm wavelength in the ultraviolet spectrum. Using 0.4 ml of the Lba-CO complex solution in a 1-cm pathlength UV cuvette and comparing it an absorbance blank of PBS at pH 7.0 one determines the concentration of leghemoglobin using the resulting absorbance and the known extinction coefficient for the Lba-CO complex.

EXAMPLE 2

Two kilograms of roots containing active nitrogen fixation nodules were obtained from Lotus plant (*Lotus japonicus*) grown hydroponically in vermiculite inoculated with bacteria from the Genus *Brachyorhizobium*. Approximately 100 grams of the nitrogen fixation nodules were cleaned and removed and the resulting nodules were extracted by macerating the nodules in a Waring blender with 200 mls of 80% aqueous ethanol. The resulting heterogeneous mixture was filtered as described above. The ethanol was removed via low tempertuare vacuum evaporation and replaced with a comparable amount of deionized water providing a root nodule extract containing approximately 0.6 mg/ml of leghemoglobin as analyzed using the method described in Example 1.

EXAMPLE 3

Samples of root nodules obtained from Alfalfa (*Medicago sativa*) were treated in a similar fashion as described above in Example 1. HPLC analysis of these extracts for the presence of the cytokinins zeatin (a pure sample of zeatin was obtained from Serva (Islandia, NY) provided concentrations comparable to those reported in the literature, in particular, Hashizume, T. et al., *Agric. Biol. Chem* 1985; 49: 3481-3484. The extract was analyzed using the method described in Example 1 and was found to comprise approximately 0.5 mg/ml of leghemoglobin.

EXAMPLE 4

An aqueous extract was prepared from root nodules harvested from the plant Lupine (*Lupinus ?*) using the method described in Example 1. This extract was found to contain 0.4 mg/ml of leghemoglobin.

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EXAMPLE 5

Samples of the various aqueous root nodule extracts were incorporated into a liposome comprising phospholipid and lecithin obtained from soybean beans. The extract was slurried together with the phospholipid and lecithin components and the mixture was homogenized using a high-pressure homogenizer obtained from Hydraulic Engineering Corporation (Brea, CA). The milky white mixture contained the nitrogen fixation root nodule extract encapsulated with the liposomal components.

EXAMPLE 6

A 1:1 mixture of the root nodule extract from Example 1 and a mixture of maltodextrin encapsulating oligosaccharides available as a 67% mixture of maltodextrin sugars and water from Roquette America (Keokuc, IA) was spray-dried using a commercial spray dryer. The resulting encapsulated powder contained approximately 0.15 mg/ml of leghemoglobin as determined by taking a sample and redissolving it in water and analyzing the mixture for leghemoglobin using the method described in Example 1.

EXAMPLE 7

A sample of the aqueous soybean root nodule extract from Example 1 was placed into an aqueous mixture of Baker's Yeast growth media obtained from Red Star Yeast (Milwaukee, WI). The media was inoculated with an active yeast culture also obtained from Red Star and the mixture was allowed to ferment under controlled aerobic conditions to provide a Live Yeast Cell Derivative (LYCD) obtained using stress conditions as described in US Patent 2,239,345. The resulting yeast lysate continued to test positive for the presence of leghemoglobin as demonstrated by using the method referenced in Example 1.

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Examples 6-9 illustrate skin care compositions according to the present invention that can be prepared using various root nodule extracts as disclosed in examples 1-4.

EXAMPLE 6

This example illustrates a high internal phase water-in-oil emulsion incorporating the root nodule extract prepared as disclosed in Example 1.

Ingredient	**wt%**
1,3 –dimethyl-2-imidazolidinone	0.2
Brij 92[1]	5.0
Bentone 38	0.5
$MgSO_4 \cdot 7H_2O$	0.3
DMDM Hydantoin	0.01
Root Nodule Extract	10.0
Water	to 100

[1] Brij 92 is polyoxyethylene (2) oleyl ether

EXAMPLE 7

This example illustrates an oil-in-water cream incorporating the root nodule extract prepared as disclosed in Example 4.

Ingredient	**wt%**
Mineral Oil	4
1,3 –dimethyl-2-imidazolidinone	1
Brij 56[1]	4
Alfol 16RD[2]	4
Triethanolamine	0.75
Butane-1,3-diol	3

Xanthan gum	0.3
Methyl, Propyl and Butyl Paraben	0.01
Root Nodule Extract	10.0
Water	to 100

[1]Brij 56 is cetyl alcohol POE (10)

[2]Alfol 16RD is cetyl alcohol

EXAMPLE 8

This example illustrates an alcoholic lotion incorporating a root nodule extract prepared as disclosed in Example 2.

Ingredient	**wt%**
1,3 –dimethyl-2-imidazolidinone	0.3
Ethanol	40
Root Nodule Extract	10.0
Water	to 100

EXAMPLE 9

This example illustrates a sub-micron emulsion concentrate that contains a root nodule extract prepared as described in Example 3.

Ingredient	**wt%**
Trimethylolpropane Tricaprylate/Tricaprate	18.0
Glycerin	8.0
Cetearyl alcohol	2.0
Ceteareth 20	2.0
Glyceryl stearate	2.0
BHT	0.01

Root Nodule Extract	10.0
Water	to 100

EXAMPLE 10

To test the ability of a root nodule extract containing leghemoglobin to control nitric oxide levels in human fibroblasts, the following assay was preformed.

Preparation of Fibroblasts

Fibroblasts were seeded into the individual wells of a 6 well plate with an initial cell density of 3,500 cells/cm^2 in a total of 2 ml of fibroblast growth medium (FGM). The seeded cells were incubated overnight in an incubator at 37±2°C and 5±1% CO_2. On the following day the FGM was removed via aspiration to eliminate any non-adherent cells and replaced with 2 ml of fresh FGM. The media was changed again every 48 hours of incubation until the cells were fully confluent.

Application of Test Material

The media from the confluent fibroblasts was replaced with the extract described in Example 1, diluted in FGM to yield a final concentration of 1%. Gamma-interferon was used as a positive control (approx 300 U/ml) while untreated cells were used as a negative control. Each condition was tested with 6 replicates. The cells were incubated for 48 hours at 37±2°C and 5±1% CO_2. After the 48-hour incubation the cell culture medium was collected and stored frozen for subsequent assay of nitrite.

Enzymatic Conversion of Nitrate to Nitrite

A 300 µl aliquot of each cell culture medium sample was mixed with 10 µl of a nitrate reductase solution (0.1 U/ml nitrate reductase enzyme, 5 µM FAD, 30 µM NADPH). This mixture was incubated in a water bath (37±2°C) for at least 15 minutes. To remove any residual NADPH that was not consumed in this initial reaction (the

NADPH will interfere with the subsequent Griess Reaction), 10 µl of a lactate dehydrogenase solution (100 U/ml lactate dehydrogenase and 0.3 mM sodium pyruvate) was added and this mixture was allowed to incubate in a water bath (37±2°C) for at least 5 minutes.

Nitrite Assay: Griess Reaction

A 100 µl aliquot of each cell culture medium sample was added to a well in a 96-well plate. To each aliquot, 50 µl of sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added and the plate was incubated at room temperature for 5 to 10 minutes (protected from light). After this first incubation, 50 µl of NED solution (0.1% N-1-napthylethylenediamine dihydrochloride in DIH_20) was added to each well and the plate was allowed to incubate at room temperature for 5 to 10 minutes (protected from light). After incubating, the absorbance values for each well of the 96-well plate were read at 540 nm.

Nitrite Standard Curve

A nitrite standard curve was generated by performing a series of dilutions with the nitrite standard to generate the following series of concentrations: 100 µM, 50 µM, 25 µM, 12.5 µM, 6.25 µM, 3.13 µM, 1.56 µM, and 0. These samples were diluted in cell culture medium.

Nitrite Assay

To derive the standard curve for the nitrite assay, the absorbance versus the nitrite concentration in µM for the standards was plotted. A linear regression was performed to establish the line that best fits these data points. Mean absorbance values for the test materials and untreated samples were used to estimate the amount of nitrite present in each sample.

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Using the analysis method it was determined that the extract from the soybean root nodules was able to reduce nitric oxide levels in human fibroblasts by 60% compared to untreated controls.

EXAMPLE 11

To test the ability of a root nodule extract to improve human skin fibroblast viability, a sample of the root nodule extract from Example 4 was tested using the following analytical procedure.

Human fibroblasts were grown to confluence using the method described in Example 10. The fibroblasts were treated with test material following the procedure described in Example 10. After 48 hours of exposure to the test material, the following test was run.

MTT Assay

The cell culture medium was removed and the fibroblasts were washed twice with PBS to remove any remaining test material. After the final wash, FGM supplemented with 2 mg/ml MTT was added to each well and the cells were incubated for approximately 2 hours at approx. 37±2°C and 5±1% CO_2. After the 2-hour incubation, the FGM/MTT solution was removed and the cells were washed again once with PBS and then 2 ml of isopropyl alcohol was added to the well to extract the purple formazin crystals. Two hundred microliters of the isopropyl extracts was transferred to a 96-well plate and the plate was read at 540 nm using isopropyl alcohol as a blank.

MTT Assay

The Mean MTT absorbance values were calculated for the negative control wells. Since these cells were not treated this mean value was used to represent 100% viability **(UNTREATED)**. For the absorbance values in the wells treated with the test material

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(**TREATED** samples), the percentage of viable cells was calculated using this equation: [(individual **TREATED** absorbance)/(mean **UNTREATED** absorbance)] x 100. Using this analytical method, it was determined that the cells treated with the extract from Example 4 had a 30% greater viability than untreated cells showing the extract improved the cell viability of the human skin fibroblasts.

EXAMPLE 12

To test the possibility that a root nodule extract might promote the growth of soluble human elastin, the following study was conducted. A soy root nodule extract was prepared as described in Example 1. The extract and an isotonic saline placebo were buffered to neutral pH with PBS.

Neonatal human fibroblast cells were placed in fibroblast growth medium (FGM) as described above in Example 10 and seeded into culture flasks. The flasks were incubated at 37 °C and 5% CO_2 until the cells were fully confluent. The media was changed 24 hours after the initial seeding of the flask to remove any cells that did not survive and then every 48 hours after that. Upon reaching confluency the FGM was removed and the cells were washed once with 9 mls of HEPES Buffered Saline Solution (HEPES-BSS) to remove any residual proteins from the FGM that can neutralize trypsin. Following removal of the HEPES-BSS, 6 ml of Trypsin/EDTA was used to cover the fibroblast monolayer. The trypsin digests the cellular adhesion proteins that hold the fibroblasts to the culture flask while the EDTA chelates any residual calcium to further inactivate the adhesion proteins. Trypsinization did not exceed 3 minutes. After gathering of the cells, 12 ml of trypsin neutralizing solution was added and the released cells were transferred to 15-ml centrifugation tubes. The flask was rinsed with 6 mls of HEPES-BSS to collect residual cells that were added to the previous wash. The centrifuge tubes were then spun at 220 x g for 5 minutes to pellet the cells. The supernatant was removed via

aspiration and the cells was resuspended in 4 ml of FGM and seeded into 6-well plates. A small aliquot was taken to count the number of cells. The cells were then diluted with FGM to reach a cell density of 17,500 cells/ml. The seeded cells were incubated overnight as described above. On the following day, the FGM was removed via aspiration to eliminate any non-adherent cells and replaced with 2 mls of fresh FGM. The media was changed again every 48 hours of incubation until the cells are fully confluent.

The media of the confluent fibroblasts was replaced with either fresh media or media containing the root nodule extract from Example 1. The cells are then incubated for 48 hours at the same conditions described above.

After the 48-hour incubation period the cell culture medium was collected and the insoluble elastin was extracted from the cells by the addition of 1 ml of 0.25M oxalic acid to the wells that house the fibroblasts. The cells were freed from the plastic wells using a cell scraper and the suspended cells/oxalic acid mixture was added to centrifuge tubes. The tubes were incubated at 95 °C for 60 minutes to solubilize the cells. The tubes were then quickly cooled to RT and centrifuged at 3000 rpm for 10 minutes. The supernatants were combined so that both soluble and insoluble elastins were tested. The supernatant was placed overnight in dialysis tubing with a low molecular weight cut-off (~15,000 MW) to both concentrate the elastin into a smaller volume and to remove the oxalic acid.

A 300 ml aliquot of the sample above was mixed with 1.0 ml of cold Fastin Precipitating Reagent (available from Biocolor, Belfast, Northern Ireland) in a 1.5 ml microcentrifuge tube. The mixture was allowed to incubate for 24 hours at 4 °C to precipitate elastin. The cooled microcentrifuge tubes were spun at 8,000 x g for 10 minutes to pack the precipitated elastin. The supernatant was removed by inverting the tube to drain it and then by gently tapping it on an absorbent paper towel.

One (1) ml of Fastin Dye Reagent along with 200 µl of 90% saturated ammonium sulphate was added to each tube. The microcentrifuge tubes were then mixed with a vortex mixer and allowed to incubate for 60 minutes at RT with gentle mechanical agitation. The Fastin Dye Reagent interacts with the amino acids of elastin specifically to form a colored elastin/dye complex that precipitates in the presence of the ammonium sulphate. The microcentrifuge tubes were spun at 8,000 x g for 10 minutes to separate the precipitated elastin/dye complex from the unbound dye. The supernatant was carefully removed and the tubes were inverted to drain any residual supernatant.

One (1) ml of Fastin Destain reagent was added to each microcentrifuge tube. The tubes were capped and vortexed as described previously. A 100 ml aliquot from each sample tube was transferred to a 96-well plate and read at 513 nm with a microplate reader. The amount of soluble and insoluble elastin was determined by comparison against a standardization curve prepared from elastin samples supplied by the manufacturer of the Fastin Dye Kit. Using the described methodology, it was found that the cells grown in the presence of the root nodule extract contained statistically greater quantities of elastin than the cells grown in the untreated controls. Fibroblast cell viability was determined as described in Example 11 and the cells were shown to be viable under the conditions of the experiment.

EXAMPLE 13

To test the possibility that an extract from a root nodule containing leghemoglobin can control the production of cyclic-GMP in human skin fibroblasts, the following test was run using the extract produced in Example 2.

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cGMP Assay: Fibroblast Cell Culture Preparation and Treatment

Human dermal fibroblasts were seeded at 7.5 x 10^4 cells/ml in 1 ml of cell culture medium into the wells of a 12-well tissue culture plate (three wells were prepared for each treatment). After seeding, the cells were incubated overnight at 37±2°C and 5±1% CO_2. On the next day, the culture medium was removed via aspiration and the cells were incubated for 10 minutes in 400 µl of prestimulation buffer (Stock 800 mM IBMX: 100 mg IBMX dissolved in 563 µl DMSO; prestimulation buffer: 9.4 µl 800 mM IBMX added to 10 ml phosphate buffered saline). After this pretreatment, a 200 µl volume of phosphate buffered saline (negative control), test material + spermine NONOate (300 mM), or spermine NONOate (300 mM, positive control) were added to each well. It should be noted that there is a dilution at this point so materials to be added were prepared at 3x their intended final concentration (final concentration of spermine NONOate was 100 mM). The cells were then incubated for 1.5 hours at 37±2°C and 5±1% CO_2. At the end of this treatment period 200 µl of lysis buffer was added to each well and 12-well plate was placed on a rocking platform for 10 minutes at room temperature. The cell lysate was then collected and assayed immediately for cGMP. In addition to the fibroblasts, blank wells were run (wells without cells in them) containing known amounts of cGMP. These samples were treated and collected as described above and used to generate a standard curve for the cGMP assay.

Catchpoint cGMP Assay

The catchpoint cGMP assay is a competitive fluorescence based ELISA assay. In this type of assay, as the amount of cGMP in a sample increases, the fluorescent signal will decrease. The assay procedure is as follows:

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1. Fifty (50) µl of each sample to be assayed were added to respective wells in a clear bottom black 96-well plate (these samples include the cGMP samples to be used for a standard curve). The wells in the well plate were previously coated with an anti-rabbit IgG.
2. Fifty (50) µl of a rabbit anti-cGMP antibody were then added to the wells used above.
3. Fifty (50) µl of a cGMP-horse radish peroxidase (HRP) conjugate were then added to each well used above and the well plate was incubated for 2 hours at room temperature on a rocking platform.
4. After the incubation period, the well plate was washed four times with 300 µl of wash buffer (supplied with the kit: 0.02M Tris [pH 7.4], 150 mM CaCl, 0.05% Tween 20, and 0.05% Proclin).
5. After removing the final wash via aspiration, 100 µl of Stoplight Red fluorescent substrate solution (with 1 mM H_2O_2) was added to each well used in the well plate and the plate was incubated for at least 1 hour (10 minutes is the minimum incubation time, but the signal is stable for up to 24 hours). During this incubation period the 96-well plate was protected from light.
6. After the incubation period, the 96-well plate was read using a Fluoroskan Ascent F/L plate reader at these setting:
    a. Excitation filter: 530 nm
    b. Emission filter: 590 nm
    c. Integration time: 50 msec

CGMP Assay

To quantify the amount of cGMP present, a standard curve was generated using known concentrations cGMP. A regression analysis was then performed to establish the

line that best fits these data points. Cyclic CMP content in the samples treated via the various conditions was then estimated via this standard curve. Using this analysis method, it was found that fibroblasts treated with 100 mM of Spermine NONOate showed an increased production of c-GMP of almost 80%. Treatment of the same fibroblasts with 100 mM of Sperimine NONOate containing 10% of the root nodule extract from Example 2 reduced the levels of c-GMP back down to levels noted for untreated cells showing the extract has an ability to control c-GMP levels in human fibroblasts.

EXAMPLE 14

To test the possibility that an extract from a root nodule containing leghemoglobin could influence melanogenesis (tanning) in human skin, the following in vitro test was performed using the extract taken from Example 2.

Tissue Preparation

Upon arrival, the MatTek MelanoDerm tissues were stored at 4°C until used. Prior to use, the tissues to be used were removed from the agarose-shipping tray and placed into a 6-well plate containing 0.9 ml of assay medium (37±2°C). All of the agarose was removed from the outside of the tissue culture insert since any residual agarose may prevent the assay medium from reaching the tissue. The tissues were allowed to incubate for at least 1 hour at 37±2°C and 5±1% CO_2. After this initial incubation, the assay medium was replaced with 5.0 ml of fresh medium (37±2°C) and the tissues inserts were placed on 2 sterile stainless steel washers to raise the level of the bottom of the insert such that it touched the surface of the assay medium.

Application of test material

Twenty-five (25) µl or mg of test material was applied directly onto the surface of the tissue. In addition to the test material, a positive (10^{-7}M alpha melanocyte stimulating hormone and 3 ng/ml bFGF in the assay medium) and negative (no treatment) control was also applied to respective tissues. The 6-well plates were then incubated at 37±2°C and 5±1% CO_2 and identical sets of plates were incubated for 10 days and 17 days. During this incubation period, the tissues were rinsed and new test material was applied and the assay medium changed every other day.

Macroscopic observation

At the end of the incubation period, the assay medium in some of the 6-well plates was replaced with 0.9 ml of PBS and the stainless steel washers were removed. The 6-well plates containing the tissue were placed under an inverted microscope and the tissues were evaluated to determine if the test material had exerted any cytotoxic effects on the melanocytes. Toxic effects would be indicated by melanocytes that had a rounded up and/or non-dendritic morphology. After the macroscopic evaluation, the Melanoderm tissues were removed from the culture inserts and stored at -75±5°C until assayed for melanin content.

Melanin extraction from tissues

Two frozen tissues for each treatment and time point were pooled and homogenized in 0.45 ml of 1% SDS containing 0.05 mM EDTA and 10 mM Tris (pH 6.8). Twenty (20) µl of a 5 mg/ml Proteinase K solution was then added to each homogenate and the mixture was allowed to digest overnight in a water bath set at 45±2°C. After the overnight digestion, an additional 20 µl of 5 mg/ml Proteinase K was added to each homogenate and the mixture was allowed to digest for an additional 4 hours (or longer-

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until no clumps of tissue remain). After the digestion, 50 µl of 500 mM sodium carbonate was added to make the digest basic, followed by the addition of 10 µl of 30% hydrogen peroxide. The samples were then incubated at 80±2°C for 30±5 minutes and then cooled to room temperature. After cooling, 100 µl of a chloroform/methanol (2:1) mixture was added to each sample. The sample was then centrifuged for 10 minutes at 10,000 x g). The top phase of each sample was collected and its absorbance at 405 nm was determined via a 96-well plate reader.

Melanin Standard

Known concentrations of synthetic melanin were taken through the extraction process described above. The absorbance values of these known concentrations were used to generate a standard curve of melanin concentrations.

The absorbance values for the melanin standards were plotted against their respective concentrations to generate a standard curve. The equation that best fits this curve was then determined via regression analysis and this equation was used to calculate the melanin concentrations of the unknown samples. The tissue melanin concentrations were then compared using a two-factor analysis of variance (using treatment and time point as the two factors). It was found that the tissue sample treated with the extract taken from Example 2 showed a statistically significant reduction in melanin concentration verses an untreated control.

While the invention has been described above with reference to specific embodiments thereof, it is apparent that many changes, modifications, and variations can be made without departing from the inventive concept disclosed herein. Accordingly, it is

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intended to embrace all such changes, modifications and variations that fall within the spirit and broad scope of the appended claims.

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WHAT IS CLAIMED IS:

1. A personal care composition characterized by containing leghemoglobin and at least one preservative selected from the group consisting of alcohols, glycols, parabens, quaternary nitrogen-containing compounds, isothiazolinones, aldehyde-releasing agents, antioxidants and halogenated compounds.

2. The composition of claim 1 in which the leghemoglobin is in the form of a nitrogen fixation root nodule extract providing a leghemoglobin concentration in the composition of between 0.0001% and about 10% based upon the total weight of the composition.

3. The composition of claim 1 additionally containing at least one root nodule extract component selected from the group consisting of plant growth regulators, amino acids, vitamins, proteins, enzymes, minerals carbohydrates, exopolysaccharides, and combinations thereof.

4. The composition of claim 1 which additionally contains water.

5. The composition of claim 1 characterized by having a consistency selected from the group consisting of lotions, ointments, creams, sprays, spritzes, aqueous or aqueous alcoholic mixtures, gels, mousses, patches, pads, masks, moistened cloths and wipes, solid sticks, clear sticks, lipsticks, aerosol creams, anhydrous powders, talcs, tonics, oils, emulsions, and bath salts.

Motif Exhibit 1148, Page 752 of 3341

6. A method of preparing the personal care composition of claim 1 characterized by the steps of:

(a) extracting the leghemoglobin from root nodules infected with bacteria selected from the group consisting of Cyanobacteria, Anaerobic bacteria, Purple sulfur bacteria, Purple non-sulfur bacteria, Green sulfur bacteria, legumes bacteria, and combinations thereof, in order to provide a leghemoglobin extract, and

(b) combining the leghemoglobin extract with a preservative to provide the personal care composition.

7. The method of claim 6 wherein the leghemoglobin is obtained from the roots of plants infected with bacteria from the Genus *Rhizobium* or *Brachyorhizobium*.

8. The method of claim 6 in which the leghemoglobin is taken from a plant root selected from those found in perennial legumes, annual legumes, legumeous woody plants, and combinations thereof.

9. The method of claim 6 in which the leghemoglobin is taken from the roots of annual legumes.

10. The composition of claim 2 in which the nitrogen fixation root nodule extract comprises a growth media for other microorganisms or confluent skin cultures.

11. The composition of claim 10 in which the microorganisms are yeast.

12. The composition of claim 1 wherein said leghemoglobin is encapsulated within another component in order to provide a time-release characteristic to said leghemoglobin.

13. The composition of claim 12 wherein said other component is selected from the group consisting of liposomes, niosomes, sub-micron emulsions, polymeric encapsulants, gels, creams, lotions, and combinations thereof.

14. The composition of claim 3 that comprises an enzyme selected from the group consisting of nitrogenases, superoxide dismutases, catalases, nitric oxide synthases, sucrose synthases, peroxidases, glutathiones and combinations thereof.

15. The composition of claim 3 that comprises a plant growth regulator selected from the group consisting of kinetin, zeatin, other furfuryl-type molecules, complimentary plant growth regulators such as auxins, and combinations thereof.

16. The composition of claim 3 wherein said component comprises exopolysaccharides, alone or in combination with lipopolysaccharides or capsular polysaccharides, or a combination thereof.

17. A method for binding free radicals on the skin that comprises contacting the skin with leghemoglobin, thereby causing said leghemoglobin to act as a scavenger for the free radicals.

18. The method of claim 17 wherein the leghemoglobin acts as a scavenger for free radicals selected from the group consisting of reactive oxygen, nitric oxide, and combinations thereof.

19. A method for removing free radicals from the skin that comprises contacting the skin with leghemoglobin in order to cause free radicals on the skin to bind with the leghemoglobin, thereby removing said free radicals from the skin.

20. The method of claim 19 wherein said leghemoglobin is provided by means of personal care composition comprising the leghemoglobin and at least one preservative selected from the group consisting of alcohols, glycols, parabens, hydantoins, quaternary nitrogen-containing compounds, isothiazolinones, aldehyde-releasing agents, and halogenated compounds.

21. The method of claim 20 wherein said leghemoglobin is in the form of a nitrogen fixation root nodule extract providing a leghemoglobin concentration in the composition of between 0.0001% and about 10% based upon the total weight of the composition.

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(54) Title: PERSONAL CARE COMPOSITION CONTAINING LEGHEMOGLOBIN

(57) Abstract: A personal care composition comprising leghemoglobin and at least one preservative selected from the group consisting of alcohols, glycols, parabens, hydantoins, quaternary nitrogen-containing compounds, isothiazolinones, aldehyde-releasing agents, and halogenated compounds. Preferably, the leghemoglobin is a nitrogen fixation root nodule extract providing a leghemoglobin concentration in the composition of between 0.0001% and about 10% based upon the total weight of the composition. Also disclosed is a method for preparing the personal care composition.

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STN, WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,126,263 A (COCKING) 30 June 1992 (30.06.1992), col. 10, lines 8-37.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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(54) Title: PLANT LIPASE

(57) Abstract: The invention relates to nucleic acid molecules encoding plant lipase polypeptides; polypeptides and fragments thereof having lipase activity; transgenic cells expressing said lipase; bioreactors which utilise said lipase nucleic acid molecules and polypeptides; and methods to isolate said lipase nucleic acid molecules.

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Plant Lipase

The invention relates to nucleic acid molecules encoding plant lipase polypeptides; polypeptides and fragments thereof having lipase activity; transgenic cells expressing said lipase and bioreactors which utilise said lipase nucleic acid molecules and polypeptides.

Lipases are enzymes that degrade lipids. The reaction catalysed is the hydrolysis of a fatty acid ester bond in triacylglycerol to release glycerol and fatty acids. These proteins have important industrial uses as biocatalysts, for example in the food, textile, medical and pharmaceutical industries. Free fatty acids are important in providing flavour and texture to many foods (e.g. dairy products such as cheese and butter). Lipases are produced by plants, animals, bacteria and moulds. Typically plant lipases are not used commercially although animal, bacterial and mould lipases are used extensively.

For example, a lipase has been purified and characterised from *Aspergillus niger*, (see Tombs and Blake Biochem. Biophys, 1982, 700: 81-89) which is a dimer of two 25kD subunits which is glycosylated. Iwai *et al* (in Lipases: Borgstrom and Brockman, Elsevier, 1984) describes an extracellular lipase isolated from *Aspergillus niger* with a molecular weight of approximately 38kDa which is monomeric with a pI of 4.3 and a pH optimum for activity of 5.6. Torossian and Bell (Biotech. Appl. Biochem 1991, 12: 205-211) describe a crude extract prepared from a *Aspergillus niger* cell extract with a molecular weight of 37kDa and a pI of 4.0. One of the few examples of plant lipases used commercially is disclosed in US3, 368, 903 which was prepared from rape seeds to provide a crude extract which was used in the baking industry.

Plants that bear oilseeds such as soybean, rapeseed and sunflower store trigycerides in their seeds which provide an energy store for germination and subsequent seedling development. In the seeds the oil globules form oil bodies in the cytoplasm that are

membrane bound organelles that have protein in the membrane surface. An example of a protein found associated with oil bodies is an oleosin. The oleosins are a family of proteins that have divergent amino and carboxyl termini with more highly conserved lipophilic central domain. These proteins are associated with oil bodies and it is thought that the association is due in part to the lipophilic domain.

The primary seed storage reserve of many higher plants is triacylglycerol (TAG), which is found in membrane-bound oil bodies. During germination TAG reserves are broken down and the carbon skeletons used to support post-germinative growth. The initial step in the process is catalysed by TAG lipase (EC: 3.1.1.3), which hydrolyses TAG at the oil/water interface to yield free fatty acids and glycerol. In most seeds TAG lipase activity is only detectable upon germination and increases concomitantly with the disappearance of TAG.

The free fatty acids released by TAG lipase are subsequently converted to sucrose via the sequential action of β-oxidation, the glyoxylate cycle and gluconeogenesis. These pathways have been quite intensively studied in plants and many of the genes concerned have been cloned and characterised. In contrast, little is known about TAG lipase. TAG lipase activity has been studied at the biochemical level in a variety of seeds and proteins have been purified. However no gene has so far been cloned from a plant that has been proven to encode a 'true' TAG lipase. This enzyme is distinguished from other esterases by its preference for an insoluble aggregated substrate and its capacity to hydrolyse long chain fatty acid esters, such as triolein.

We have cloned two conserved plant lipases referred to as OBL 1 and OBL 2 from castor bean that have different lipid specificity's; OBL 1 is an acid lipase and OBL 2 is a neutral lipase.

According to a first aspect of the invention there is provided an isolated nucleic acid molecule, or part thereof, which encodes a polypeptide wherein said nucleic acid molecule is selected from the group consisting of:

i) a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 1a or 2a;

ii) a nucleic acid molecule which hybridises to the nucleic acid molecule in (i) and which has lipase activity;

iii) a nucleic acid molecule which differs from the nucleic acid molecules of (i) and (ii) due to the degeneracy in the genetic code.

In a preferred embodiment of the invention said nucleic acid molecule hybridises under stringent hybridisation conditions to a nucleic acid molecule as represented in Figure 1a or 2a. Preferably said nucleic acid molecule consists of the nucleic acid sequence represented in Figure 1a or 2a.

In a further preferred embodiment of the invention said lipase is an acid lipase.

In an alternative preferred embodiment of the invention said lipase is a neutral lipase.

In a preferred embodiment of the invention said nucleic acid encodes a polypeptide with an oil body targeting domain.

In a further preferred embodiment of the invention said nucleic acid molecule that encodes a targeting domain comprises a nucleic acid sequence that encodes an amino acid sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence which encodes an amino acid sequence as represented in Figure 3;

ii) a nucleic acid molecule which hybridises to the nucleic acid molecule in (i) and which specifically targets a polypeptide comprising said targeting domain to an oil body; and

iii) a nucleic acid molecule which differs from the nucleic acid molecules of (i) and (ii) due to the degeneracy in the genetic code.

In a preferred embodiment of the invention said targeting domain is encoded by a nucleic acid molecule comprising a nucleic acid sequence which hybridises under stringent hybridisation conditions to a nucleic acid molecule which encodes an amino acid sequence as represented in Figure 3.

Stringent hybridisation/washing conditions are well known in the art. For example, nucleic acid hybrids that are stable after washing in 0.1x SSC,0.1% SDS at 60°C. It is well known in the art that optimal hybridisation conditions can be calculated if the sequence of the nucleic acid is known. For example, hybridisation conditions can be determined by the GC content of the nucleic acid subject to hybridisation. Please see Sambrook et al (1989) Molecular Cloning; A Laboratory Approach. A common formula for calculating the stringency conditions required to achieve hybridisation between nucleic acid molecules of a specified homology is:

$T_m = 81.5^0\ C + 16.6\ Log\ [Na^+] + 0.41[\ \%\ G + C] - 0.63$ (%formamide).

Typically, hybridisation conditions uses 4 – 6 x SSPE (20x SSPE contains 175.3g NaCl, 88.2g $NaH_2PO_4\ H_2O$ and 7.4g EDTA dissolved to 1 litre and the pH adjusted to 7.4); 5-10x Denhardts solution (50x Denhardts solution contains 5g Ficoll (type 400, Pharmacia), 5g polyvinylpyrrolidone and 5g bovine serum albumen; 100µg-1.0mg/ml sonicated salmon/herring DNA; 0.1-1.0% sodium dodecyl sulphate; optionally 40-60% deionised formamide. Hybridisation temperature will vary depending on the GC content of the nucleic acid target sequence but will typically be between 42^0- 65^0 C.

In a preferred embodiment of the invention said nucleic acid molecule comprises a nucleic acid sequence that has at least 30% homology to the nucleic acid sequence represented in Figure 1a or 2a or a nucleic acid sequence which encodes an amino acid sequence as represented by Figure 3. Preferably said homology is at least 40%; 50%; 60%; 70%; 80%; 90%; or at least 99% identity with the nucleic acid sequence

represented in Figures 1a or 2a or a nucleic acid sequence which encodes a amino acid sequence as represented in Figure 3.

According to a further aspect of the invention there is provided an isolated polypeptide which polypeptide is represented by an amino acid sequence as shown in Figure 1b or 2b, or a variant polypeptide which is modified by addition, deletion or substitution of at least one amino acid residue wherein said polypeptide has lipase activity.

A variant polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, truncations which may be present in any combination. Among preferred variants are those that vary from a reference polypeptide by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid by another amino acid of like characteristics. The following non-limiting list of amino acids are considered conservative replacements (similar): a) alanine, serine, and threonine; b) glutamic acid and aspartic acid; c) asparagine and glutamine d) arginine and lysine; e) isoleucine, leucine, methionine and valine and f) phenylalanine, tyrosine and tryptophan. Most highly preferred are variants which retain or enhance the same biological function and activity as the reference polypeptide from which it varies. In addition deletion may be of entire functional domains. For example, the removal of a targeting domain from a polypeptide such that the lipase activity is retained by the deleted polypeptide.

A functionally equivalent polypeptide is a variant wherein one in which one or more amino acid residues are substituted with conserved or non-conserved amino acid residues, or one in which one or more amino acid residues includes a substituent group. Conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr; exchange of the acidic residues Asp and Glu; substitution between amide residues Asn and Gln; exchange of the basic residues Lys and Arg; and replacements among aromatic residues Phe and Tyr.

In addition, the invention features polypeptide sequences having at least 75% identity with the polypeptide sequences as herein disclosed, or fragments and functionally equivalent polypeptides thereof. In one embodiment, the polypeptides have at least 85% identity, more preferably at least 90% identity, even more preferably at least 95% identity, still more preferably at least 97% identity, and most preferably at least 99% identity with the amino acid sequences illustrated herein.

According to a further aspect of the invention there is provided a vector comprising a nucleic acid molecule according to the invention. Preferably said vector is an expression vector adapted for the expression of a polypeptide according to the invention.

Preferably the nucleic acid in the vector is operably linked to an appropriate promoter or other regulatory elements for transcription in a host cell such as a prokaryotic, (e.g. bacterial), or eukaryotic (e.g. fungal, plant, mammalian or insect cell). The vector may be a bi-functional expression vector which functions in multiple hosts. In the example of nucleic acids encoding polypeptides according to the invention this may contain its native promoter or other regulatory elements and in the case of cDNA this may be under the control of an appropriate promoter or other regulatory elements for expression in the host cell.

By "promoter" is meant a nucleotide sequence upstream from the transcriptional initiation site and which contains all the regulatory regions required for transcription. Suitable promoters include constitutive, tissue-specific, inducible, developmental or other promoters for expression in plant cells comprised in plants depending on design. Such promoters include viral, fungal, bacterial, animal and plant-derived promoters capable of functioning in plant cells.

Constitutive promoters include, for example CaMV 35S promoter (Odell *et al* (1985) Nature 313, 9810-812); rice actin (McElroy *et al* (1990) Plant Cell 2: 163-171); ubiquitin (Christian *et al* . (1989) Plant Mol. Biol. 18 (675-689); pEMU (Last *et al*

(1991) Theor Appl. Genet. 81: 581-588); MAS (Velten *et al* (1984) EMBO J. 3. 2723-2730); ALS promoter (U.S. Application Seriel No. 08/409,297), and the like. Other constitutive promoters include those in U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680, 5,268,463; and 5,608,142.

Chemical-regulated promoters can be used to modulate the expression of a gene in a plant through the application of an exogenous chemical regulator. Depending upon the objective, the promoter may be a chemical-inducible promoter, where application of the chemical induced gene expression, or a chemical-repressible promoter, where application of the chemical represses gene expression. Chemical-inducible promoters are known in the art and include, but are not limited to, the maize In2-2 promoter, which is activated by benzenesulfonamide herbicide safeners, the maize GST promoter, which is activated by hydrophobic electrophilic compounds that are used as pre-emergent herbicides, and the tobacco PR-1a promoter, which is activated by salicylic acid. Other chemical-regulated promoters of interest include steroid-responsive promoters (see, for example, the glucocorticoid-inducible promoter in Schena *et al* (1991) Proc. Natl. Acad. Sci. USA 88: 10421-10425 and McNellie *et al.* (1998) Plant J. 14(2): 247-257) and tetracycline-inducible and tetracycline-repressible promoters (see, for example, Gatz *et al.* (1991) Mol. Gen. Genet. 227: 229-237, and US Patent Nos. 5,814,618 and 5,789,156, herein incorporated by reference.

Where enhanced expression in particular tissues is desired, tissue-specific promoters can be utilised. Tissue-specific promoters include those described by Yamamoto *et al.* (1997) Plant J. 12(2): 255-265; Kawamata *et al* (1997) Plant Cell Physiol. 38(7): 792-803; Hansen *et al* (1997) Mol. Gen. Genet. 254(3): 337-343; Russell *et al.* (1997) Transgenic Res. 6(2): 157-168; Rinehart *et al* (1996) Plant Physiol. 112(3): 1331-1341; Van Camp *et al* (1996) Plant Physiol. 112(2): 525-535; Canevascni *et al* (1996) Plant Physiol. 112(2): 513-524; Yamamoto *et al* (1994) Plant Cell Physiol. 35(5): 773-778; Lam (1994) Results Probl. Cell Differ. 20: 181-196; Orozco *et al*

(1993) Plant Mol. Biol. 23(6): 1129-1138; Mutsuoka *et al* (1993) Proc. Natl. Acad. Sci. USA 90(20): 9586-9590; and Guevara-Garcia *et al* (1993) Plant J. 4(3): 495-50.

In a preferred embodiment of the invention said tissue specific promoter is a promoter which is active during the accumulation of oil in developing oil seeds, (for example see Broun *et al.* (1998) Plant J. 13(2): 201-210.

"Operably linked" means joined as part of the same nucleic acid molecule, suitably positioned and oriented for transcription to be initiated from the promoter. DNA operably linked to a promoter is "under transcriptional initiation regulation" of the promoter.

In a preferred embodiment the promoter is an inducible promoter or a developmentally regulated promoter.

Particular vectors are nucleic acid constructs which operate as plant vectors. Specific procedures and vectors previously used with wide success upon plants are described by Guerineau and Mullineaux (1993) (Plant transformation and expression vectors. In: Plant Molecular Biology Labfax (Croy RRD ed) Oxford, BIOS Scientific Publishers, pp 121-148. Suitable vectors may include plant viral-derived vectors (see e.g. EP-A-194809).

Vectors may also include selectable genetic marker such as those that confer selectable phenotypes such as resistance to herbicides (e.g. kanamycin, hygromycin, phosphinotricin, chlorsulfuron, methotrexate, gentamycin, spectinomycin, imidazolinones and glyphosate).

Alternatively, or in addition, said vectors are vectors suitable for mammalian cell transfection or yeast cell transfection. In the latter example multi-copy vectors such as 2μ episomal vectors are preferred. Alternatively yeast CEN vectors and integrating vectors such as YIP vectors are suitable for transformation of yeast species such as *Saccharomyces cerevisiae* and *Pichia* spp.

According to a further aspect of the invention there is provided a cell transfected or transformed with at least one nucleic acid molecule or vector according to the invention.

In a preferred embodiment of the invention said cell is eukaryotic cell.

Preferably said eukaryotic cell is selected from the group consisting of: mammalian cells (e.g. Chinese Hamster Ovary cells); yeast cells (e.g. *Saccharomyces spp*, *Pichia* spp); algal cells (e.g *Phaeodactylum tricornutum, Chlamydomonas reinhardtii*); insect cells (e.g. *Spodoptera spp)* or plant cells.

In a preferred embodiment of the invention said cell is a plant cell.

In a preferred embodiment of the invention said plant is selected from: corn (*Zea mays*), canola (*Brassica napus*, *Brassica rapa* ssp.), flax (*Linum usitatissimum*), alfalfa (*Medicago sativa*), rice (*Oryza sativa*), rye (*Secale cerale*), sorghum (*Sorghum bicolor*, *Sorghum vulgare*), sunflower (*Helianthus annus*), wheat (*Tritium aestivum*), soybean (*Glycine max*), tobacco (*Nicotiana tabacum*), potato (*Solanum tuberosum*), peanuts (*Arachis hypogaea*), cotton (*Gossypium hirsutum*), sweet potato (*Iopmoea batatus*), cassava (*Manihot esculenta*), coffee (Cofea spp.), coconut (*Cocos nucifera*), pineapple (*Anana comosus*), citris tree (Citrus spp.) cocoa (*Theobroma cacao*), tea (*Camellia senensis*), banana (*Musa* spp.), avacado (*Persea americana*), fig (*Ficus casica*), guava (*Psidium guajava*), mango (*Mangifer indica*), olive (*Olea europaea*), papaya (*Carica papaya*), cashew (*Anacardium occidentale*), macadamia (*Macadamia intergrifolia*), almond (*Prunus amygdalus*), sugar beets (*Beta vulgaris*), oats, barley, vegetables and ornamentals.

Preferably, plants of the present invention are crop plants (for example, cereals and pulses, maize, wheat, potatoes, tapioca, rice, sorghum, millet, cassava, barley, pea),

and other root, tuber or seed crops. Important seed crops are oil-seed rape, sugar beet, maize, sunflower, soybean,sorghum, and flax (linseed). Horticultural plants to which the present invention may be applied may include lettuce, endive, and vegetable brassicas including cabbage, broccoli, and cauliflower. The present invention may be applied in tobacco, cucurbits, carrot, strawberry, sunflower, tomato, pepper.

Grain plants that provide seeds of interest include oil-seed plants and leguminous plants. Seeds of interest include grain seeds, such as corn, wheat, barley, rice, sorghum, rye, etc. Oil seed plants include cotton, soybean, safflower, sunflower, Brassica, maize, alfalfa, palm, coconut, etc. Leguminous plants include beans and peas. Beans include guar, locust bean, fenugreek, soybean, garden beans, cowpea, mungbean, lima bean, fava been, lentils, chickpea, etc.

In a further preferred embodiment of the invention said cell is a prokaryotic cell.

According to a further aspect of the invention there is provided a seed comprising a plant cell according to the invention. Preferably said seed is from an oil seed plant.

In a preferred embodiment of the invention said cell over-expresses the nucleic acid molecule according to the invention when compared to a non-transgenic reference cell of the same species. Preferably said cell has increased lipase activity.

In a further preferred embodiment of the invention said cell over-expresses said nucleic acid molecule by at least 2-fold above basal level expression. Preferably said cell over-expresses by at least 5-fold; 10-fold, 20-fold, 30-fold, 40-fold, or 50-fold. Preferably said cell expresses said nucleic acid by at least 100-fold above basal level expression when compared to a non-transgenic cell of the same species.

It will be apparent that means to increase the activity of a polypeptide encoded by a nucleic acid molecule are known to the skilled artisan. For example, and not by limitation, increasing the gene dosage by providing a cell with multiple copies of said

gene. Alternatively or in addition, a gene(s) may be placed under the control of a powerful promoter sequence or an inducible promoter sequence to elevate expression of mRNA encoded by said gene. The modulation of mRNA stability is also a mechanism used to alter the steady state levels of an mRNA molecule, typically via alteration to the 5' or 3' untranslated regions of the mRNA.

In an alternative preferred embodiment of the invention said cell is modified such that the expression of the nucleic acid molecule according to the invention is decreased when compared to a non-transgenic reference cell of the same species.

In a preferred embodiment of the invention said cell is modified to reduce the expression of said nucleic acid molecule wherein lipase activity is reduced by at least 10% when compared to a non-transgenic reference cell of the same species. Preferably said activity is reduced by between about 10%-90%. More preferably said activity is reduced by at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or at least 90%.

In a preferred embodiment of the invention said transgenic cell is null for a nucleic acid molecule comprising a sequence selected from the group consisting of:

i) the nucleic acid molecule comprising a sequence as represented by Figure 1a or 2a;

ii) nucleic acids which hybridise to the sequences of (i) above and which have lipase activity; and

iii) nucleic acid sequences which are degenerate as a result of the genetic code to the sequences defined in (i) and (ii) above.

Null refers to a cell that includes a non-functional copy of the nucleic acid sequence described above wherein the activity of the polypeptide encoded by said nucleic acid is ablated. Methods to provide such a cell are well known in the art and include the use of antisense genes to regulate the expression of specific targets; insertional mutagenesis using T-DNA; the introduction of point mutations and small deletions

into open reading frames and regulatory sequences; and double stranded inhibitory RNA (RNAi).

A number of techniques have been developed in recent years that purport to specifically ablate genes and/or gene products. A recent technique to specifically ablate gene function is through the introduction of double stranded RNA, also referred to as inhibitory RNA (RNAi), into a cell that results in the destruction of mRNA complementary to the sequence included in the RNAi molecule. The RNAi molecule comprises two complementary strands of RNA (a sense strand and an antisense strand) annealed to each other to form a double stranded RNA molecule. The RNAi molecule is typically derived from exonic or coding sequence of the gene which is to be ablated. Surprisingly, only a few molecules of RNAi are required to block gene expression that implies the mechanism is catalytic. The site of action appears to be nuclear as little if any RNAi is detectable in the cytoplasm of cells indicating that RNAi exerts its effect during mRNA synthesis or processing.

An alternative embodiment of RNAi involves the synthesis of so called "stem loop RNAi" molecules that are synthesised from expression cassettes carried in vectors. The DNA molecule encoding the stem-loop RNA is constructed in two parts, a first part that is derived from a gene the regulation of which is desired. The second part is provided with a DNA sequence that is complementary to the sequence of the first part. The cassette is typically under the control of a promoter that transcribes the DNA into RNA. The complementary nature of the first and second parts of the RNA molecule results in base pairing over at least part of the length of the RNA molecule to form a double stranded hairpin RNA structure or stem-loop. The first and second parts can be provided with a linker sequence. Stem loop RNAi has been successfully used in plants to ablate specific mRNA's and thereby affect the phenotype of the plant , see, Smith *et al* (2000) Nature 407, 319-320.

In a preferred embodiment of the invention said cell is transfected with a nucleic acid molecule comprising an expression cassette which cassette comprises a nucleic acid

sequence which encodes at least part of a lipase as herein described wherein said cassette is adapted such that both sense and antisense nucleic acid molecules are transcribed from said cassette.

In a preferred embodiment of the invention said cassette is provided with at least two promoters adapted to transcribe sense and antisense strands of said nucleic acid molecule.

In a further preferred embodiment of the invention said cassette comprises a nucleic acid molecule wherein said molecule comprises a first part linked to a second part wherein said first and second parts are complementary over at least part of their sequence and further wherein transcription of said nucleic acid molecule produces an RNA molecule which forms a double stranded region by complementary base pairing of said first and second parts.

According to a further aspect of the invention there is provided a nucleic acid molecule comprising an expression cassette which cassette comprises a nucleic acid sequence which encodes at least part of a lipase as hereindescribed wherein said cassette is adapted such that both sense and antisense nucleic acid molecules are transcribed from said cassette.

In a preferred embodiment of the invention said cassette comprises a nucleic acid molecule wherein said molecule comprises a first part linked to a second part wherein said first and second parts are complementary over at least part of their sequence and further wherein transcription of said nucleic acid molecule produces an RNA molecule which forms a double stranded region by complementary base pairing of said first and second parts.

In a preferred embodiment of the invention said first and second parts are linked by at least one nucleotide base. In a further preferred embodiment of the invention said first and second parts are linked by 2, 3, 4, 5, 6, 7, 8, 9, or 10 nucleotide bases. In a yet further preferred embodiment of the invention said linker is at least 10 nucleotide bases.

In a further preferred embodiment of the invention the length of the RNA molecule is between 10 nucleotide bases (nb) and 1000nb. Preferably said RNA molecule is at least 100nb; 200nb; 300nb; 400nb; 500nb; 600nb; 700nb; 800nb; 900nb; or 1000nb in length. More preferably still said RNA molecule is at least 1000nb in length. Preferably still said RNA molecule is 21nb in length.

According to a further aspect of the invention there is provided a method to manufacture a polypeptide according to the invention comprising the steps of:

i) providing a cell according to the invention and growth conditions conducive to the production of a polypeptide according to the invention; and optionally

ii) purifying said polypeptide from said cell or growth media.

According to a yet further aspect of the invention there is provided the use of a polypeptide encoded by a nucleic acid according to the invention as a target for the discovery of agents that inhibit the lipase activity of said polypeptide.

According to a further aspect of the invention there is provided a screening method for the identification of an agent with the ability to inhibit plant growth and/or viability comprising the steps of:

i) providing a polypeptide encoded by the nucleic acid selected from the following group;

a) a nucleic acid represented by Fig 1a or 2a;

b) nucleic acids which hybridise to the sequences of (i) above and which have lipase activity; and

c) nucleic acid sequences which are degenerate as a result of the genetic code to the sequences defined in (a) and (b) above;

ii) providing at least one candidate agent;

iii) forming a preparation which is a combination of (i) and (ii);

iv) determining the interaction of the polypeptide and said candidate agent; and

testing the effect of the agent on the growth and/or viability of plants.

In a preferred method of the invention said agent is a herbicide.

According to a further aspect of the invention there is provided a method to isolate a plant nucleic acid molecule encoding at least part of a polypeptide which has lipase activity comprising the steps of:

i) providing a preparation comprising isolated plant nucleic acid, a pair of oligonucleotide primer molecules which anneal to a part of said plant nucleic acid, buffering factors, polymerase chain reaction cofactors and a thermophilic DNA polymerase;

ii) providing polymerase chain reaction conditions which allow the amplification by said primer molecules of a part of a plant lipase nucleic acid.

In a preferred method of the invention said plant nucleic acid is cDNA.

In an alternative preferred method of the invention said nucleic acid is genomic DNA.

In a preferred method of the invention said lipase in an oil body lipase.

In a preferred method of the invention at least one first primer molecule anneals to a part of said plant nucleic acid molecule encoding said lipase between about amino acid residue + 245 and +253 and at least one second primer molecule anneals between about amino acid residue +414 and +421 of the consensus sequence shown in Figure 15.

In a preferred method of the invention said first primer molecule, or reverse sequence complement thereof is selected from the group consisting of;

RtY agY ttY aga ggI acH gaR c
RtY agY ttY aga ggI acH gaY c
RtY agY ttY aga ggc acH gaR c
RtY agY ttY aga ggc acH gaY c

and said second primer molecule, or reverse sequence complement thereof, is selected from the group consisting of:

gtB taY aaY aaY gaY atg gtI c
gtB taY tgY aaY gaY atg gtI c
gtB taY aaY aaY gaY RtI gtI c
gtB taY tgY aaY gaY RtI gtI c

According to a further aspect of the invention there is provided first and second oligonucleotide primer molecules, or reverse sequence complements thereof wherein said first molecule is selected from the group consisting of;

RtY agY ttY aga ggI acH gaR c
RtY agY ttY aga ggI acH gaY c
RtY agY ttY aga ggc acH gaR c
RtY agY ttY aga ggc acH gaY c

and said second molecule, or reverse sequence complement thereof, is selected from the group consisting of ;

gtB taY aaY aaY gaY atg gtI c
gtB taY tgY aaY gaY atg gtI c
gtB taY aaY aaY gaY RtI gtI c
gtB taY tgY aaY gaY RtI gtI c

According to an aspect of the invention there is provided an oil body lipase isolated by the method according to the in invention.

According to a further aspect of the invention there is provided a kit comprising; oligonucleotide primer molecules according to the invention and polymerase chain reaction buffer, co-factors (e.g. divalent cation, dNTP's,) and thermophilic DNA polymerase.

In a preferred embodiment of the invention said kit further comprises buffers suitable for the extraction of nucleic acid from plant tissue.

In a further preferred embodiment of the invention said kit further comprises enzymes and co-factors typically used in the synthesis of cDNA.

In a yet further preferred embodiment of the invention said kit further comprises control nucleic acid wherein said control nucleic acid encodes an oil body lipase.

An embodiment of the invention will now be described by example only and with reference to the following figures:

Figure 1a is the nucleic acid sequence of OBL1; Figure 1b is the amino acid sequence of OBL1;

Figure 2a is the nucleic acid sequence of OBL 2; Figure 2b is the amino acid sequence of OBL2;

Figure 3 is the amino acid sequence of the hydrophobic domain of OBL 1;

Figure 4 is a summary of the isolation procedure for castor bean oil bodies;

Figure 5 compares the amino acid sequence of tryptic peptide fragments with other plant lipase fragments and including degenerate primers;

Figure 6 shows the amino acid sequence of OBL1 and a homology comparison with related fungal lipase polypeptides;

Figure 7 represents a hydrophobicity plot for OBL 1;

Figure 8 illustrates proteolytic cleavage of OBL 1 and fractionation;

Figure 9 illustrates the lipase activity of OBL1 expressed in *E.coli*;

Figure 10 is a sequence comparison of lipase-like genes from *Arabidopsis spp;*

Figure 11 illustrates PCR primers used to sub-clone homologues of OBL-1;

Figure 12 illustrates the amino acid sequence of OBL2;

Figure 13 illustrates the expression patterns of OBL1 and OBL 2 during germination and post-germinative growth;

Figure 14 is an amino acid comparison of OBL 1, OBL2 and including homologues of these proteins; and

Figure 15a is an amino acid comparison of oil body lipase sequences including regions of said lipase to which oligonucleotide primer molecules are designed; Figure 15b illustrates examples of degenerate primers used in the amplification of oil body lipase nucleic acid sequences.

Materials and Methods

Materials- Castor beans (*Ricinus communis* var. gibsonii) were supplied by Chiltern Seeds (Ulverston, Cumbria, UK). The beans were soaked in running water for 1 d and germinated in the dark on moist vermiculite at 30°C. All reagents were obtained from Sigma-Aldrich Co. (Gillingham, UK), except for glycerol tri[1-^{14}C]oleate that was from Amersham Biosciences UK Ltd. (Little Chalfont, UK) and *Flavobacterium meningsepticum* propyl endopeptidase (PEP) from Seikagaku America (Falmouth, USA).

Preparation of oil body membranes and proteolytic treatment – Oil bodies were isolated from the endosperm of imbibed castor beans and the membranes delipidated using the methods of Hills et al., (2). Peripheral proteins were removed from the oil bodies using urea buffer, according to Millichip et al., (3). The membrane fraction was solublized in SDS-loading buffer, heated at 70°C for 10 min and the polypeptides separated on a 14% SDS-PAGE gel as described by Laemmli (5) and stained using 0.2% (w/v) Coomassie blue R-250 in methanol: acetic acid: water (4:1:5 v/v/v). For proteolytic treatments purified urea washed oil bodies were incubated in 0.1M phosphate buffer (pH 7) containing 0.2 U ml^{-1} of PEP for 1h at 37°C. The oil bodies were recovered by centrifugation.

Tryptic digestion and peptide sequence analysis - In-gel tryptic digestion was performed by washing the gel pieces in Eppendorf tubes three times with 50% (v:v) aqueous acetonitrile containing 25 mM ammonium bicarbonate and drying the gel pieces in a vacuum concentrator for 30 min. Sequencing-grade modified porcine trypsin (Promega) was dissolved in the 50 mM acetic acid supplied by the manufacturer, then diluted 5-fold by adding 25 mM ammonium bicarbonate containing 0.1% (v:v) octyl-ß-D-glucopyranoside (Sigma) to give a final trypsin concentration is 0.02 µg/µL. Gel pieces were rehydrated by adding 10 µL of trypsin solution, and after 30 min enough trypsin solution was added to cover the gel. Digests were incubated overnight at 37°C.

Tryptic digests were applied directly to the MALDI target plate. Dried peptides were redissolved in 0.5 μL of formic acid followed by addition of 9.5 μL of water, and one or more 0.5 μL aliquots were spotted and dried onto the MALDI target plate. A matrix solution containing 10 mg/mL 4-hydroxy-α-cyano-cinnamic acid (Sigma) in 50% aqueous (v:v) acetonitrile containing 0.1% TFA (v:v) was freshly prepared. Serial 2-fold dilutions of matrix solution were spotted onto the MALDI target plate, and the dilution that gave a uniform layer of crystals when viewed in the mass spectrometer was selected for use with samples. A 0.5 μL aliquot of matrix solution was pipetted onto the dried peptide spot. In some cases, the matrix was added directly to the sample droplet on the target plate before drying.

Positive-ion MALDI mass spectra were obtained using an Applied Biosystems 4700 Proteomics Analyzer (CTS version, Applied Biosystems, Foster City, CA, USA) in reflectron mode with an accelerating voltage of 20 kV. MS spectra were acquired with a total of 1000 laser pulses over a mass range of m/z 800-4000. Final mass spectra were the summation of 20 sub-spectra, each acquired with 50 laser pulses, and internally calibrated using the tryptic peptides at m/z 842.509 and 2211.104. Monoisotopic masses were obtained from centroids of raw, unsmoothed data.

For CID-MS/MS, a Source 1 accelerating voltage of 8 kV, a collision energy of 1 kV, and a Source 2 accelerating voltage of 15 kV were used. Air was used as the collision gas at the instrument's 'medium' pressure setting with a recharge threshold of 9.9×10^{-7} torr, which produced a Source 2 pressure of about 1×10^{-6} torr. The precursor mass window was set to +/-10 "Da", and the metastable suppressor was enabled. The default calibration was used for MS/MS spectra.

Mass spectral data obtained in batch mode were submitted to database searching using a locally-running copy of the Mascot program (Matrix Science Ltd., version1.7). Batch-acquired MS and MS/MS spectral data were submitted to a combined peptide mass fingerprint and MS/MS ion search through the Applied

Biosystems GPS Explorer software interface (version 1.0) to Mascot. Search criteria included: Maximum missed cleavages, 1; Variable modifications, Oxidation (M); Peptide tolerance, 25 ppm; MS/MS tolerance, 0.2 Da. Peptide sequence tags were generated from CID-MS/MS spectra by manual interpretation or using a *de novo* sequencing program supplied by Applied Biosystems.

RNA extraction, cDNA synthesis and PCR - Total RNA from various tissues was isolated using the RNeasy kit from Qiagen. The synthesis of single stranded cDNA was carried out using SuperScript™ II RNase H⁻ reverse transcriptase from Invitrogen. Degenerate primers corresponding to peptide sequences 1 and 2 were designed (5'-ttgatagtirtyagyttyaga and 5'-ctgtccraatgtrtaiarctt) and used to amplify a fragment of the acid lipase (*RcOBL1*) cDNA from imbibed seed endosperm. Gene specific primers (5'-gaccacttggtatgggcatatgatgg and 5'-catgtcattgcagtaaaccaccctga) were then used to obtain the full-length cDNA sequence by 3'- and 5'-RACE using the SMART™ RACE cDNA Amplification kit from BD Biosciences, following the manufacturers protocols. Primers to a castor actin-like gene (*RcACT*) (5'-cgttctctccttgtatgccagtggtc and 5'-gagctgctcttggcagtctcaagttc) were used as a constitutive control for RT-PCR experiments on various tissues.

A partial clone of *RcOBL2* was obtained by performing PCR on cDNA from four-day old endosperm using different combinations of four pairs of degenerate primers designed using alignments of multiple *OBL-like* genes (5'- rtyagyttyagaggiachgarc or 5'-rtyagyttyagaggiachgayc or 5'-rtyagyttyagaggcachgarc or 5'-rtyagyttyagaggcachgayc with 5'-giaccatrtcrttrttrtabac or 5'-giaccatrtcrttrcartabac or 5'-giaciayrtcrttrttrtabac or 5'-giaciayrtcrttrcartabac). Gene specific primers (5'-taggtctgggcaacagaagtgacgctac and 5'-tgcccaaatgtgtatatgttcagcaacc) were then used to obtain the full-length *RcOBL2* cDNA sequence by 3'- and 5'-RACE using the SMART™ RACE cDNA Amplification kit from BD Biosciences, following the manufacturers protocols.

Expression of RcOBL1 and determination of lipase activity – A fragment of the *RcOBL1* cDNA that lacks the hydrophobic N-terminus was amplified using primers 5'-gaattcgtgtcgcaccaggcagacgaagtgatttca and 5'-tctagactagtaaccttgtgccatcattttcagag and cloned into the pCR 2.1-TOPO vector from Invitrogen. The insert was then excised using EcoR1 and Xba1 and cloned into the pMAL-c2E vector from New England Biolabs. The cMBP fusion protein was expressed in BL21-Codon Plus-RIL cells from Stratagene. The cells were cultured at 37°C and induced using 0.4 mM IPTG. Lipase activity was measured using ^{14}C-labelled triolein according to the method of Fuchs et al., (1). Protein content was determined as described by Bradford (4) using BSA as a standard.

ABBREVIATIONS

The abbreviations used are: TAG, triacylglycerol; CHAPS, 3-cholamidopropyl-dimethylammonio-1-propanesulphonate; PEP, propyl endopeptidase; TFA, trifluoroacetic acid; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; CID, collision-induced dissociation; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; RACE-PCR, rapid amplification if cDNA ends-PCR; PAGE, polyacrylamide gel electrophoresis; MBP, maltose binding protein.

EXAMPLE 1

Identification of an oil body lipase (OBL) from castor seeds – Castor beans contain an acid lipase activity, which is associated with the oil body membrane (8). To identify this enzyme oil bodies were isolated from the endosperm of soaked castor beans by floatation centrifugation (10) and stripped of peripheral proteins using denaturing urea buffer (11). The remaining polypeptides that are intergral to the oil body membrane were separated by SDS-PAGE (Fig. 4). A 58kDa band was excised, subjected to tryptic digestion and the resulting peptides analysed by MALDI and CID-MS/MS. The sequence of two peptides was interpreted from the CID-MS/MS spectra (Fig. 4).

Degenerate primers were designed, based on the peptide sequences and a cDNA fragment was amplified by RT-PCR from imbibed seed RNA. Gene specific primers were then used to obtain a full-length cDNA sequence by 3'- and 5'-RACE. This sequence was designated oil body lipase 1 (OBL1) and submitted to the Genbank database (accession number XXX). The cDNA is 1870 bp and contains a 1578 bp putative open reading frame. The deduced RcOBL1 protein is 525 amino acids (Fig. 6), has a calculated mass of 59.6 kDa and a predicted pI 6.68. The mass of six peptides derived from a tryptic digest of the ~58kDa band matched those predicted for RcOBL1, confirming the clones identity.

A similarity search revealed that the C-terminal region of RcOBL1 shares a low level of identity (28-31%) with TAG lipases from the fungi *Rhizomucor miehei, Rhizopus oryzae* and *Humicola lanuginosa*(Fig 6).

Lipases from different organisms can vary considerably in their primary amino acid sequence however they are all 'serine esterases' and exhibit a common structural feature called an α/β fold which consists of a central β-sheet composed of parallel strands that are linked via α-helices [15]. The nucleophilic serine forms part of a catalytic triad (S-[DE]-H) and is located in a sharp turn (nucleophilic elbow). A conserved signature surrounds the serine residue (PROSITE accession number PS00120: [LIV]-x-[LIVFY]-[LIVMST]-G-[HYWV]-S-x-G-[GSTAC]). The RcOBL1 amino acid sequence (Fig. 6) contains both a putative catalytic triad (S340, D404, H497) and all but the first residue of the conserved signature (FVVTGHSLGG).

EXAMPLE 2

RcOBL1 contains an N-terminal oil body anchor domain - A hydrophobicity plot of RcOBL1 showed that near the N-terminus there is an unusually long stretch of ~60 hydrophobic amino acids (Fig. 7). Oil body proteins such as oleosin (Fig. 7) contain a characteristic 'H domain', consisting of ~70 hydrophobic residues, which anchors the protein to the membrane (16). Although the N-terminus of RcOBL1 does not show

significant sequence homology to oleosins the extent of the hydrophobic region suggests that it might play an analogous role. To address this purified urea washed oil bodies were digested with propyl endopeptidase (PEP), which is predicted to cleave RcOBL1 specifically at P224. The oil body and soluble fractions were then separated by centrifugation and analysed by SDS-PAGE (Fig. 8). The N-terminal fragment of RcOBL1 (~24 kDa) containing the hydrophobic region was retained in the oil body membrane. In contrast the C-terminal fragment (~36 kDa) containing the lipase catalytic domain was solublized. These data suggest that the hydrophobic region acts as an oil body membrane anchor.

EXAMPLE 3

RcOBL1 is the acid lipase - To confirm that RcOBL1 encodes the acid lipase the enzyme was expressed heterologusly in *E. coli*. To maximize the solubility of the recombinant protein the hydrophobic N-terminus was removed and the C-terminus fused to maltose binding protein (MBP). Acid lipase activity was measured in extracts from *E. coli* by following the hydrolysis of ^{14}C-triolein at pH 4.2. No activity was detected in cells that did not harbour the expression vector (data not shown). However in those that did, induction of cMBP-RcOBL1 expression by IPTG resulted in a 20-fold increase in acid lipase activity (Fig. 9). When the pH of the assay was varied the optimum was found to be between 4 and 4.5 (Fig. 9). This value is the same as that reported for lipase activity from purified castor seed oil body membranes (8).

EXAMPLE 4

RT-PCR was used to show that *RcOBL1* transcripts are present in the endosperm of imbibed seed but that the level declines following germination (Fig. 13). The expression of *RcOBL1* was not detected in the embryo of imbibed seed or in the leaves, stems or roots of mature plants (data not shown). The catalytic properties of

the acid lipase and its distribution have been defined previously in some detail (8-10). A pH optima of ~4.2 and localization to the seed endosperm can be considered as diagnostic.

EXAMPLE 5

RcOBL1-like proteins are present in many plants- An investigation of all available higher plant sequences indicates that there are RcOBL1 homologues present in numerous species including; *Arabidopsis thaliana*, soybean (*Glycine max*), tomato (*Lycopersicon esculentum*), Lettuce (*Lactuca sativa*), rice (*Oryza sativa*) and wheat (*Triticum aestivum*). A phylogenetic analysis was performed of *Arabidopsis* proteins with homology to fungal lipases. *Arabidopsis* contains five OBL1-like proteins (Fig. 5A) of which At3g14360 is most similar to RcOBL1 (~44% identity). All five proteins contain both the membrane anchor domain and the lipase catalytic domain, suggesting that they are also oil body lipases (Fig. 14).

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Claims

1. An isolated nucleic acid molecule, or part thereof, that encodes a polypeptide wherein said nucleic acid molecule is selected from the group consisting of:

i) a nucleic acid molecule comprising a nucleic acid sequence as represented in Figure 1a or 2a;

ii) a nucleic acid molecule which hybridises to the nucleic acid molecule in (i) and which has lipase activity;

iii) a nucleic acid molecule which differs from the nucleic acid molecules of (i) and (ii) due to the degeneracy in the genetic code.

2. A nucleic acid molecule according to Claim 1 wherein said molecule hybridises under stringent hybridisation conditions to a nucleic acid molecule as represented in Figure 1a or 2a.

3. A nucleic acid molecule according to Claim 1 or 2 wherein said nucleic acid molecule consists of the nucleic acid sequence represented in Figure 1a or 2a.

4. A nucleic acid molecule according to any of Claims 1-3 wherein said nucleic acid molecule encodes a polypeptide with an oil body targeting domain.

5. A nucleic acid molecule according to Claim 4 wherein said nucleic acid molecule which encodes a targeting domain comprising a nucleic acid sequence selected from the group consisting of:

i) a nucleic acid molecule consisting of a nucleic acid sequence which encodes an amino acid sequence as represented in Figure 3;

ii) a nucleic acid molecule which hybridises to the nucleic acid molecule in (i) and which specifically targets a polypeptide comprising said targeting domain to an oil body; and

iii) a nucleic acid molecule which differs from the nucleic acid molecules of (i) and (ii) due to the degeneracy in the genetic code.

6. A nucleic acid molecule according to Claim 5 comprising a nucleic acid sequence that hybridises under stringent hybridisation conditions to a nucleic acid molecule that encodes an amino acid sequence as represented in Figure 3.

7. An isolated polypeptide which polypeptide is represented by an amino acid sequence as shown in Figure 1b or 2b, or a variant polypeptide which is modified by addition, deletion or substitution of at least one amino acid residue wherein said polypeptide has lipase activity.

8. An isolated polypeptide according to Claim 7 comprising an amino acid sequence as represented in Figure 3 wherein said amino acid sequence is an oil body targeting domain.

9. A vector comprising a nucleic acid molecule according to any of Claims 1-6.

10. A cell transfected or transformed with at least one nucleic acid molecule according to any of Claims 1-6 or vector according to Claim 9.

11. A cell according to Claim 10 wherein said cell is eukaryotic cell.

12. A cell according to Claim 11 wherein said eukaryotic cell is selected from the group consisting of: a mammalian cell; a yeast cell; an algal cell; an insect cell; or a plant cell.

13. A cell according to Claim 12 wherein said cell is a plant cell.

14. A cell according to Claim 10 wherein said cell is a prokaryotic cell.

15. A seed comprising a plant cell according to Claim 13.

16. A seed according to Claim 15 wherein said seed is from an oil seed plant.

17. A cell according to any of Claims 10-14 wherein said cell over-expresses the nucleic acid molecule according to any of Claims 1-6 when compared to a non-transgenic reference cell of the same species.

18. A cell according to any of Claims 10-14 wherein said cell is modified such that the expression of the nucleic acid molecule according to any of Claims 1-6 is decreased when compared to a non-transgenic reference cell of the same species.

19. A cell according to Claim 18 wherein said cell is null for a nucleic acid molecule comprising a sequence selected from the group consisting of:

i) the nucleic acid molecule comprising a sequence as represented by Figure 1a or 2a;

ii) nucleic acids which hybridise to the sequences of (i) above and which have lipase activity; and

iii) nucleic acid sequences which are degenerate as a result of the genetic code to the sequences defined in (i) and (ii) above.

20. A cell according to Claim 17 which is transfected with a nucleic acid molecule comprising an expression cassette which cassette comprises a nucleic acid sequence according to any of Claims 1-6 wherein said cassette is adapted such that both sense and antisense nucleic acid molecules are transcribed from said cassette.

21. A cell according to Claim 20 wherein said cassette is provided with at least two promoters adapted to transcribe sense and antisense strands of said nucleic acid molecule.

22. A cell according to Claim 20 wherein said cassette comprises a nucleic acid molecule wherein said molecule comprises a first part linked to a second part wherein said first and second parts are complementary over at least part of their sequence and further wherein transcription of said nucleic acid molecule produces an RNA molecule which forms a double stranded region by complementary base pairing of said first and second parts.

23. A nucleic acid molecule comprising an expression cassette which cassette comprises a nucleic acid molecule according to any of Claims 1-6 wherein said cassette is adapted such that both sense and antisense nucleic acid molecules are transcribed from said cassette.

24. A nucleic acid molecule according to Claim 23 wherein said molecule comprises a first part linked to a second part wherein said first and second parts are complementary over at least part of their sequence and further wherein transcription of said nucleic acid molecule produces an RNA molecule which forms a double stranded region by complementary base pairing of said first and second parts.

25. A method to manufacture a lipase polypeptide comprising the steps of:

i) providing a cell according any of Claims 10-14 and growth conditions conducive to the production of a polypeptide according to Claim 7 or 8; and optionally

iii) purifying said polypeptide from said cell or growth media.

26. The use of a polypeptide encoded by a nucleic acid according to any of Claims 1-6 as a target for the discovery of agents that inhibit the lipase activity of said polypeptide.

27. A screening method for the identification of an agent with the ability to inhibit plant growth and/or viability comprising the steps of:

i) providing a polypeptide encoded by the nucleic acid selected from the following group;

a) a nucleic acid represented by Fig 1a or 2a;

b) nucleic acids which hybridise to the sequences of (i) above and which have lipase activity; and

c) nucleic acid sequences which are degenerate as a result of the genetic code to the sequences defined in (a) and (b) above;

ii) providing at least one candidate agent;

iii) forming a preparation which is a combination of (i) and (ii);

iv) determining the interaction of the polypeptide and said candidate agent; and testing the effect of the agent on the growth and/or viability of plants.

28. A method according to Claim 27 wherein said agent is a herbicide.

29. A method to isolate a plant nucleic acid molecule encoding at least part of a polypeptide that has lipase activity comprising the steps of:

i) providing a preparation comprising isolated plant nucleic acid, a pair of oligonucleotide primer molecules which anneal to a part of said plant nucleic acid, buffering factors, polymerase chain reaction co-factors and a thermophilic DNA polymerase;

iii) providing polymerase chain reaction conditions which allow the amplification by said primer molecules of a part of an oil body plant lipase nucleic acid.

30. A method according to Claim 29 wherein said plant nucleic acid is cDNA.

31. A method according to Claim 29 wherein said nucleic acid is genomic DNA.

32. A method according to any of Claims 29-31 wherein at least one first primer molecule anneals to a part of said plant nucleic acid molecule encoding said lipase between about amino acid residue + 245 and +253 and at least one second primer molecule anneals between about amino acid residue +414 and +421 of the consensus sequence shown in Figure 15.

33. A method according to Claim 32 wherein said first primer molecule, or reverse sequence complement thereof, is selected from the group consisting of;

RtY agY ttY aga ggI acH gaR c
RtY agY ttY aga ggI acH gaY c
RtY agY ttY aga ggc acH gaR c
RtY agY ttY aga ggc acH gaY c

and said second primer molecule, or reverse sequence complement thereof, is selected from the group consisting of:

gtB taY aaY aaY gaY atg gtI c
gtB taY tgY aaY gaY atg gtI c
gtB taY aaY aaY gaY RtI gtI c
gtB taY tgY aaY gaY RtI gtI c

34. A pair of oligonucleotide primer molecules wherein at least one first primer molecule anneals to a part of a plant nucleic acid molecule encoding a lipase between about amino acid residue + 245 and +253 and at least one second primer molecule that anneals between about amino acid residue +414 and +421 of the consensus sequence shown in Figure 15.

35. A pair of oligonucleotide primer molecules according to Claim 34 wherein a first oligonucleotide primer molecule, or reverse sequence complement thereof, is selected from the group consisting of;

RtY agY ttY aga ggI acH gaR c
RtY agY ttY aga ggI acH gaY c
RtY agY ttY aga ggc acH gaR c
RtY agY ttY aga ggc acH gaY c

and a second oligonucleotide primer molecule, or reverse sequence complement thereof, is selected from the group consisting of ;

gtB taY aaY aaY gaY atg gtI c
gtB taY tgY aaY gaY atg gtI c
gtB taY aaY aaY gaY RtI gtI c
gtB taY tgY aaY gaY RtI gtI c

36. An oil body lipase isolated by the method according to any of Claims 29-35.

37. A kit comprising; oligonucleotide primer molecules according to Claim 34 or 35, polymerase chain reaction buffer, co-factors and thermophilic DNA polymerase.

38. A kit according to Claim 37 wherein said kit further comprises buffers suitable for the extraction of nucleic acid from plant tissue.

39. A kit according to Claim 37 or 38 wherein said kit further comprises enzymes and co-factors typically used in the synthesis of cDNA.

40. A kit according to any of Claims 37-39 wherein said kit further comprises control nucleic acid wherein said control nucleic acid encodes an oil body lipase

Figure 1a

CTAAAGCCATGGATGATGCTGGTAAAATCACTTCCACAAGCCATCTCATAGTGAGCCCAGATGAAGGAAC
CTTTTTGGACCTGTTCAAGCACATTGTGCTGAGTGATTTGGGCAGTGGAGCCAAATTCTTTAGGGCTTCA
GATCAGAGAGTGCCTGCTACGGCAGCATATTATAGCAGGTGGCCTGTTTCAGTTTTCATTTGCAAAATAC
TTCAACTTTTCCAGATGCCAGCCGCGATGCTTGGTCATCTTACTGATTTCTTGCTCAACTTCTATTATCA
GAATCATGGCTTCCTTGGCATACTCAGAAACATCTTCTTAATAAGACTGAAGATACCAAAAAGAGGTGAA
GCCGACTTTATAAGCACGATAGGGTATTTAGATTCACGAATGGACCTTCACGGGACGCCAATGGTGTCGC
ACCAGGCAGACGAAGTGATTTCAAATGCAGATAATCCAAGCCTGAAAGAAGGGCACAATTCAAAGATAAA
AGGAGCCCTTGGGAACCGATCTCTCATGGATCTTTGTATCATGGCGTCAAAGCTTGCTTATGAAAATACC
AAAGTTGTTGAAAGAGTAGTTGCCGAACATTGGAAGATGCATTTCGTGGCTGACTATGGGGGCATGAATT
ATTTCCAAGATGCAAGGAACACTCATGCGTTCATCTTTTGTGACAAGCCAAAAGATGCAAACTTGATAGT
GATCAGCTTCAGAGGCACAGGACCTTTTAGTATACCAAATTGGTGTACTGATTTTGATTTCTCCTTAGTT
GGGTTGGGAGACGCAGGAAGTGTCCATGTTGGATTCTTAGAAGCAATGGGTTTGGGTCACAGAAATTCTA
TTTCCAGCTTTGAGACTAGCATTAACACAAAGTCGCCAGGAAGCATAACCGAATTAAGGAAAGAGTCCGA
GATGGCTCCGGACCACTTGGTATGGGCATATGATGGTGTTTACTTTCTTGCGGCATCGACGCTCAAGGGA
TTACTAAAAGACCACAAGAACGCAAAATTTGTAGTCACTGGGCATAGCTTAGGTGGTGCACTTGCTATAC
TGTTCACATGCATTCTTGAGATACAGCAGGAGACAGAGGTGCTTGACAGACTGCTAAATGTATACACATT
CGGACAGCCTAGGATTGGGAACTATAATCTTGGTTACTTCATGCAGAACCGTCTCAATTTTCCAGAACGT
AGGTATTTCAGGGTGGTTTACTGCAATGACATGGTTCCTAGGGTGCCTTTCGATGATGTCTTCTTCACTT
TCGAGCATTTCGGAACCTGCATTTACTATGATAGCCGCTTCTTTGGCTACTTTACCAAAGAGGAGCCCAG
CAGAAACCCTTTCGGAATAGAAAATGCCATCAGTGCGCACATCACCGCCTGGTGGGAGCTCTGGAGAAGT
TTCATATTAAATCACGTATATGGCGCAGAATACAAGGAGACCTGGGAATCCAGAATGTTCAGGATATTGG
GACTGTTTCTCCCTGGTGTTGCAGCTCATAGTCCTGTGAATTATGTCAATTCTGTCAGGCTTGGAAGGGA
GCTTGCAATTCCCTTGATGTCTCTGAAAATGATGGCACAAGGTTACTAGAATTATCGTTATAAAGTCTAA
GAGATGATCATTAATGATAAATGGTTCACTCTTTGCCAAAAAAAGAAAAATAATCAAAAGGCTTACGCTA
TTGTAATAAAAGGATAGCTGTTTCATGAACAGGTCGCCTAGGGTTGTGGTGTGGAGCTTTGATATGCATA
TATGCATATATGGCCTGTTTGTTTGTCAGTTTGTTTTTCTCTTTAAACAAAATGAAATGCGGTAGTTCAA
TAAAAAGGAACGTTGAGTAGTTTTTGGGTTGCCAAAAAAAAAAAAAAAAA

Figure 1b>RcOBL1

MDDAGKITSTSHLIVSPDEGTFLDLFKHIVLSDLGSGAKFFRASDQRVPATAAYYSRWPVSVFICKILQL
FQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLIRLKIPKRGEADFISTIGYLDSRMDLHGTPMVSHQA
DEVISNADNPSLKEGHNSKIKGALGNRSLMDLCIMASKLAYENTKVVERVVAEHWKMHFVADYGGMNYFQ
DARNTHAFIFCDKPKDANLIVISFRGTGPFSIPNWCTDFDFSLVGLGDAGSVHVGFLEAMGLGHRNSISS
FETSINTKSPGSITELRKESEMAPDHLVWAYDGVYFLAASTLKGLLKDHKNAKFVVTGHSLGGALAILFT
CILEIQQETEVLDRLLNVYTFGQPRIGNYNLGYFMQNRLNFPERRYFRVVYCNDMVPRVPFDDVFFTFEH
FGTCIYYDSRFFGYFTKEEPSRNPFGIENAISAHITAWWELWRSFILNHVYGAEYKETWESRMFRILGLF
LPGVAAHSPVNYVNSVRLGRELAIPLMSLKMMAQGY

Figure 2

GATCTTCAAAGTAGTTTTGAACTCTCTGCTGAGGGAAAAAAAGAGTGAGGGAAGTGAGAAACCAAAAGCC
ATGGCTGCTTCTGCTACTACTAGCAATAATATTGCTCCAAACTTCTTGGTTGTTGACCCAAAAAAGGGAA
GAAAAAGAGACATATTCAAGTATTTGGTGAGGAAAGATGTGAAGAGTGGAATGAGTTTCTTGGATAGTTC
AGAGGAAGGAGTTAAAGGTGGCGCAGCAGTTGATCATAGGTGGATTTTATTGGTTTCTATCATCATTCGG
AGGGTTCTTGCGCTTATTGATACCCCATTAAAGTACCTTGGATATGTCATTGATTTCTTTCTCAACCTTA
TCTCCCAAAATAGTGGATTCTCTGGCATACTCAACAACTTTCTCCATGGAAACCTGAAGATACCGAGGAG
AGGAACAGAGAATTTTATAAGCACGATTGGGCAATTGGATGGGCGAATAGACCTTTATAGAACTACAATA
TTATCGGAGAAAGTAGATGATTCTGTTGCTACTGATGTTAACAACATTAAAGCAGAACTGGGTAATCGAT
ATCTCATGGATCTTTGTATCATGGCAGCCAAACTTGTCTATGAGAATGAGAAAGTTGCTCAAAATGTTGT
TGATCGTCACTGGAAGATGCATTTTGTGGCTTTCTACAACTGCTGGAATGAGTACCAAAAGCAAAACAAC
ACCCAAGTGTTCATATGTTGTGACAAGCCAAAGGATGCAAATTTGATAGTGGTCAGCTTTAGAGGAACAG
AACCATTTAATGCACAAGATTGGAGTACGGATTTTGATTTCTCGTGGTATGAAATCCCAAAAGTTGGAAA
GATCCATATTGGATTCTTAGAAGCTTTAGGTCTGGGCAACAGAAGTGACGCTACCACTTTCCAAACTCAC
CTTCAGAGGAAACATACAGGTTTCTTCCATCTAAATGGTGAGTCTGAAGGCAATATGACGGAATGGGCAA
AGAAGAGTGCATACTATGCTGTCGCGTTGAAGCTAAAGAGCTTACTGAAAGAACACAGGAATGCTAAATT
TATAGTCACTGGACATAGTTTAGGTGGAGCACTTGCAATATTGTTCCCGTCAATACTGGTTATACAGGAG
GAGACAGAGATGCTAAACAGGTTGCTGAACATATACACATTTGGGCAGCCAAGAATTGGAGATGCACAGC
TTGGAACTTTCATGGAGTCCCACTTGAATTATCCAGTTACTAGATACTTCAGGGTTGTTTACTGCAACGA
TATGGTGCCTAGAGTGCCTTTCGATGACAAGATTTTCGCTTTCAAGCATTTCGGTACATGTCTTTACTAT
GATAGCCGCTACTTTGGCCGATTTATGGATGAGGAGCCGAACAGAAATTATTTTGGACTGAGACACATAA
TTCCAATGCGGGTGAATGCATTATGGGAACTATTCAGAAGTTTTATGATAACCCATGCACATGGACCTGA
CTACCAGGAGAGTTGGTTCTGCACTCTTTCCAGGGTAGCAGGACTGGTGCTTCCTGGTGTTGCTGCTCAT
AGTCCTATAGATTATGTTAATTCAGTTAGGCTTGGAAAGGAGAGAGTAGCTCCAATGACATCCTTGAAAA
GCTTCGCTCGCAAGTCATAAATCTGGGTTGCACTTGTACTCTTCTTCATGGATGAGACACTGAACACAAA
GGAAAATAATAACAGGGTGCAGTTTAAAATGATCATAAGGGAAATAAATCTTATATTTCTTACTCTTACG
GAAATTTGATAATCTGTGACCTTGTGGTTGTGGGTAGTTCCAATTTAATTTCTTTTTCTTTTCAATAAAA
ATCCTGTACTTCGGTGATAATATGAATTATAGTGTGACTTTTTTGGTTGCCCATAGAAAAAAAAAAAAAA
AAAAAAAAAA

Figure 2b>RcOBL2

MAASATTSNNIAPNFLVVDPKKGRKRDIFKYLVRKDVKSGMSFLDSSEEGVKGGAAVDHRWILLVSIIIR
RVLALIDTPLKYLGYVIDFFLNLISQNSGFSGILNNFLHGNLKIPRRGTENFISTIGQLDGRIDLYRTTI
LSEKVDDSVATDVNNIKAELGNRYLMDLCIMAAKLVYENEKVAQNVVDRHWKMHFVAFYNCWNEYQKQNN
TQVFICCDKPKDANLIVVSFRGTEPFNAQDWSTDFDFSWYEIPKVGKIHIGFLEALGLGNRSDATTFQTH
LQRKHTGFFHLNGESEGNMTEWAKKSAYYAVALKLKSLLKEHRNAKFIVTGHSLGGALAILFPSILVIQE
ETEMLNRLLNIYTFGQPRIGDAQLGTFMESHLNYPVTRYFRVVYCNDMVPRVPFDDKIFAFKHFGTCLYY
DSRYFGRFMDEEPNRNYFGLRHIIPMRVNALWELFRSFMITHAHGPDYQESWFCTLSRVAGLVLPGVAAH
SPIDYVNSVRLGKERVAPMTSLKSFARKS

Figure 3 >RcOBL1 core hydrophobic region (50-105)

ATAAYYSRWPVSVFICKILQLFQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLI

Figure 4

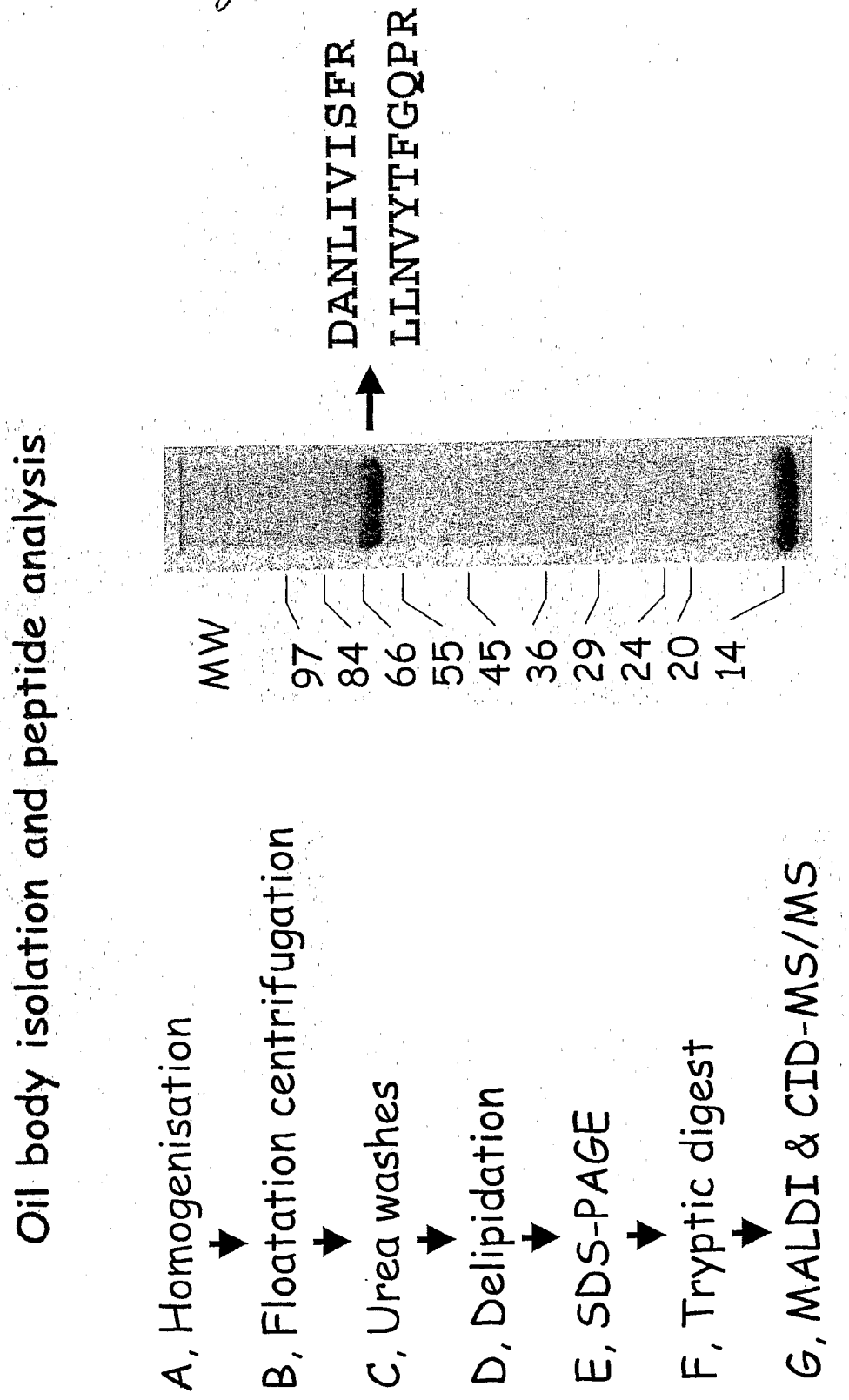

Oil body isolation and peptide analysis
A, Homogenisation
B, Floatation centrifugation
C, Urea washes
D, Delipidation
E, SDS-PAGE
F, Tryptic digest
G, MALDI & CID-MS/MS
MW
97
84
66
55
45
36
29
24
20
14
DANLIVISFR
LLNVYTFGQPR

Figure 5

Homology with *lipase-like* genes

Peptide 1	DANLIVISFR	Peptide 2	LLNVYTFGQPR
At3g14360	...DANLIVISFR...		...LLGVYTFGQPR...
Lettuce EST	...DANLIVISFR...		
Peach EST	...DANLILISFR...		
Sunflower EST			...LEGVYTFGQPR...
Soya EST			...LEGVYTFGQPR...

Degenerate primers:

5'-ttgatagtirtyagyttyaga and 5'-ctgtccraatgtrtaiarctt

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Figure 6

Castor oil body lipase 1 (OBL1)

```
MDDAGKITSTSHLIVSPDEGTFLDLFKHIVLSDLGSGAKFFRASDQRVPATAAYYSRWPV 60
SVFICKILQLFQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLIRLKIPKRGEADFIST 120
IGYLDSRMDLHGTPMVSHQADEVISNADNPSLKEGHNSKIKGALGNRSLMDLCIMASKLA 180
YENTKVVERVVAEHWKMHFVADYGGMNYFQDARNTHAFIFCDKPKDANLIVISFRGTGPF 240
SIPNWCTDFDFSLVGLGDAGSVHVGFLEAMGLGHRNSISSFETSINTKSPGSITELRKES 300
EMAPDHLVWAYDGVYFLAASTLKGLLKDHKNAKFVVTGHSLGGALAILFTCILEIQQETE 360
VLDRLLNVYTFGQPRIGNYNLGYFMQNRLNFPERRYFRVVYCNDMVPRVPFDDVFFTFEH 420
FGTCIYYDSRFFGYFTKEEPSRNPFGIENAISAHITAWWELWRSFILNHVYGAEYKETWE 480
SRMFRILGLFLPGVAAHSPVNYVNSVRLGRELAIPLMSLKMMAQGY
```

230-422 31% identical to *Rhizomucor miehei* lipase
222-410 29% identical to *Rhizopus oryzae* lipase
229-410 28% identical to *Humicola lanuginosa* lipase

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Figure 7

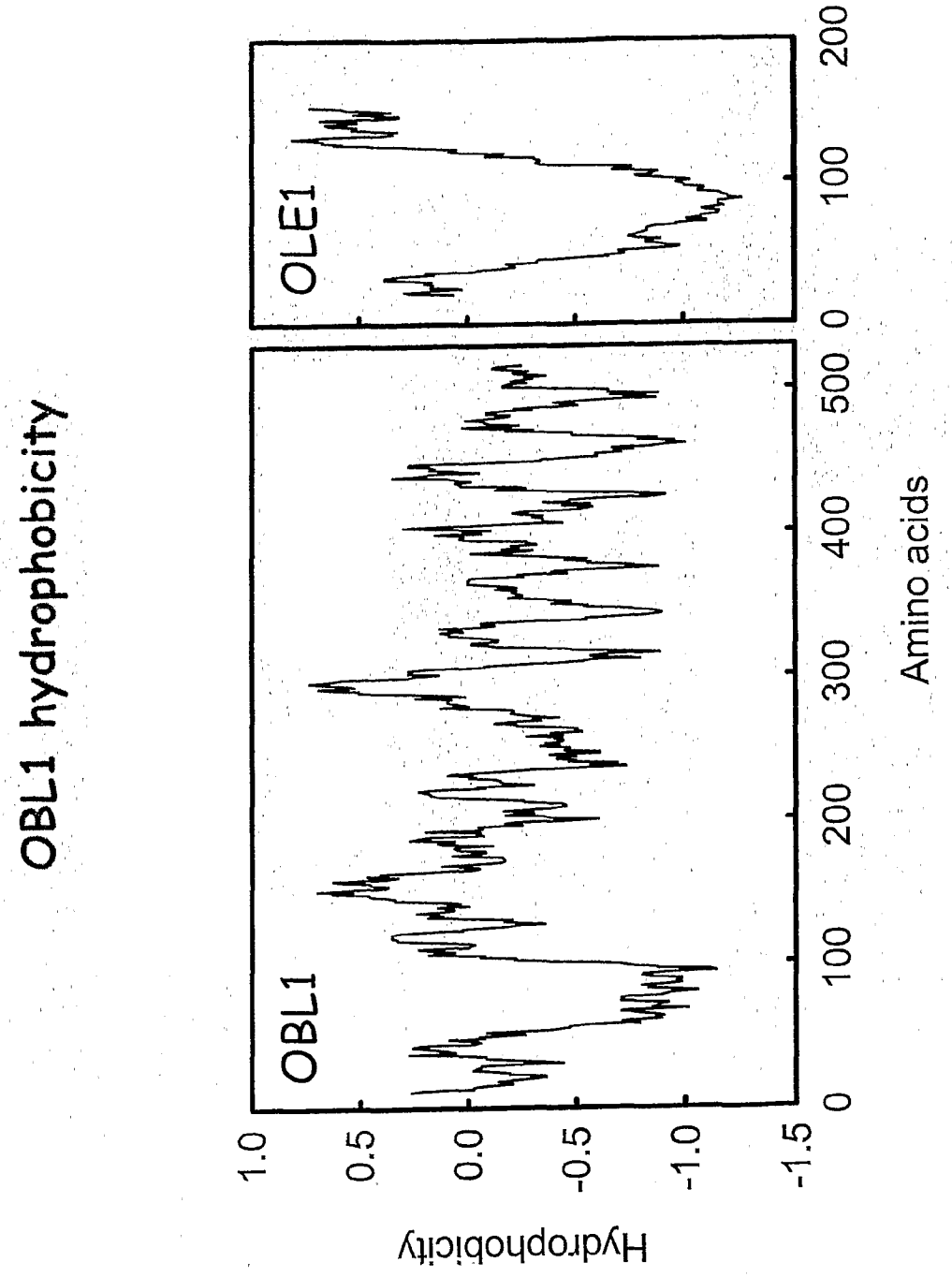

OBL1 hydrophobicity
OLE1
OBL1
1.0
0.5
0.0
-0.5
-1.0
-1.5
Hydrophobicity
0
100
200
300
400
500
0
100
200
Amino acids

Figure 8

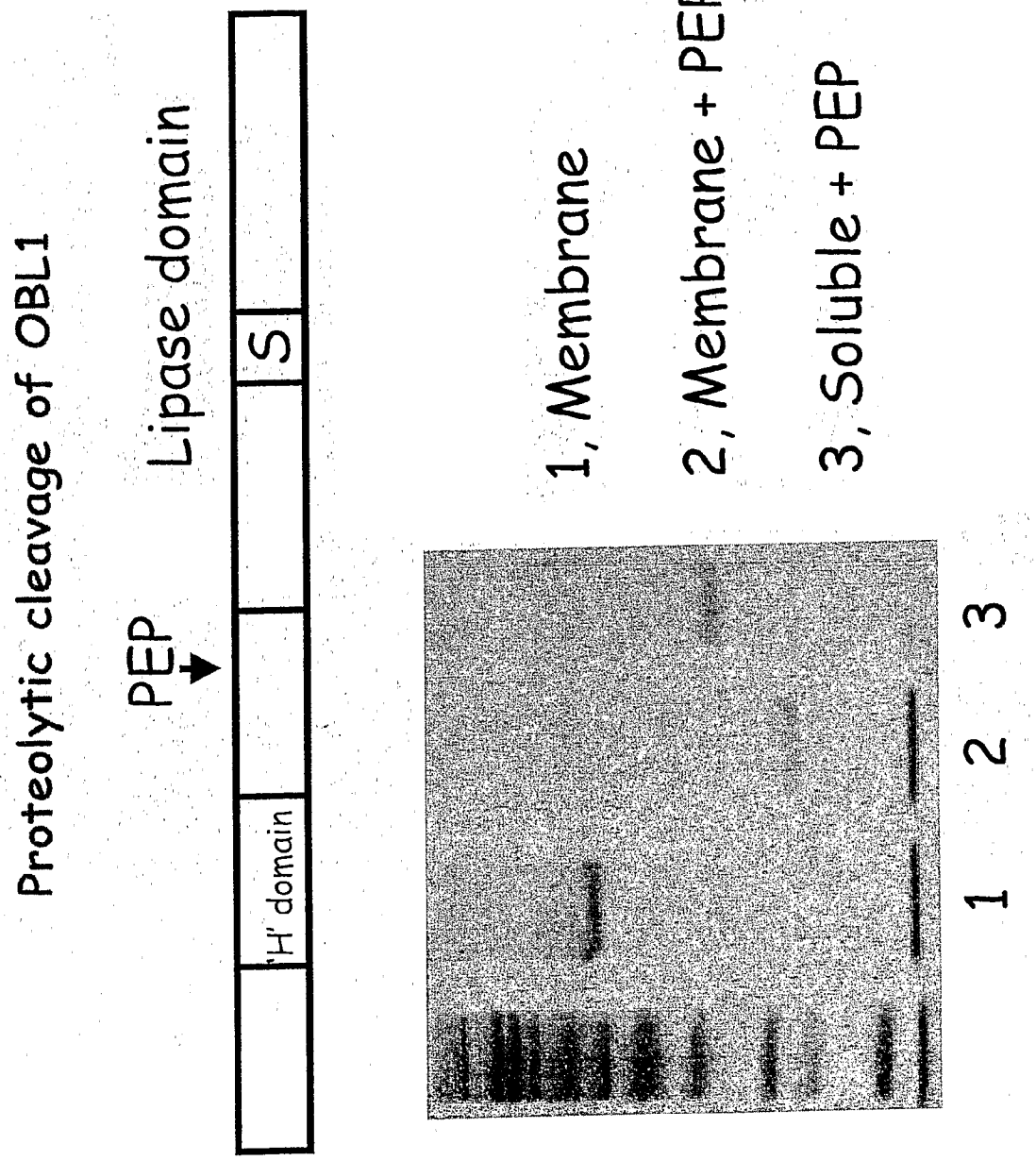

Proteolytic cleavage of OBL1
Lipase domain
S
PEP
'H' domain
1, Membrane
2, Membrane + PEP
3, Soluble + PEP
1 2 3

Figure 9

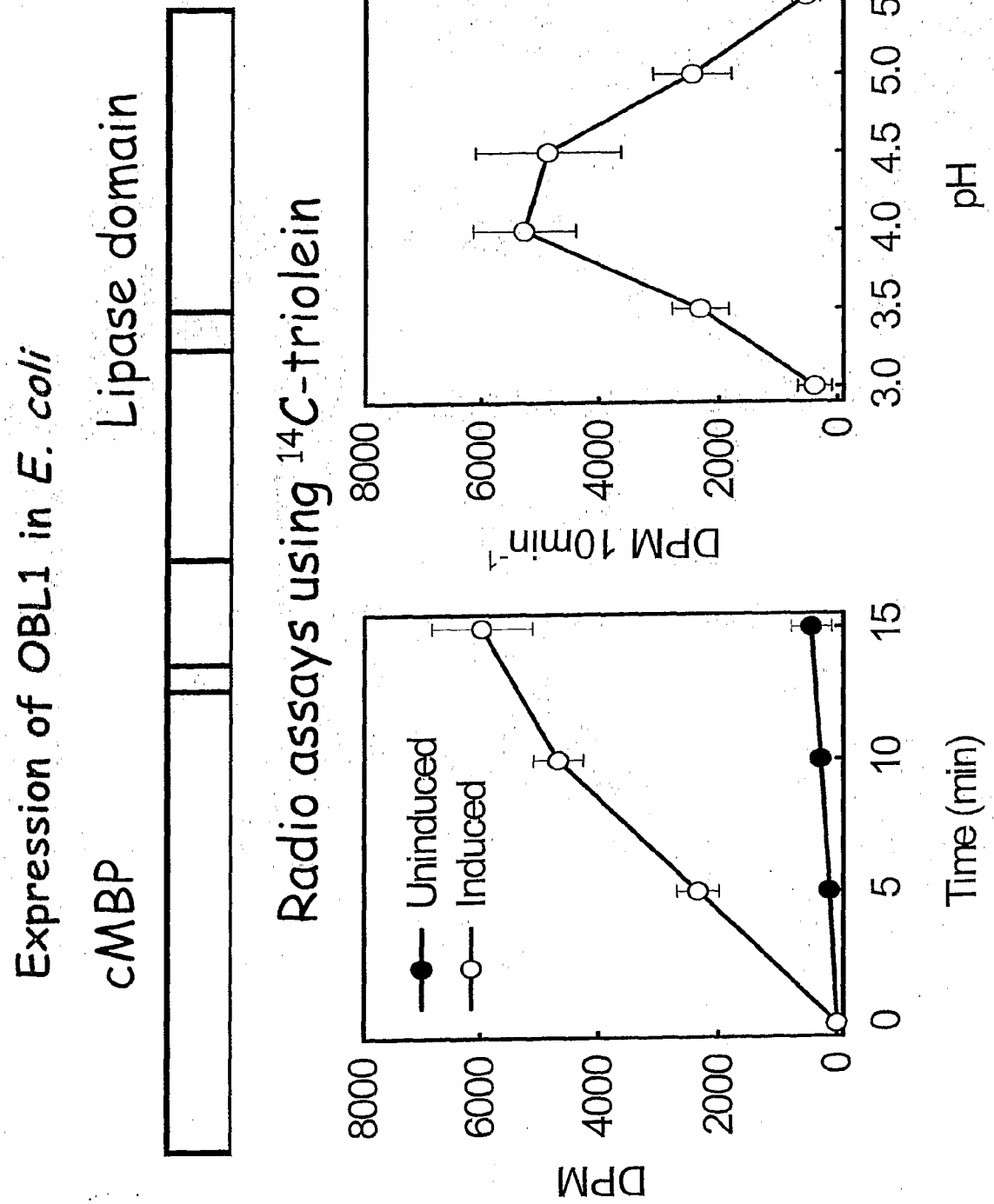

Expression of OBL1 in E. coli
cMBP
Lipase domain
Radio assays using ^{14}C-triolein
DPM
8000
6000
4000
2000
0
Uninduced
Induced
0
5
10
15
Time (min)
DPM 10min^{-1}
8000
6000
4000
2000
0
3.0
3.5
4.0
4.5
5.0
5.5
pH

Figure 10

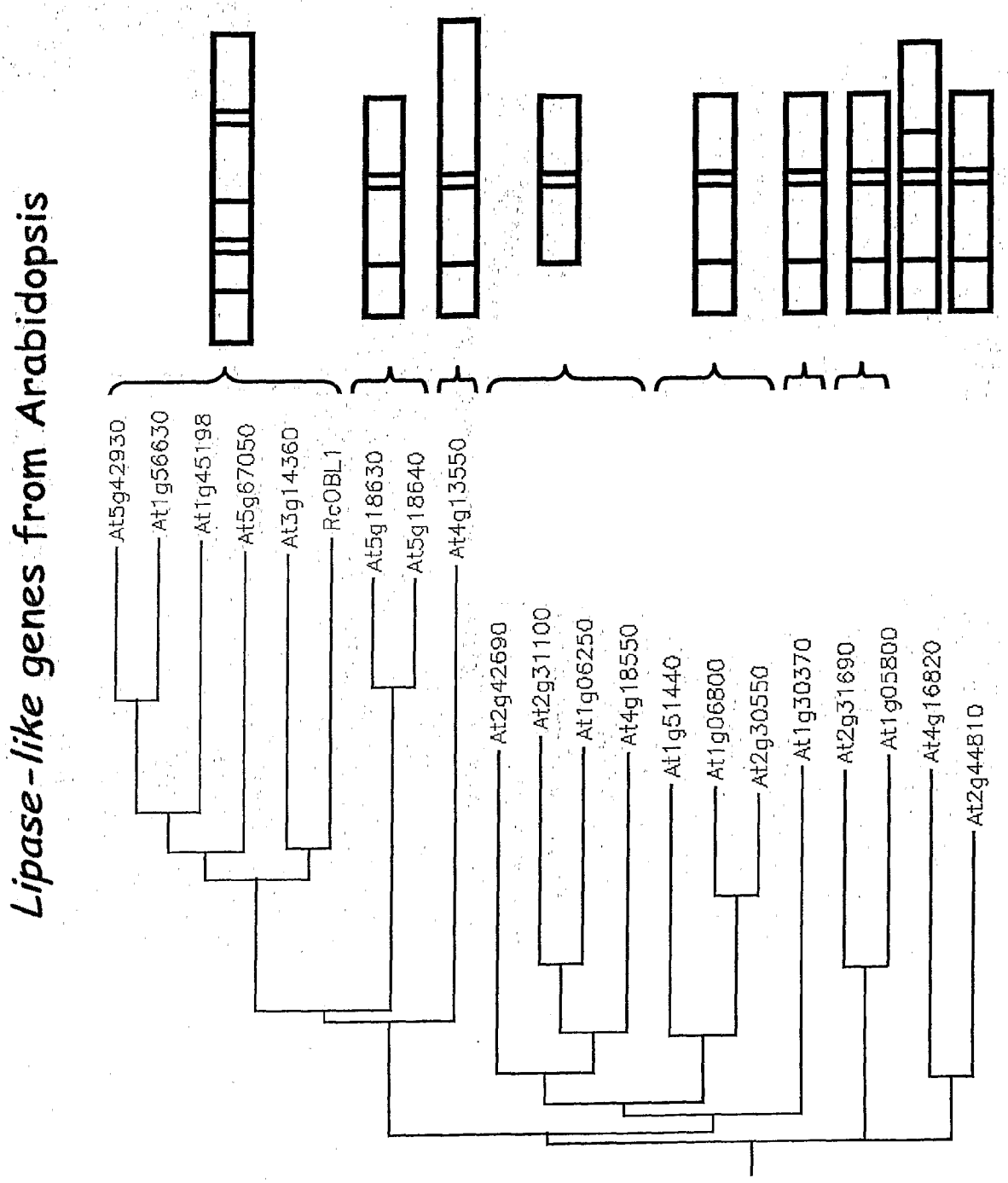
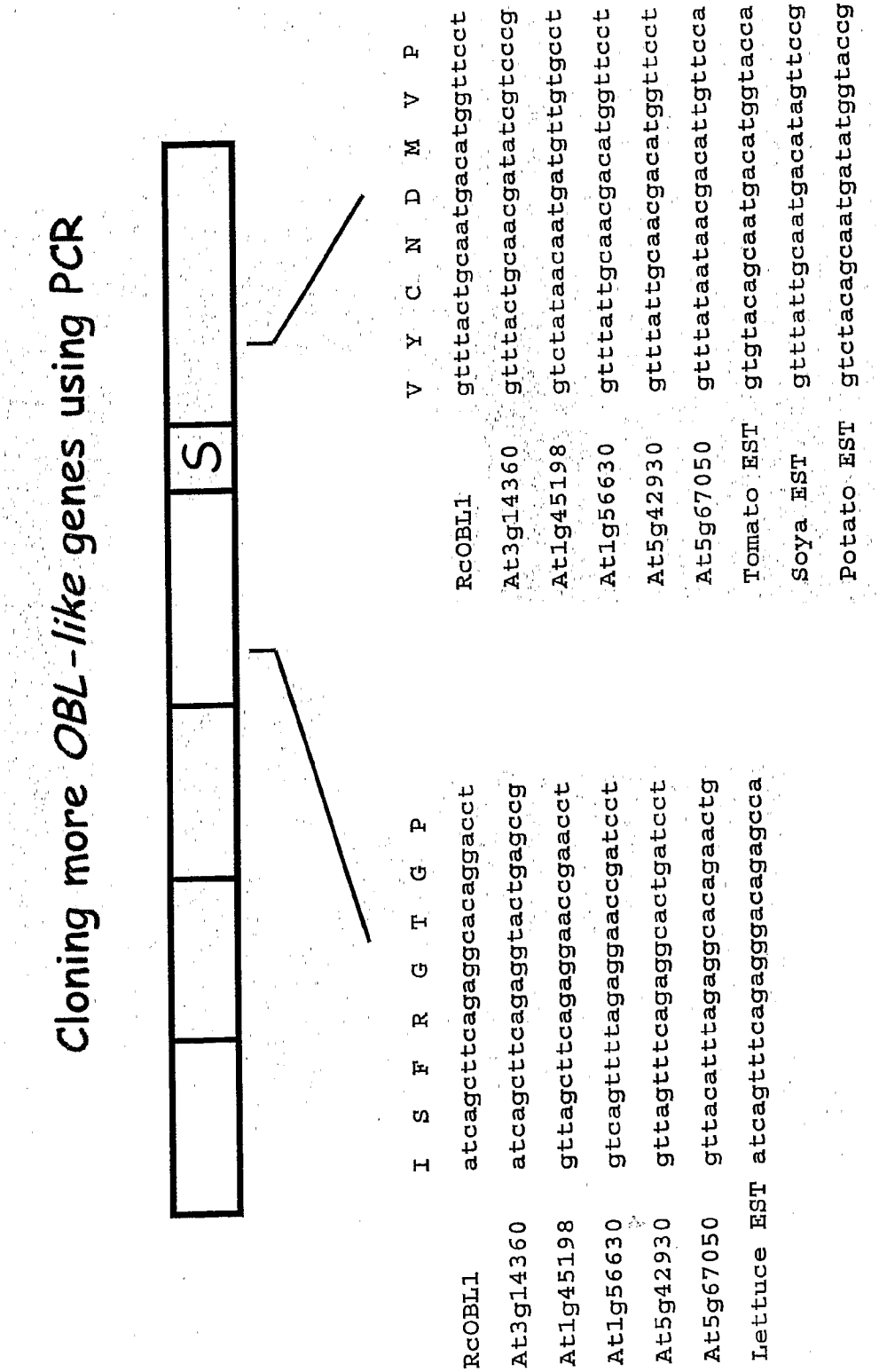

Lipase-like genes from Arabidopsis
At5g42930
At1g56630
At1g45198
At5g67050
At3g14360
RcOBL1
At5g18630
At5g18640
At4g13550
At2g42690
At2g31100
At1g06250
At4g18550
At1g51440
At1g06800
At2g30550
At1g30370
At2g31690
At1g05800
At4g16820
At2g44810

Figure 11


Cloning more OBL-like genes using PCR
S
I S F R G T G P
RcOBL1 atcagcttcagaggcacaggacct
At3g14360 atcagcttcagaggtactgagccg
At1g45198 gttagcttcagaggaaccgaacct
At1g56630 gtcagttttagaggaaccgatcct
At5g42930 gttagtttcagaggcactgatcct
At5g67050 gttacatttagaggcacagaactg
Lettuce EST atcagtttcagagggacagagcca
V Y C N D M V P
RcOBL1 gtttactgcaatgacatggttcct
At3g14360 gtttactgcaacgatatcgtcccg
At1g45198 gtctataacaatgatgtttgtgcct
At1g56630 gtttattgcaacgacatggttcct
At5g42930 gtttattgcaacgacatggttcct
At5g67050 gtttataataacgacattgttcca
Tomato EST gtgtacagcaatgacatggtacca
Soya EST gtttattgcaatgacatagttccg
Potato EST gtctacagcaatgatatggtaccg

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Castor *OBL2*

```
MAASATTSNNIAPNFLVVDPKKGRKRDIFKYLVRKDVKSGMSFLDSSEEGVKGGAAVDHR 60
WILLVSIIIRRVLALIDTPLKYLGYVIDFFLNLISQNSGFSGILNNFLHGNLKIPRRGTE 120
NFISTIGQLDGRIDLYRTTILSEKVDDSVATDVNNIKAELGNRYLMDLCIMAAKLVYENE 180
KVAQNVVDRHWKMHFVAFYNCWNEYQKQNNTQVFICCDKPKDANLIVVSFRGTEPFNAQD 240
WSTDFDFSWYEIPKVGKIHIGFLEALGLGNRSDATTFQTHLQRKHTGFFHLNGESEGNMT 300
EWAKKSAYYAVALKLKSLLKEHRNAKFIVTGHSLGGALAILFPSILVIQEETEMLNRLLN 360
IYTFGQPRIGDAQLGTFMESHLNYPVTRYFRVVYCNDMVPRVPFDDKIFAFKHFGTCLYY 420
DSRYFGRFMDEEPNRNYFGLRHIIPMRVNALWELFRSFMITHAHGPDYQESWFCTLSRVA 480
GLVLPGVAAHSPIDYVNSVRLGKERVAPMTSLKSFARKS
```

OBL1 and OBL2 are 53% identical

Figure 12

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Figure 13

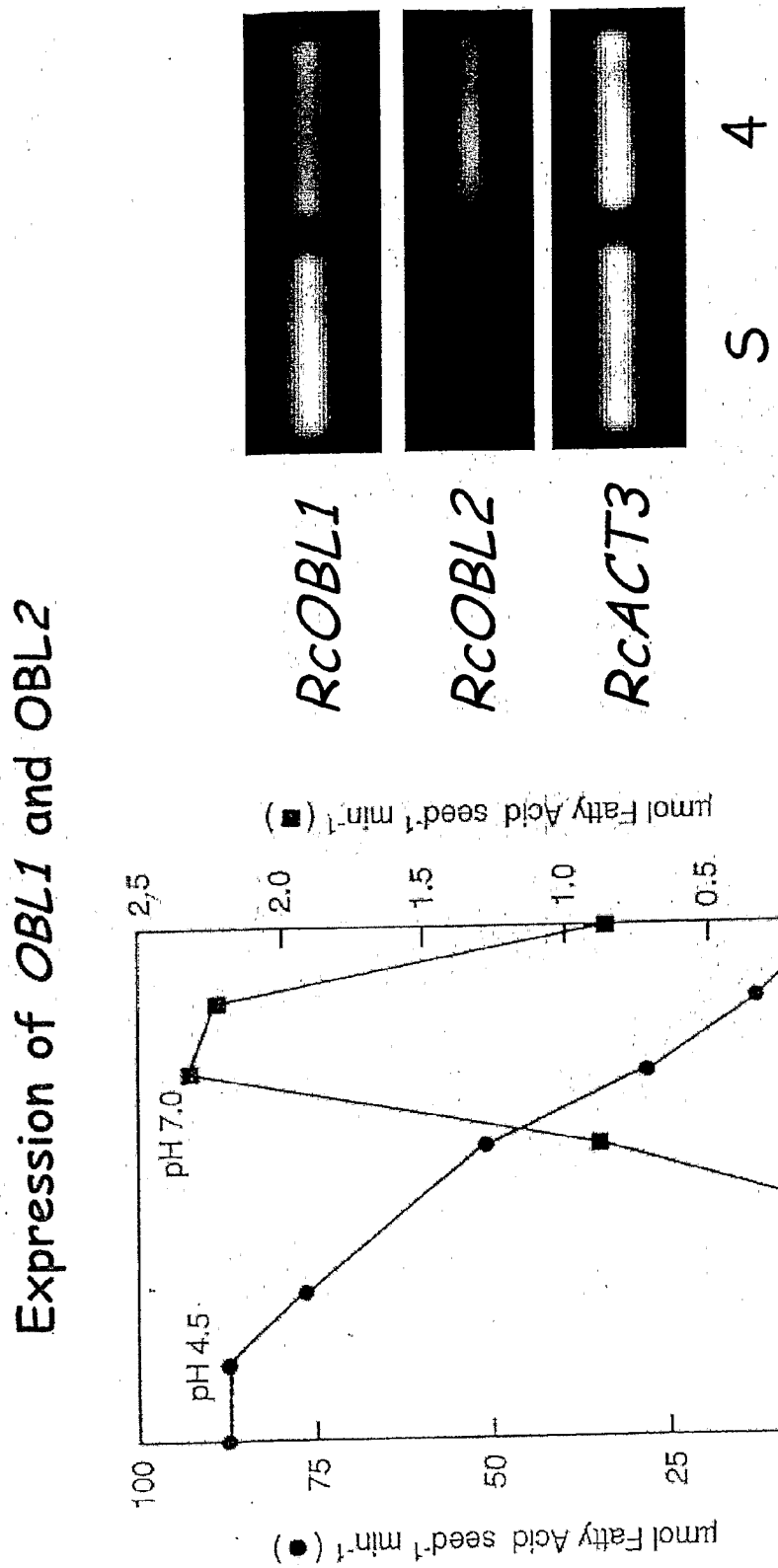

Expression of OBL1 and OBL2
RcOBL1
RcOBL2
RcACT3
S 4
µmol Fatty Acid seed-1 min-1 (■)
2.5
2.0
1.5
1.0
0.5
0
pH 7.0
pH 4.5
100
75
50
25
0
D S 1 2 3 4 5 6
Days After Planting
µmol Fatty Acid seed-1 min-1 (●)

Fig. 14

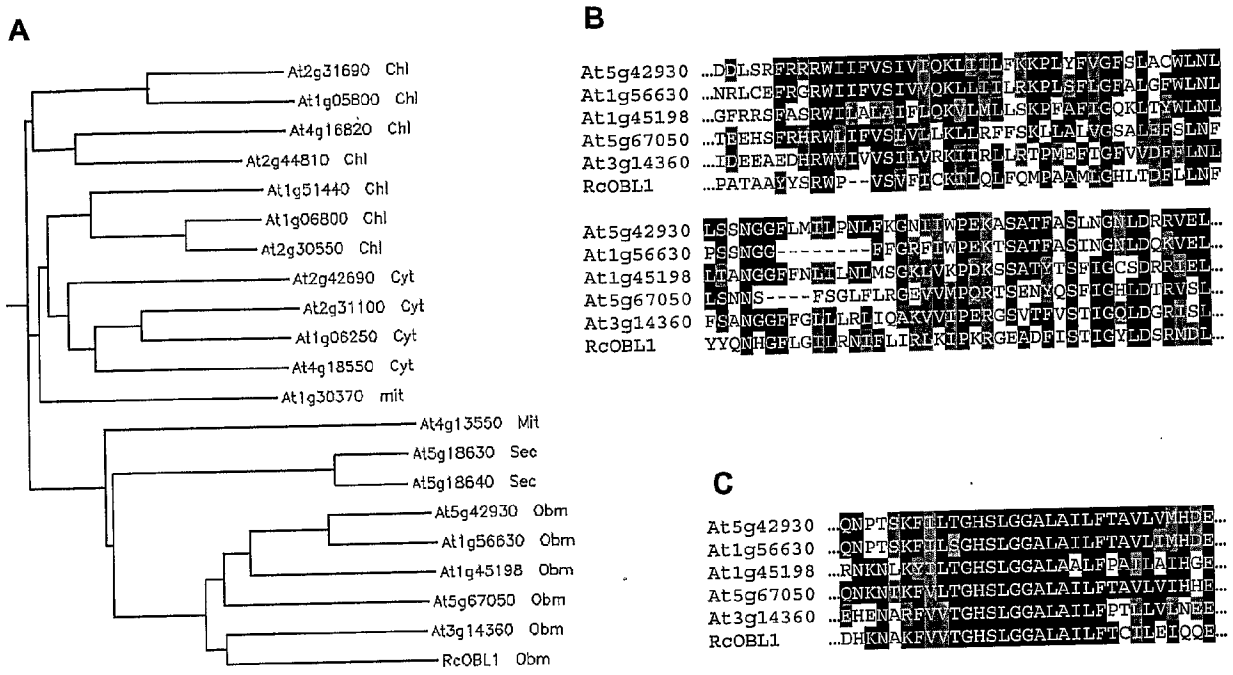

A
At2g31690 Chl
At1g05800 Chl
At4g16820 Chl
At2g44810 Chl
At1g51440 Chl
At1g06800 Chl
At2g30550 Chl
At2g42690 Cyt
At2g31100 Cyt
At1g06250 Cyt
At4g18550 Cyt
At1g30370 mit
At4g13550 Mit
At5g18630 Sec
At5g18640 Sec
At5g42930 Obm
At1g56630 Obm
At1g45198 Obm
At5g67050 Obm
At3g14360 Obm
RcOBL1 Obm
B
At5g42930
At1g56630
At1g45198
At5g67050
At3g14360
RcOBL1
C
At5g42930
At1g56630
At1g45198
At5g67050
At3g14360
RcOBL1

Figure 15A

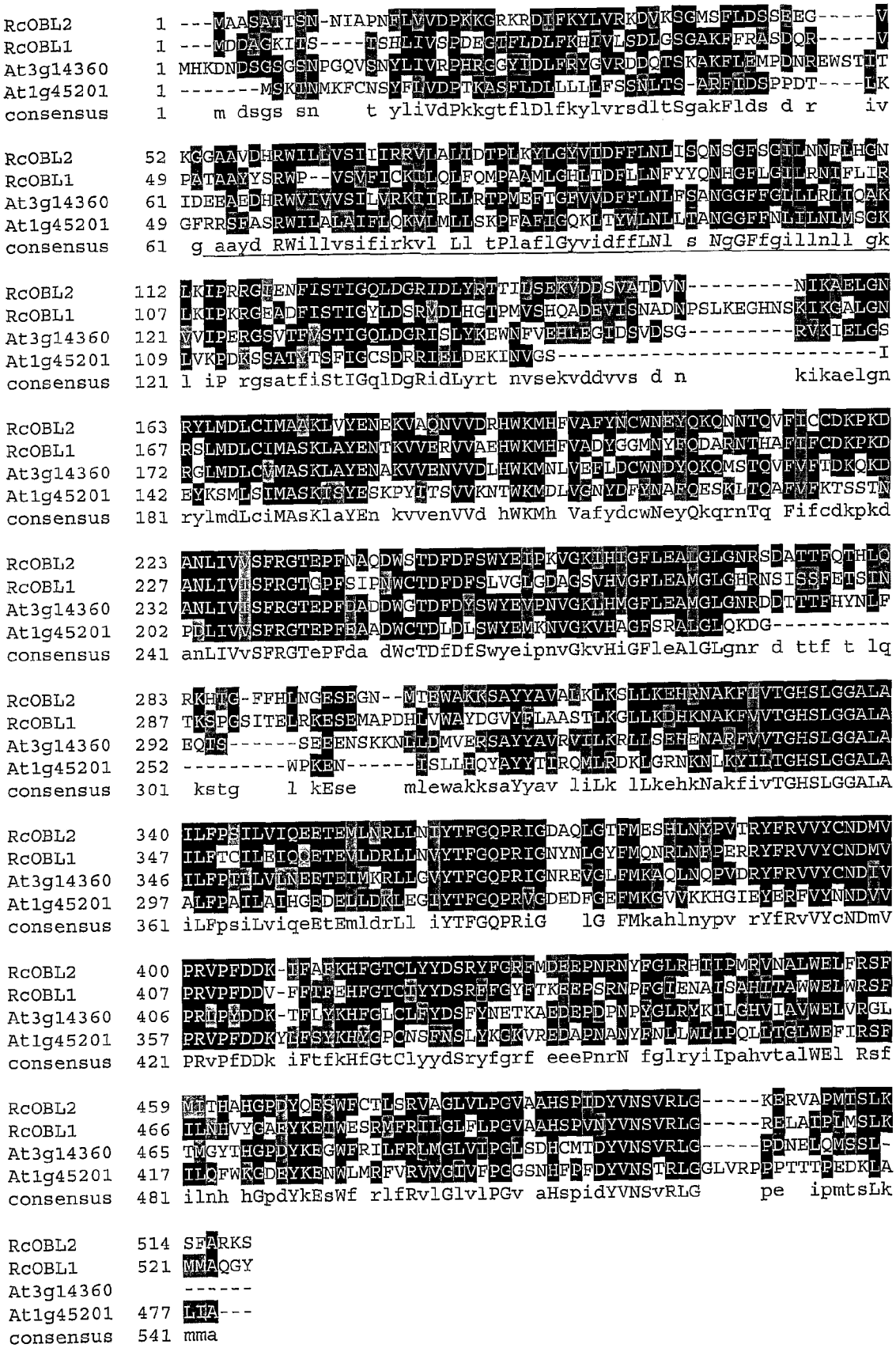

RcOBL2 1 ---MAASATTSN-NIAPNFLVVDPKKGRKRDIFKYLVRKDVKSGMSFLDSSEEG-----V
RcOBL1 1 ---MDDAGKITS----TSHLIVSPDEGTFLDLFKHIVLSDLGSGAKFFRASDQR-----V
At3g14360 1 MHKDNDSGSGSNPGQVSNYLIVRPHRCGYIDLFRYGVRDDQTSKAKFLEMPDNREWSTIT
At1g45201 1 -------MSKTNMKFCNSYFLVDFTKASFLDLLLLLFSSNLTS-ARFIDSPPDT----LK
consensus 1 m dsgs sn t yliVdPkkgtflDlfkylvrsdltSgakFlds d r iv
RcOBL2 52 KGGAAVDHRWILLVSIIIRRVLALIDTPLKYLGYVIDFFLNLISQNSGFSGILNNFLHGN
RcOBL1 49 PATAAMYSRWP--VSVFICKTLQLFQMPAAMLGHLTDFLLNFYYQNHGFLGILRNIFLIR
At3g14360 61 IDEEAEDHRWVIVVSILVRKTIRLLRTPMEFTGFVVDFFLNLFSANGGFFGLLLRLIQAK
At1g45201 49 GFRRSFASRWILALAIFLQKVLMLLSKPFAFIGQKLTYWLNLLTANGGFFNLILNLMSGK
consensus 61 g aayd RWillvsifirkvl Ll tPlaflGyvidffLNl s NgGFfgillnll gk
RcOBL2 112 LKIPRRGIENFISTIGQLDGRIDLYRTTILSEKVDDSVATDVN---------NIKAELGN
RcOBL1 107 LKIPKRGEADFISTIGYLDSRVDLHGTPMVSHQADEVISNADNPSLKEGHNSKIKGALGN
At3g14360 121 VVIPERGSVTFVSTIGQLDGRISLYKEWNFVEHLEGIDSVDSG---------RVKIELGS
At1g45201 109 LVKPDKSSATYTSFIGCSDRRIELDEKINVGS--------------------------I
consensus 121 l iP rgsatfiStIGqlDgRidLyrt nvsekvddvvs d n kikaelgn
RcOBL2 163 RYLMDLCIMAAKLVYENEKVAQNVVDRHWKMHFVAFYNCWNEYQKQNNTQVFICCDKPKD
RcOBL1 167 RSLMDLCIMASKLAYENTKVVERVVAEHWKMHFVADYGGMNYFQDARNTHAFIFCDKPKD
At3g14360 172 RGLMDLCVMASKLAYENAKVVENVVDLHWKMNLVEFLDCWNDYQKQMSTQVFVFTDKQKD
At1g45201 142 EYKSMLSIMASKISYESKPYITSVVKNTWKMDLVGNYDFYNAFQESKLTQAFVFKTSSTN
consensus 181 rylmdLciMAsKlaYEn kvvenVVd hWKMh VafydcwNeyQkqrnTq Fifcdkpkd
RcOBL2 223 ANLIVVSFRGTEPFNAQDWSTDFDFSWYEIPKVGKIHIGFLEALGLGNRSDATTFQTHLQ
RcOBL1 227 ANLIVISFRGTGPFSIPNWCTDFDFSLVGLGDAGSVHVGFLEAMGLGHRNSISSFETSIN
At3g14360 232 ANLIVISFRGTEPFDADDWGTDFDYSWYEVPNVGKLHMGFLEAMGLGNRDDTTTFHYNLF
At1g45201 202 PDLIVVSFRGTEPFEAADWCTDLDLSWYEMKNVGKVHAGFSRALGLQKDG----------
consensus 241 anLIVvSFRGTePFda dWcTDfDfSwyeipnvGkvHiGFleAlGLgnr d ttf t lq
RcOBL2 283 RKHTG-FFHLNGESEGN--MTEWAKKSAYYAVALKLKSLLKEHRNAKFIVTGHSLGGALA
RcOBL1 287 TKSPGSITELRKESEMAPDHLVWAYDGVYFLAASTLKGLLKDHKNAKFVVTGHSLGGALA
At3g14360 292 EQTS------SEEENSKKNLLDMVERSAYYAVRVILKRLLSEHENARFVVTGHSLGGALA
At1g45201 252 ---------WPKEN------TSLLHQYAYYTLRQMLRDKLGRNKNLKYIITGHSLGGALA
consensus 301 kstg l kEse mlewakksaYyav liLk lLkehkNakfivTGHSLGGALA
RcOBL2 340 ILFPSILVIQEETEMLNRLLNIYTFGQPRIGDAQLGTFMESHLNYPVTRYFRVVYCNDMV
RcOBL1 347 ILFTCILEIQQETEVLDRLLNVYTFGQPRIGNYNLGYFMQNRLNFPERRYFRVVYCNDMV
At3g14360 346 ILFPIILVLNEETETMKRLLGVYTFGQPRIGNREVGLFMKAQLNQPVDRYFRVVYCNDIV
At1g45201 297 ALFPAILAIHGEDELLDKLEGIYTFGQPRVGDEDFGEFMKGVVKKHGIEYERFVYNNDVV
consensus 361 iLFpsiLviqeEtEmldrLl iYTFGQPRiG lG FMkahlnypv rYfRvVYcNDmV
RcOBL2 400 PRVPFDDK-IFAFKHFGTCLYYDSRYFGRFMDEEPNRNYFGLRHTIPMRVNALWELFRSF
RcOBL1 407 PRVPFDDV-FFTFEHFGTCIYYDSRFFGYFTKEEPSRNPFGIENAISAHITAWWELWRSF
At3g14360 406 PRIPYDDK-TFLYKHFGLCLEYDSFYNETKAEDEPDPNPYGLRYKILGHVIAVWELVRGL
At1g45201 357 PRVPFDDKYLFSYKHYGPCNSFNSLYKGKVREDAPNANYFNLLWLIPQLLTGLWEFIRSF
consensus 421 PRvPfDDk iFtfkHfGtClyydSryfgrf eeePnrN fglryiIpahvtalWEl Rsf
RcOBL2 459 MITHAHGPDYQESWFCTLSRVAGLVLPGVAAHSPIDYVNSVRLG-----KERVAPMTSLK
RcOBL1 466 ILNHVYGAEYKETWESRMFRILGLFLPGVAAHSPVNYVNSVRLG-----RELAIPLMSLK
At3g14360 465 TMGYTHGPDYKEGWFRILFRLMGLVIPGLSDHCMTDYVNSVRLG-----PDNELQMSSL-
At1g45201 417 ILQFWKGDEYKENWLMRFVRVVGIVFPGGSNHFPFDYVNSTRLGGLVRPPPTTTPEDKLA
consensus 481 ilnh hGpdYkEsWf rlfRvlGlvlPGv aHspidYVNSvRLG pe ipmtsLk
RcOBL2 514 SFARKS
RcOBL1 521 MMAQGY
At3g14360 ------
At1g45201 477 LIA---
consensus 541 mma

Figure 15B

Sense primers:

	L--	I--	I--	V--	S--	F--	R--	G--	T--	D--	P--	F--
At1g45201	ctc	atc	gtc	gtt	agc	ttc	aga	gga	acc	gaa	cct	ttc
At3g14360	ttg	ata	gta	atc	agc	ttc	aga	ggt	act	gag	ccg	ttt
Lettuce est	ttg	ata	gtg	atc	agt	ttc	aga	ggg	aca	gag	cca	ttc
At1g56630	ctt	atc	gtc	gtc	agt	ttt	aga	gga	acc	gat	cct	ttc
At5g42930	ctc	atc	atc	gtt	agt	ttc	aga	ggc	act	gat	cct	ttc

RtY agY ttY aga ggI acH gaR c
RtY agY ttY aga ggI acH gaY c
RtY agY ttY aga ggc acH gaR c
RtY agY ttY aga ggc acH gaY c

Antisense primers:

	V--	Y--	C--	N--	D--	M--	V--	P--	R--	V--	P--
RcOBL1	gtt	tac	tgc	aat	gac	atg	gtt	cct	agg	gtg	cct
At3g14360	gtt	tac	tgc	aac	gat	atc	gtc	ccg	aga	ttg	ccc
At1g45198	gtc	tat	aac	aat	gat	gtt	gtg	cct	aga	gtg	ccc
At5g42930	gtt	tat	tgc	aac	gac	atg	gtt	cct	agg	ttg	cct
At1g56630	gtt	tat	tgc	aac	gac	atg	gtt	cct	agg	ttg	cct
At5g67050	gtt	tat	aat	aac	gac	att	gtt	cca	aga	ttg	ccg
tomato est	gtg	tac	agc	aat	gac	atg	gta	cca	aga	ttg	cca
soya est	gtt	tat	tgc	aat	gac	ata	gtt	ccg	agg	ttg	ccc
potato est	gtc	tac	agc	aat	gat	atg	gta	ccg	aga	atg	cca

Primer1 gtB taY aaY aaY gaY atg gtI c (rev. comp. g Iac cat Rtc Rtt Rtt Rta Bac)
Primer2 gtB taY tgY aaY gaY atg gtI c (rev. comp. g Iac cat Rtc Rtt Rca Rta Bac)
Primer3 gtB taY aaY aaY gaY RtI gtI c (rev. comp. g Iac IaY Rtc Rtt Rtt Rta Bac)
Primer4 gtB taY tgY aaY gaY RtI gtI c (rev. comp. g Iac IaY Rtc Rtt Rca Rta Bac)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/002215

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/82 C12N9/20 C12N5/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBASE, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ALTAF A ET AL: "Acid lipase of castor bean lipid bodies: Isolation and characterisation" JOURNAL OF PLANT BIOCHEMISTRY AND BIOTECHNOLOGY, vol. 6, no. 1, 1997, pages 13-18, XP009037294 ISSN: 0971-7811 the whole document ----- -/--	1-40

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
1 October 2004	18/10/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mundel, C

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Form PCT/ISA/210 (second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB2004/002215

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WANG ET AL: "Cloning and expression of phosphatidylcholine-hydrolyzing phospholipase D from Ricunius communis L" JOURNAL OF BIOLOGICAL CHEMISTRY, AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, US, vol. 269, no. 32, 12 August 1994 (1994-08-12), pages 20315-20317, XP002087693 ISSN: 0021-9258 abstract	1,7,9, 10,14, 17,25
X	WO 99/55883 A (RAFALSKI J ANTONI ; DU PONT (US); CAHOON EDGAR B (US); CAHOON REBECCA) 4 November 1999 (1999-11-04) abstract page 2, line 4 - line 28 sequences 26,29-32 page 15, line 28 - page 16, line 7	1,7, 9-18,25

2

Form PCT/ISA/210 (continuation of second sheet) (January 2004)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2004/002215

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. type of material

   [X] a sequence listing

   [ ] table(s) related to the sequence listing

   b. format of material

   [X] in written format

   [X] in computer readable form

   c. time of filing/furnishing

   [ ] contained in the international application as filed

   [ ] filed together with the international application in computer readable form

   [X] furnished subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB2004/002215

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 9955883 A	04-11-1999	AU	3967599 A	16-11-1999
		BR	9909605 A	11-09-2001
		EP	1075527 A2	14-02-2001
		WO	9955883 A2	04-11-1999
		US	2004148653 A1	29-07-2004

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: ICE CONFECTION AND ITS MANUFACTURING PROCESS

(57) Abstract: An ice confection containing; (i) at least 2% by wt. fat; (ii) at least 10% by wt. of a sugar or sugars; and (iii) protein, which is present at a level of less than 2 % by weight; wherein some or all of the fat and protein are present as oil bodies.

WO 2005/013713 A1

Ice Confection and its manufacturing process

Technical field of the invention

The invention relates to ice confections and their manufacturing process, in particular to low cost ice confections which contain oil bodies.

Background to the invention

Frozen confections or "ice confections" such as ice cream are well known. However, standard ice cream is too expensive for many consumers to eat every day. Also, the presence of high levels of saturated fat, common to many ice confections, is unattractive to many consumers from a health perspective for an "every day" product.

Typically ice cream will contain, by weight of the composition, 10-18 % fat, 7-11.5 % milk solids not fat (MSNF), 15-18% sugars and other ingredients such as stabilisers, emulsifiers and flavourings (Ice Cream, Fourth Edition by W.S. Arbuckle, Pub. Van Nostrand Reinhold, New York, 1986, p 381). However, the precise composition of ice cream products varies from market to market. One reason for this is that the legal definition of ice cream (in terms of ingredients and formulation) differs from country to country.

A significant portion of the overall cost of these formulations is the expense of the ingredients, and a particularly high percentage of this cost is the cost of MSNF. MSNF contains casein micelles and whey proteins which contribute to the stabilisation of the fat emulsion and the air phase; MSNF also contains lactose. It is the

stabilisation of the air phase which makes it possible for ice cream to have a typical overrun of around 100% and, as a result, a light texture.

The stabilisation of the fat emulsion is important as it is the presence of this emulsion which delivers the 'creamy' mouth feel characteristic of ice cream products.

To reduce the cost of an ice cream confection, it would therefore be desirable to produce confections which contain less MSNF, or in which this ingredient is entirely absent. In addition, for every day confections it would be advantageous if the product could include polyunsaturated fats, which are perceived as a 'healthy' alternative to the saturated fats commonly found in ice confection products.

Cheaper alternatives to ice cream include milk ices and water ices. Milk ices typically contain around 2 wt% fat and 3-5 wt% MSNF. However, as a result, the overrun of these products is typically only about 10-30%; therefore they do not have a light texture. Water ices provide an even cheaper ice confection product, typically containing none of the costly fat or MSNF. They are unaerated, and hence typically have an overrun of 10 % or less; they are neither creamy nor light in texture.

Schlegel et al. (EP 1 180 330) describes a low cost ice confection where the MSNF is replaced with starch to reduce the cost of the product without loss of product creaminess. The ice confection comprises fatty matter, sweetening agent, MSNF, water and starch, and the total amount of starch and MSNF is in the range 2.5 wt% to 18 wt%.

"Ice cream" (i.e. ice confections claiming to have some of the properties of traditional ice cream) compositions containing

no MSNF are also known in the art. Many of these formulations are aimed at providing 'ingredient free' products aimed at consumers with food intolerances or allergies. For example, Minoru et al. (JP 11 253 104) discloses a protein free "ice cream" comprising butter oil as a lipid source; a saccharide sweetener and melting point depressant; and a polysaccharide. The polysaccharide is present in this composition in place of protein.

Gonsalves et al. (US 5,384,145) relates to low fat frozen toppings which are non-dairy, aerated, exhibit a high over run (greater than 250%) and have a high solids content (38-50 wt%). These compositions comprise 8-15 wt% fat. This formulation is stabilised through precise control of the ratio of emulsifier to fat and water soluble protein.

Riviere et al. (WO 97/30600) relates to a soft frozen dessert comprising a fat with a low freezing point (sunflower oil); dairy protein, sugars and stabilisers.

None of the documents above relate to an ice confection which is both low cost and which will be perceived by the general consumer as a 'healthy' alternative to other ice confections. Moreover, none relate to an ice confection that has been designed to appeal as an "everyday" product by virtue of both its low cost and the presence of a healthy oil phase, containing for example unsaturated fats, anti-oxidants or vitamins such as vitamin E. Oil bodies are a low cost alternative to traditional ingredients, in part because they are pre-emulsified. This eliminates the need for an emulsification step during processing and reduces or eliminates the amount of MSNF (or other protein ingredient) that needs to be added to the formulation. In addition, the fats found in oil bodies tend to be unsaturated, and often contain vitamins which are not present in typical 'fat'

mixtures. For example, oil bodies extracted from sunflower seeds contain oil which is about 70% polyunsaturated, and also vitamin E. Therefore, the addition of oil bodies to ice confection products also provides a health aspect which has not been offered before. The resultant ice confection products also have a highly acceptable taste.

Methods for the extraction of oil bodies from a range of plant seeds are known in the art. For example, Deckers et al. (US 6,146,645) concerns the extraction of oil bodies from plant seeds, for example sunflower seeds, and the use of oil bodies in a range of industries including the food industry. Ice cream compositions are said to be a possible end utility for the oil bodies so produced, but no further details are provided. The contents of this document, particularly in as far they relate to the detection, nature of, preparation and processing of oil bodies are specifically incorporated herein by reference.

Wakabayashi et al. (EP 0 883 997) discusses the extraction of lipid/protein complexes (oil bodies) from seeds. Again, the contents of this document, particularly in as far they relate to the detection, nature of, preparation and processing of oil bodies are specifically incorporated herein by reference.

Methods for the extraction of lipid/protein isolates from a range of other plant sources and their use in ice confections are also known in the art. It is possible that some of these extracts or isolates contain oil bodies. For example, Juillerat et al. (US 6,383,550) discloses the extraction of lipid and protein extracts from fruit kernels and their use in food products such as ice cream. It is possible that this extract would contain oil bodies. However, the extract described therein has a lipid/protein ratio of 0.05 to 3.5. Only one example of an ice cream product is described (example

7), and this is thought to have a protein content of approximately 4%.

Goodnight Jr et al. (US 4,088,795) discloses the removal of soluble carbohydrate from an oil seed-lipid containing emulsion in order that the emulsion, when used in food products, is more easily digested.

There continues, however, to be a need for 'every day' ice confections which are inexpensive and relatively healthy. To achieve this it is desirable to develop an ice confection which has some or all of the characteristics of ice cream, but which contains reduced amounts of MSNF, or perhaps in some embodiments no MSNF at all; and contains low amounts of (or no) other protein ingredients. Moreover, the total protein content in the ice confection should be kept to a workable minimum so that the ingredient costs are low.

The applicant has found that the use of oil bodies in ice confection products can provide this.

In a preferred aspect, the use of oil bodies in ice confection compositions containing an aerating agent can produce ice confection products which have a light texture and creamy mouth feel, but which contain reduced levels of or even no MSNF.

The products of the invention typically have a low total solids content, and provide health benefits as oil bodies typically comprise polyunsaturated oils, such as are found for example in sunflower oil, which are healthier than the saturated fats often used in frozen confectionery products. In addition, oil bodies are less refined than the purified oil on which they are based, allowing desirable components such as vitamin E to be present in the final formulation.

One advantageous aspect of the invention is therefore the production of an ice confection which contains reduced amounts of MSNF (e.g. at most 5% by weight of the composition), or no MSNF at all.

A further advantage is to be able to produce frozen aerated confectionery products which improve on deficiencies of prior art products, and which furthermore may be both cheaper to produce and healthier.

Brief description of the invention

It is a first object of the present invention to provide an ice confection containing;

i) at least 2% by wt. fat;

ii) at least 10% by wt. of a sugar or sugars; and

iii) proteins, present at a level of less than 2%;

wherein some or all of the fat and protein are present as oil bodies.

The presence of oil bodies in the composition reduces or eliminates the need to include MSNF, as the oil bodies are pre-emulsified. The resultant products are typically much cheaper to manufacture. The presence of non-oil body fats require the addition of a separate emulsifying agent so that a product of acceptable texture may be produced.

In a preferred embodiment, an aerating agent is added to the formulation so that an aerated ice confection can be produced. In this embodiment, the overrun of the composition is at least 30%, preferably at least 50%, more preferably in the range 75% to 150%. Preferably the overrun is no more than 200%. However for certain envisaged ice confection product forms, the overrun can be up to 30%.

More preferably the aerating agent is a polyglycerol ester of fatty acids. In another more preferred embodiment, the aerating agent comprises monoglycerides.

More preferably also, some of the protein is oleosin. Even more preferably, some of the protein is sunflower oleosin.

In another more preferred embodiment of the invention the oil bodies are derived from a source selected from the group consisting of sunflower, rapeseed, soybean, oil palm, cotton seed, ground nut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, maize, sesame, chick pea, avocado, or any mixture thereof. Even more preferably, the oil bodies are derived from a source selected from the group consisting of sunflower, soybean, avocado or rapeseed or any mixture thereof. Even more preferably the oil bodies are derived from sunflower.

Preferably also, the oil bodies are present at a level of 0.5 % to 20 % by weight of the ice confection. More preferably the oil bodies are present at a level of 2 % to 10 % by weight of the ice confection.

Preferably also, proteins are present at a level of at least 0.2% by weight of the ice confection. More preferably, proteins arepresent at a level of at least 0.5% by weight of the ice confection.

Preferably also, fat is present at a level of 2 % to 10 % by weight of the ice confection. More preferably, fat is present at a level of 2 % to 6 % by weight of the ice confection.

Preferably also, sugar is present at a level of 10 % to 20 % by weight of the ice confection.

Preferably, the ice confection additionally comprises a stabilizer. More preferably, the stabilizer is present at a level of 0.05 % to 1 % by weight of the ice confection. More preferably also, the stabilizer is selected from the group consisting of locust bean gum, kappa carrageenan or guar gum, or any mixture thereof.

Preferably also, the ice confection additionally comprises a fruit puree.

It is a second object of the present invention to provide a method for preparing an ice confection including the steps of;

a) preparing an oil body preparation;
b) mixing non-oil body components together at elevated temperature;
c) adding the oil body preparation;
d) pasteurisation;
e) cooling;
f) aeration; and
g) freezing the confection.

In a preferred embodiment, the aerating agent is added separately from the other non-oil body components after cooling and prior to aeration.

Detailed description of the invention

The overrun of an ice cream (or other aerated ice confection) is defined as the increase in volume of the ice cream over the volume of the unaerated and unfrozen mix due to the incorporation of air and the formation of ice. This is expressed as a percentage of the volume of the mix. The percentage overrun can be calculated by weight using the formula given in "Ice Cream, Fourth Edition by W.S. Arbuckle, Pub. Van Nostrand Reinhold, New York, 1986, p 187."

$$\text{overrun \%} = \frac{\text{weight of 1 gal mix - weight of 1 gal ice cream}}{\text{weight of 1 gal ice cream}} \times 100$$

This formula uses weight per gallon, but it is equally correct to use the weight of any other volume, so long as the same measure is used throughout the calculations. The important quantity in these calculations is the density.

The formula can therefore be rewritten as:

$$\text{overrun \%} = \frac{\text{density of mix - density of ice cream}}{\text{density of ice cream}} \times 100$$

Overrun can be determined most accurately at the point of manufacture as described below. However, where this is not possible overrun can be estimated using the Archimedes' principle. It is understood that when a body is added to a volume of water, the increase in weight is equal to the upthrust and hence weight of water displaced. Taking the density of water as 1 gcm^{-3}, the weight of water displaced is used to determine the volume of water displaced and thus the volume of ice cream immersed in the beaker. From the mass and volume of the product, the density of the ice cream can be calculated.

With the exception of percentages cited in relation to the overrun of the composition, all percentages, unless otherwise stated refer to the approximate percentage by weight of the total composition.

Compositions according to the invention have been found to show improvements on prior products; in particular they are cheaper to make, and may be healthier than earlier products. They may also provide benefits to allergy sufferers as it is

possible that these products will be 'dairy free'. They may be cheaper to manufacture as the emulsification/ homogenisation step is not essential due to the presence of pre-emulsified oil-bodies.

Oil bodies have been previously described and defined in the art. For instance, Deckers et al. (US 6,146,645) writes as follows: "In the seeds of oilseed crops, such as soy bean, rapeseed, sunflower and palm, the water insoluble oil fraction is stored in discrete subcellular structures variously known in the art as oil bodies, oleosomes, lipid bodies or spheresomes. Besides a mixture of oils (triacylglycerides*) which chemically are defined as glycerol esters of fatty acids, oil bodies comprise phospholipids and a number of associated proteins, collectively termed oil body proteins. From a structural point of view, oil bodies are considered to be a triacylglyceride matrix encapsulated by a monolayer of phospholipids in which oil body proteins are embedded".

* More usually known as triacylglycerols (TAGs) or fatty acid triglycerides.

The term 'oil body preparation' as used herein refers to the product of a process of extraction from a natural source, for example as in Example 1 below.

The term 'oil body' as used herein refers to the lipid-protein complex present in an oil body preparation. Moreover, where water is present in the preparation an allowance is made when calculating the mass of oil body added to an ice confection (for more details refer to examples).

Therefore the terms 'oil bodies' and 'oil body preparation' exclude the un-processed seeds.

Oil bodies suitable for use in the invention include those derived from sunflower, rapeseed, soybean, oil palm, cotton seed, ground nut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, maize, sesame, chick pea, avocado, or other sources containing similar amounts of protein and oil as would be obvious to the skilled person. In some embodiments, conveniently the oil body is derived from oil crop or vegetable (e.g. non-fruit) sources. In other embodiments, preferably the oil bodies of the invention are derived from sunflower seeds, soybean, avocado or rapeseed; most preferably, the oil bodies are derived from sunflower seeds.

Preferably, the oil bodies used in this invention have an oil/protein ratio (=lipid/protein ratio) by weight of greater than 3.5, and preferably less than 20.

The oil bodies will typically be present in the range 0.5-20 wt% of the composition, preferably in the range 2-10 wt%.

The protein component of the oil body will comprise at most 2%, preferably no more than 1% of the total composition. Protein will always be present in the ice compositions of the invention. Typically protein will be present in the composition at a level of at least 0.2%, more often at a level of at least 0.5% or 0.6% of the composition.

The fat component of the oil body will typically comprise at least 0.5 wt%, preferably 2-10 wt%, most preferably 2-6 wt% of the total composition.

Abundant oil body-associated proteins in the oil bodies as used in the invention are oleosins [A.H.C. Huang (1992) Annu Rev Plant Physiol Plant Mol Biol 43: 177-200]. Oleosins are relatively small (15-25kD) amphipathic proteins [D.J. Murphy

(1993) INFORM Vol 4 no. 8 p922]. Sequence information derived from different oleosins shows a strong homology within the central hydrophobic region, but very little similarity in the other two domains (Murphy, 1993). Oleosins may play a role in the stabilisation of the emulsions formed by oil bodies.

The presence of oil bodies can be detected in ice confections for example by the presence of oleosin protein. This is usually not present in refined oil source, for example sunflower oil. Methods for doing this are described below. Methods suitable for detecting the presence of triacylglycerols and other components that are characteristic of sunflower oil are also known.

Detection of oleosin by amino acid sequencing

McCarthy et al. (WO 01/36648) relates to recombinant genes which code for oleosin proteins found in cacao. The genes are used to manufacture emulsifiers, encapsulating agents and flavour components which may be useful in, among others, the food industry.

Amino acid sequences for oleosins from sunflower seeds and other oil seeds have been published and are available through sequence databases such as SwissProt and PIR (1). The sequences of oleosins from different species are related, in particular the central, hydrophobic domain is the region most conserved between species (2). Therefore, the oleosin protein can be identified by amino acid sequencing. Fragments of amino acid sequence obtained from the product (as described below) can be compared with the published sequences using database searching and sequence comparison facilities that are well-known in the art, such as ExPasy or SRS. If the stretches of sequence from the product closely match a published sequence,

it indicates that oleosin from that oil seed is present in the product.

The protein component of an ice cream product can be separated from the other ingredients by melting the ice cream, diluting with water and carrying out a cold acetone precipitation. Following centrifugation the supernatant is discarded leaving the pelleted protein material. After drying off traces of acetone, the protein is re-solubilised into SDS sample buffer and prepared for SDS-PAGE (Polyacrylamide gel electrophoresis) by heating at 60°C for 10 minutes or boiling for 1-2 minutes. The sample can then be run on SDS-PAGE alongside molecular weight standards, and the protein bands visualised with a stain such as coomassie blue. Using this procedure, two oleosin protein bands are typically seen. These bands correspond to the two oleosin isomers (approximate molecular weights 19.5kD and 20.5kD).

As the oleosin proteins are blocked to N-terminal sequencing (3), the protein bands are digested using an in-gel digestion technique and a suitable proteolytic enzyme such as trypsin or endoproteinase Lys-C. The protein fragments are then separated from each other using reverse phase chromatography, and the individual fragments sequenced using standard protein sequencing equipment. The short pieces of internal amino acid sequence thus obtained are compared with the published oleosin protein sequences as described above.

A general review article describing commonly used methods for preparing proteins for sequencing, including the strategies outlined above can be found in ref. (4).

1. Examples of sunflower seed oleosin sequences include the following accession numbers: SwissProt P29529 and PIR S70453.

2. Napier J.A., Beadoin F., Tatham A.S., Alexander L.G., Shewry P.R. (2001) Adv. in Bot. Res. 35 111-138.
3. Millichip M., Tatham A.S., Jackson F., Griffiths G., Shewry P.R., Stobart A.K. (1996) Biochem. J. 314 333-337.
4. Patterson S.D., (1994) Anal. Biochem. 221 1-15.

Detection of oil bodies by specific antibodies

Antibodies specific for oleosin are known in the art [S.S.K. Tai et al. (2002) Biosci. Biotechnol. Biochem. 66 (10) 2146-2153]. These can be used to detect oil bodies in an ice confection. First the ice confection is allowed to melt and then the oil bodies are recovered by centrifugation as described above. Then a small sample of the oil bodies is suspended and diluted in water or aqueous buffer and visualised by immuno-fluorescent microscopy using the oleosin-specific antibody and staining reagents and procedures that are well known.

Detection of TAG and other components that are characteristic of sunflower oil

Triacylglycerol (TAG) profiles for sunflower oil are easily determined by GC analysis. In addition, phospholipid (PL) profiles can be determined using HPLC. The levels of sterols, triterpene alcohols and tocopherols which are found in sunflower oil can all be determined by GC/HPLC and with Mass Spectrometry detection.

Useful references include:

1. For data on sunflower oil for fatty acids/triglycerides/ PL/minor components: Lipid Handbook by F.D. Gunstone, J. Harwood and F.B. Padley, Pub. Chapman and Hall, 1986, Chap. 3.3, 35, p 101.

2. Also for sunflower oil fatty acid profiles: Codex Alimentarius Commission, Codex Committee on oils and fats.
3. For the determination of seed oil content and fatty acid composition in sunflower oil through the analysis of intact seeds, husked seeds, meal and oil by near IR reflectance spectroscopy: Perez-Vich B., Velasco L., Fernandez-Martinez J.M., J. Am. Oil Chem. Soc., 75 (5), 547 - 555.
4. For Phospholipid (PL) Profiles: Chapman G.W., J. Am. Oil Chem. Soc., 59, 299.
5. For sterols/triterpene alcohols and tocopherols: Sterols are found in the Lipid Handbook by F.D. Gunstone, J. Harwood and F.B. Padley, Pub. Chapman and Hall, 1986, Table 3.163 (adapted from Itoh et al. J. Am. Oil Chem. Soc. 1973). Tocopherols may be found in the Lipid Handbook table 3.167, p 129. Or in Analysis of oilseeds, fats and fatty foods, Elsevier, London, p 315

Proteins which may also be present in the inventive composition included skimmed milk proteins, soy protein, wheat protein, barley protein, lupin protein and mixtures thereof. Preferably any additional protein (i.e. not associated with oil bodies) will not comprise more than about 1 wt% of the composition.

The sugar of the invention will typically be a mono-, di-, or oligo-saccharide or sugar alcohol for instance, sucrose, dextrose, glucose, purified lactose, lactose monohydrate, glucose syrup, invert sugar, corn syrup, fructose or mixtures thereof. Where greater freezing point depression is required, so that the ice confection produced is softer, lower molecular weight molecules such as fructose may be selected. Preferably a blend of sugars is used, more preferably one of the sugars is sucrose. The sugar must be present in at least 10 wt%, preferably 10-20 wt%, most preferably 12-18 wt% of the composition.

Method for evaluating candidate aerating agents

When the ice confection product is aerated, a suitable aerating agent (also known as a foaming agent) is required. Aerating agents suitable for the invention need to be both effective at aerating (i.e. they are active at low concentrations) and compatible with maintaining the integrity of the oil body structure. This combination of properties is thought to be a new technical requirement, and therefore it is not possible to use existing methodologies to define aerating agents that are suitable. A method for determining whether an aerating is suitable is described below.

Candidate aerating agents can be readily tested for suitability by making up a range of base mixes consisting of:

Sucrose (Tate & Lyle)	18%
Guar gum (Willy Benecke)	0.3%
Oil bodies (Sunflower seed source)*	1 - 5%
	(1% and 5% should be tried)
Candidate aerating agent	0.05 - 1%
	(0.05%, 0.2%, 0.5%, and 1% should be tried)
Water (de-ionised)	to 100%

* Made by the method of Example 1

Eight candidate formulations are thus assessed.

1-2 litres of each mix is made up in water at 60-70°C, the oil body preparation is added last. Then the mix is heated to 80°C and the oil body preparation is dispersed using a Silverson L4R homogeniser. The Pasteurised oil body mix is then cooled

to 4°C. Aeration (i.e. "whipping") is carried out using a Hobart mixer (Hobart corp. model N50 CE) using 1-2 litres of mix and with the mixer set on full speed. Overrun is monitored by making a single measurement every 30 seconds using the overrun cup method (see section on methods for determining overrun). Aeration is continued until an overrun of 100% is reached or until the overrun stops increasing, whichever is sooner.

Aerating agents suitable for the invention produce an overrun of at least 30% in at least one of the mixes. The aerated mix is then poured into stainless steel moulds and frozen at -18°C in a glycol bath. After freezing, the moulds are immersed in warm water (25°C - 30°C) to release the frozen products from the moulds. The overrun is measured using the Archimedes' method (see section on methods for determining overrun). Aerating agents suitable for the invention produce an overrun of at least 30% in at least one of the frozen products.

An examples of a suitable and preferred aerating agent is PGE 55 (a polyglycerol ester of fatty acids, available from Danisco), known as food ingredient E475 in the EU. Preferably, the PGE will be present in the range 0.2-1 wt%, more preferably 0.5-1 wt% of the composition in aerated products. Another example of a suitable and preferred aerating agent is Myverol 18-04K (a distilled 95% monoglyceride prepared from vegetable oils, available from Quest International). Other sources of monoglyceride provide aerating agents suitable for the invention. Preferably the monoglyceride is present in the range 0.2-1 wt%, more preferably 0.5-1 wt%. The term "monoglyceride" as used herein means an ester of glycerol with one fatty acid molecule.

Water is an essential component of the composition; preferably water will be present in at least 70 wt% of the composition.

The frozen confection products of the invention may comprise various optional components.

Stabilisers that may be used include proteins such as gelatin; plant extrudates such as gum arabic, gum ghatti, gum karaya, gum tragacanth; seed gums such as locust bean gum, guar gum, psyyllium seed gum, quince seed gum or tamarind seed gum; seaweed extracts such as agar, alganates, carrageenan or furcelleran; pectins such as low methoxyl or high methoxyl-type pectins; cellulose derivatives such as sodium carboxymethyl cellulose, microcrystalline cellulose, methyl and methylethyl celluloses, or hydroxylpropyl and hydroxypropylmethyl celluloses; and microbial gums such as dextran, xanthan or β-1,3-glucan. Preferably, the stabiliser is selected from locust bean gum, kappa carrageenan, guar gum or mixtures thereof. Preferably the stabilisers are present at a level of 0.05-1 wt% of the composition.

In addition, the composition of the invention may contain flavouring and/or colouring. Typical flavourings include mint, vanilla, chocolate, coffee, or fruit flavours. Preferably, the flavouring or colouring will be present at a level of less than 1 wt% of the composition. Pieces of nut, chocolate, ginger, biscuit, fruit, fruit puree, or other ingredients or additives commonly added to ice cream or other ice confections may also be included. The term "fruit puree" as used herein means a homogeneous product which has been prepared from whole or peeled fruit, which has been pulped by a suitable physical process. The puree may or may not have had a portion of the water physically removed, may or may not have had sugars added and may or may not have been heat treated.

Examples

In the following, compositions demonstration various facets of the invention were prepared. Properties of the compositions such as fat content, water content, overrun and protein content were determined as set forth below.

Methods for determining overrun

Determining overrun at the point of manufacture

The density of the unaerated mix is determined by weighing a standard overrun cup of mix at approximately 4°C, subtracting the mass of the cup and dividing by the known volume of the cup (density = mass/volume). A minimum of three repeat measurements is taken. The density of the (aerated) ice cream is determined by repeating the procedure on the same overrun cup with freshly extruded ice cream (at approximately -2°C to -7°C). Again a minimum of three repeat measurements is taken. With a knowledge of the density of both unaerated mix and aerated ice cream, the overrun can be calculated using the equation given above.

Determining overrun of a finished product

The density of a finished ice cream (or other aerated ice confection) product can also be estimated by making use of the Archimedes' principle as described in "A-level Physics, Third Edition, by R. Muncaster, Pub. Stanley Thornes Ltd., Cheltenham, 1989".

First a sample of ice cream is weighed in air to determine its mass. Then the volume of the same sample is determined using the Archimedes' principle as described below. The sample of ice cream is held carefully in a beaker of chilled water just

below the surface of the water by a fork (or a knife) inserted into the end of the product. The beaker is placed on a balance throughout the experiment and the increase in weight on immersing the product is recorded. By Archimedes' principle, the increase in weight is equal to the upthrust and hence weight of water displaced. Taking the density of water as 1 gcm^{-3}, the weight of water displaced is used to determine the volume of water displaced and thus the volume of ice cream immersed in the beaker. From the mass and volume of the product, the density of the ice cream can be calculated. A minimum of three repeat measurements is taken.

The density of the unaerated mix can either be assumed to be 1.1 g/cm^3 or can be estimated by melting the ice cream until the air-phase is lost and then determining the density in an overrun cup at 4°C as described above. With a knowledge of the density of both unaerated mix and aerated ice cream, the overrun can be calculated using the equation on page 6.

A method for determining fat content

For the purposes of this method, the terms "fat" and "oil" are regarded as being one and the same, in terms of molecular composition. Fat (or oil) content can be determined by the "Weibul" acid hydrolysis procedure. This is a recognised BS Method (No.4401) Ref. Official, Standardised and Recommended Methods of Analysis SAC, 1973 2nd Ed. p 160. The sample is boiled with approximately 6M hydrochloric acid to release 'bound' fat and the digest is filtered through a double filter paper using filter aid. Fat was retained by the filter paper and aid. After washing and drying, the residue is extracted with light petroleum spirit using a Soxhlet extractor. Descriptions of the performance of the method and a comparison with other methods can be found in these references

a) Weibul, Staatsbled van het Koninkrijk Der Hederlander pl - 16, 1919, No. 581.

b) W Stoldt, Z Undersuchung & Lebensmittel, 1937, 73. 329.

c) Nottbolm &, Baumann, Z Undersuchung & Lebensmittel 1931, 62. 164.

d) ISO 1143-1973

A method for determining protein content

Protein content can be determined by measuring the nitrogen present in the sample. This can be done by using equipment that is manufactured for the purpose: the "Macro N" (Foss-Heraeus). In this procedure, the sample under test is completely burned at temperatures in excess of 1000°C in the presence of oxygen. The resultant combustion gases are swept through a series of absorption tubes by a stream of carbon dioxide, this procedure removes unwanted gases, finally the carbon dioxide and nitrogen mixture are passed through a thermal conductivity detector where the nitrogen is quantified. Nitrogen content is converted to protein content using a conversion factor based on the average nitrogen of the amino acids found in particular foods.

A suitable conversion factor to use for analysing the protein content in ice confections that are made according to this invention is 6.25, although other conversion factors could be used - based on the particular protein source that is being analysed.

The procedure is published in the following article. Ian D Smith, Analytical Proceedings, 1991, 28. 320 - 324. "Evaluation of the Foss-Heraeus Macro N for the Determination of Nitrogen in a Wide Range of Foodstuffs, Ingredients and Biological Materials and Comparison with the Kjelfoss".

A method for determining water content

The method involves the measurement of weight loss due to evaporation of water. A fan assisted, thermostatically controlled air oven is used at a temperature of 100°C. The procedure described is similar to Official and Standardised methods recommended by:

a) The Association of Official Agricultural Chemists USA, 'Official Methods of Analysis' 12th Edition, 1975,

b) The Fertiliser and Feedingstuffs Regulations HMSO, Statutory Inst. No 840, 1976.

c) ISO 1026-1982, ISO 1442-1973.

15-20 gram of dry sand and a small glass rod are placed in an aluminium foil cup. This assembly is weighed (= W1). A sample of melted ice cream (approximately 5 gram) is added to the cup and weighed again (= W2). The melted ice cream is then mixed into the sand with the glass rod. The cup is then placed on a steam bath and evaporated to dryness (takes 30 minutes);the sample is stirred with the rod throughout this procedure. The sample is then placed in an oven for 2.5 hours that has been pre-set at 100°C. The cup is then placed in a dessicator to cool before weighing (= W3).

Water content is given by:

$$\%\text{Water w/w} = \frac{W2 - W3}{W2 - W1} \times 100$$

Where: W1 = Weight of cup (including sand and glass rod).
W2 = Weight of cup + wet sample.
W3 = Weight of cup + dried sample.

Example 1. A method for producing oil bodies

A total of 1.7 kg of de-hulled sunflower seeds was ground in a food-processor until no large particles were present. The ground seeds were homogenised in two volumes of cold grinding buffer (0.6 M sucrose and 1.0 M NaCl) using a Waring blender (a commercial heavy duty blender) at low speed. The homogenate was filtered through a 500μm pore size sieve to remove large particles and seed skins. After sieving, the homogenate was centrifuged at 10,000xg for 30 minutes at 4°C in order to remove large particles, insoluble proteins and separate the oil bodies from the aqueous soluble seed proteins. The floating oil body layer was skimmed off by using a metal spatula and added to one volume of floating buffer (0.6 M sucrose).

After homogenisation in the Waring blender at low speed, the mixture was sieved through a 150μm pore-size sieve to obtain an emulsion with oil bodies less than 150μm in size. The homogenised oil bodies were centrifuged again as described above. The skimmed oil bodies were washed twice in one volume of floating buffer and after each wash step centrifuged as described. The final oil body preparation was placed in a sealed plastic container and stored at 4°C until used.

Approximately 1kg of oil body preparation was produced. This was determined to have a water content of approximately 35% using the method described above. Therefore the "dry" oil body content of the preparation was approximately 65% by weight. It is important to measure this for each oil body preparation so that it is known how much oil body is added to each mix (see below). When the method for producing oil bodies was repeated several times it was found that the water content in different oil body preparations varied slightly (between 30% and 40%).

Therefore the "dry" oil body content varied between 60% and 70%.

Example 2. A method for making an aerated ice confection in a shop or in a small manufacturing unit

A mix was prepared with the following composition:

Ingredient	Weight %
Sucrose	12
Locust Bean Gum	0.35
Kappa Carrageenan	0.02
Glucose Syrup 42DE	8
PGE 55*	1
Oil body preparation (prepared as described in example 1) †	7.5
Flavour	0.1
Colour	0.05
Water (de-ionised)	70.98

* PGE 55 is polyglycerol ester 55 (having a melting point of 55°C) available from Danisco

† Water content of this oil body preparation was approximately 35%. Therefore the oil body content in the mix was approximately 4.9% (7.5% x 0.65).

The mix was prepared by dissolving dry ingredients in water at 60-70°C and then adding the oil body. The mix was heated in a stainless steel pan on a hot plate to 80°C at which point the oil body preparation was dispersed in the mix using an homogeniser (Silverson L4R), heated to 80°C and Pasteurised. The mix was then cooled to approximately 4°C by placing it in a chill store.

Aeration was carried out using a Hobart mixer (Hobart Corp. Model: N50 CE). 1-2 litres of the mix was whipped by setting the mixer on full speed. Approximately 100% overrun was achieved in less than 3 minutes. Overrun was determined with the overrun cup method as described above. The aerated mix was poured into stainless steel moulds and wooden sticks were inserted into the mix. The moulds were placed in a glycol bath at -18°C until the mix was frozen.

After freezing, the moulds were immersed in warm water (25°C-30°C) to release the frozen products from the moulds. The products were put in packets and stored at -25°C in a freezer.

Example 3. A method for making an aerated ice confection in a factory

A mix was prepared with the following composition:

Ingredient	Weight %
Sucrose	18.0
Guar gum	0.3
PGE 55*	0.5
Vanillin	0.05
Oil body preparation (prepared as described in example 1)†	7.1
Water	74.05

* PGE 55 is polyglycerol ester 55 available from Danisco.

† Water content of this oil body preparation was approximately 31%. Therefore the oil body content in the mix was approximately 4.9% (7.1% x 0.69).

All the ingredients except the oil body were mixed together using a high shear mixer for approximately 5 minutes, the

water being added at a temperature of approximately 80°C. The temperature of the mix was above 60°C after mixing. The mix was passed through to a plate heat exchanger for Pasteurisation at 82°C for 25 seconds. The mix was then cooled to approximately 4°C in the plate heat exchanger and stored at approximately 4°C overnight in churns in a chill store.

The mix was heated to 60°C-70°C, then the oil body preparation was added, then the mix was heated to approximately 80°C (to Pasteurise it) and dispersed using a homogeniser (Silverson L4R). The Pasteurised oil body mix was then cooled to approximately 4°C.

The oil body mix was re-homogenised (using the Silverson homogeniser) immediately prior to use, aerated and frozen in a Technohoy MF75 scraped surface heat exchanger fitted with a C29800 open dasher. The mix was extruded at a temperature of between -2°C and -3.3°C into plastic cups.

The overrun at extrusion was determined using the overrun cup method as described above. The overrun was found to be approximately 75%.

The products (in plastic cups) were then hardened in a blast freezer at -35°C and stored at -25°C.

Example 4: Analysis of hardened ice confection

The hardened ice confection produced in Example 3 was analysed using the methods described above.

Results of the analysis

Fat content:	4.1%	
Protein content:	0.6%	
Overrun	90%	(estimate using the Archimedes' method)
Water content	77%	

Example 5. A method for making an unaerated ice confection in a shop or in a small manufacturing unit

A mix was prepared with the following composition:

Ingredient	Weight %
Sucrose	18
Guar Gum	0.15
Oil body preparation (prepared as described in example 1)†	7.5%
Water (de-ionised)	74.35

† Water content of this oil body preparation was approximately 35%.Therefore the oil body content in the mix was approximately 4.9% by weight.

The mix was prepared by dissolving dry ingredients in water at 60-70°C and then adding the oil body. The mix was heated in a stainless steel pan on a hot plate to 80°C. Then the mix was placed in a food blender and blended on maximum power for 1 minute to disperse the oil body. The mix was then cooled to approximately 4°C by placing it in a chill store. The mix was then poured into stainless steel moulds and wooden sticks were inserted into the mix. The moulds were placed in a glycol bath at -18°C until the mix was frozen.

After freezing, the moulds were immersed in warm water (25°C-30°C) to release the frozen products from the moulds. The products were put in packets and stored at -25°C in a freezer.

Examples 6 - 13 evaluate a number of aerating agents using the method for evaluating candidate aerating agents set out above. The oil body preparations were prepared according to Example 1. The overrun of the aerated mix was measured as a function of time using the method described above in the section entitled "Determining overrun at the point of manufacture".

Example 6 PGE-55 added before the oil body preparation

PGE-55 is polyglycerol ester 55 available from Danisco.

Ingredient	Weight %
Sucrose	18
Guar	0.3
PGE-55	0.5
Oil body preparation	7.7
De-ionised water	73.5

The water content of the oil body preparation was 35.3%. Therefore the oil body content in the mix was 7.7% x (1-0.353) = 5.0%. The mass of the overrun cup was 409.1g and the mass of a full cup of unaerated mix was 136.1g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	529.6	120.5	12.9
1	524.3	115.2	18.1
1.5	525	115.9	17.4
2	518.8	109.7	24.1
3	515.8	106.7	27.6
4	512.5	103.4	31.6
6	514.2	105.1	29.5
8	513.5	104.4	30.4
10	510.2	101.1	34.6
12	503.3	94.2	44.5
14	504.7	95.6	42.4
16	505.4	96.3	41.3
18	503.4	94.3	44.3
20	501.9	92.8	46.7
24	497.7	88.6	53.6
32	495.2	86.1	58.1
42	494.1	85	60.1
50	496.2	87.1	56.3

Example 7 PGE-55 added after the oil body preparation

Ingredient	Weight %
Sucrose	18
Guar 0.3	
PGE 55	0.5
Oil body preparation	8.1
De-ionised water	71.6
De-ionised water (to dissolve PGE-55)	1.5

The water content of the oil body preparation was 38%. Therefore the oil body content in the mix was 8.1% x (1-0.38) = 5.0%. The mix was prepared using the method for evaluating candidate aerating agents set out above, except that PGE-55 was omitted from the initial heated mix. PGE-55 was instead dissolved in a portion of the de-ionised water (1.5%) and heated to 80°C. The PGE-55 solution was then cooled to approximately 4°C by placing it in a chill store. The chilled PGE-55 solution was added to the chilled mix immediately prior to aeration. The mass of the overrun cup was 407.7g and the mass of a full cup of unaerated mix was 130.8g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	514.5	106.8	22.5
1	496.3	88.6	47.6
1.5	488	80.3	62.9
2	479.4	71.7	82.4
2.5	475.4	67.7	93.2
3	466.6	58.9	122.1

Example 8 Mono-Di HP 40-1 (0.9%)

Mono-Di HP 40-1 is a mono-diglyceride made from edible, fully hydrogenated palm based oil available from Danisco.

Ingredient	Weight %
Sucrose	18
Guar	0.3
Mono-Di HP 40-1	0.9
Oil body preparation	7.7
Water	73.1

The water content of the oil body preparation was 35.3%. Therefore the oil body content in the mix was 7.7% x (1-0.353)

= 5.0%. The mass of the overrun cup was 409.1g and the mass of a full cup of unaerated mix was 132.1g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	524.9	115.8	14.1
1	514.1	105	25.8
1.5	518.7	109.6	20.5
2	513.5	104.4	26.5
2.5	516.0	106.9	23.6
3.5	516.8	107.7	22.7
5	513.9	104.8	26.0
7	512.6	103.5	27.6
10	510.4	101.3	30.4
15	512.2	103.1	28.1
20	513.3	104.2	26.8

Example 9 Mono-Di HP 40-1 (0.3%)

Ingredient	Weight %
Sucrose	18
Guar	0.3
Mono-Di HP 40-1	0.3
Oil body preparation	7.7
Water	73.7

The water content of the oil body preparation was 35.3%. Therefore the oil body content in the mix was 7.7% x (1-0.353) = 5.0%. The mass of the overrun cup was 409.1g and the mass of a full cup of unaerated mix was 127.5g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	525.9	116.8	9.2
1	524.9	115.8	10.1
2	518.0	108.9	17.1
4	517.7	108.6	17.4
6	518.1	109	17.0
8	519.8	110.7	15.2
10	514.9	105.8	20.5
12	521.3	112.2	13.6
14	520.4	111.3	14.6

Example 10 Myverol 18-04 K (1%)

Myverol 18-04 K is a kosher approved distilled monoglyceride which is prepared from vegetable oils and fats available, and is available from Quest International.

Ingredient	Weight %
Sucrose	18
Guar	0.3
Myverol 18-04 K	1
Oil body preparation	7.8
Water	72.9

The water content of the oil body preparation was 36%. Therefore the oil body content in the mix was 7.8% x (1-0.36) = 5.0%. The mass of the overrun cup was 407.4g and the mass of a full cup of unaerated mix was127.3g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	468.0	60.6	110.1

Example 11 Myverol 18-04 K (0.3%)

Ingredient	Weight %
Sucrose	18
Guar	0.3
Myverol 18-04 K 0.3	
Oil body preparation	7.7
Water	73.7

The water content of the oil body preparation was 36%. Therefore the oil body content in the mix was 7.8% x (1-0.36) = 5.0%. The mass of the overrun cup was 409.1g and the mass of a full cup of unaerated mix was 132.2g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	515.0	105.9	24.8
1	511.0	101.9	29.7
1.5	505.5	96.4	37.1
2	504.3	95.2	38.9
3	497.5	88.4	49.5
4	495.2	86.1	53.5
5	493.0	83.9	57.6
7	486.8	77.7	70.1
9	484.4	75.3	75.6
11	478.8	69.7	89.7
13	473.9	64.8	104.0

Example 12 Myverol 18-04 K (0.1%)

Ingredient	Weight %
Sucrose	18
Guar	0.3
Myverol 18-04 K 0.1	
Oil body preparation	7.7
Water	73.9

The water content of the oil body preparation was 35.1%. Therefore the oil body content in the mix was 7.7% x (1-0.351) = 5.0%. The mass of the overrun cup was 409.1g and the mass of a full cup of unaerated mix was 127.5g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	523.0	113.9	11.9
1	518.0	108.9	17.1
2	519.3	110.2	15.7
4	514.6	105.5	20.9
6	515.9	106.8	19.4
8	514.0	104.9	21.5
12	513.6	104.5	22.0
18	516.1	107.0	19.2

Example 13 Versa-Whip 500 (0.5%)

Versa-Whip 500 is a food grade modified soy protein, available from Quest International.

Ingredient	Weight %
Sucrose	18
Guar	0.3
Versa-Whip 500	0.5
Oil body preparation	7.7
Water	73.5

The water content of the oil body preparation was 35.3%. Therefore the oil body content in the mix was 7.7% x (1-0.353) = 5.0%. The mass of the overrun cup was 407.4g and the mass of a full cup of unaerated mix was 131.4g.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	538.8	131.4	0.0
20	538.8	131.4	0.0

Example 14 Preparation of an iced confection using myverol and fruit puree

Ingredient	Weight %
Sucrose	18
Guar	0.3
Myverol 18-04 K	0.3
Banana puree (62% solids)	40
Banana flavour	0.2
Oil body preparation	7.8
Water	33.4

The banana puree was obtained from SVZ International (www.svz.com)

The water content of the oil body preparation was 36%. Therefore the oil body content in the mix was 7.8% x (1-0.36) = 5.0%. The mass of the overrun cup was 407.4g and the mass of a full cup of unaerated mix was 126.8g. The mix was prepared and products were made as described in Example 2, except that the banana puree and banana flavour was added to the chilled mix just prior to aeration.

Time (minutes)	Mass of full cup of aerated mix (g)	Mass of aerated mix (g)	Overrun (%)
0.5	489.3	81.9	54.8
1	478.3	70.9	78.8
1.5	471.3	63.9	98.4
2	464.6	57.2	121.7

Example 15 Analysis of protein and fat content of ice confections

Ice confections were prepared from the aerated mixes of examples 7, 8, 9, 11, 12, and 14 following the procedure described in Example 2. The fat content and protein content of the products were determined as described in the sections entitled "A method for determining fat content" and "A method for determining protein content" above. The results were as follows.

Example	Aerating agent	Fat content (% w/w)	Protein content (% w/w)
7	0.5% PGE	3.9	0.6
8	0.9% HP-40	4.0	0.5
9	0.3% HP-40	3.7	0.4
11	0.3% Myverol	3.7	0.6
12	0.1% Myverol	3.6	0.5
14	0.3% Myverol (+ banana puree)	4.0	1.4

Example 16. Detection of oleosin in an iced confection

Aerated and unaerated ice confections were prepared according to examples 3 and 5 respectively. The following procedure was used for both samples. In order to extract intact oil bodies, 1-2g of the confection was placed in an eppendorf tube and allowed to melt. The sample was then centrifuged at 13,500rpm for 5 minutes in a Microcentaur centrifuge. The resulting 'fat pad' on the surface of the sample was transferred into a fresh eppendorf tube.

In order to remove non-oleosin proteins (such as sunflower seed proteins and milk proteins) the samples were washed with urea, following the procedure of reference 3 of page 12. 1ml of 9M urea was added to the fat pads, mixed by vortexing thoroughly and incubated in the fridge for 2 hours. The sample was centrifuged and the fat pad was skimmed off. Two further urea washes were performed.

In order to remove the fat from the intact oil bodies and to precipitate the oleosins, 1ml of acetone chilled to -25°C was added to the fat pad, and the sample was incubated on ice for 1 hour. The sample was centrifuged at 13500rpm in a Microcentaur centrifuge. The precipitate was retained and the supernatant was discarded. Two further washes with chilled acetone were carried out. The pellet was left to air-dry overnight.

Samples were then prepared for SDS-PAGE. All reagents were used according to the manufacturer's instructions. 0.001g of the dry powder was re-solubilised in 0.5ml sample buffer (from Invitrogen), and incubated at room temperature for 30 minutes. The sample reducing agent (from Invitrogen) was then added and the sample was boiled for 2 minutes. 25□l of the resulting oleosin sample solution was loaded into to each of 8 wells of

a 10% bis-tris NuPAGE gel (also from Invitrogen). Precision Plus Protein Standards (from Bio-Rad) were used as molecular weight markers in another well. The gel was then run using MES running buffer.

Two lanes, one containing the molecular weight standards and the other the oleosin sample were cut off the gel and stained with colloidal coomassie blue, or Simply Blue Safestain (both from Invitrogen) in order to identify the location of the protein in the sample. A pair of strongly stained protein bands with apparent molecular weights of between 15 and 20kD were observed, corresponding to the two oleosin isoforms.

The proteins from the remaining unstained lanes were further purified by eluting them from the gel. The area on the gel corresponding to the location of the oleosin was excised, and minced up in an eppendorf tube. 1ml of 5mM tris-HCl buffer at pH8 with 5mM EDTA and 0.25% Tween-20 was added. The solution was vortexed thoroughly and then incubated on a shaker for 3 hours, with occasional vortexing. After spinning for 5 minutes at 13,500rpm in a Microcentaur centrifuge the supernatant liquid was recovered. The gel was washed twice more with 1ml of buffer. In order to precipitate the protein, chilled acetone was added to the combined supernatants, and the sample was incubated in a freezer overnight. The precipitate was pelleted by centrifugation at 4,000rpm for 10 minutes and the supernatant discarded. The pellet was dried and resolubilised with 160□l sample buffer, and then prepared and run on SDS-PAGE again.

The gel was stained as before. A small glass capillary tube was used to excise three spots from each protein band. These were placed in a 200□l PCR type tube with just enough water to cover them, frozen and transported on dry ice to the sequencing facility.

Sequencing of the protein was carried out at the John Innes Centre Proteomics facility. Tryptic digests of the samples were prepared, followed by QToF amino acid sequencing carried out using a Micromass® Q-ToF 2 mass spectrometer. Searching for matches with published amino acid sequences was carried out using the Mascot search engine (available through Matrix Science, www.matrixscience.com) which uses mass spectrometry data to identify proteins from primary sequence databases. Details of the search parameters are listed below.

Database: SPtrEMBL sptrembl20031031 (1295042 sequences
413813148 residues)
Taxonomy: Viridiplantae (Green Plants) (140955 sequences)
Type of search: MS/MS Ion Search
Enzyme: Trypsin
Fixed modifications: Carbamidomethyl (C)
Variable modifications: Oxidation (M)
Mass values: Monoisotopic
Protein Mass: Unrestricted
Peptide Mass Tolerance: ± 0.25 Da
Fragment Mass Tolerance: ± 0.25 Da
Max Missed Cleavages: 2

Five peptides from each sample gave close matches to published oleosin sequences. This demonstrates that the proteins isolated both from the aerated ice cream type product (where milk proteins were also initially present) and from the unaerated water ice type product (where the oil bodies are the only source of protein) were oleosins.

Claims

1. An ice confection containing;
i) at least 2% by wt. fat;
ii) at least 10% by wt. of a sugar or sugars; and
iii) proteins present at a level of less than 2% by weight;
wherein some or all of the fat and protein are present as oil bodies.

2. The ice confection of claim 1 further comprising 0.05% to 1% by wt. of an aerating agent, wherein the overrun is at least 30%, preferably more than 50%.

3. The ice confection of claim 2, wherein the confection has an overrun of no more than 200%.

4. The ice confection of claim 2 wherein the confection has an overrun of between 75 % and 150 %.

5. The ice confection of claim 2 wherein the aerating agent is a polyglycerol ester of fatty acids.

6. The ice confection of claim 2 wherein the aerating agent comprises monoglycerides.

7. The ice confection any of the preceding claims wherein some of the protein is oleosin.

8. The ice confection of claim 7 wherein some of the protein is sunflower oleosin.

9. The ice confection product of any claim 1 wherein the oil bodies are derived from a source selected from the group consisting of sunflower, rapeseed, soybean, oil palm, cotton seed, ground nut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, maize, sesame, chick pea, avocado, or any mixture thereof.

10. The ice confection of claim 9 wherein the oil bodies are derived from a source selected from the group consisting of sunflower, soybean, avocado or rapeseed or any mixture thereof.

11. The ice confection of claim 10 wherein the oil bodies are derived from sunflower.

12. The ice confection of claim 1 wherein the oil bodies are present at a level of 0.5 % to 20 % by weight of the ice confection.

13. The ice confection of claim 12 wherein the oil bodies are present at a level of 2 % to 10 % by weight of the ice confection.

14. A method for preparing an ice confection including the steps of;

a) preparing an oil body preparation;
b) mixing non-oil body components together at elevated temperature;
c) adding the oil body preparation;
d) pasteurisation;
e) cooling;
f) aeration; and
g) freezing the confection.

15. A method for preparing an ice confection according to claim 14 wherein, between steps e) and f) an aerated agent is added.

INTERNATIONAL SEARCH REPORT

International Application No
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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A23G9/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A23G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP 0 441 494 A (PFIZER) 14 August 1991 (1991-08-14) claims; example 12	1,8-15
A	EP 0 308 091 A (GEN MILLS INC) 22 March 1989 (1989-03-22) the whole document	1,14
A	WO 92/13465 A (CONAGRA INC) 20 August 1992 (1992-08-20) the whole document	1,14
A	US 2002/197375 A1 (ADOLPHI ULRICH ET AL) 26 December 2002 (2002-12-26) the whole document	1,14

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP2004/008502

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title:** METHOD FOR THE PREPARATION OF COSMETIC EMULSIONS

(57) **Abstract:** A novel method is disclosed for the preparation of oil-in-water emulsions suitable for cosmetic or topical dermatological products. The method comprises the step of mixing at least one cosmetically active ingredient with an aqueous emulsion of washed vegetable oil bodies (oleosomes) and the temperature of the products does never exceed about 40°C during the steps of the method.

WO 2005/097059 A1

METHOD FOR THE PREPARATION OF COSMETIC EMULSIONS

FIELD OF THE INVENTION

This invention relates to a method for the preparation of oil-in-water emulsions suitable for cosmetic or topical dermatological products.

BACKGROUND OF THE INVENTION

Oil-in-water (O/W) emulsions typically contain between 5 and 40% by weight of oil and/or oil soluble (hydrophobic) ingredients and between 60 and 95% by weight of water and/or water soluble (hydrophilic) ingredients.

O/W emulsions are normally prepared by combining the oil phase and the water phase together at high temperature (80–85 °C) and with vigorous agitation in the presence of an emulsifier until the oil droplets are completely dispersed within the water. The emulsifying agent is generally part of the oil phase, and is present at a level of between 10 and 30% by weight of the total oil phase. The resulting emulsion must then be continuously agitated during the cooling process in order to prevent separation (of the emulsion) into the original two phases. Typically agitation is required until the emulsion has cooled to a temperature of 40 °C or below. The process of heating the oil and water phases in order to achieve the emulsion is very time and energy consuming as is the cooling down phase. Moreover, in products for topical application the presence of the emulsifier in the finished emulsion presents a number of drawbacks:

1) The presence of the emulsifier reduces the water repellency of the formulation once it has been applied to the skin. This is particularly important for sunscreen applications and color cosmetics.
2) In skin care products, the presence of the emulsifier increases the irritancy potential of the formulation, in that it has a delipidizing effect on the skin.

Vegetable oil bodies (oleosomes) or oil microspheres are discrete spheres which occur naturally in the seeds of oilseed crops. They consist of an outer coat of phospholipids and proteins (oleosins) and an internal liquid, semi-solid, or low melting solid collection of the triglycerides associated with the individual plant seed. For example, safflower oil microspheres (oleosomes) would contain the triglycerides with the fatty acid content normally associated with that plant seed, i.e., 6.5% palmitic, 2.5% stearic, 11.5% oleic, 79% linoleic, and 0.5% linolenic acids. Size and properties of these microspheres are dependent on the seed type, but for

safflower seeds the microspheres are typically 3 μm in diameter and can be obtained as a 70–75 wt.% emulsion in water.

The preparation and several applications of oil bodies (oleosomes) are described in a number of patents assigned to the Canadian company Sembiosys Genetics Inc., e.g. U.S. Patent No. 6,146,645. Three of these patents are discussed in the following.

Deckers et al., U.S. Patent No. 6,183,762, disclose the preparation of cosmetic compositions for various applications. However, in each one of the applications described, the oil and water phases of the emulsion are heated to temperatures of 70 °C or higher whereupon they are added together with agitation. After the emulsion has been prepared and cooling has taken place (to 40 °C or lower) then the oil body (microsphere) emulsion is added.

In U.S. Patent No. 6,582,710, the same inventors claim the use of vegetable oil microspheres (oleosomes) in cosmetic applications. Again all of the formulations are prepared in the traditional way, i.e. through the process of heating the oil and water phases independently, combining them together with agitation, and then cooling to 40 °C before addition of the oleosomes.

Another patent by the same inventors, U.S. Patent No. 6,599,513, describes the use of oil bodies to achieve finished formulations applicable to cosmetics. Again all of the Examples comprise a heating step.

SUMMARY OF THE INVENTION

We have found that we can prepare stable, cosmetically acceptable emulsions simply by adding the requisite water or oil soluble ingredients directly to the dispersion of washed oil bodies (oleosomes) with stirring. No lengthy heating period is required and obviously no cooling period is needed either; the process comprises no heating step at all. The advantages of such an approach in terms of energy savings, preparation time, and in increased product throughput should be obvious.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the cosmetic or topical dermatological emulsions are prepared according to the present invention by adding the cosmetically or dermatologically active ingredients and, optionally, water to a dispersion of washed oil bodies (oleosomes) under stirring without any heating, at a temperature never exceeding about 40 °C, to obtain an oil-in-water emulsion containing at least 60% by weight of water. The order of addition is not critical and the ingredients may be added one at a time or in premixed form, e.g. water-soluble

components as aqueous solution or mutually miscible liquid ingredients as a mixture. Additional oil soluble ingredients (e.g. UV chromophores, vitamins, etc.) as well as other adjuvants and additives can also be added to the dispersion of oil bodies. The stirring can be effected by essentially any sort of stirrer or impeller operated at appropriate speed.

The cosmetic or topical dermatological products that can be prepared by the method of the present invention are essentially all products comprising oil-in-water emulsions in lotion, cream or paste form, including, but not limited to, moisturizing preparations, ointments, skin reparatives, sunscreens, skin lighteners, and decorative cosmetics such as color foundation creams, eye shadow creams and tanner creams.

Accordingly, they may comprise ingredients designed to exfoliate, repair, color, lighten, moisturize, smooth, condition, protect, prevent the moisture loss of, reverse the damage of, and clean and rejuvenate the skin and hair.

Such ingredients may include but are not limited to alcohols, esters, fatty acids, hydrocarbons, silicones, amines, enzymes, salts of amines and salts of fatty acids.

Other ingredients which could be added would include thickeners, barrier agents, UV chromophores, emollients, emulsifiers, conditioners, vitamins, etc.

Preferably, the emulsion is a cosmetic product is selected from the group consisting of moisturizers, ointments, cleansers, makeup removers, toners, night treatments, skin reparatives, sunscreens, skin lighteners, color foundations, eye shadows and tanners.

In one preferred embodiment, the product is a lotion.

In another preferred embodiment, the product is a cream.

The vegetable oil bodies (oleosomes) can be obtained from any plant cells, including cells from pollens, spores, seeds and vegetative plant organs in which oil bodies or oil body-like organelles are present. Preferably, the vegetable oil bodies are obtained from plant seeds or fruits, more preferably from the group of plant species consisting of rapeseed, soybean, sunflower, oil palm, cottonseed, peanut, walnut, coconut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, avocado and maize. Most preferably, the vegetable oil bodies are obtained from safflower seeds.

The preparation of the vegetable oil bodies (oleosomes) is disclosed in several patents, for example, U.S. Pat. No. 6,146,645. The preparation process comprises at least one washing step to accomplish removal of contaminating seed material, which is essential for obtaining a dispersion having properties suitable for being used in cosmetics. Furthermore, said removal of contaminating material reduces the risk of allergic reactions when the cosmetic preparation is applied to the skin.

The method of the present invention requires no additional emollients, emulsifiers etc. which constitutes a substantial advantage over the methods and products of prior art. In a preferred embodiment, no additional emulsifier is used.

The oleosomes are preferably used in an amount of from 2 wt.% to 15 wt.%, more preferably 5 wt.% to 12 wt.%, of a 70–75 wt.% emulsion in water, based on the total weight of the cosmetic or topical dermatological product.

The cosmetic or topical dermatological product produced according to the method of the present invention are remarkably stable and do not show any phase separation even after several months at elevated temperatures of e.g. 45 °C.

The following non-limiting examples are illustrative of the present invention:

EXAMPLES

General procedure:

All preparations have been carried out using a 2 L beaker and a Heidolph® RZR50 stirrer equipped with an anchor-type impeller of 10 cm diameter The stirring speed is given in min^{-1} ($1\ min^{-1} = 1$ revolution per minute ≡ 1 rpm). The batch size was 1 kg in each example.

Materials:

All materials used in the examples are commercially available, in particular:

Stearic acid, octyl methoxycinnamate, paraffin oil, cetyl alcohol, urea, vitamin A, vitamin E, glycerol, arbutin, polysorbate 80 and hydroxyethylcellulose are standard chemicals obtainable from several suppliers. Decaglycerol L DE (Natrulon® H 10), L-carnitine, Geogard® 361, Lonzest® 143S (myristyl propionate), sorbitan monooleate and Aldo® MCT (medium chain triglycerides) are supplied by Lonza Inc., Allendale, NJ, USA. Novemer® EC-1 is available from Noveon Inc., Cleveland, OH, USA. Almond fragrance 901A79 is available from Flavor & Fragrance Specialties Inc., Mahwah, NJ, USA. Safflower (*Carthamus tinctorius* L.) oil microspheres (oleosomes) are available from Sembiosys Genetics Inc., Calgary, Canada. Kelzan®-T is available from CP Kelco, Wilmington, DE, USA. The pigments used in examples 8 to 13, i.e., green color pigment, red cosmetic iron oxide, brown iron oxide and titanium dioxide are supplied by Whittaker, Clark & Daniels Inc., South Plainfield, NJ, USA. Papain is available from Enzybel S.A., Villers-le-Bouillet, Belgium and bromelain from Marcor Development Corp., Carlstadt, NJ, USA. *Rumex occidentalis* extract is obtainable from Atrium Biotechnologies Inc., Fairfield, NJ, USA.

An example of a typical sunscreen formulation prepared in the traditional way is shown below in Comparative Example 1. The process for preparation of a similar sunscreen via a "cold process" approach is shown in Example 1. Examples 2 to 15 relate to various cosmetic formulations.

Comparative Example 1

Sunscreen (SPF ≅ 13)

Ingredients	**% Weight**
Stearic Acid	6.00
Octyl Methoxycinnamate	8.00
Paraffin Oil	3.00
Cetyl Alcohol	2.00
Myristyl Propionate (Lonzest® 143S)	3.00
Medium Chain Triglycerides (Aldo® MCT)	3.00
Sorbitan Monooleate	5.05
Polysorbate 80	3.95
Water	65.60
Geogard® 361 (Preservative)	0.40
Novemer® EC-1	1.00
Almond Fragrance # 901A79	q.s.

SPF = sun protection factor, q.s. = quantum sufficit, "≅" = approximately

Procedure

Stearic acid, octyl methoxycinnamate, paraffin oil, cetyl alcohol, myristyl propionate, medium chain triglycerides, sorbitan monooleate and polysorbate 80 are mixed (phase 1) and heated to 80–85 °C with stirring to avoid charring and overheating. Water (phase 2) is heated to 80–85 °C. Phase 1 is added to phase 2 with vigorous stirring over a 30 minute time period. The temperature is maintained at 80 °C for an additional 30 min while agitation continues. The emulsion is then cooled while stirring vigorously until a temperature of 40 °C or less is achieved, whereupon the preservative, Novemer® and the fragrance is added. Total process time is approximately 4.5 hours.

Example 1

Sunscreen (SPF ≅ 15)

Ingredients	% Weight
Octyl Methoxycinnamate	8.00
Water	76.60
Safflower Oleosomes (70%)	8.00
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 10,240 mPa·s The formulation passed stability testing at 45 °C for two months.

Thus the formulation of Example 1 in which safflower oleosomes were substituted for the diluents, emollients and emulsifiers that were used in the Comparative Example 1 was prepared with no heating, no cooling, in much less time, and with far fewer ingredients.

Example 2

Emulsifier Free Sunscreen

Ingredients	**% Weight**
Octyl Methoxycinnamate	8.00
Water	76.60
Safflower Oleosomes (70%)	8.00
Decaglycerol L DE (Natrulon® H 10)	5.00
Hydroxyethylcellulose (Natrosol®)	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes and mix at 500 min^{-1} for 5 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add Natrosol® (presolubilized) and stir at 500 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 15 min. Total preparation time is ~30 min, viscosity 22,800 mPa·s. The formulation passed stability testing at 40 °C for two months.

Many other cosmetic or topical dermatological products can be prepared in stable form via the cold process route, for example reparative creams/lotions, moisturizing creams/lotions, lightener creams, ointment creams, exfoliating creams, eye shadow creams, color foundation creams or tanner creams (Examples 3–15).

Example 3

Reparative Cream

Ingredients	% Weight
Octyl Methoxycinnamate	8.00
Water	78.60
Safflower Oleosomes (70%)	7.00
L-Carnitine	1.00
Decaglycerol L DE (Natrulon® H 10)	3.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes along with the L-carnitine and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 17,800 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 4

Moisturizing Cream

Ingredients	% Weight
Octyl Methoxycinnamate	8.00
Water	69.60
Safflower Oleosomes (70%)	10.00
Urea	5.00
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with urea and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 17,800 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 5

Lightener Cream

Ingredients	**% Weight**
Water	84.00
Safflower Oleosomes (70%)	7.00
Arbutin	1.00
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with arbutin and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 13,220 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 6

Ointment Cream with Vitamin A and E

Ingredients	**% Weight**
Water	84.00
Safflower Oleosomes (70%)	10.00
Vitamin A	0.5
Vitamin E	0.5
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with vitamin A and E and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 12,320 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 7

Botanical Exfoliating Cream

Ingredients	% Weight
Water	72.60
Safflower Oleosomes (70%)	10.00
Enzyme concentrate[1)]	2.00
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Octyl Methoxycinnamate	8.00
Almond Fragrance # 901A79	q.s.

[1)] 18.9 mg papain (62000 PU) + 4.5 mg bromelain (3300 GDU) per 2 g solution (solvent: 50/50 mixture of decaglycerol and water)

Procedure

Add the water to the safflower oleosomes with papain and bromelain and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 17,310 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 8

Eye Shadow Cream

Ingredients	% Weight
Water	80.30
Safflower Oleosomes (70%)	12.00
Green Color Pigment A2-2502	0.30
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with green color pigment and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 17,820 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 9

Color Foundation Cream

Ingredients	**% Weight**
Water	80.10
Safflower Oleosomes (70%)	12.00
Red Cosmetic Iron Oxide	0.50
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with red iron oxide color pigment and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 17,200 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 10

Color Foundation/Tanner Cream

Ingredients	% Weight
Water	80.10
Safflower Oleosomes (70%)	12.00
Brown Iron Oxide # E 172	0.50
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with brown iron oxide color pigment and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 16,200 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 11

Color Blush/Eye Shadow Cream

Ingredients	% Weight
Water	82.35
Safflower Oleosomes (70%)	10.00
Red Cosmetic Iron Oxide	0.25
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with red cosmetic iron oxide color pigment and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at

500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 16,890 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 12

Color Blush/Eye Shadow Cream

Ingredients	**% Weight**
Water	77.60
Safflower Oleosomes (70%)	10.00
Red Cosmetic Iron Oxide	0.25
Decaglycerol L DE (Natrulon® H 10)	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with red cosmetic iron oxide color pigment and mix at 500 min^{-1} for 10 min. Add the decaglycerol to the mixture with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 16,890 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 13

Emulsifier Free Lightener/Exfoliating Cream

Ingredients	**% Weight**
Water	77.60
Safflower Oleosomes (70%)	10.00
Urea	1.00
Glycerol	2.00
Rumex occidentalis Extract	1.00
Kelzan®–T	3.00
Titanium Dioxide (Whittaker, Clark & Daniels # 3328)	5.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with urea and mix at 500 min^{-1} for 10 min. Add the glycerol and titanium dioxide and *Rumex occidentalis* extract with stirring for 2 min at 500 min^{-1}. Add the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Kelzan®-T and stir at 400 min^{-1} until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~23 min, viscosity 14,500 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 14

Reparative Cream For Dry Skin

Ingredients	**% Weight**
Octyl Methoxycinnamate	8.00
Water	73.60
Safflower Oleosomes (70%)	10.00
L-Carnitine	1.00
Decaglyceryl decaoleate	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes along with the L-carnitine and mix at 500 min^{-1} for 10 min. Add the decaglycerol decaoleate to the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 8 min. Add the Novemer® EC-1 and stir at 400 min^{-1} (5 min at 500 min^{-1}) until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~26 min, viscosity 17,800 mPa·s. The formulation passed stability testing at 45 °C for two months.

Example 15

Moisturizing Cream For Dry Skin

Ingredients	**% Weight**
Octyl Methoxycinnamate	8.00
Water	69.60
Safflower Oleosomes (70%)	10.00
Urea	5.00
Decaglyceryl decaoleate	5.00
Novemer® EC-1	2.00
Geogard® 361 (Preservative)	0.40
Almond Fragrance # 901A79	q.s.

Procedure

Add the water to the safflower oleosomes with urea and mix at 500 min^{-1} for 10 min. Add the decaglyceryl decaoleate to the methoxycinnamate and the preservative to the mixture and stir at 500 min^{-1} for 10 min. Add the Novemer® EC-1 and stir at 400 min^{-1} (2 min) until the mixture begins to thicken and then increase the stirrer speed to 600 min^{-1} and stir for an additional 3 min. Total preparation time is ~25 min, viscosity 17,800 mPa·s. The formulation passed stability testing at 45 °C for two months.

Claims:

1. A method for preparing a cosmetic or topical dermatological product comprising an oil-in-water emulsion containing at least 60% by weight of water, the method comprising the step of mixing at least one cosmetically or dermatologically active ingredient with an aqueous emulsion of washed vegetable oil bodies (oleosomes), wherein the temperature of said ingredients and said emulsion does never exceed about 40 °C.

2. The method of claim 1, wherein the product is a cosmetic product is selected from the group consisting of moisturizers, ointments, cleansers, makeup removers, toners, night treatments, skin reparatives, sunscreens, skin lighteners, color foundations, eye shadows and tanners.

3. The method of claim 1 or 2, wherein the product is a lotion.

4. The method of claim 1 or 2, wherein the product is a cream.

5. The method of any of claims 1 to 4, wherein the vegetable oil bodies (oleosomes) are selected from the group consisting of oil bodies obtained from rapeseed, soybean, sunflower, oil palm, cottonseed, peanut, walnut, coconut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, avocado and maize.

6. The method of claim 5, wherein the vegetable oil bodies (oleosomes) are obtained from safflower.

7. The method of any of claims 1 to 6, wherein no additional emulsifier is used.

8. The method of any of claims 1 to 7, wherein the vegetable oil bodies (oleosomes) are used in an amount of from 2 wt.% to 15 wt.% of a 70–75 wt.% aqueous emulsion, based on the total weight of the cosmetic or topical dermatological product.

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2005/003501

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K7/48 A61K7/02 A61K7/021 A61K7/42

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	examples 2,4,7 claims 30-40	1-8
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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

International Application No
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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(54) Title: ICE CONFECTION

(57) Abstract: An ice confection containing; i) at least 2% by wt. polyunsaturated fat; ii) at least 3% by wt. of protein; iii) at least 5% by wt. of a sugar or sugars; iv) 50% to 88% by wt. of water characterized in that at least 50% of the fat is present as oil bodies.

WO 2006/042608 A1

ICE CONFECTION

Technical field of the invention

The invention relates to ice confections, in particular to healthy ice confections which contain oil bodies.

Background to the invention

Frozen confections or "ice confections" such as ice cream are well known. Typically ice cream will contain, by weight of the composition, 10–18 % fat, 7-11.5 % milk solids not fat (MSNF, which contains casein micelles, whey proteins and lactose), 15–18% sugars and other ingredients such as stabilisers, emulsifiers and flavourings, and is aerated to an overrun of typically 100%.

The fats normally used in ice cream, for example butterfat or coconut oil, contain high levels of saturated fatty acids. It would be advantageous if the saturated fats commonly found in ice confection products could be replaced with polyunsaturated fats which are believed to be more healthy. WO 97/30600 discloses unaerated frozen desserts made with sunflower oil which is high in polyunsaturated fats.

However, it is difficult to make ice confections with fats which are high in polyunsaturated fatty acids because polyunsaturated fats are liquid at the processing temperature. This can result in an unstable air phase and hence a poor quality ice cream. Japanese Patent Application 57/036944 describes the production of aerated ice cream with oils such as safflower oil and sunflower oil. It was found necessary to emulsify the oil with a specific emulsifier, namely a sucrose fatty acid ester. However, such additives can detract from the natural and healthy perception of the product by consumers.

There is therefore a need for ice confections which contain polyunsaturated fats but which do not suffer from the drawbacks of poor aeration or the need for additives. It has now been discovered that the use of oil bodies in ice confection products can provide this.

Tests and definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. Definitions and

descriptions of various terms and techniques used in frozen confectionery manufacture are found in "Ice Cream", Fourth Edition by W.S. Arbuckle, Pub. Van Nostrand Reinhold, New York, 1986.

All percentages, unless otherwise stated, refer to the percentage by weight of the total composition, with the exception of percentages cited in relation to the overrun.

Oil Bodies

The fat in the seeds of oilseed crops, such as soy bean, rapeseed, sunflower and palm, is naturally emulsified in discrete subcellular structures known as oil bodies (alternatively oleosomes, lipid bodies or spheresomes). The fat is encapsulated by a monolayer of phospholipids in which proteins (known as oleosins) are embedded. The extraction of oil bodies from a range of plant seeds is known in the art, for example, Deckers et al. (US 6,146,645) and Wakabayashi et al. (EP 0 883 997).

The term 'oil body' as used herein refers to the lipid-oleosin protein complex. The term 'oil body' does not include the oil droplets in conventional ice cream emulsions in which the fat is emulsified with emulsifiers and / or proteins (such as mono-/di-glycerides and milk proteins) that are not present in the seeds.

The term 'oil body preparation' as used herein refers to the product of a process of extraction of intact oil bodies from a natural source, as in Example 1 below. The terms 'oil body' and 'oil body preparation' do not include the seeds per se.

Overrun

The overrun of an aerated ice confection is defined as

$$\text{overrun \%} = \frac{\text{density of mix - density of ice confection}}{\text{density of ice confection}} \times 100$$

The density of an ice cream (or other aerated ice confection) is measured by making use of the Archimedes' principle as described in "A-level Physics", Third Edition, by R. Muncaster, Pub. Stanley Thornes Ltd., Cheltenham, 1989. First a sample of ice cream is weighed in air to determine its mass. Then the volume of the same sample is determined. The sample of ice cream is held carefully in a beaker of chilled water just

below the surface of the water by a fork (or a knife) inserted into the end of the sample. The beaker is placed on a balance throughout the experiment and the increase in weight on immersing the sample is recorded. By Archimedes' principle, the increase in weight is equal to the upthrust and hence to the weight of water displaced. Taking the density of water as 1 gcm^{-3}, the weight of water displaced is used to determine the volume of water displaced and thus the volume of the ice cream sample immersed in the beaker. From the mass and volume of the product, the density of the ice cream is calculated (density = mass/volume). A minimum of three repeat measurements is taken.

The density of the unaerated mix is measured as follows. First the ice cream is melted until the air-phase is lost. Then a standard overrun cup (of known mass and volume) is filled with unaerated mix at approximately 4°C and weighed. Subtracting the mass of the cup and dividing by the known volume of the cup gives the density of the mix. A minimum of three repeat measurements is taken.

With a knowledge of the density of both unaerated mix and aerated ice cream, the overrun is calculated using the above equation.

Method for determining fat content

Fat content is determined by the "Weibul" acid hydrolysis procedure. This is a recognised BS Method (No.4401) Ref. Official, Standardised and Recommended Methods of Analysis SAC, 1973 2nd Ed. p 160. The sample is boiled with approximately 6M hydrochloric acid to release 'bound' fat and the digest is filtered through a double filter paper using filter aid. Fat is retained by the filter paper and aid. After washing and drying, the residue is extracted with light petroleum spirit using a Soxhlet extractor. Descriptions of the performance of the method and a comparison with other methods can be found in the following references

a) Weibul, Staatsbled van het Koninkrijk Der Hederlander pl – 16, 1919, No. 581.
b) W Stoldt, Z Undersuchung & Lebensmittel, 1937, 73. 329.
c) Nottbolm &, Baumann, Z Undersuchung & Lebensmittel 1931, 62. 164.
d) ISO 1143-1973

Method for determining protein content

Protein content is determined by measuring the nitrogen present in the sample. This is done by using equipment that is manufactured for the purpose: the "Macro N" (Foss-Heraeus). In this procedure, the sample under test is completely burned at temperatures in excess of 1000°C in the presence of oxygen. The resultant combustion gases are swept through a series of absorption tubes by a stream of carbon dioxide; this procedure removes unwanted gases, finally the carbon dioxide and nitrogen mixture are passed through a thermal conductivity detector where the nitrogen is quantified. Nitrogen content is converted to protein content using a conversion factor based on the average nitrogen content of the amino acids found in particular foods.

A suitable conversion factor to use for analysing the protein content in ice confections that are made according to this invention is 6.25, although other conversion factors could be used - based on the particular protein source that is being analysed. The procedure is published in the following article.

e) Ian D Smith, Analytical Proceedings, 1991, 28. 320 - 324. "Evaluation of the Foss-Heraeus Macro N for the Determination of Nitrogen in a Wide Range of Foodstuffs, Ingredients and Biological Materials and Comparison with the Kjelfoss".

Method for determining water content

The method involves the measurement of weight loss due to evaporation of water. A fan assisted, thermostatically controlled air oven is used at a temperature of 100°C. The procedure described is similar to Official and Standardised methods recommended by:

f) The Association of Official Agricultural Chemists USA, 'Official Methods of Analysis' 12th Edition, 1975,

g) The Fertiliser and Feedingstuffs Regulations HMSO, Statutory Inst. No 840, 1976.

h) ISO 1026-1982, ISO 1442-1973.

15-20 gram of dry sand and a small glass rod are placed in an aluminium foil cup. This assembly is weighed (= W1). A sample (approximately 5 gram) of e.g. melted ice cream is added to the cup and weighed again (= W2). The melted ice cream is then mixed into

the sand with the glass rod. The cup is then placed on a steam bath and evaporated to dryness (takes 30 minutes); the sample is stirred with the rod throughout this procedure. The sample is then placed in an oven for 2.5 hours that has been pre-set at 100°C. The cup is then placed in a dessicator to cool before weighing (= W3).

Water content is given by:

$$\%\text{Water w/w} = \frac{W2 - W3}{W2 - W1} \times 100$$

Where: W1 = Weight of cup (including sand and glass rod).
W2 = Weight of cup + wet sample.
W3 = Weight of cup + dried sample.

Detection of oil bodies

The presence of oil bodies is detected in ice confections by the presence of oleosin protein (which is usually not present in refined fats such as sunflower oil). The presence of sunflower oil is detected by the presence of triacylglycerols and other characteristic components. Suitable methods are described below.

Detection of oleosin by amino acid sequencing

Amino acid sequences for oleosins from sunflower seeds and other oil seeds have been published and are available through sequence databases such as SwissProt and PIR (1). The sequences of oleosins from different species are related, in particular the central, hydrophobic domain is the region most conserved between species (2). Therefore, the oleosin protein can be identified by amino acid sequencing. Fragments of amino acid sequence obtained from the product (as described below) are compared with the published sequences using database searching and sequence comparison facilities that are well-known in the art, such as ExPasy or SRS. If the stretches of sequence from the product closely match a published sequence, it indicates that oleosin from that oil seed is present in the product.

The protein component of an ice cream product is separated from the other ingredients as follows:

In order to extract intact oil bodies from the ice cream, 1-2g of the confection is placed in an eppendorf tube and allowed to melt. The sample is then centrifuged at 13,500rpm for 5 minutes. The resulting 'fat pad' on the surface of the sample is transferred into a fresh eppendorf tube.

In order to remove non-oleosin proteins (such as sunflower seed proteins and milk proteins) the sample is washed with urea. 1ml of 9M urea is added to the fat pad, mixed by vortexing thoroughly and incubated in the fridge for 2 hours. The sample is then centrifuged and the fat pad is skimmed off. Two further urea washes are performed.

In order to remove the fat from the intact oil bodies and to precipitate the oleosins, 1ml of acetone chilled to -25°C is added to the fat pad, and the sample is incubated on ice for 1 hour. The sample is then centrifuged at 13500rpm. The precipitate is retained and the supernatant is discarded. Two further washes with chilled acetone are then carried out and the pellet is left to air-dry overnight.

In order to prepare the sample for SDS-PAGE (Polyacrylamide gel electrophoresis), 0.001g of the dry powder is re-solubilised in 0.5ml sample buffer, and incubated at room temperature for 30 minutes. The sample reducing agent is then added and the sample is boiled for 2 minutes. The sample can then be run on SDS-PAGE alongside molecular weight standards, and the protein bands visualised with a stain such as coomassie blue. 25µl of the oleosin sample solution is loaded into to each of 8 wells of a 10% bis-tris NuPAGE gel. The gel is then run using MES running buffer. Using this procedure, two oleosin protein bands are typically seen. These bands correspond to the two oleosin isomers (approximate molecular weights 19.5kD and 20.5kD).

As the oleosin proteins are blocked to N-terminal sequencing (3), the protein bands are digested using an in-gel digestion technique and a suitable proteolytic enzyme such as trypsin or endoproteinase Lys-C. The protein fragments are then separated from each other using reverse phase chromatography, and the individual fragments sequenced using standard protein sequencing equipment. The short pieces of internal amino acid sequence thus obtained are compared with the published oleosin protein sequences as described above.

A general review article describing commonly used methods for preparing proteins for sequencing, including the strategies outlined above can be found in ref. (4).

1. Examples of sunflower seed oleosin sequences include the following accession numbers: SwissProt P29529 and PIR S70453.
2. Napier J.A., Beadoin F., Tatham A.S., Alexander L.G., Shewry P.R. (2001) Adv. in Bot. Res. **35** 111-138.
3. Millichip M., Tatham A.S., Jackson F., Griffiths G., Shewry P.R., Stobart A.K. (1996) Biochem. J. **314** 333-337.
4. Patterson S.D., (1994) Anal. Biochem. **221** 1-15.

Detection of triacylglycerol and other components that are characteristic of sunflower oil

Triacylglycerol profiles for sunflower oil are easily determined by GC analysis. In addition, phospholipid (PL) profiles are determined using HPLC. For data on fatty acids/triacylglycerols/ PL/minor components in sunflower oil see the Lipid Handbook by F.D. Gunstone, J. Harwood and F.B. Padley, Pub. Chapman and Hall, 1986, Chap. 3.3, **35**, p 101; and also for sunflower oil fatty acid profiles see Codex Alimentarius Commission, Codex Committee on oils and fats. For the determination of seed oil content and fatty acid composition in sunflower oil through the analysis of intact seeds, husked seeds, meal and oil by near IR reflectance spectroscopy: Perez-Vich B., Velasco L., Fernandez-Martinez J.M., J. Am. Oil Chem. Soc., **75** (5), 547 – 555. For Phospholipid (PL) Profiles see Chapman G.W., J. Am. Oil Chem. Soc., **59**, 299.

The levels of sterols, triterpene alcohols and tocopherols which are found in sunflower oil can all be determined by GC/HPLC and with Mass Spectrometry detection. Data on sterols are found in the Lipid Handbook by F.D. Gunstone, J. Harwood and F.B. Padley, Pub. Chapman and Hall, 1986, Table 3.163 (adapted from Itoh et al. J. Am. Oil Chem. Soc. 1973). Tocopherols may be found in the Lipid Handbook table 3.167, p 129, or in Analysis of oilseeds, fats and fatty foods, Elsevier, London, p 315.

Brief description of the invention

We have found that frozen confections can be formulated with polyunsaturated fats without the use of additives by using oil bodies as a source of the fat. Thus, in a first aspect, the present invention provides an ice confection containing;

i) at least 2% by wt. polyunsaturated fat;

ii) at least 3% by wt. of protein;

iii) at least 5% by wt. of a sugar or sugars;

iv) 50% to 88% by wt. of water

characterized in that at least 50% of the fat is present as oil bodies.

Oil bodies provide a source of healthy polyunsaturated fats for ice confection products. Furthermore liquid fats obtained from sources such as sunflower oil are normally refined in order to remove pro-oxidants, which would otherwise increase the tendency of the fat to become rancid over time, especially if the fat is in proximity to air, as is the case in ice cream. However, refining also reduces the amount of desirable ingredients, for example, vitamin E. In contrast oil bodies, which are an unrefined source of oil, retain these desirable natural ingredients, providing a further health aspect. The resultant ice confection products also have a highly acceptable taste and do not become rancid on storage. Preferably at least 80%, more preferably at least 95%, most preferably all of the fat is present as oil bodies.

Preferably the ice confection has an overrun of more than 20%, more preferably more than 50%, most preferably more than 75%. Equally preferably the ice confection has an overrun of less than 200%, more preferably less than 150%, most preferably less than 120%. It has been found that when the fat is present as oil bodies, aerated ice confections that have a stable air structure are produced even though the fat is liquid at standard processing temperatures.

Preferably, the ice confection comprises at least one emulsifier. Examples of known emulsifiers include mono- and di-glycerides of saturated or unsaturated fatty acids (for example monoglyceryl palmitate), polyoxyethylene derivatives of hexahydric alcohols (usually sorbitol), glycols, glycol esters, polyglycerol esters, sorbitan esters, stearoyl lactylate, lactic acid esters, citric acid esters, acetylated monoglyceride, diacetyl tartaric acid esters, polyoxyethylene sorbitan esters, lecithin and egg yolk and mixtures thereof. It has been found that the presence of emulsifier improves the storage stability and meltdown of the ice confection.

Preferably the emulsifier is a mono-/diglyceride of saturated fatty acids with a monoglyceride content of at least 40%.

Preferably the emulsifier is present at between 0.1 and 1.5% by weight.

Preferably the oil bodies are derived from a source selected from the group consisting of the seeds of sunflower, rapeseed, soybean, oil palm, cotton seed, ground nut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, maize, sesame, chick pea, avocado; or from avocado fruit, or any mixture thereof. More preferably the oil bodies are derived from a source selected from the group consisting of the seeds of sunflower, soybean, avocado or rapeseed, or from avocado fruit, or any mixture thereof. Most preferably the oil bodies are derived from sunflower seed.

Preferably the ice confection contains oil bodies at a level of 0.5 % to 20 %, more preferably 2 % to 11 % by weight of the ice confection.

Preferably, the oil bodies used in this invention have an oil/protein ratio (=lipid/protein ratio) by weight of greater than 3.5, and preferably less than 20.

Proteins which may be present in the ice confection (in addition to oleosin proteins) include milk proteins, soy protein, wheat protein, barley protein, lupin protein and mixtures thereof. Particularly preferred are milk proteins owing to their superior flavour, heat stability and surface activity. Suitable sources of milk protein include milk, concentrated milk, milk powders, whey, whey powders and whey protein concentrates/isolates. In order to aid in emulsification and/or aeration during manufacture of the frozen confection it is preferable that the protein content (in addition to oleosin proteins) is greater than 3% by weight of the frozen confection, more preferably greater than 4%. In order to prevent the texture of the confection from becoming chalky, it is also preferable that the protein content (in addition to oleosin proteins) is less than 8%, more preferably less than 7% by weight of the frozen confection.

Sugars are present in the ice confections of the invention. The sugar is typically a mono-, di-, or oligo-saccharide or sugar alcohol for instance, sucrose, dextrose, fructose, lactose (for example from milk solids), purified lactose, lactose monohydrate, glucose syrup, invert sugar, corn syrup, fructose, erythritol arabitol, xylitol, sorbitol, glycerol, mannitol, lactitol and maltitol or mixtures thereof. In order to provide freezing point depression and contribute to the sweetness of the confection it is preferred that the confection comprises at least 5% sugars, more preferably at least 8% and most preferably at least 10% by

weight of the frozen confection. To avoid the confection being too sweet, it is preferred that the confection comprises at most 25% sugars, more preferably at most 20% and most preferably at most 18% by weight of the frozen confection. Where large freezing point depression is required, so that the ice confection produced is soft, low molecular weight molecules such as fructose may be selected. Some or all of the sugar may be provided from fruit puree. Preferably a blend of sugars is used; more preferably one of the sugars is sucrose.

Stabilisers may be included in the ice confections of the invention. Stabilisers that may be used include proteins such as gelatin; plant extrudates such as gum arabic, gum ghatti, gum karaya, gum tragacanth; seed gums such as locust bean gum, guar gum, psyyllium seed gum, quince seed gum or tamarind seed gum; seaweed extracts such as agar, alganates, carrageenan or furcelleran; pectins such as low methoxyl or high methoxyl-type pectins; cellulose derivatives such as sodium carboxymethyl cellulose, microcrystalline cellulose, methyl and methylethyl celluloses, or hydroxylpropyl and hydroxypropylmethyl celluloses; and microbial gums such as dextran, xanthan or β-1,3-glucan. Preferably, the stabiliser is selected from locust bean gum, kappa carrageenan, guar gum or mixtures thereof. Preferably the stabilisers are present at a level of 0.05-1 wt% of the composition.

In addition, the ice confection may contain flavouring and/or colouring. Typical flavourings include mint, vanilla, chocolate, coffee, or fruit flavours. Preferably, the flavouring or colouring will be present at a level of less than 1 wt% of the composition. Pieces of nut, chocolate, ginger, biscuit, fruit, fruit puree, or other ingredients or additives commonly added to ice cream or other ice confections may also be included.

Detailed Description of the invention

The present invention will be further described in the following examples and by reference to the drawing, wherein Figure 1 represents a plot of the meltdown results for ice creams containing 5% oil bodies with and without emulsifier. In the following examples, compositions demonstrating various facets of the invention were prepared.

Example 1. A method for producing an oil body preparation

A total of 1.7 kg of de-hulled sunflower seeds was ground in a food-processor until no large particles were present. The ground seeds were homogenised in two volumes of cold grinding buffer (0.6 M sucrose and 1.0 M NaCl) using a Waring blender (a commercial heavy duty blender) at low speed. The homogenate was filtered through a 500µm pore size sieve to remove large particles and seed skins. After sieving, the homogenate was centrifuged at 10,000xg for 30 minutes at 4°C in order to remove large particles, insoluble proteins and separate the oil bodies from the aqueous soluble seed proteins. The floating oil body layer was skimmed off by using a metal spatula and added to one volume of floating buffer (0.6 M sucrose).

After homogenisation in the Waring blender at low speed, the mixture was sieved through a 150µm pore-size sieve to obtain an emulsion with oil bodies less than 150µm in size. The homogenised oil bodies were centrifuged again as described above. The skimmed oil bodies were washed twice in one volume of floating buffer and after each wash step centrifuged as described. The final oil body preparation was placed in a sealed plastic container and stored at 4°C until used.

Approximately 1kg of oil body preparation was produced. This was determined to have a water content of approximately 35% using the method described above. Therefore the "dry" oil body content of the preparation was approximately 65% by weight. It is important to measure this for each oil body preparation so that it is known how much oil body is added to each mix (see below). When the method for producing oil bodies was repeated several times it was found that the water content in different oil body preparations varied slightly (between 30% and 40%). Therefore the "dry" oil body content varied between 60% and 70%.

Example 2. Production of frozen confections with oil bodies as the only source of fat

Ice cream mixes were prepared as shown in Table 1, based on a standard ice cream formulation taken from Ice Cream, Fourth Edition by W.S. Arbuckle, Pub. Van Nostrand Reinhold, New York, 1986.

Table 1: Ice cream formulations (amounts are expressed as dry weight %)

Ingredient				
Skimmed milk powder	11.5	11.5	11.5	11.5
Sucrose	15.0	15.0	15.0	15.0
Locust bean gum	0.144	0.144	0.144	0.144
Carrageenan L100	0.016	0.016	0.016	0.016
Vanillin	0.012	0.012	0.012	0.012
Oil bodies	5.0	5.0	10.0	10.0
HP 40	0	0.3	0	0.3
Water	to 100%	to 100%	to 100%	to 100%

HP40 is a saturated mono-diglyceride (40% monoglyceride) supplied by Danisco.

The mixes were prepared as follows: the ingredients other than the oil body preparation were mixed, stirred and heated to 60-70 °C. The oil body preparation was then added, so that the amount of oil bodies in the final mix was 5% or 10% as required. Since water is present in the oil body preparation an allowance was made when calculating the mass of oil body added to an ice confection. For example, to make a mix containing 5% oil bodies an amount of 7.7% oil body preparation (which consists of 65% oil bodies and 35% water) was used. Similarly the amount of water in the oil body preparation was taken into account when preparing the mix, so that the required concentrations were achieved after the oil body preparation was added.

Then the mix was heated to approximately 80°C to pasteurise it and dispersed using a homogeniser (Silverson L4R). The mix was then left to cool overnight at approximately 4°C. The oil body mix was re-homogenised (using the Silverson homogeniser) immediately prior to freezing.

The mixes were aerated and frozen in a Technohoy MF75 scraped surface heat exchanger fitted with a C29800 open dasher. The mixes were extruded at between -5°C and –6°C with 100% overrun through a fluted nozzle into pots or 500 ml cardboard boxes. The products were blast frozen at –35°C for 3-4 hours then stored at –25°C. Ice creams were produced satisfactorily both with and without the emulsifier.

The ice creams had an acceptable texture. The ice creams with 10% oil bodies were thicker and had more body than those with 5%.

Example 3

The ability of products to resist melting and serum leakage when exposed to ambient temperatures for an extended period of time was measured as follows. A block of ice cream (500 ml) was taken from a –25°C cold store, placed on a wire mesh (3.0 mm square holes, 1.0 mm thick wire) on a balance and the mass of the block was recorded. The mesh with the block was then placed above a funnel and collection container in a controlled temperature environment at +20°C. The mass collected in the container (i.e. mass lost from the product) was measured automatically by computer every minute for 250 minutes. 3 repeats of each sample were measured simultaneously. The results were plotted as average percentage mass loss as a function of time.

The meltdown results for the 5% oil body samples with and without emulsifier are shown in Figure 1. The effect of the emulsifier on the oil body ice cream was to reduce the percentage mass lost over 250 minutes thereby improving the meltdown.

Example 4: Effect of temperature abuse

Samples produced as described in Example 2 were temperature abused by cycling them between –10°C and –20°C in a temperature controlled cabinet every 12 hours for 3 weeks.

Samples with no emulsifier lost some air during abuse: the ice cream had visibly shrunk away from the edge of the pot and had decreased in height. However, samples with emulsifier retained their shape during temperature cycling and did not lose overrun. Thus the presence of the emulsifier improved the storage stability of the ice cream. No rancidity was detected on tasting in samples with or without emulsifier.

The various features of the embodiments of the present invention referred to in individual sections above apply, as appropriate, to other sections *mutatis mutandis*. Consequently features specified in one section may be combined with features specified in other sections as appropriate.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and products of the invention will be apparent to those skilled in the art without departing from the scope of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are apparent to those skilled in the relevant fields are intended to be within the scope of the following claims.

Claims

1. An ice confection containing;
i) at least 2% by wt. polyunsaturated fat;
ii) at least 3% by wt. of protein;
iii) at least 5% by wt. of a sugar or sugars;
iv) 50% to 88% by wt. of water
characterized in that at least 50% of the fat is present as oil bodies.

2. The ice confection of claim 1 wherein at least 80% of the fat is present as oil bodies.

3. The ice confection of claim 1 or claim 2 wherein the overrun is more than 20%.

4. The ice confection of claim 3, wherein overrun is less than 200%.

5. The ice confection of claim 4 wherein the confection has an overrun of more than 75% and less than 120%.

6. The ice confection of any preceding claim which further comprises at least one emulsifier.

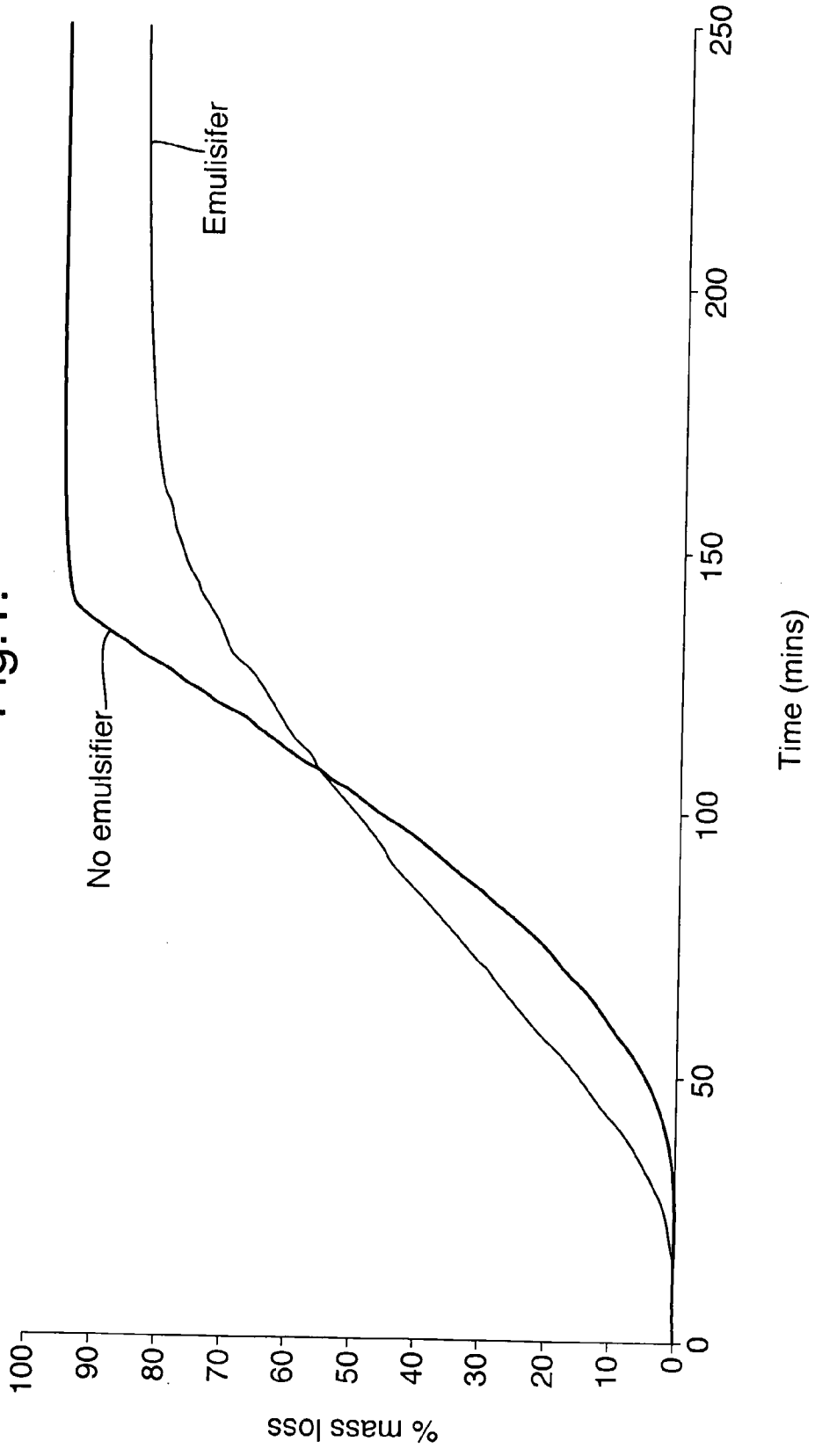
7. The ice confection of claim 6 wherein the emulsifier is present at between 0.1 and 1.5% by weight.

8. The ice confection of any preceding claim wherein the oil bodies are derived from a source selected from the group consisting of the seeds of sunflower, rapeseed, soybean, oil palm, cotton seed, ground nut, castor, safflower, mustard, coriander, squash, linseed, brazil nut, jojoba, maize, sesame, chick pea and avocado; or from avocado fruit, or any mixture thereof.

9. The ice confection of claim 9 wherein the oil bodies are derived from sunflower seeds.

10. The ice confection of any preceding claim wherein the oil bodies are present at a level of 0.5 % to 20 % by weight of the ice confection.

Fig.1.


No emulsifier
Emulisifer
% mass loss
Time (mins)
0
10
20
30
40
50
60
70
80
90
100
0
50
100
150
200
250

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2005/010165

A. CLASSIFICATION OF SUBJECT MATTER
A23G9/32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, FSTA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 04, 30 April 1996 (1996-04-30) -& JP 07 313066 A (KAO CORP), 5 December 1995 (1995-12-05) abstract	1-10
A	PATENT ABSTRACTS OF JAPAN vol. 006, no. 106 (C-108), 16 June 1982 (1982-06-16) -& JP 57 036944 A (LOTTE CO LTD), 27 February 1982 (1982-02-27) cited in the application abstract	1-10

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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International application No.
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6 146 645 A (DECKERS ET AL) 14 November 2000 (2000-11-14) cited in the application column 12; claims	1-10
A	US 6 558 729 B1 (RIVIERE PHILIPPE ET AL) 6 May 2003 (2003-05-06) cited in the application the whole document	1-10

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2005/010165

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(54) Title: SATIETY ENHANCING FOOD PRODUCTS

(57) Abstract: The present invention relates to an food product comprising at least 0.1 wt.% of oil bodies, said oil bodies having a volume weighted mean diameter within the range of 0.1- 100 m and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked. Furthermore the invention concerns the use of such cross-linked oil bodies in the treatment or prevention of overweight. The inventors have found that resistance of oil bodies against digestion may be improved dramatically by cross-linking the structural proteins contained therein. It is believed that the cross-linked oil bodies according to the present invention have a positive influence on appetite suppression because the oil contained in the oil bodies will reach the distal gut and thus inhibit transit through the proximal small intestine by means of the so called ileal brake mechanism.

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SATIETY ENHANCING FOOD PRODUCTS

TECHNICAL FIELD OF THE INVENTION

The present invention relates to satiety enhancing food products. More particularly, the present invention relates to such satiety enhancing food products containing oil bodies.

BACKGROUND OF THE INVENTION

Obesity is defined as an excess of body fat relative to lean body mass. Not only is obesity regarded as unattractive, it is also a well-established risk factor for a number of potentially life-threatening diseases such as atherosclerosis, hypertension, diabetes, stroke, pulmonary embolism, sleep apnea, and cancer. Furthermore, it complicates numerous chronic conditions such as respiratory diseases, osteoarthritis, osteoporosis, gall bladder disease, and dyslipidemias. The enormity of this problem is best reflected in the fact that death rates escalate with increasing body weight. More than 50% of all-cause mortality is attributable to obesity-related conditions once the body mass index (BMI) exceeds 30 kg/m^2, as seen in 35 million Americans (Lee, JAMA 268:2045-2049, 1992).

The accumulation or maintenance of body fat bears a direct relationship to caloric intake. Comprehensive treatment programs, therefore, focused on behaviour modifications to reduce caloric intake and increase physical activity using a myriad of systems. These methods have limited efficacy and are associated with extremely high recidivism rates.

Obesity has also been treated by administering specific agents, for example, anorectic agents, to obese subjects.

However, anorectic agents such as dextroamphetamine, the combination of the non-amphetamine drugs phentermine and fenfluramine (Phen-Fen), and dexfenfluramine (Redux) alone, are associated with serious side effects. Indigestible materials such as olestra (OLEAN), mineral oil or neopentyl esters have been proposed as substitutes for dietary fat. Garcinia acid and derivatives thereof have been described as treating obesity by interfering with fatty acid synthesis. Swellable cross-linked vinyl pyridine resins have been described as appetite suppressants via the mechanism of providing non-nutritive bulk.

Surgical interventions, such as gastric partitioning procedures, jejunoileal bypass, and vagotomy, have also been developed to treat severe obesity. Although these surgical procedures are somewhat more effective in the long run, the acute risk benefit ratio has reserved these invasive procedures for morbidly obese patients. Therefore, this approach is not an alternative for the majority of overweight patients unless and until they become profoundly obese and are suffering the attendant complications.

Meal replacer products have also been proposed as part of a healthy diet in order to control or reduce body weight. For example, U.S. Pat. No. 5,688,547 discloses a nutritional meal replacement composition comprising dietary fibre, protein, a cellulose gum and gel. These meal replacer products are generally products that are intended to be consumed as a single-serving food product, such as a bar, drink etc to replace one or two meals per day. The meal replacer products are designed such that on the one hand they provide a restricted caloric intake, but on the other hand they provide a healthy balance of nutritional ingredients and are convenient to incorporate into an individual's daily diet.

However, a general problem with products intended to be used in a weight loss or weight maintenance plan, e.g. meal

replacer products or low-calorie snacks, is that feelings of hunger may occur sooner than desired after consumption and/or the feeling of satiety obtained may not be as great as desired. Both of these considerations may render it difficult for the individual to adhere to the plan or it may make it and/or the products used therein less appealing to consumers.

Recognising the demand for effective and convenient satiety-inducing food products, research has been carried out to try to address the problems associated with the above approaches to controlling or reducing body weight.

One approach to addressing the aforementioned problems has been to investigate the use of satiety agents in food products in order to increase the satiety effect obtained from consuming a food product comprising the satiety agents.

WO 99/02041 discloses a food composition giving a prolonged feeling of satiety and comprising a mixture of specific triglyceride oils and a food emulsifier.

Another approach to reduce the feeling of hunger which has been suggested is to use the principle of the ileal brake. The ileal brake principle itself is described by Gregg W. Van Citters in The Ileal Brake: A fifteen-year progress report, Current Gastronenterology Reports 1999, I:404-409 and which concerns the delivery of satiety agents to parts of the gut e.g the ileum, duodenum or jejunum.

However, the satiety effects obtained by the above compositions are often sub-optimal. Furthermore, many of the satiety enhancing agents proposed in the prior art can only be applied in particular types of food products and/or their use is associated with regulatory issues.

Thus, there still is a need for a variety of food compositions that provide a good satiety effect for consumers, especially those wishing to control their calorie intake and/or body weight.

Peng CC et al in Biotechnol. Prog. Vol 19 (2003), pages 1623-1626 describe size and stability of reconstituted sesame oil bodies. Reconstituted oil bodies are seen as carrier for hydrophobic molecules such as neutraceuticals and cosmetic lipids. No food application is described, nor any use of cross-linked oil bodies in satiety enhancement.

US2004/258803 describes the use of encapsulated satiety agents. No use of cross-linked oil bodies in satiety enhancement is disclosed.

SUMMARY OF THE INVENTION

The present inventors have unexpectedly discovered that the aforementioned need can be met by providing food products containing a significant amount of oil bodies in which the structural proteins have been cross-linked.

Cross-linking of naturally derived and reconstituted oil bodies with glutaraldehyde and genipin has been described by Peng et al. (Biotechnol. Prog. 2003, 19, 16523-1626). The authors report that the cross-linking was found to improve the thermostability of the oil bodies.

Oil bodies are the discrete intracellular organelles in which triacylglycerides are stored in, for instance, plant seeds. Plant oil bodies are spherical particles about 0.5-4 µm in diameter and are found in all oilseed plants (i.e. Canola, flax, sunflower, soybean and corn). The oil-bodies consist primarily of central droplet of oil (triglycerides) that is surrounded by a coat consisting of a phospholipid monolayer and a structural protein.

Oil bodies are remarkably stable both inside cells and in isolated preparations. In both situations the oil bodies occur as individual entities. Even when pressed against one another during seed desiccation or flotation centrifugation, oil bodies hardly aggregate or coalesce. The structural proteins that together with the phospholipid monolayer surround the central lipid core of the oil bodies, are believed to protect the phospholipids in the monolayer by making them inaccessible to external phospholipase A2 and phospholipase C. Structural proteins found in plant oil bodies include oleosins and caleosins.

The structural protein oleosin is a relatively small protein (15 to 26 kD) and typically contains a central stretch of about 72 hydrophobic residues that is inserted into the lipid core whereas the amphiphatic N-terminal and C-terminal are located at the surface of the oil body. Both terminal portions of the oleosin can be either short (e.g. 4 residues) or very long (e.g. 1200 residues). The central hydrophobic stretch of 72 uninterrupted hydrophobic residues is the hallmark of an oleosin. No other known protein in any organism has such a long hydrophobic stretch (Hsieh et al., Plant Physiol. 2004 November; 136(3): 3427-3434).

The key structural features of caleosins are an N-terminal region with a single Ca^{2+}- binding EF hand domain, a central hydrophobic region able to form a single bilayer span, and a C-terminal region with several putative protein kinase phosphorylation sites (D.J. Murphy, LIPID ASSOCIATED PROTEINS IN PLANTS, 16th Int. Plant Lipid Symposium, Budapest, June 2004, pages 1-8). Caleosins lack an N-terminal signal peptide, but do have a central, hydrophobic region of typically more than 30 residues. Like oleosins, caleosins contain a proline-rich region with the potential to form a "proline know" motif.

In vitro studies under simulated human GI tract conditions conducted by the inventors showed that non-cross-linked oil bodies are largely stable under gastric conditions and that degradation starts upon addition of intestinal enzymes. Consequently, even if the stability of oil bodies during digestion is higher than that of oil droplets contained in simple oil-in-water emulsions, the are not sufficiently stable to survive until the distal part of the intestine (ileum).

The inventors have found that resistance of oil bodies against digestion may be improved dramatically by cross-linking the structural proteins contained therein. Although the inventors do not wish to be bound by theory, it is believed that the cross-linked oil bodies according to the present invention have a positive influence on appetite suppression because the oil contained in the oil bodies will reach the distal gut and thus inhibit transit through the proximal small intestine by means of the so called ileal brake mechanism. The term "ileal brake" refers to a physiological state of the lower small intestine that slows down the normal digestive process. Specifically, it is an endocrine-induced intestinal change in which the contents (e.g., nutrients) of the lower small intestine (ileum) influence upper gastrointestinal activity such as by decreasing gastric and pancreatic secretions and preventing gastric emptying.

DETAILED DESCRIPTION OF THE INVENTION

Accordingly, one aspect of the invention relates to an food product comprising at least 0.1 wt.% of oil bodies, said oil bodies having a volume weighted mean diameter within the range of 0.1-100 µm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins,

wherein said structural proteins have been cross-linked. The oil bodies according to the present invention will be referred hereinafter as "cross-linked oil bodies" or "the oil bodies".

The aforementioned lipid matrix represents the core of the oil body and essentially consists of lipids. According to a preferred embodiment, the lipid matrix contains at least 80 wt.%, more preferably at least 90 wt.%, even more preferably at least 95 wt.% and most preferably at least 98 wt.% of glycerides selected from the group consisting of triglycerides, diglycerides, monoglycerides, phosphoglycerides and combinations thereof. In a preferred embodiment, the glycerides are selected from the group consisting of triglycerides and diglycerides. Most preferably, the glyceride is triglyceride oil. Besides glycerides of various structures and fatty acid chain length the lipid matrix may also contain other minor components that are dissolved in said matrix.

Examples of lipids that may be present in the lipid matrix include: triglycerides of various structures and fatty acid chain lengths and other minor components that are dissolved in said matrix.

In order to achieve a significant resistance against intestinal digestion, it is preferred that at least 5% of the structural proteins in the oil bodies have been cross-linked. Even more preferably, at least 20% of the structural protein is cross-linked, most preferably at least 40% is cross-linked. It should be understood that the term "cross-linking" as used herein refers to the introduction of a covalently bonded link between two different protein molecules or between different parts of the same protein molecule.

The degree of cross-linking as referred to herein is defined as the average number of amino acid residues per structural protein molecule that is conjugated with (covalently bound to) a cross-linking agent. Analytical methods suitable

for determining the degree of cross-linking can be found in the relevant scientific literature. Different methods may be applied to determine the degree of cross-linking, depending on the nature of the cross-linking agent. A suitable method for determining the degree of cross-linking is the amino acid analysis described in US 2005/0130261, which is incorporated herein by reference.

Another methodology for determining the degree of cross-linking makes use of LC-MS/MS. Sample preparation includes hydrolysis of the proteinaceous material, e.g. by treatment with 6 mol/L hydrochloric acid at 110 °C for 24 hours. The protein hydrolysate so obtained will essentially consist of single amino acids and amino acids that are linked together by a cross-linking agent. The latter components can easily be identified on the basis of their relatively high molecular mass.

Typically, the cross-linked oil bodies according to the present invention exhibit a degree of cross-linking of at least 0.3, preferably of at least 1.0, more preferably of at least 3.0 and most preferably of at least 5.0.

According to a particularly preferred embodiment, a significant fraction of the structural proteins in the oil bodies contain intermolecular cross-links that link these structural proteins to one or more other structural proteins within the same oil body or another oil body. Advantageously, the degree of intermolecular cross-linking is at least 0.1, more preferably at least 0.3 and most preferably at least 1.0. Here the degree of intermolecular cross-linking is defined as the average number of amino acid residues per structural protein molecule that is covalently bound to another structural protein molecule by means of a cross-linking agent.

Cross-linking of the structural protein in the oil bodies may suitably be achieved with any method known in the art,

provided the cross-linked material can be considered food-grade. The structural proteins may, for instance, be cross-linked by carbonylic cross-linkers, phenolic cross-linkers and/or enzymatic cross-linkers.

The term "carbonylic cross-linkers" covers cross-linking agents that form covalent bonds with protein residues through a reactive carbonyl group. Preferred carbonylic cross-linkers are ketoses, polyfunctional aldehydic or phenolic cross-linkers. A typical example of a polyfunctional aldehydic cross-linker is glutaraldehyde (pentane-1,5-dial). A typical example of a ketose cross-linker is dihydroxy-acetone (1,3-dihydroxy-2-propanone).

Typical examples of phenolic acid cross-linkers are phenolic acid cross-linkers and polyphenolic cross-linkers. Preferred phenolic acid cross-linkers include chlorogenic acid, caffeic acid, ferulic acid, coumaric acid and mixtures and derivatives thereof. Polyphenolic cross-linkers that can suitably be employed include polyphenols from plant sources such as polyphenols from grapes (e.g. anthocyanidins), coffee, tea, grains, cocoa and chocolate, fruits and berries.

Enzymatic cross-linkers that may suitably be employed include transglutaminases and/or oxidoreductases (e.g. peroxidase, laccase, monoamine oxidase or polyphenol oxidase).

The oil bodies employed in according with the present invention may be obtained from plant sources or microbial sources. Preferably, the oil bodies are obtained from plant seeds. Suitable plant seed sources include flax, safflower, rapeseed, soybean, maize and sunflower.

The food product according to the present invention advantageously comprises 0.3-10 wt.% of the cross-linked oil bodies. Even more preferably, said food product contains 0.5-8 wt.% of said cross-linked oil bodies.

The volume weighted mean diameter of the cross-linked oil bodies typically is within the range of 0.2-50 µm, preferably of 0.3-20 µm, especially within the range of 0.5-6 µm. The phospholipid layer surrounding the lipid core of the oil body usually is a monolayer.

Glycerides advantageously constitute the bulk of the cross-linked oil bodies of the present invention. Typically, the oil bodies contain at least 90 wt.% of glycerides selected from the group consisting of triglycerides, diglycerides and monoglycerides. Even more preferably the oil bodies contain at least 90 wt.% of triglyceridesl The glycerides contained in the lipid matrix preferably contain at least 20 wt.% of polyunsaturated fatty acids. According to a particularly preferred embodiment, said lipid matrix contains at least 20 wt.% of ω-3 polyunsaturated fatty acids by weight of the total amount of fatty acids contained therein.

Phospholipids are normally present in a concentration of 0.2-5 wt.%, whereas the (cross-linked) structural proteins usually represent 0.2-7 wt.% of the oil bodies.

The structural protein present in the cross-linked oil bodies of the present invention preferably has a hydrophilic N-terminal portion and/or a hydrophilic C-terminal portion; and a central stretch of at least 50, preferably at least 70 uninterrupted hydrophobic residues. According to an even more preferred embodiment, the central stretch of hydrophobic residues of the structural protein is inserted into the lipid core and the amphiphatic N-terminal and/or amphiphatic C-terminal are located at the surface of the oil bodies, with positively charged residues embedded in a phospholipid monolayer and the negatively charged ones exposed to the exterior. In accordance with a particularly preferred embodiment, the structural protein contains both an amphiphatic N-terminal and an amphiphatic C-terminal with are both located

at the surface of the oil bodies. More preferably, the structural protein employed in accordance with the present invention is selected from the group consisting of oleosin, caleosin or combinations thereof. Most preferably, the structural protein is oleosin.

The structural proteins present in the oil bodies of the present invention typically have a molecular size of 10-100 kD, more preferably of 12-70 kD. It is noted that these molecular sizes refer to the individual proteins, i.e. not to the aggregates formed by two or more cross-linked proteins.

The lipid matrix in the oil bodies of the present inventions was found to be surprisingly stable against lipid oxidation. Even if the oil bodies are employed in an food product that contains significant levels of pro-oxidative metals, such as iron, copper and/or zinc, oxidation rates remain surprisingly low. Thus, the food product of the present invention may suitably contain at least at least 10 mg/kg of a transition metal selected from iron, copper or zinc, especially in the form of a metal salt. Most preferably the food product contains at least 10 mg/kg of iron. The exceptional stability of the oil bodies is especially appreciated in case the oil bodies contain a significant level of polyunsaturated fatty acids, notably ω-3 polyunsaturated fatty acids.

Furthermore, the stability of the present oil bodies is particularly manifest in food products that contain at least 1 wt.% of water, especially food products that contain at least 1 wt.% of an aqueous phase containing dispersed oil bodies. Even more preferably, the present food product contains at least 5 wt.%, most preferably at least 10 wt.% of a continuous or dispersed aqueous phase containing the dispersed oil bodies.

The food product according to the present invention is advantageously selected from the group consisting of beverages (e.g. nutritional beverages and dairy drinks), meal replacers,

spreads, nutrition bars, pasta products, ice cream, desserts, dairy products (e.g. yogurt, quark, cheese), dressings, sauces, soups, instant powders, fillings, dips and breakfast type cereal products (e.g. porridge). According to a particularly preferred embodiment, the food product is a beverage, meal replacer, a nutrition bar or an instant powder. The term "instant powder" refers to an essentially dry powder that prior to consumption can be reconstituted with water to produce, for instance, a beverage or a soup.

The food composition of the present invention may suitably contain additional ingredients such as carbohydrates, additional proteins, additional oil, emulsifiers, herbs, spices, anti-oxidants, preservatives, flavourings, colourings, vitamins and minerals. Furthermore, the food composition may optionally comprise, in suitable amounts, one or more agents which may beneficially influence (post-prandial) energy metabolism and substrate utilisation, for example caffeine and flavonoids (including tea catechins, capsaicinoids and carnitine).

According to a preferred embodiment, the food composition contains added vitamins selected from at least one of: Vitamin A, Vitamin B1, Vitamin B2 (riboflavin), Vitamin B3 (Niacinamide), Vitamin B5 (d-Ca-pantothenate), Vitamin B6, Vitamin B11, Vitamin B12 (Cyanocobalamin), Vitamin C (Ascorbic acid), Vitamin D, Vitamin E (Tocopherol),Vitamin H (Biotin), and Vitamin K.

The composition also preferably comprises added minerals selected from at least one of; calcium, magnesium, potassium, zinc, iron, cobalt, nickel, copper, iodine, manganese, molybdenum, phosphorus, selenium and chromium. Calcium is preferably present in the food compositions in amounts of from 5 to 50% of the European Commission Directive 96/8/EC of 26 Feb. 1996 on foods intended for use in energy-restricted diets

for weight reduction, more preferably about 10 to 35%, most preferably 15 to 35% per serving. Any suitable calcium source may be used.

It is preferred that the food compositions comprise potassium, especially in an amount of at least 300 mg of potassium per serving of the food composition, more preferably 400-1000, most preferably 450-700 mg. Any suitable potassium source may be used.

One or more of the above-mentioned vitamins and minerals are preferably present at amounts of from 5 to 45% of the amounts given in the above European Commission Directive 96/8/EC, especially 5 to 40%, most especially 10 to 30%.

The food compositions according to the present invention preferably have a caloric content in the range of from 50 kilocalories (kcals) to 500 kcals, more preferably 100 kcals to 400 kcals per serving. However, it will be understood that the caloric content per serving will vary according to the type of food composition. For a dairy or soy based beverage or dessert the caloric content is typically in the range of from 50 kcals to 400 kcals, more preferably 100 or 150 kcals to 350 kcals, most preferably 200 kcals to 350 kcals per serving. For a soup the calorie content is typically in the range of from 50 kcals to 350 kcals, more preferably 100 kcals to 250 kcals. These products may be consumed either to replace a meal (a meal replacer product) or as a snack product which is not intended to replace a meal.

If the food composition is a meal replacer product the caloric content per serving is typically in the range of from 150 to 350 Kcal. If the food composition is a product which is intended to be eaten as a snack product (i.e. not intended by itself to replace a whole meal) the caloric content per serving is typically in the range of from 50 to 150 Kcal.

The size of a serving of the food composition will depend upon the type of composition. A serving of the food composition as referred to herein refers to the amount of the food composition that is intended to be consumed as a single portion. For beverages and soups, the typical serving size is in the range of from 100 to 500 ml, preferably 150 to 400 ml, such as 200 to 350 ml. For desserts the typical serving size is in the range of from 75 g to 300 g, preferably 100 g to 250 g, such as 125 g to 200 g.

Another aspect of the present invention relates to the use of the cross-linked oil bodies as defined herein before in the treatment or prevention of overweight. Furthermore the present invention relates to the use of the cross-linked oil bodies in weight loss. In addition the use of the cross-linked oil bodies in satiety enhancement is envisioned. In accordance with a preferred embodiment, these cross-linked oil bodies are consumed in an amount of at least 100 mg, more preferably at least 300 mg and most preferably at least 500 mg per consumption event. As will be appreciated from the above, the cross-linked oil bodies are advantageously orally ingested in the form of a food product, e.g. a beverage or meal replacer such as a bar, soup, or shake. It will be understood that the present invention may suitably be employed to treat or prevent overweight in animals, such as mammals. Most preferably, the present invention is used to treat or prevent overweight in humans.

The terminology "treatment or prevention of overweight" encompasses the treatment or prevention of obesity as well as the treatment or prevention of any diseases or disorders that are directly associated with being overweight or obese. The present invention may advantageously be used to improve compliance with a pre-defined dietary plan to control, reduce or maintain body weight. Subjects will find it easier to comply

with such a dietary plan if they consume the cross-linked oil bodies of the present invention as such consumption will strongly decrease the temptation to snack or over-eat. As will be understood from what has been described herein before, the cross-linked oil bodies are suitably consumed in the form of an food product such as a beverage, a nutritional bar or a meal replacer.

In accordance with a preferred embodiment, the cross-linked oil bodies are consumed during a meal (breakfast, lunch or dinner). In this embodiment, the oil bodies are advantageously provided by a meal component or by a beverage that is consumed during the meal. The consumption of oil bodies during the meal offers the advantage that the satiety effect of the meal will be prolonged. Consumption of a composition according to the invention is intended to enhance and/or prolong the feeling of satiety for the consumer and/or extend the time interval between meals and/or reduce the amount of calories consumed in the following meal. This in turn aids the individual concerned to better adhere to a weight loss or weight control plan.

According to another preferred embodiment, the cross-linked oil bodies are consumed between 2 meals (e.g. at least 1 hour before a meal) in order to reduce food intake during the meal. In this embodiment, the cross-linked oil bodies are suitably consumed in the form of a snack or a beverage.

The food composition of the present invention may be consumed as desired. Preferably, the composition is consumed at least daily in order to provide advantageous satiety effects, more preferably it is consumed at least twice daily.

The invention is further illustrated by means of the following examples.

EXAMPLES

Examples 1-6

Oil bodies obtained from different plant seeds were subjected to a cross-linking treatment with glutaric dialdehyde (pentane-1,5-dial). The oil bodies were obtained from commercial sources or extracted from seeds as described below. Oil bodies from the following seed sources were included in the experiment.

Safflower oil bodies	Natrulon™ OSF, ex Lonza, USA
Rapeseed	Supplied by Spack, the Netherlands
Sesame seed	Supplied by Natudis, the Netherlands
Sunflower seed	Supplied by Natudis, the Netherlands
Linseed	Supplied by Natudis, the Netherlands
Pine seed	Supplied by Jacob Hooy, the Netherlands

Oil bodies were extracted from the seeds using the following procedure. Seeds were ground to flour using a water-cooled laboratory M20 universal mill (ex IKA, Germany). Fifty gram of the flour was mixed with 500 ml of aqueous grinding solution (0.6M sucrose) in a Waring blender at a speed of 22,000 rpm for one minute. The extraction solution which contained the oil bodies and different solutes, such as storage proteins, was separated from the fibres and other debris using a laboratory sieve with a mesh size of 150 µm. The liquid phase was further processed in a Sorvall centrifuge, equipped with a GSA rotor, for 30 minutes at 8,000 rpm in order to isolate the oil bodies. A separation in three phases was observed. At the bottom a layer of sediment, consisting mainly of fibres and proteins was formed. In the middle an aqueous phase consisting of water and solutes was formed. The top layer consisted of a

creamy phase consisting of oil bodies mixed with water and some impurities.

The cream top layer was carefully removed with a spoon and subjected to three more liquid-liquid separation steps successively using three different aqueous buffer solutions 1, 2 and 3.

Floating buffer 1	0.2M sucrose / 1.0M NaCl
Floating buffer 2	0.1M sucrose / 1.0M NaCl
Floating buffer 3	0.2M NaCl

The isolated and commercial oil bodies were cross-linked by means of glutaric dialdehyde by means of the following method. Twenty-five gram of oil body slurry was carefully mixed with 250 ml of demineralised water in a beaker. To this mixture 100 µl (low degree of cross-linking) or 1000 µl (high degree of cross-linking) of glutaric dialdehyde solution was added. The beaker was covered with foil to prevent light from affecting the cross-linking reaction. Under constant stirring (50 rpm) at room temperature, the cross-linking reaction was performed overnight. Next, the diluted cross-linked oil body mixture was centrifuged at 5000 rpm for 30 minutes and a slurry containing the cross-linked oil bodies was recovered.

Lipolysis tests were carried out under intestinal conditions to determine the digestion resistance of the cross-linked oil bodies in comparison to non-cross-linked oil bodies. For each lipolysis assay 40 ml of incubation buffer, 1.0g of bile (ex SIGMA, USA) and 1.0g of oil cross-linked oil body slurry were mixed. The incubation buffer contained 40 mM NaCl, 2mM tris(hydroxymethyl)-aminomethane, 80 mM $CaCl_2$ and the pH of the buffer had been adjusted to 7.0 with 0.1 M NaOH solution. The lipolysis reaction was started with the addition of 25 mg of pancreatin powder (ex SIGMA, USA). Pancreatin contains a broad mixture of different types of enzymes, proteases and

lipases being predominant. When added to oil bodies, the proteases in the pancreatin will hydrolyse the oleosins and the phospholipases will start to hydrolyse the phospholipid membrane layer. Subsequently, the oil contained in the oil bodies is hydrolysed by the lipases.

During the incubation period of 60 minutes, automatic addition of sodium hydroxide solution was performed to maintain pH at 7.0. Temperature during the lipolysis assay was kept constant at 37 °C. The molar amount of sodium hydroxide added is equal to the molar amount of fatty acids formed due to triglyceride and phospholipid hydrolysis. For the calculation of the degree of hydrolysis it is assumed that pancreatic lipase hydrolyses 1 mole of triglycerides into 2 moles of fatty acids and 1 mole of monoglyceride.

The results obtained form the lipolysis assay are summarised below.

Table 1: degree of hydrolysis observed in sunflower oil bodies (GDA = glutaric dialdehyde)

	10 min.	30 min.	50 min.
Sunflower (control)	59	100	100
Sunflower low GDA cross-link	34	76	98
Sunflower high GDA cross-link	23	58	81

Table 2: degree of hydrolysis observed in canola oil bodies

	10 min.	30 min.	50 min.
Canola (control)	68	100	100
Canola low GDA cross-link	37	76	96
Canola high GDA cross-link	14	32	45

Table 3: degree of hydrolysis observed in linseed oil bodies

	10 min.	30 min.	50 min.
Linseed (control)	51	100	100

Linseed low GDA cross-link	13	35	57
Linseed high GDA cross-link	12	28	39

Table 4: degree of hydrolysis observed in sesame oil bodies
Please leave the sesame oil bodies out of the patent! It seems that something went wrong with the crosslinking protocol.

	10 min.	30 min.	50 min.
Sesame (control)			
Sesame low GDA cross-link			
Sesame high GDA cross-link			

Table 5: degree of hydrolysis observed in pine tree oil bodies

	10 min.	30 min.	50 min.
Pine (control)	31	75	90
Pine low GDA cross-link	26	52	N.D.
Pine high GDA cross-link	19	44	57

Table 6: degree of hydrolysis observed in safflower oil bodies (Natrulon OSF)

	10 min.	30 min.	50 min.
Safflower (control)	55	97	100
Safflower low GDA cross-link	14	31	47
Safflower high GDA cross-link	10	21	30

Example 7

Oil bodies from safflower (Natrulon OSF) were also cross-linked by means of transglutaminase using the same procedure as described above, except that instead of glutaric dialdehyde solution, transglutaminase (type WM, ex Ajinomoto, Germany) was added in concentrations of 0.1 to 5 wt.% to 25 gram of oil body slurry. Furthermore, pH of the diluted oil body mixture was

adjusted to 7.2 with 0.1M sodium hydroxide solution prior to cross-linking.

Table 7: degree of hydrolysis observed in safflower oil bodies (Natrulon OSF)

	10 min.	30 min.	50 min.
Safflower (control)	48	88	90
Safflower - 0.1% transglutaminase cross-link	16	53	66
Safflower - 0.5% transglutaminase cross-link	17	53	65
Safflower - 5% transglutaminase cross-link	16	52	66

These results show that, as regards protecting the oil bodies against lipolysis, cross-linking of safflower oil bodies with transglutaminase was less effective than cross-linking of these same oil bodies with glutaric dialdehyde.

Claims

1. A food product comprising at least 0.1 wt.% of oil bodies, said oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked.

2. A food product according to claim 1 wherein the food product is a satiety enhancing food product

3. A food product according to any of the previous claims comprising at least 10 mg/kg of a transition metal selected from iron, copper or zinc.

4. A food product according to any of the previous claims wherein at least 5% of the structural proteins in the oil bodies have been cross-linked.

5. A food product according to claim 1 or 2, wherein the structural proteins have been cross-linked by carbonylic cross-linkers, phenolic cross-linkers and/or enzymatic cross-linkers.

6. A food product according to to any of the previous claims, comprising 0.3-10 wt.% of the oil bodies.

7. A food product according to any one of the preceding claims, wherein the oil bodies contain at least 90 wt.% of glycerides selected from the group consisting of

triglycerides, diglycerides and monoglycerides, 0.2-5 wt.% of phospholipids and 0.2-7 wt.% of structural proteins.

8. A food product according to any one of the preceding claims, wherein the structural protein has a hydrophilic N-terminal portion and/or a hydrophilic C-terminal portion; and a central stretch of at least 50, preferably at least 70 uninterrupted hydrophobic residues.

9. A food product according to any one of the preceding claims, wherein the structural protein is selected from the group consisting of oleosin, caleosin and combinations thereof.

10. A food product according to any one of the preceding claims, said food product being selected from the group consisting of beverages, meal replacers, spreads, nutrition bars, pasta products, ice cream, desserts, dairy products, dressings, sauces, soups, instant powders, fillings, dips and breakfast type cereal products.

11. Use of oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked, in the treatment or prevention of overweight.

12. Use of oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked, in a weight-loss treatment.

13. Use of oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked, to enhance satiety.

14. Use of oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked in the manufacture of a food product for use in weight-loss treatment.

15. Use of oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked in the manufacture of a food product that enhances satiety.

16. Use of oil bodies having a volume weighted mean diameter within the range of 0.1-100 μm and comprising a lipid matrix surrounded by a layer of phospholipids embedded with structural proteins, wherein said structural proteins have been cross-linked in the manufacture of a food product for use in the treatment or prevention of overweight.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/052520

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23D7/005 A23D7/01 A23L1/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A23D A23L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, EMBASE, BIOSIS, FSTA, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PENG C-C ET AL: "Size and stability of reconstituted sesame oil bodies" BIOTECHNOLOGY PROGRESS, XX, XX, vol. 19, 6 August 2003 (2003-08-06), pages 1623-1626, XP002392398 ISSN: 8756-7938 cited in the application	1-10
A	page 1623, left-hand column, paragraph 2 figure 5 page 1626, left-hand column, paragraph 2	11-16
X	US 2002/037303 A1 (DECKERS HARM M [CA] ET AL) 28 March 2002 (2002-03-28) page 9, paragraph 104 page 1, paragraph 144 - paragraph 147 page 15, paragraph 164 page 16, paragraph 175	1-10
A	examples 17-21	11-16

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 2007	26/09/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Smeets, Dieter

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/052520

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 11-13
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 11-13 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/052520

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2002037303 A1	28-03-2002	US 2002114820 A1	22-08-2002
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		WO 8703198 A1	04-06-1987

Espacenet

Bibliographic data: WO2007118751 (A2) — 2007-10-25

METHOD FOR MODIFYING THE ATP/ADP RATIO IN CELLS

No documents available for this priority number.

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Classification: **- international:** ***C07K14/415; C12N15/82***
- cooperative: **C07K14/805; C12N15/8247**

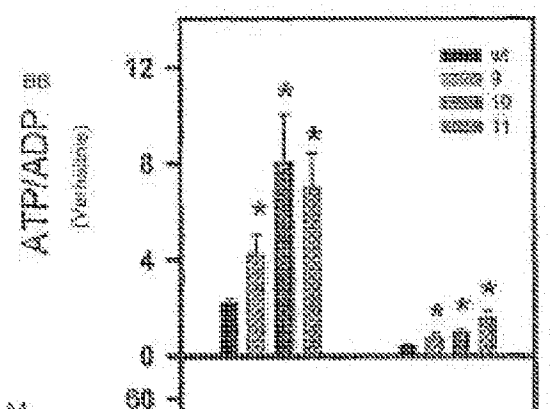
Application number: WO2007EP52655 20070320

Priority number(s): EP20060112638 20060413

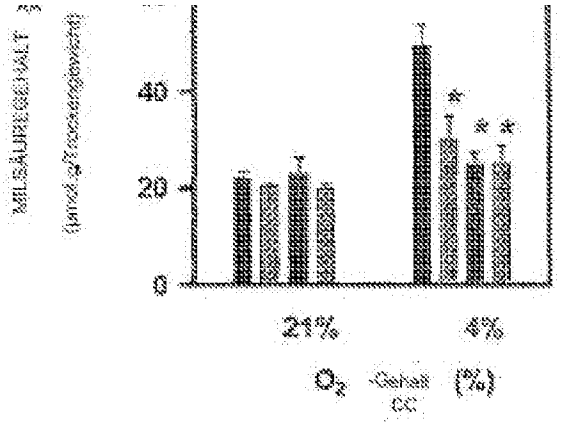
Also published as: WO2007118751 (A3) US2010064384 (A1) EP2007894 (A2) CA2650061 (A1)

Abstract of WO2007118751 (A2)

The invention relates to a method for modifying the ATP/ADP ratio in a cell, tissue, organ, microorganism or plant by modifying the hemoprotein activity in the cell. The invention also relates to the use of said method.



Last updated: 11.12.2013 Worldwide Database 5.8.15; 92p



AA... LACTIC ACID CONTENT
(µmol g/dry weight)
BB... ATP/ADP
(ratio)
CC... O2 content (%)

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

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06112638.9 13. April 2006 (13.04.2006) EP

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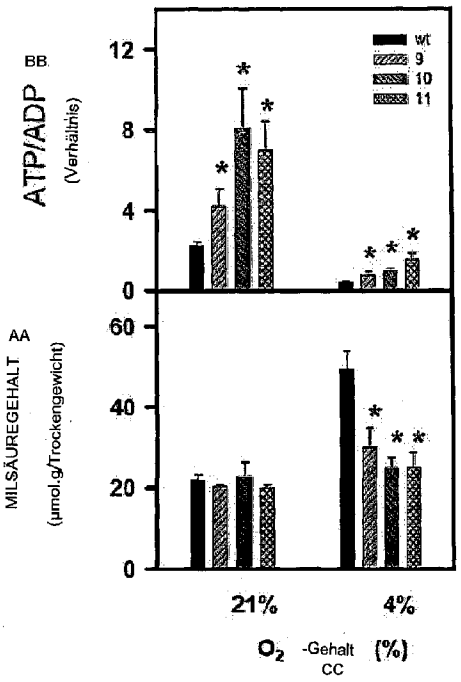
(74) Anwalt: **FITZNER, Uwe**; Hauser Ring 10, 40878 Ratingen (DE).

(81) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare nationale Schutzrechtsart)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,

[Fortsetzung auf der nächsten Seite]

(54) Title: METHOD FOR MODIFYING THE ATP/ADP RATIO IN CELLS

(54) Bezeichnung: VERFAHREN ZUR VERÄNDERUNG DES ATP-ADP-VERHÄLTNISSES IN ZELLEN



AA... LACTIC ACID CONTENT
(µmol.g/dry weight)
BB... ATP/ADP
(ratio)
CC... O2 content (%)

(57) Abstract: The invention relates to a method for modifying the ATP/ADP ratio in a cell, tissue, organ, microorganism or plant by modifying the hemoprotein activity in the cell. The invention also relates to the use of said method.

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur Veränderung des ATP/ADP-Verhältnisses in einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze durch Änderung der Hemprotein Aktivität in der Zelle sowie dessen Verwendung.

WO 2007/118751 A2

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IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Bestimmungsstaaten** *(soweit nicht anders angegeben, für jede verfügbare regionale Schutzrechtsart)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), eurasisches (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:

— *ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts*

— *mit dem Sequenzprotokollteil der Beschreibung in elektronischer Form getrennt veröffentlicht; auf Antrag vom Internationalen Büro erhältlich*

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

Verfahren zur Veränderung des ATP-ADP-Verhältnisses in Zellen

Die Erfindung betrifft ein Verfahren zur Veränderung des ATP/ADP-Verhältnisses in einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze durch Änderung der Hemprotein Aktivität in der Zelle sowie dessen Verwendung.

Adenosintriphosphat (ATP) wird sowohl im Prozeß der Photosynthese als auch bei der Atmung aus ADP (Adenosindiphosphat) und energiereichen Phosphatsbindungen gebildet. Dabei handelt es sich um endergonische Reaktionen. Das energiereiche ATP wird mittels ATPasen hydrolisiert, wobei Energie frei wird. Da die meisten Prozesse in der Zelle endergonisch verlaufen werden sie erst durch die Kopplung mit einer zweiten exergonischen Reaktion möglich, bei der es sich in den meisten Fällen die Hydrolyse von ATP handelt.

Die Hydrolyse von ATP zu ADP dient dabei als treibende Kraft bei vielen biochemischen Prozessen wie z. B. aktiver Transport durch Membranen, Biosynthese von Lipiden, Proteinen, Kohlenhydraten oder Nukleinsäuren.
Somit ist ATP in der Zelle ein Energieüberträger der die Energie für letztendlich jede Aktivität der Zelle oder des Organismus liefert . Jeder Organismus verwendet somit ATP als primäre Energiequelle. Dadurch kommt ATP eine Schlüsselrolle in den Stoffwechsel der Zelle zu.

ATP hat aber andererseits eine sehr kurze Halbwertszeit ("Lebensdauer") und deshalb praktisch keine Speicherkapazität.

Das ATP-ADP-Verhältnis ist ein wichtiger Parameter des Energiemetabolismus.
Ein hohes ATP-ADP-Verhältnis bedeutet einen Energieüberschuß. Bei Energiemangel in der Zelle verbrauchen sich die intrazellulären ATP Reserven und das ATP-ADP-Verhältnis wird in Richtung ADP verschoben.

Die Expression von Hemoglobin oder verwandter Proteine ist aus dem Stand der Technik bekannt.
Aus dem US Patent 6,372,961 ist die Expression von Genen kodieren für Hemoglobin bekannt, wodurch der Sauerstoff Metabolismus in Pflanzen erhöht wird. Dieser erhöhte Sauerstoff oder ATP Gehalt kann die Biosynthese in den Pflanzen beeinflussen.
Aus der WO 98/12913 ist ein Verfahren zur Erhöhung der Sauerstoff-Assimilation bekannt welches auf Expression von Hemoglobin - Proteinen basiert. Ferner wird in dieser Druckschrift offenbart, daß eine Erhöhung der Produktion von Sekundärmetaboliten

auf einer gleichzeitigen Erhöhung der ATP-Konzentration zurückgeführt werden kann.
Des weiteren ist aus der WO 00/00597 bekannt, daß die Expression von nichtsymbiotischen Hemoglobin in Zellen zu einer Erhöhung des ATP - Gehalts führt.
Die Expression von Hemoglobin und Verwandtenproteinen wurde gemäß WO 99/02687 A eingesetzt um den Eisengehalt in Zellen zu erhöhen.
In der WO 2004/057946 A wird durch die Expression von Leghemoglobin ein höherer Ertrag von Stärke und Öl in Pflanzen erreicht.
Aus der Druckschrift WO 2004/087755 ist ein Verfahren bekannt zur Erhöhung der Streßresistenz von Pflanzen und der daraus gewonnenen Ausbeute beruhend auf der Expression von pflanzlichen der Klasse zwei.
Die Expression von Leghemoglobin in Pflanzenzellen ist ferner aus Barata et al: (Plant Science; Vol.155; June 2000, 193-202) bekannt, worin die Sauerstoff-Verfügbarkeit untersucht wird.

Eine Erhöhung des ATP-ADP-Verhältnisses ist aus dem Stand der Technik nicht bekannt.

Aufgabe der vorliegenden Erfindung ist es ein Verfahren bereitzustellen, durch welches der Zelle bzw. dem Organismus mehr ATP und somit mehr Energie zur Verfügung steht. Insbesondere sollte ATP auch als Energiespeicher genutzt werden, das heißt es soll eine Erhöhung des ATP-ADP-Verhältnisses erzielt werden.

Eine weitere Aufgabe der vorliegenden Erfindung ist es die so zur Verfügung gestellte Energie gezielt für die Synthese von Fettsäuren, insbesondere alpha-Linolensäure (cis, cis, cis-9,12,15-Octadecatriensäuere)

Diese Aufgaben werden dadurch gelöst, daß im erfindungsgemäßen Verfahren zur Veränderung des ATP-ADP-Verhältnisses in einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze die Aktivität mindestens eines Hemproteins verändert wird.

Überraschenderweise konnte festgestellt werden, daß durch die Veränderung der Aktivitäten mindestens eines Hemproteins Zellen, Organe, Gewebe, Mikroorganismen oder Pflanzen hergestellt werden, die ein erhöhtes ATP-ADP-Verhältnis aufweisen.

Unter dem ATP-ADP-Verhältnis ist das Verhältnis der Konzentration von ATP zu der Konzentration von ADP zu verstehen. Die Konzentrationen können mit den gängigen, dem Fachmann bekannten Verfahren bestimmt werden, z.B. mittels 31-P-NMR-Spektroskopie in intrazellulärer Messung oder wie nachfolgend in den Beispielen beschrieben.

Im Sinne der vorliegenden Erfindung umfaßt der Begriff Zelle: Zellen, Teile von Pflanzen wie Organe oder Gewebe so wie ganze Pflanzen und Mikroorganismen.

Hemproteine sind Proteine die in der Lage sind Sauerstoff über eine prosthetische Gruppe zu binden, wie zum Beispiel nichtsymbiontisches Hemoglobin, Myoglobin oder Leghemoglobin, bevorzugt Leghemoglobin und nichtsymbiontisches Hemoglobin, besonders bevorzugt Leghemoglobin.

"Aktivität eines Hemproteins", bedeutet die Fähigkeit des Polypeptids Sauerstoff an die prosthetische Gruppe (hem) zu binden. Darunter werden erfindungsgemäß Eisen-II-Komplexe des Protoporphyrins verstanden.

Eine Änderung der Aktivitäten eines Hemproteins in einer Zelle meint die Fähigkeit mehr oder weniger Sauerstoff in der Zelle zu binden im Vergleich zu Zellen des Wildtyps derselben Gattung und Art auf welche die erfindunggemäßen Verfahren nicht angewendet wurde, unter ansonsten gleichen Rahmenbedingungen (wie beispielsweise Kulturbedingungen, Alter der Zellen etc.). Die Änderung, Erhöhung oder Erniedrigung, bevorzugt Erhöhung, im Vergleich zu dem Wildtyp beträgt dabei mindestens 1%, 2%, 5%, 10 %, bevorzugt mindestens 10 % oder mindestens 20 %, besonders bevorzugt um mindestens 40 % oder 60 %, ganz besonders bevorzugt um mindestens 70 % oder 80 %, am meisten bevorzugt um mindestens 90 %, 95 % oder mehr.
In einer Ausführung der vorliegenden Erfindung beträgt das ATP-ADP-Verhältnis mindestens 200%, bevorzugt 300%, besonders bevorzugt mindestens 400% oder mehr bezogen auf das ATP-ADP-Verhältnis des Wildtyps.

Der Vergleich wird bevorzugt unter analogen Bedingungen durch geführt. "Analoge Bedingungen" bedeutet, dass alle Rahmenbedingungen wie beispielsweise Kultur- oder Zuchtbedingungen, Assaybedingungen (wie Puffer, Temperatur, Substrate, Konzentration etc.) zwischen den zu vergleichenden Versuchen identisch gehalten werden und die Ansätze sich allein durch die Aktivität von Hemproteinen unterscheiden.

Verändern bedeutet erfindungsgemäß eine de-novo-Einführung der Aktivität eines erfindungsgemäßen Polypeptids in eine Zelle, Gewebe , Organ, Mikroorganismus oder Pflanze oder eine Erniedrigung oder, bevorzugt, eine Erhöhung einer schon vorhandenen Aktivität des erfindungsgemäßen Polypeptids.In einer Ausführungsform der vorliegenden Erfindung wird die Konzentration der Hemproteine erhöht.

Die Änderung der Aktivität eines Hemproteins kann durch eine Veränderung der Struktur der Proteine es zielt werden, durch Änderung der Stabilität der Hemproteine oder durch Änderung der Konzentration der Hemproteine in einer Zelle.

Eine bevorzugte Variante der vorliegenden Erfindung ist dadurch gekennzeichnet daß die Aktivitäten eines Hemproteins, bevorzugt eines nichtsymbiontischen Hemoglobins oder eines Leghemoglobins erhöht wird.

Besonders bevorzugt wird die Aktivität eines Polypeptids erhöht, welches kodiert wird von einem Nukleinsäuremolekül umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäuresequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt.

In einer bevorzugten Ausführung erfolgt die Erhöhung der Aktivitäten des erfindungsgemäßen Hemproteins durch Expression, bevorzugt Überexpression im Vergleich zum Wildtyp wie oben beschrieben, mindestens eines Nukleinsäuremoleküls umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäuresequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt.

„Nukleinsäuren" meint Biopolymere aus Nukleotiden, die über Phosphodiesterbindungen miteinander verknüpft sind (Polynukleotide, Polynukleinsäuren). Je nach dem Zuckertyp in den Nukleotiden (Ribose oder Desoxyribose), unterscheidet man zwischen den beiden Klassen der Ribonukleinsäuren (RNA) und den Desoxyribonukleinsäuren (DNA).

Die Begriffe "Protein" und Polypeptid" sind gleichbedeutend und gegenseitig austauschbar im Sinne der vorliegenden Erfindung.

In einer bevorzugen Ausführung der vorliegenden Erfindung werden durch Expression eines nichtsymbiontischen Hemoglobins transformierte Zellen, bevorzugt Pflanzen hergestellt, die ein erhöhtes ATP-ADP-Verhältnis aufweisen.

Nichtsymbiontsches Hemoglobin gehört zur Familie der Hemoglobin-Proteine, deren Funktion die reversible Sauerstoffbindung und Versorgung ist. Im Gegensatz zum Leghemoglobin kommt es nicht in den Wurzelknöllchen von Hülsenfrüchten (Leguminosen). Sie sind u.a. in die Detoxifizierung von Nitritoxid und in die Erkennung der Sauerstoffverfügbarkeit involviert.

In einer weiteren bevorzugten Ausfürung der vorliegenden Erfindung werden durch Expression eines Leghemoglobins transformierte Zellen, bevorzugt Pflanzen hergestellt, die ein erhöhtes ATP-ADP-Verhältnis aufweisen.

Das Leghemoglobin gehört zur Familie der Hemoglobin-Proteine, deren Funktion die reversible Sauerstoffbindung und Versorgung ist. Es stammt aus Wurzelknöllchen von Hülsenfrüchten (Leguminosen) und ist eine isolierbare rote Substanz, die dem Myoglobin der Wirbeltiere ähnelt. Durch die reversible Bindung von O_2 kann Leghemoglobin den hohen Sauerstoff-Bedarf bei der Stickstoff-Fixierung durch die Knöllchenbakterien gewährleisten. Das Apoprotein wird von den Pflanzenzellen und das hem von den Bakterien gebildet (Quelle: CD Römpp Chemie Lexikon – Version 1.0, Stuttgart/New York: Georg Thieme Verlag 1995).

Unter Expression wird in der vorliegenden Anmeldung die Übertragung einer genetischen Information ausgehend von DNA oder RNA in ein Genprodukt (Polypeptid oder Protein, hier Leghemoglobin) verstanden und soll auch den Begriff der Überexpression beinhalten, womit eine verstärkte Expression gemeint ist, so dass das Fremdprotein oder das natürlich vorkommende Protein verstärkt produziert werden oder einen großen Teil des gesamten Protein-Gehaltes der Wirtszelle ausmachen.

Die Expression der erfindungsgemäßen Hemproteine wird durch Transformation von Zellen erzielt.
"Transformation" beschreibt einen Prozess zur Einführung heterologer DNA in eine pro- oder eukaryontische Zelle. Mit einer transformierten Zelle ist nicht nur das Produkt das Transformationsprozesses an sich beschrieben, sondern auch alle transgenen Nachkommen des durch die Transformation hergestellten transgenen Organismus. Unter Transformation wird also die Übertragung einer genetischen Information in einen Organismus, insbesondere Pflanze verstanden. Darunter sollen alle dem Fachmann bekannten Möglichkeiten zur Einschleusung der Information fallen, z. B. Mikroinjektion, Elektroporation, Teilchenbeschuss (Partikelbombardement), Agrobakterien oder Che-

mikalien vermittelte Aufnahme (z. B. Polyethylenglycol-vermittelter DNA-Aufnahme oder über die Siliziumcarbonatfaser-Technik). Die genetische Information kann z. B. als DNA, RNA, Plasmid und auf sonstige Weise in die Zellen eingebracht und entweder durch Rekombination ins Wirtsgenom eingebaut, in freier Form oder unabhängig als Plasmid vorliegen.

Die Transformation kann mittels Vektoren enthaltend die obengenannten Nukleinsäuremoleküle durchgeführt werden, bevorzugt Vektoren enthaltend Expressionskassetten welche die die oben genannten Nukleinsäure Moleküle beinhalten.

Eine Expressionskassette enthält eine erfindungsgemäße Nukleinsäuresequenz funktionell verknüpft mit mindestens einem genetischen Kontrollelement, wie einem Promotor, sowie vorteilhaft mit einem weiteren Kontrollelement, wie einem Terminator. Die Nukleinsäuresequenz der Expressionskasette kann beispielsweise eine genomische oder eine komplementäre DNA-Sequenz oder eine RNA-Sequenz sowie halb- oder vollsynthetische Analoga davon sein. Diese Sequenzen können in linearer oder zirkulärer Form, extra-chromosomal oder integriert in das Genom vorliegen. Die entsprechenden Nukleinsäuresequenzen können synthetisch hergestellt oder natürlich gewonnen werden oder eine Mischung aus synthetischen und natürlichen DNA-Bestandteilen enthalten, sowie aus verschiedenen heterologen Genabschnitten verschiedener Organismen bestehen.

Der Begriff der genetischen Kontrollsequenzen ist breit zu verstehen und meint all solche Sequenzen, die einen Einfluss auf das Zustandekommen oder die Funktion der erfindungsgemäßen Expressionskassette haben. Genetische Kontrollsequenzen modifizieren zum Beispiel die Transkription und Translation in prokaryotischen oder eukaryotischen Organismen. Vorzugsweise umfassen die erfindungsgemäßen Expressionskasset-ten 5'-stromaufwärts von der jeweiligen transgen zu exprimierenden Nukleinsäuresequenz einen Promotor mit einer der vorab beschriebenenen Spezifität und 3'-stromabwärts eine Terminatorsequenz als zusätzliche genetische Kontrollsequenz, sowie gegebenenfalls weitere übliche regulative Elemente, und zwar jeweils funktionell verknüpft mit der transgen zu exprimierenden Nukleinsäuresequenz.

In einer Ausführungsform der vorliegenden Erfindung werden Homologe der erfindungsgemäßen Nukleinsäuremoleküle eingesetzt.

"Homologie" zwischen zwei Nukleinsäuresequenzen oder Polypeptidsequenzen wird durch die Identität der Nukleinsäuresequenz/Polypeptidsequenz über die jeweils gesamte Sequenzlänge definiert, die durch Vergleich mit Hilfe des BESTFIT-Alignments (nach Needleman and Wunsch 1970, J. Mol. Biol. 48; 443-453) unter Einstellung folgender Parameter für Aminosäuren

Gap Weight: 50 Length Weight: 3
Average Match: 10.000 Average Mismatch: -9.000

berechnet wird,

und die folgenden Parameter für Nukleinsäuren

Gap Weight: 50 Length Weight: 3
Average Match: 10.000 Average Mismatch: 0.000

Anstelle des Begriff “homolog” oder “Homologie” wird im Folgenden auch gleichbedeutend der Begriff "Identität" verwendet.

In einer Ausführung der vorliegenden Erfindung werden funktionelle Äquivalente der SEQ ID NO: 1, 3, 5, verwendet. Erfindungsgemäße funktionelle Äquivalente der SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 werden durch Rückübersetzung einer Aminosäuresequenz abgeleitet, die eine Identität von mindestens 40%, 50%, 60%, 61%, 62%, 63%, 64%, 65% oder 66% vorzugsweise mindestens 67%, 68%, 69%, 70%, 71%, 72% oder 73% vorzugsweise mindestens 74%, 75%, 76%, 77%, 78%, 79% oder 80% bevorzugt mindestens 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 89%, 90%, 91%, 92% oder 93% besonders bevorzugt mindestens 94%, 95%, 96%, 97%, 98% oder 99% mit SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist. Funktionelle Äquivalente der SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 werden durch eine Aminosäuresequenz kodiert, welche eine Identität mit der SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 von mindestens 40%, 50%, 60%, 61%, 62%, 63%, 64%, 65% oder 66% vorzugsweise mindestens 67%, 68%, 69%, 70%, 71%, 72% oder 73% vorzugsweise mindestens 74%, 75%, 76%, 77%, 78%, 79% oder 80% bevorzugt mindestens 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 89%, 90%, 91%, 92% oder 93% besonders bevorzugt mindestens 94%, 95%, 96%, 97%, 98% oder 99% aufweist.

“Funktionelle Äquivalente” beschreiben hier Nukleinsäuresequenzen, die unter Standardbedingungen mit einer Nukleinsäuresequenz oder Teilen einer Nukleinsäuresequenz zu hybridisieren und befähigt sind, die Expression der Hemproteine in einer Zelle oder einem Organismus zu bewirken.

Zur Hybrisierung werden vorteilhaft kurze Oligonukleotide mit einer Länge von etwa 10-50 bp, vorzugsweise 15-40 bp beispielsweise der konservierten oder sonstigen Bereiche, die über Vergleiche mit anderen verwandten Genen in dem Fachmann bekann-

ter Weise ermittelt werden können, verwendet. Es können aber auch längere Fragmente der erfindungsgemäßen Nukleinsäuren mit einer Länge von 100-500 bp oder die vollständigen Sequenzen für die Hybridisierung verwendet werden. Je nach der verwendeten Nukleinsäure/Oligonukleotid, der Länge des Fragmentes oder der vollständige Sequenz oder je nachdem welche Nukleinsäureart, d.h. DNA oder RNA, für die Hybridisierung verwendet werden, variieren diese Standardbedingungen. So liegen beispielsweise die Schmelztemperaturen für DNA:DNA–Hybride ca 10°C niedriger als die von DNA:RNA–Hybriden gleicher Länge.

Unter Standardhybridisierungsbbedingungen sind beispielsweise je nach Nukleinsäure Temperaturen zwischen 42 und 58°C in einer wäßrigen Pufferlösung mit einer Konzentration zwischen 0,1 bis 5 x SSC (1 X SSC = 0,15 M NaCl, 15 mM Natriumcitrat, pH 7,2) oder zusätzlich in Gegenwart von 50 % Formamid wie beispielsweise 42°C in 5 x SSC, 50% Formamid zu verstehen. Vorteilhafterweise liegen die Hybridisierungsbedingungen für DNA:DNA–Hybride bei 0,1 x SSC und Temperaturen zwischen etwa 20°C bis 65°C, bevorzugt zwischen etwa 30°C bis 45°C. Für DNA:RNA–Hybride liegen die Hybridisierungsbedingungen vorteilhaft bei 0,1 x SSC und Temperaturen zwischen etwa 30°C bis 65°C, bevorzugt zwischen etwa 45°C bis 55°C. Diese angegebenen Temperaturen für die Hybridisierung sind beispielhaft kalkulierte Schmelztemperaturwerte für eine Nukleinsäure mit einer Länge von ca. 100 Nukleotiden und einem G + C–Gehalt von 50% in Abwesenheit von Formamid. Die experimentellen Bedingungen für die DNA-Hybridisierung sind in einschlägigen Lehrbüchern der Genetik, wie beispielsweise Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989, beschrieben und lassen sich nach dem Fachmann bekannten Formeln beispielsweise abhängig von der Länge der Nukleinsäuren, der Art der Hybride oder dem G + C–Gehalt berechnen. Weitere Informationen zur Hybridisierung kann der Fachmann folgenden Lehrbüchern entnehmen: Ausubel et al. (eds), 1985, "Current Protocols in Molecular Biology", John Wiley & Sons, New York; Hames and Higgins (eds), 1985, "Nucleic Acids Hybridization: A Practical Approach", IRL Press at Oxford University Press, Oxford; Brown (ed), 1991, Essential Molecular Biology: A Practical Approach, IRL Press at Oxford University Press, Oxford.

Unter einem funktionellen Äquivalent versteht man weiterhin auch Nukleinsäuresequenzen die mit einer bestimmten Nukleinsäuresequenz („ursprüngliche Nukleinsäuresequenz) bis zu einem definierten Prozentsatz homolog bzw. identisch sind und die gleiche Aktivität der ursprünglichen Nukleinsäuresequenzen aufweisen, ferner insbesondere auch natürliche oder künstliche Mutationen dieser Nukleinsäuresequenzen. Entsprechende Definitionen finden sich an geeigneten Stellen der Beschreibung.

"Mutationen" von Nuklein- oder Aminosäuresequenzen umfassen Substitutionen (=Ersetzungen), Additionen (Hinzufügung), Deletionen (Löschung), Inversion (Veränderungen) oder Insertionen (Einfügungen) eines oder mehrerer Nukleotidreste, wodurch sich auch die entsprechende Aminosäuresequenz des Zielproteins mittels Substitution, Insertion oder Deletion einer oder mehrerer Aminosäuren verändern kann, wobei jedoch insgesamt die funktionellen Eigenschaften des Zielproteins im wesentlichen beibehalten werden.

Es werden weiterhin auch solche Nukleotidsequenzen unter den Begriffes des funktionellen Äquivalentes durch die vorliegende Erfindung mit umfasst, welche man durch Modifikation der Nukleinsäuresequenzen SEQ ID NO 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 erhält. Beispielhaft können solche Modifikationen durch dem Fachmann geläufige Techniken, wie "Site Directed Mutagenesis", "Error Prone PCR", "DNA-shuffling" (Nature 370, 1994, pp.389-391) oder "Staggered Extension Process" (Nature Biotechnol. 16, 1998, pp.258-261) erzeugt werden. Ziel einer solchen Modifikation kann z.B. die Einfügung weiterer Restriktionsenzymschnittstellen, die Entfernung von DNA zur Verkürzung der Sequenz, der Austausch von Nukleotiden zur Codon-Optimierung oder das Hinzufügen weiterer Sequenzen sein. Proteine, die über modifizierte Nukleinsäuresequenzen kodiert werden, müssen trotz abweichender Nukleinsäuresequenz noch die gewünschten Funktionen besitzen.

Funktionelle Äquivalente umfassen somit natürlich vorkommende Varianten der hierin beschriebenen Sequenzen sowie künstliche, z.B. durch chemische Synthese erhaltene, an den Kodon-Gebrauch adaptierte Nukleinsäuresequenzen bzw. die davon abgeleiteten Aminosäuresequenzen.

Unter Nukleotidsequenz werden alle Nukleotidsequenzen verstanden, die (i) exakt den dargestellten Sequenzen entsprechen; oder (ii) mindestens eine Nukleotidsequenz umfaßen, die innerhalb des Bereichs der Degeneration des genetischen Codes den dargestellten Sequenzen entspricht; oder (iii) mindestens eine Nukleotidsequenz umfasst, die mit einer zur Nukleotidsequenz (i) oder (ii) komplemetären Nukleotidsequenz hybridisiert, und gegebenenfalls (iiii) funktionsneutrale Sinnmutationen in (i) umfasst. Dabei bedeutet der Begriff "funktionsneutrale Sinnmutationen" den Austausch chemisch ähnlicher Aminosäuren, wie z. B. Glycin durch Alanin oder Serin durch Threonin.

Unter modifizierten Formen sind erfindungsgemäß Proteine zu verstehen, bei denen Änderungen in der Sequenz, beispielsweise am N- und/oder C-Teminus des Polypeptids oder im Bereich konservierter Aminosäuren vorliegen, ohne jedoch die Funktion

des Proteins zu beeinträchtigen. Diese Veränderungen können in Form von Aminosäureaustauschen nach bekannten Methoden vorgenommen werden.

Erfindungsgemäß sind auch die den kodierenden Bereichen (Strukturgenen) vorausgehenden (5`-oder upstream) und/oder nachfolgenden (3`-oder downstream) Sequenzbereiche eingeschlossen. Insbesondere sind hierin Sequenzbereiche mit regulatorischer Funktion inbegriffen. Sie können die Transkription, die RNA-Stabilität oder das RNA Processing sowie die Translation beeinflussen. Beispiele für regulatorische Sequenzen sind u. a. Promotoren, Enhancer, Operatoren, Teminatoren oder Translationsverstärker.

Ein weiterer Gegenstand der vorliegenden Erfindung ist ein Nukleinsäuremolekül das für ein Polypeptid kodiert welches ein Polypeptid umfasst, welches kodiert wird von einem Nukleinsäuremolekül umfassend ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO

46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt.

Weiterer Gegenstand der vorliegenden Erfindung ist ein Polypeptid, welches kodiert wird von einem Nukleinsäuremolekül umfassend ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt.

Ferner ist Gegenstand der vorliegenden Erfindung ein Nukleinsäuremolekül das für ein Polypeptid kodiert welches ein Polypeptid umfasst, welches kodiert wird von einem Nukleinsäuremolekül

das sich in ein, zwei, drei, vier, fünf, sechs, sieben, acht, neun, zehn oder mehreren Nukleinsäuren unterscheidet von einem Nukleinsäuremolekül umfassend ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäuresequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;

und für ein Polypeptid mit Aktivität eines Hemproteins kodiert.

Ferner ist Gegenstand der vorliegenden Erfindung ein Polypeptid Aktivität eines Hemproteins, welches kodiert wird von einem Nukleinsäuremolekül

das sich in ein, zwei, drei, vier, fünf, sechs, sieben, acht, neun, zehn oder mehreren Nukleinsäuren unterscheidet von einem Nukleinsäuremolekül umfassend ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ

ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;

und für ein Polypeptid mit Aktivität eines Hemproteins kodiert.

Ein weiterer Gegenstand der vorliegenden Erfindung ist eine DNA Expressionskassette enthaltend eine Nukleinsäuresequenz wie oben beschrieben.

Ein weiterer Gegenstand der vorliegenden Erfindung ist ein Vektor enthaltend eine Expressionskassette enthaltend eine Nukleinsäuresequenz wie oben beschrieben.

Ferner ist Gegenstand der vorliegenden Erfindung eine Zelle im Sinne der Erfindung, bevorzugt ein monokotyledoner Organismus oder ein dikotyledoner Organismus mit einer erhöhten Aktivität mindestens eines Hemproteins beruhend auf der Expression einer Nukleinsäuresequenz wie oben beschrieben.

Ein Gegenstand der vorliegenden Erfindung ist ferner eine Zelle hergestellt mit dem erfindungsgemäßen Verfahren.

In einer Ausführung der vorliegenden Erfindung wird durch die Änderung der Aktivität eines Hemproteins, neben der Erhöhung des ATP-ADP-Verhältnisses, der Ölgehalt in

den Zellen erhöht.

Als Ölgehalt wird der Gesamt- Fettsäuregehalt in den erfindungsgemäßen Zellen bezeichnet.

"Öl" umfasst im. Sinne der Erfindung neutrale und/oder polare Lipide und Mischungen derselben. Beispielhaft jedoch nicht einschränkend seien die in Tabelle 1 aufgeführten zu nennen.

Tabelle 1: Pflanzliche Lipidklassen

Neutrale Lipide	Triacylglycerol (TAG)
	Diacylglycerol (DAG)
	Monoacylglycerol (MAG)
Polare Lipide	Monogalactosyldiacylglycerol (MGDG)
	Digalactosyldiacylglycerol (DGDG)
	Phosphatidylglycerol (PG)
	Phosphatidylcholin (PC)
	Phosphatidylethanolamin (PE)
	Phosphatidylinositol (PI)
	Phosphatidylserin (PS)
	Sulfoquinovosyldiacylglycerol

Neutrale Lipide meint bevorzugt Triacylglyceride. Sowohl die neutralen als auch die polaren Lipide können ein breites Spektrum an verschiedenen Fettsäuren enthalten. Beispielhaft jedoch nicht einschränkend seien die in Tabelle 2 aufgeführten Fettsäuren zu nennen.

Tabelle 2: Übersicht über verschiedene Fettsäuren (Auswahl)

[1] Kettenlänge:Anzahl der Doppelbindungen

* nicht natürlicherweise in Pflanzen vorkommend

Nomenklatur[1]	Name

16:0	Palmitinsäure
16:1	Palmitoleinsäure
16:3	Roughaninsäure
18:0	Stearinsäure
18:1	Ölsäure
18:2	Linolsäure
18:3	Linolensäure
-18:3	Gamma-Linolensäure*
20:0	Arachidinsäure
22:6	Docosahexanonsäure (DHA) *
20:2	Eicosadienonsäure
20:4	Arachidonsäure (AA) *
20:5	Eicosapentaenosäure (EPA) *
22:1	Erucasäure

Für weitergehende Informationen wird ebenfalls auf das Römpp Chemie Lexikon – CD Version 2.0, Stuttgart/New York: Georg Thieme Verlag 1999 verwiesen.

In einer bevorzugten Variante wird dabei der Gehalt an ungesättigten Fettsäuren erhöht, insbesondere an Linolensäure.

Durch die Erhöhung des Gesamtölgehalts der erfindungsgemäßen Zelle wird der Gesamtgehalt an Proteinen jedoch nicht oder nur kaum verringert. Das heißt, der Gesamtgehalt an Fettsäuren, ausgedrückt in Masse auf Masse Trockengewicht, ist gegenüber jenem des Wildtyps signifikant erhöht. Der Gesamtgehalt an Proteinen im Vergleich zum jenem des Wildtyps, ebenfalls in Masse auf Masse Trockengewicht ausgedrückt, bleibt jedoch konstant oder wird minimal verringert. Die Verringerung ist prozentuell, bezogen auf den Wildtyp, kleiner als die Erhöhung des Ölgehalts.

Die Erhöhung des ATP-ADP-Verhältnis, also die Erhöhung des Energiestatus durch Speicherung der Energie in ATP, bleibt bei Zellen die aufgrund des erfindungsgemäßen Verfahrens eine erhöhte Aktivität von Hemproteinen zeigen, konstant. Das heißt,

das ATP-ADP-Verhältnis der Zellen wird durch eine Veränderung der Außenbedingungen nicht beeinflußt.
Unter Außenbedingungen versteht man im Sinne der Erfindung die Kulturbedingungen für Zellen, Gewebe, Organe, Mikroorganismen oder Pflanzen. Dies können z. B. Mediumszusammensetzung, Temperatur, Zusammensetzung der Atmosphäre oder andere, den Wildtyp beeinflussenden Faktoren sein.

In einer Ausführung der vorliegenden Erfindung beträgt das ATP-ADP-Verhältnis der Zellen mit erfindungsgemäßen erhöhter Aktivität von Hämoproteins bei einer Absenkung der Sauerstoffkonzentration in der umgebenden Atmosphäre auf 4% mindestens 200 %, 300 %, bevorzugt 400%, besonders bevorzugt mindestens 500% oder mehr bezogen auf das ATP-ADP-Verhältnis des Wildtyps.
Zusätzlich beträgt die unter diesen anaeroben Kulturbedingungen gebildete Lactamenge maximal 80%, bevorzugt 75%, 70%, besonders bevorzugt 65%, 60%, 55%, 50% oder weniger bezogen auf die Lactatmenge des Wildtyps.

In einer weiteren Ausführung der Erfindung ist die Veränderung der Hemprotein – Aktivität, das erhöhte ATP-ADP-Verhältnis, der erhöhte Ölgehalt und/oder die verringerte Latatmenge ein stabiles Merkmal der erfindungsgemäßen Zellen, welches über mehrere Generationen erhalten bleibt, bevorzugt bis zu der T2, besonders bevorzugt zur T3-Generation.

In einer bevorzugten Variante der vorliegenden Erfindung sind die erfindungsgemäßen Zellen Pflanzenzellen, Organe, Teile von Pflanzen oder ganze Pflanzen.

"Pflanzen" im Rahmen der Erfindung meint alle dicotyledonen oder monokyledonen Pflanzen.“Pflanzen“ im Sinne der Erfindung sind Pflanzenzellen, -gewebe, -organe oder ganzen Pflanzen wie Samen, Knollen, Blüten, Pollen, Früchte, Sämlinge, Wurzeln, Blätter, Stengel oder sonstige Pflanzenteile zu verstehen. Außerdem ist unter Pflanzen Vermehrungsmaterial wie Samen, Früchte, Sämlinge, Stecklinge, Knollen, Schnitte oder Wurzelstöcke zu verstehen.

Eingeschlossen unter dem Begriff "Pflanzen" sind ferner die reifen Pflanzen, Saatgut, Sprosse und Keimlinge, sowie davon abgeleitete Teile, Vermehrungsgut, Pflanzenorgane, Gewebe, Protoplasten, Kallus und andere Kulturen, zum Beispiel Zellkulturen, sowie alle anderen Arten von Gruppierungen von Pflanzenzellen zu funktionellen oder strukturellen Einheiten. Reife Pflanzen meint Pflanzen zu jedem beliebigen Entwicklungsstadium jenseits des Keimlings. Keimling meint eine junge, unreife Pflanze in einem frühen Entwicklungsstadium.

"Pflanze" umfaßt auch einjährige und mehrjährige dicotyledone oder monokotyledone Pflanzen und schließt, beispielhaft, jedoch nicht einschränkend, solche der Gattungen, Bromus, Asparagus, Pennisetum, Lolium, Oryza, Zea, Avena, Hordeum, Secale, Triticum, Sorghum und Saccharum ein.

In einer bevorzugten Ausführungsform wird das Verfahren auf monokotyle Pflanzen, beispielsweise aus der Familie der Poaceae, besonders bevorzugt auf die Gattungen Oryza, Zea, Avena, Hordeum, Secale, Triticum, Sorghum, und Saccharum, ganz besonders bevorzugt auf Pflanzen mit landwirtschaftlicher Bedeu-tung, wie z.B. Hordeum vulgare (Gerste), Triticum aestivum (Weizen), Triticum aestivum subsp.spelta (Dinkel), Triticale, Avena sative (Hafer), Secale cereale (Roggen), Sorghum bicolor (Hirse), Zea mays (Mais), Saccha-rum officinarum (Zuckerrohr) oder Oryza sative (Reis) angewendet.

Bevorzugte monokotyle Pflanzen sind insbesondere ausgewählt aus den monokotylen Kulturpflanzen, wie zum Beispiel der Familie der Gramineae wie Reis, Mais, Weizen oder andere Getreidearten wie Gerste, Hirse, Roggen, Triticale oder Hafer sowie dem Zuckerrohr sowie alle Arten von Gräsern. Besonders bevorzugt aus der Familie der Gramineae sind Reis, Mais, Weizen und Gerste.

Eine transformierte Pflanze im Sinne der Erfindung ist also eine gentechnisch veränderte Pflanze.

Erfindungsgemäß eignen sich alle Pflanzen zur Durchführung des erfindungsgemäßen Verfahrens. Bevorzugt werden Kartoffeln, Arabidopsis thaliana, Raps, Sojabohnen, Erdnüsse, Mais, Maniok, Purgiernuss, Yams, Reis, Sonnenblumen, Roggen, Gerste, Hopfen, Hafer, Hart und Weichweizen, Lupinen, Erbsen, Klee, Rüben, Kohl, Reben usw. wie sie z. B. aus der Verordnung über das Artenverzeichnis zum Saatgutverkehrsgesetz (Blatt für PMZ 1986 S. 3, zuletzt geändert Blatt für PMZ 2002 S. 68) entnehmbar sind, eingesetzt.

1. Bevorzugte dikotyledone Pflanzen sind insbesondere ausgewählt aus den dikotylen Kulturpflanzen, wie zum Beispiel

- Asteraceae wie Sonnenblume, Tagetes oder Calendula,
- Compositae, besonders die Gattung Lactuca, ganz besonders die Art sativa (Salat),
- Cruciferae, besonders die Gattung Brassica, ganz besonders die Arten napus (Raps), campestris (Rübe), oleracea cv Tastie (Kohl), oleracea cv Snowball Y (Blumenkohl) und oleracea cv Emperor (Broccoli) und weitere Kohlarten; und

der Gattung Arabidopsis, ganz besonders die Art thaliana sowie Kresse oder Canola,

- Cucurbitaceae wie Melone, Kürbis oder Zucchini,
- Leguminosae besonders die Gattung Glycine, ganz besonders die Art Glycine max (Sojabohne) sowie Alfalfa, Erbse, Bohnengewächsen oder Erdnuss.
- Rubiaceae, bevorzugt der Unterklasse Lamiidae wie beispielsweise Coffea arabica oder Coffea liberica (Kaffeestrauch),
- Solanaceae besonders die Gattung Lycopersicon, ganz besonders die Art esculentum (Tomate) und die Gattung Solanum, ganz besonders die Art tuberosum (Kartoffel) und melongena (Aubergine) sowie Tabak oder Paprika,
- Sterculiaceae, bevorzugt der Unterklasse Dilleniidae wie beispielsweise Theobroma cacao (Kakaostrauch),
- Theaceae, bevorzugt der Unterklasse Dilleniidae wie beispielsweise Camellia sinensis oder Thea sinensis (Teestrauch),
- Umbelliferae, besonders die Gattung Daucus (ganz besonders die Art carota (Karrotte)) und Apium (ganz besonders die Art graveolens dulce (Selarie)) und andere mehr; und die Gattung Capsicum, ganz besonders die Art annum (Pfeffer),
- sowie Lein, Soja, Baumwolle, Hanf, Flachs, Gurke, Spinat, Möhre, Zuckerrübe und den verschiedenen Baum-, Nuss- und Weinarten, insbesondere Banane und Kiwi.

Umfasst sind ferner Schmuckpflanzen, Nutz- oder Zierbäume, Blumen, Schnittblumen, Sträucher oder Rasen. Beispielhaft aber nicht einschränkend seien zu nennen Angiospermen, Bryophyten wie zum Beispiel Hepaticae (Leberblümchen) und Musci (Moose); Pteridophyten wie Farne, Schachtelhalm und Lycopoden; Gymnospermen wie Koniferen, Cycaden, Ginkgo und Gnetalen, die Familien der Rosaceae wie Rose, Ericaceae wie Rhododendrons und Azaleen, Euphorbiaceae wie Weihnachtssterne und Kroton, Caryophyllaceae wie Nelken, Solanaceae wie Petunien, Gesneriaceae wie das Usambaraveilchen, Balsaminaceae wie das Springkraut, Orchidaceae wie Orchideen, Iridaceae wie Gladiolen, Iris, Freesie und Krokus, Compositae wie Ringelblume, Geraniaceae wie Geranien, Liliaceae wie der Drachenbaum, Moraceae wie Ficus, Araceae wie Philodendron und andere mehr.

Besonders interessant ist der Einsatz von Ölpflanzen, d.h. Pflanzen, die bereits natürlicherweise einen hohen Ölgehalt aufweisen und/oder zu industriellen Gewinnung von Ölen verwendet werden. Diese Pflanzen können einen hohen Ölgehalt und/oder aber eine besondere, industriell interessante Fettsäurezusammensetzung aufweisen. Bevorzugt sind Pflanzen, die einen Lipidanteil von mindestens 1 Gew.-% aufweisen. Ölpflanzen umfassen beispielhaft: Bovago oficinalis (Borretsch); Brassica Arten wie B. campestris, B. napus, B. rapa (Senf oder Raps); Cannabis sativa (Hanf); Curthamus tinctorius (Färberdiestel); Cocos nucifera (Kokusnuss); Crambe abyssinica (Krambe); Cuphea Arten (Cuphea Arten liefern fettsäuren von mittlerer Kettenlänge insbesondere für industrielle Anwendungen); Elaeis guinensis (Afrikanische Ölpalme); Ekeis oleiferu (Amerikanische Ölpalme); Glycine max (Sojabohne); Gossypium hirisfum (Amerikanische Baumwolle); Gossypium barbadense (Ägyptische Baumwolle); Gossypium herbaceum (Asiatische Baumwolle Cotton); Helianthus annus (Sonnenblume); Jatropha curcas (Purgiernuss oder Brechnuss); Linum usitatissimum (Lein oder Flachs); Oenethera biennis (Evening primrose); Ozea europea (Olive); Oryza sativa (Rice); Ricinus communis (Castor); Sesamum indicum (Sesam); Glycine max (Sojabohne); Triticum Arten (Weizen); Zea maize (Mais) sowie verschiedene Nussarten wie beispielsweise Walnuss oder Mandel.

Wenn es sich bei den verwendeten Pflanzen um solche zur Gattung der Leguminosen (Hülsenfrüchte) gehörenden Pflanzen handelt, so fällt unter die Erfindung die Expression von Fremdproteinen Leghemoglobinen oder in der Natur nicht-symbiontisch vorkommenden Hemoglobinen oder die Veränderung der Pflanzen derart, dass sie das natürlich vorkommende Leghemoglobin oder nicht-symbiontisches Hemoglobin überexprimieren.

Höchst bevorzugt sind Kartoffeln, Arabidopsis thaliana, Raps und Soja.

Vorteilhaft ist es, wenn die vorgenannten Pflanzen ein Leghemoglobin ausgewählt aus der Gruppe bestehend aus Leghemoglobin aus den Pflanzen Lupinus luteus (LGB1_LUPLU, LGB2_LUPLU), Glycine max (LGBA_SOYBN, LGB2_SOYBN, LGB3_SOYBN), Medicago sativa (LGB1-4_MEDSA), Medicago trunculata (LGB1_MEDTR), Phaseolus vulgaris (LGB1_PHAVU, LGB2_PHAVU), Vicia faba (LGB1_VICFA, LGB2_VICFA), Pisum sativum (LGB1_PEA, LGB2_PEA), Vigna unguiculata (LGB1_VIGUN), Lotus japonicus (LGB_LOTJA), Psophocarpus tetragonolobus (LGB_PSOTE), Sesbania rostrata (LGB1_SESRO), Casuarina glauca (HBPA_CASGL) und Canvalaria lineata (HBP_CANLI) exprimieren. In Klammern sind die jeweiligen Swiss-Prot Datenbankeinträge vermerkt.

Besonders vorteilhaft ist es, wenn die vorgenannten Pflanzen ein nicht-symbiontisches Hemoglobin ausgewählt aus der Gruppe bestehen aus Hemoglobin aus den Pflanzen Arabidopsis thalina (AT_AHB2), Brassica napus (BN_AHB2), Linum usitatissimum (LU_AHB2), Glycine max (GM_AHB2), Helianthus annus (HA_AHB2), Triticum aestivum (TA_AHB2), Hordeum vulgare (HV_AHB2), Oryza sativa (OS_AHB2) und Zea mays (ZM_AHB2).

Besonders vorteilhaft sind Pflanzen, die die Sequenz Nr. 1 (AT-AHB2) codierend für nicht symbiontische Hemoglobin aufweisen.

In einer bevorzugten Variante der Erfindung handelt es sich um Pflanzen, die das Hemprotein speicherorganspezifisch exprimieren.

Dies sind z. B. Zwiebeln, Knollen, Samen, Körner, Nüsse, Blätter usw. Unter Speicherorgane im Sinne der Erfindung sind auch Früchte zu verstehen. Früchte sind die Sammelbezeichnung für die den Samen als Nährgewebe umhüllenden Organe der Pflanzen. Dabei ist nicht nur an die eßbaren Früchte, insbesondere an das Obst, aber auch an Hülsenfrüchte, Getreide, Nüsse, Gewürze zu denken, sondern auch an offiziell genutzte Drogen (s. Fructus, Semen). Natürlich kann auch eine Speicherung der Speicherstoffe in der gesamten Pflanze stattfinden.

Bevorzugterweise wird das Hemprotein knollenspezifisch oder samenspezifisch exprimiert.

Als Pflanzen kommen alle zuvor genannten in Betracht. Es ist besonders bevorzugt, wenn es sich um knollenproduzierende Pflanzen, insbesondere Kartoffel-Pflanzen oder um samenproduzierende Pflanzen, insbesondere Arabidopsis thaliana oder Raps handelt.

Die gewebespezifische Expression kann z. B. durch Verwendung eines gewebespezifischen Promotors erreicht werden. Eine solche gewebespezifische Expression ist beispielsweise bekannt aus der US 6,372,961 B1 Spalte 11, Zeilen 44 ff.

In einer weiteren Ausführung betrifft die vorliegende Erfindung die Verwendung der oben beschriebenen Nukleinsäuremolekülen kodierend für Polypeptide mit der Aktivität von Hemoproteinen zur Herstellung von Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze mit erhöhtem ATP-ADP-Verhältnis und/oder verändertem Ölgehalt, bevorzugt erhöhtem Fettsäure-Gehalt, bevorzugt erhöhtem Linolensäure-Gehalt

Die Erfindung wird unter Bezugnahme auf die folgenden Versuche beispielhaft beschrieben.

Beispiele

Allgemeine Methoden:

Alle Chemikalien, wenn nicht anders erwähnt, stammen von den Firmen Fluka (Buchs), Merck (Darmstadt), Roth (Karlsruhe), Serva (Heidelberg) und Sigma (Deisenhofen). Restriktionsenzyme, DNA-modifizierende Enzyme und Molekularbiologie-Kits wurden von den Firmen Amersham--Pharmacia (Freiburg), Biometra (Göttingen), Roche (Mannheim), New England Biolabs (Schwalbach), Novagen (Madison, Wisconsin, USA), Perkin--Elmer (Weiterstadt), Qiagen (Hilden), Stratagen (Amsterdam, Niederlande), Invitrogen (Karlsruhe) und Ambion (Cambridgeshire, United Kingdom). Die verwendeten Reagenzien wurden entsprechend der Angaben des Herstellers eingesetzt.

Die chemische Synthese von Oligonukleotiden kann beispielsweise, in bekannter Weise, nach der Phosphoamiditmethode (Voet, Voet, 2. Auflage, Wiley Press New York, Seite 896--897) erfolgen. Die im Rahmen der vorliegenden Erfindung durchgeführten Klonierungsschritte wie z. B. Restriktionsspaltungen, Agarosegelelektrophorese, Reinigung von DNA--Fragmenten, Transfer von Nukleinsäuren auf Nitrozellulose und Nylonmembranen, Verknüpfen von DNA--Fragmenten, Transformation von E. coli Zellen, Anzucht von Bakterien, Vermehrung von Phagen und Sequenzanalyse rekombinanter DNA werden wie bei Sambrook et al. (1989) Cold Spring Harbor Laboratory Press; ISBN 0--87969--309--6 beschrieben durchgeführt. Die Sequenzierung rekombinanter DNA--Moleküle erfolgt mit einem Laserfluoreszenz-DNA-Sequenzierer der Firma ABI nach der Methode von Sanger (Sanger et al. (1977) Proc Natl Acad Sci USA 74:5463--5467).

Beispiel 1: Klonierung der Gene AHB1 und AHB2 aus Arabidopsis thaliana

Zur Klonierung des Gens AHB2 wurde Gesamt-RNA aus 6 wochen alten Arabidopsis-Pflanzen extrahiert.Die Herstellung der entsprechenden cDNA erfolgte durch RT-PCR mit Hilfe der SUPERSCRIPT II (Invitrogen).

Für die Klonierung des AHB2-Gens wurde die isolierte Arabidopsis cDNA in einer PCR-Reaktion mit den Oligonukleotidprimern AHb2f und AHb2r eingesetzt.

Sequenz Primer Ahb2f
SEQU.ID.No :
5'-TTTGGTACCATGGGEGAGATTGGGTTTACAGAG-3'

Sequenz Primer Ahb2r

SEQU.ID.No :

5'-TTTGGATCCTTATGACCTTTCTTGTTTCATCTCGG-3'

Zusammensetzung des PCR-Ansatzes (50 uL):

5,00 µL cDNA aus Arabidopsis thaliana

5,00 µL 10x Puffer (Advantage-Polymerase)+ 25mM $MgCl_2$

5,00 µL 2mM dNTP

1,25 µL je Primer (10 pmol/uL)

0,50 µL Advantage-Polymerase

Die Advantage-Polymerase von Clontech wurden eingesetzt.

PCR-Programm:

Anfangsdenaturierung für 2 min bei 95°C, dann 35 Zyklen mit 45 sec 95°C, 45 sec 55°C und 2 min 72°C. Abschliessende Extension von 5 min bei 72°C.

Das PCR-Ansätze wurden anschließend über Agarose-Gelelektrophorese aufgetrennt und die amplifizierten DNA-Fragmente von AHB2 aus dem Gel ausgeschnitten und mit dem ‚Gelpurification'Kit von Qiagen nach Herstellerangaben aufgereinigt und mit 50 µL Elutionspuffer eluiert.

Anschließend wurden die DNA Fragment in den pCR2.1-TOPO Vektor (Invitrogen) gemäss Herstellerangaben kloniert, resultierend in dem Vektor pCR2.1-AHB2 und die Sequenz wurde durch Sequenzierung überprüft.

Anschließend wurden die codierenden Sequenzen für AHB2 in einen binären Pflanzenvektor, wie pBIN, hinter den samenspezifischen USP Promoter (Bäumein et al. (1991) Mol Gen Genet 225(3):459-467) kloniert. Hierfür wurde der Vektor pCR2.1-AHB2 mit den Restriktionsenzymen KpnI und BamHI verdaut. Die resultierenden DNA-Fragmente über Agarose-Gelelektrophorese aufgetrennt und die AHB codierenden Fragmente aus dem Gel ausgeschnitten und mit dem ‚Gelpurification'Kit von Qiagen nach Herstellerangaben aufgereinigt und mit 50 µL Elutionspuffer eluiert. Die eluierten DNA Fragment wurden mit dem mit den gleichen Enzymen verdauten binären Vektor über Nacht bei 16°C ligiert (T4 Ligase von New England Biolabs). Die Ligationsprodukte werden dann in TOP10 Zellen (Stratagene) nach Herstellerangaben transformiert und entsprechend selektioniert. Positive Klone werden mit den Primern AHb2f und AHb2r durch PCR und Sequenzierung verifiziert.

Beispiel 3: Transformation von Agrobacterium

Die Agrobacterium-vermittelte Pflanzentransformation kann zum Beispiel unter Verwendung der Agrobacterium tumefaciens-Stämme GV3101 (pMP90) (Koncz und Schell (1986) Mol Gen Genet 204: 383- 396) oder LBA4404 (Clontech) durchgeführt werden. Die Transformation kann durch Standard-Transformationstechniken durchgeführt werden (Deblaere et al.(1984) Nucl Acids Res 13:4777-4788).

Beispiel 4: Pflanzentransformation

Die Agrobacterium-vermittelte Transformation von Arabidopsis thaliana wurde unter Verwendung von Standard-Transformations- und Regenerationstechniken durchgeführt werden (Gelvin, Stanton B., Schilperoort, Robert A., Plant Molecular Biology Manual, 2. Aufl., Dordrecht: Kluwer Academic Publ., 1995, in Sect., Ringbuc Zentrale Signatur: BT11-P ISBN 0-7923-2731-4; Glick, Bernard R., Thompson, John E., Methods in Plant Molecular Biology and Biotechnology, Boca Raton: CRC Press, 1993, 360 S., ISBN 0-8493-5164-2). Die Verwendung von Antibiotika für die Agrobacterium- und Pflanzenselektion hängt von dem für die Transformation verwendeten binären Vektor und Agrobacterium-Stamm ab. Die Selektion der mit AHB2 transformierten Arabidopsis thaliana Pflanzen erfolgte mit Hygormycin.

Die Agrobakterium-vermittelte Transformation von Raps kann z. B. durch Kotyledonen- oder Hypokotyltransformation erfolgen (Moloney et al., Plant Cell Report 8 (1989) 238-242; De Block et al., Plant Physiol. 91 (1989) 694-701). Die Verwendung von Antibiotika für die Agrobacterium- und Pflanzenselektion hängt von dem für die Transformation verwendeten binären Vektor und Agrobacterium-Stamm ab.

Der Agrobacterium-vermittelte Gentransfer in Lein (Linum usitatissimum) lässt sich unter Verwendung von beispielsweise einer von Mlynarova et al. (1994) Plant Cell Report 13:282-285 beschriebenen Technik durchführen.

Die Transformation von Soja kann unter Verwendung von beispielsweise einer in EP–A–0 0424 047 (Pioneer Hi-Bred International) oder in EP–A–0 0397 687, US 5,376,543, US 5,169,770 (University Toledo) beschriebenen Technik durchgeführt werden.

Die Pflanzentransformation unter Verwendung von Teilchenbeschuss, Polyethylenglycol-vermittelter DNA-Aufnahme oder über die Siliziumcarbonatfaser-Technik ist beispielsweise beschrieben von Freeling und Walbot "The maize handbook"

(1993) ISBN 3-540-97826-7, Springer Verlag New York).

Beispiel 5: Untersuchung der Expression eines rekombinanten Genproduktes in einem transformierten Organismus

Ein geeignetes Verfahren zur Bestimmung der Menge an Transkription des Gens (ein Hinweis auf die Menge an RNA, die für die Translation des Genproduktes zur Verfügung steht) ist die Durchführung eines Northern--Blots wie unten ausgeführt (als Bezugsstelle siehe Ausubel et al. (1988) Current Protocols in Molecular Biology, Wiley: New York, oder den oben erwähnten Beispielteil), wobei ein Primer, der so gestaltet ist, dass er an das Gen von Interesse bindet, mit einer nachweisbaren Markierung (gewöhnlich radioaktiv oder chemilumineszent) markiert wird, so dass, wenn die Gesamt-RNA einer Kultur des Organismus extrahiert, auf einem Gel aufgetrennt, auf eine stabile Matrix transferiert und mit dieser Sonde inkubiert wird, die Bindung und das Ausmaß der Bindung der Sonde das Vorliegen und auch die Menge der mRNA für dieses Gen anzeigt. Diese Information zeigt den Grad der Transkription des transformierten Gens an. Zelluläre Gesamt-RNA kann aus Zellen, Geweben oder Organen mit mehreren Verfahren, die alle im Fachgebiet bekannt sind, wie zum Beispiel das von Bormann, E.R., et al. (1992) Mol. Microbiol. 6:317--326 beschriebene, präpariert werden.

Northern-Hybridisierung:

Für die RNA-Hybridisierung wurde Gesamt-RNA mit Hilfe des Concert RNA Plant Reagent (Invitrogen GmbH, Karlsruhe, Deutschland) aus reifenden Samen extrahiert. 20 µg Gesamt-RNA oder 1 µg poly(A)+-RNA wurden mittels Gelelektrophorese in Agarosegelen mit einer Stärke von 1,25 % unter Verwendung von Formaldehyd, wie beschrieben in Amasino (1986, Anal. Biochem. 152, 304) aufgetrennt, mittels Kapillaranziehung unter Verwendung von 10 x SSC auf positiv geladene Nylonmembranen (Hybond N+, Amersham, Braunschweig) übertragen, mittels UV-Licht immobilisiert und 3 Stunden bei 68°C unter Verwendung von Hybridisierungspuffer (10 % Dextransulfat Gew./Vol., 1 M NaCl, 1 % SDS, 100 mg Heringssperma-DNA) vorhybridisiert. Die Markierung der DNA-Sonde mit dem Highprime DNA labeling-Kit (Roche, Mannheim, Deutschland) erfolgte während der Vorhybridisierung unter Verwendung von alpha-^{32}P-dCTP (Amersham Pharmacia, Braunschweig, Deutschland). Die Hybridisierung wurde nach Zugabe der markierten DNA-Sonde im gleichen Puffer bei 68°C über Nacht durchgeführt. Die Waschschritte

wurden zweimal für 15 min unter Verwendung von 2 X SSC und zweimal für 30 min unter Verwendung von 1 X SSC, 1 % SDS, bei 68°C durchgeführt. Die Exposition der verschlossenen Filter wurde bei -70°C für einen Zeitraum von 1 bis 14 T durchgeführt.

Zur Untersuchung des Vorliegens oder der relativen Menge an von dieser mRNA translatiertem Protein können Standardtechniken, wie ein Western-Blot, eingesetzt werden (siehe beispielsweise Ausubel et al. (1988) Current Protocols in Molecular Biology, Wiley: New York). Bei diesem Verfahren werden die zellulären Gesamt-Proteine extrahiert, mittels Gelelektrophorese aufgetrennt, auf eine Matrix, wie Nitrozellulose, übertragen und mit einer Sonde, wie einem Antikörper, der spezifisch an das gewünschte Protein bindet, inkubiert. Diese Sonde ist gewöhnlich mit einer chemilumineszenten oder kolorimetrischen Markierung versehen, die sich leicht nachweisen lässt. Das Vorliegen und die Menge der beobachteten Markierung zeigt das Vorliegen und die Menge des gewünschten, in der Zelle vorliegenden Proteins an.

Abbildung 1 zeigt die Ergebnisse des Northern Blots von 3 unabhängigen transgenen Arabidopsis-Linien die mit dem AHB2 Konstrukt transformiert wurden sowie des Wildtyps. Die Pflanzen der Linien 9, 10 und 11 zeigen ein starkes Detektionssignal auf dem Northern Blot . Die Pflanzen exprimieren demzufolge das AHB2 Gen in reifenden Samen. In den Samenprobe des Wildtyps konnte dagegen nur ein schwaches Signal detektiert werden, welches auf der Expression des endogenen AHB2 Gens beruhte.

Beispiel 6: Analyse der Auswirkung der rekombinanten Proteine auf die Produktion des gewünschten Produktes

Die Auswirkung der genetischen Modifikation in Pflanzen oder auf die Produktion einer gewünschten Verbindung (wie einer Fettsäure) kann bestimmt werden, indem die modifizierte Pflanze unter geeigneten Bedingungen (wie den vorstehend beschriebenen) gezüchtet wird und das Medium und/oder die zellulären Komponenten auf die erhöhte Produktion des gewünschten Produktes (d.h. von Lipiden oder einer Fettsäure) untersucht wird. Diese Analysetechniken sind dem Fachmann bekannt und umfassen Spektroskopie, Dünnschichtchromatographie, Färbeverfahren verschiedener Art, enzymatische und mikrobiologische Verfahren sowie analytische Chromatographie, wie Hochleistungs-Flüssigkeitschromatographie (siehe beispielsweise Ullman, Encyclopedia of Industrial Chemistry, Bd. A2, S. 89-90 und S. 443-613, VCH: Weinheim (1985); Fallon, A., et al., (1987) "Applications of HPLC in Biochemistry" in: Laboratory Techniques in Biochemistry and Molecular Biology, Bd.

17; Rehm et al. (1993) Biotechnology, Bd. 3, Kapitel III: "Product recovery and purification", S. 469-714, VCH: Weinheim; Belter, P.A., et al. (1988) Bioseparations: downstream processing for Biotechnology, John Wiley and Sons; Kennedy, J.F., und Cabral, J.M.S. (1992) Recovery processes for biological Materials, John Wiley and Sons; Shaeiwitz, J.A., und Henry, J.D. (1988) Biochemical Separations, in: Ullmann's Encyclopedia of Industrial Chemistry, Bd. B3; Kapitel 11, S. 1-27, VCH: Weinheim; und Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

Neben den oben erwähnten Verfahren werden Pflanzenlipide aus Pflanzenmaterial wie von Cahoon et al. (1999) Proc. Natl. Acad. Sci. USA 96 (22):12935-12940, und Browse et al. (1986) Analytic Biochemistry 152:141-145, beschrieben extrahiert. Die qualitative und quantitative Lipid- oder Fettsäureanalyse ist beschrieben bei Christie, William W., Advances in Lipid Methodology, Ayr/Scotland: Oily Press (Oily Press Lipid Library; 2); Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland: Oily Press, 1989, Repr. 1992, IX, 307 S. (Oily Press Lipid Library; 1); "Progress in Lipid Research, Oxford: Pergamon Press, 1 (1952) - 16 (1977) u.d.T.: Progress in the Chemistry of Fats and Other Lipids CODEN.

Ein Beispiel ist die Analyse von Fettsäuren (Abkürzungen: FAME, Fettsäuremethylester; GC-MS, Gas-Flüssigkeitschromatographie-Massenspektrometrie; TAG, Triacylglycerin; TLC, Dünnschichtchromatographie).

Der unzweideutige Nachweis für das Vorliegen von Fettsäureprodukten kann mittels Analyse rekombinanter Organismen nach Standard-Analyseverfahren erhalten werden: GC, GC-MS oder TLC, wie verschiedentlich beschrieben von Christie und den Literaturstellen darin (1997, in: Advances on Lipid Methodology, Vierte Aufl.: Christie, Oily Press, Dundee, 119-169; 1998, Gaschromatographie-Massenspektrometrie-Verfahren, Lipide 33:343-353).

Das zu analysierende Material kann durch Ultraschallbehandlung, Mahlen in der Glasmühle, flüssigen Stickstoff und Mahlen oder über andere anwendbare Verfahren aufgebrochen werden. Das Material muss nach dem Aufbrechen zentrifugiert werden. Das Sediment wird in Aqua dest. resuspendiert, 10 min bei 100°C erhitzt, auf Eis abgekühlt und erneut zentrifugiert, gefolgt von Extraktion in 0,5 M Schwefelsäure in Methanol mit 2 % Dimethoxypropan für 1 Std. bei 90°C, was zu hydrolysierten Öl- und Lipidverbindungen führt, die transmethylierte Lipide ergeben. Diese Fettsäuremethylester werden in Petrolether extrahiert und schließlich einer GC-

Analyse unter Verwendung einer Kapillarsäule (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 mikrom, 0,32 mm) bei einem Temperaturgradienten zwischen 170°C und 240°C für 20 min und 5 min bei 240°C unterworfen. Die Identität der erhaltenen Fettsäuremethylester muss unter Verwendung von Standards, die aus kommerziellen Quellen erhältlich sind (d.h. Sigma), definiert werden.

Pflanzenmaterial wird zunächst mechanisch durch Mörsern homogenisiert, um es einer Extraktion zugänglicher zu machen.

Für die quantitative und qualtitive Ölanalyse der mit den Konstrukten ADH1 und ADH2 transformierten Arabidopsis Pflanzen wurde folgendes Protokoll angewendet:

Die Extraktion der Lipide aus Samen wird nach der Methode von Bligh & Dyer (1959) Can J Biochem Physiol 37:911 durchgeführt. Dazu werden 5 mg Arabidopsis Brassica Samen in 1,2 ml Qiagen-Microtubes (Qiagen, Hilden) auf einer Sartorius (Göttingen) Mikrowaage abgewogen. Das Samenmaterial wird mit 1 ml Chloroform/Methanol (1:1; enthält Mono-C15-glycerin von Sigma als internen Standard) in der Rätschmühle MM300 der Firma Retsch (Haan) homogenisiertund 20 min bei RT inkubiert. Nach Zentrifugation wurde der Überstand in ein neues Gefäß überführt und das Sediment erneut mit 1 ml Chloroform/Methanol (1:1) extrahiert. Die Überstände wurden vereinigt und bis zu Trockene eingeengt. Die Derivatisierung der Fettsäure erfolgte durch saure Methanolyse. Hierzu wurden die extrahierten Lipide mit 0,5 M Schwefelsäure in Methanol und 2 % (v/v) Dimethoxypropan versetzt und für 60 min. bei 80°C inkubiert. Anschließend erfolgte eine zweimalige Extraktion mit Petrolether gefolgt von Waschschritten mit 100 mM Natriumhydrogencarbonat und Wasser. Die so hergestellten Fettsäuremethylester wurden bis zur Trockene eingeengt und in einem definierten Volumen Petrolether aufgenommen. 2 µL der Fettsäuremethylester-Lösung wurden schließlich gaschromatographisch (HP 6890, Agilent Technologies) auf einer Kapillarsäule (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 m, 0.32 mm) aufgetrennt und über einen Flamenionisationsdetektor analysiert.

Die Quantifizierung des Öls erfolgte durch einen Vergleich der Signalstärken der derivatisierten Fettäuren mit denen des internen Standards Mono-C15-glycerin (Sigma).

Die Bestimmung des Fettsäureprofils erfolgte durch relativen Vergleich der Signalstärken zueinander. Die Bestimmung der unsaturierungs-/saturierungs-index (USI) erfolgte wie in Gutiérrez at al. ((2005) Food Chemistry 90, 341-346) beschrieben und reflektiert das Verhältnis von ungesättigten zu gesättigten Fettsäuren in dem

Samenöl.

Die quantitative Proteinanalyse der mit dem Konstrukt USP-AHB2 transformierten Arabidopsis Pflanzen wurde das Protokoll von Bradford (1976) angewendet. Rinderserumalbumin diente hierbei als Standard.

Tabelle 3: Ölgehalt (Gesamtfettsäuregehalt) in reifen und reifenden (13-14 DAF) Samen transgener Arabidopsis Linien, die mit dem USP-AHB2-Konstrukt transformiert worden waren sowie in reifen und reifenden (13-14 DAF) Samen untransformierter Wildyp-Pflanzen. In reifen Samen wurde der Ölgehalt über drei aufeinanderfolgende Generationen bestimmt. Die aufgeführten Werte entsprechen Mittelwerten und Standardfehelern aus 6 unabhängigen Messungen. Signifikante Änderungen zum Wildtyp (basierend auf der statistischen t-test Analyse; $p < 0.05$) sind durch Sternchen (*) hervorgehoben.

Lipidgehalt (mg TFA gDW^{-1})	WT	Linie 9	Linie 10	Linie 11
Lipidgehalt in reifem Samen				
T1 generation	324 ± 12	430 ± 20*	488 ± 79*	502 ± 34*
T2 generation	383 ± 13	451 ± 48	499 ± 21*	507 ± 30*
T3 generation	331 ± 26	380 ± 18	435 ± 17*	464 ± 25*
Lipidgehalt in sich entwickelndem Samen				
T3 generation	128 ± 11	245 ± 22*	224 ± 13*	208 ± 34*

Tabelle 3 fast beispielhaft den Verlauf der Ölgahlte in reifen Samen von 3 unabhängigen transgenen Arabidopsis Linien über 3 Generationen zusammen, die mit dem Konstrukt USP-AHB2 transformiert worden waren sowie den untransformierten Wildty-Pflanzen. Die Werte entsprechen den Mittelwerten aus 6 unabhängigen Messungen. Die Standardfehler sind ebenfalls angegeben. Signifikante Änderungen zum Wildtyp (basierend auf der statistischen t-test Analyse) sind durch Sternchen (*) hervorgehoben. In allen 3 Generationen konnte in den reifen Samen der transgenen

Linien eine deutliche Erhöhung im Ölgehalt gezeigt werden. Der erzielte Phänotyp ist dementsprechend stabil über mehrere Generationen. Darüber hinaus konnte auch in reifenden T3 Samen während der Phase der Ölspeicherung ein deutliche höhere Ölgehalt in den transgenen Linien festgestellt werden (siehe Tabelle 1).

Abbildung 2 zeigt beispielhaft die Ergbnisse für die quantitative Bestimmung der Öl- und Proteingehalte in T3 Samen von 3 unabhängigen transgenen Arabidopsis Linien (9, 10, 11), die mit dem Konstrukt USP-AHB2 transformiert worden waren sowie in den Samen der untransformierten Wildtyp-Pflanzen. Die Werte entsprechen den Mittelwerten aus 10 unabhängigen Messungen. Die Standardfehler sind ebenfalls angegeben. Signifikante Änderungen zum Wildtyp (basierend auf der statistischen t-test Analyse) sind durch Sternchen (*) hervorgehoben. In allen 3 transgenen Linien konnte eine signifikante Erhöhung des Ölgehalts um 15 % (Linie 9), 31 % Linie (Linie 10) und 40 % (Linie 11). Die unterschiedlichen Ölsteigerungen in den verschiedenen Linien korrelieren hierbei mit der in Abbildung 2 gezeigten Expressionsstärken. Im Gegensatz dazu hat die Überexpression von AHB2 keinen Einfluss auf den Ölgehalt

Abbildung 3 zeigt beispielhaft die Ergebnisse der qualitativen Ölanalyse in den reifen Samen transgener Arabidopsis Linien, die mit dem Konstrukt USP-AHB2 transformiert worden waren sowie in den Samen der untransformierten Wildtyp-Pflanzen (A. Linolsäure-Gehalt, B. Linolensäure-Gehalt, C. Linolsäure/Linolensäure-Verhältnis und D. USI (Unsaturierungs / Saturierungs Index)). Die Werte entsprechen den Mittelwerten und Standardfehlern aus 10 unabhängigen Messungen. Signifikante Änderungen zum Wildtyp (basierend auf der statistischen t-test Analyse) sind durch Sternchen (*) hervorgehoben. Samenspezifische Überexpression von AHB2 führt im Samenöl zu einer deutlichen Erhöhung von alpha-Linolensäure (C18:3) von 25 % in den Wildtyppflanze auf über 30 % in den transgenen Linien 10 und 11. Der Gehalt an Linolensäure (C18:2) Vorstufe von C18:3 ist dagegen unverändert. Dies spiegelt sich auch im Verhältnis von C18:3 zu C18:2 wieder (0,8 im Samenöl der Wildtyp-Pflanzen und >1 im Samenöl der transgenen Pflanzen. Die Überexpression von AHB2 führt folglich zu eine erhöhte Desaturierung der Fettsäuren in den Samen der transgenen Linien wie auch durch den USI wiedergegeben der von 9 in den Wildtyp-Samen auf bis zu 12 in den transgenen Samen ansteigt.

Beispiel 7: Bestimmung des ATD/ADP Verhältnisses und des Milchsäuregehalts.

Um den Einfluss von unterschiedliche Sauerstoffkonzentrationen auf den Metabolismus in den Samen von Wildtyp und AHB2-überexprimierenden Arabidopsis-Pflanzen zu untersuchen wurden die Pflanzen im Gewächshaus (21 °C/Tag und 17 °C/Nacht, 50 % Feuchtigkeit Tag und Nacht, Photoperiode 16 h Tag / 8 h Nacht, Nachtinensität 180 µmol Photons $m^{-2}s^{-1}$). Für die Inkubationsexperimente mit verschiednen Konzentrationen Sauerstoff wurde Schotentragende Stängel in eine transparente Plastiktüte verpackt in der Luft mit einem Sauerstoffgehalt von 21 % bzw. 4 % (v/v) zirkulierte. Die Luftgemische der Firma Messer Griesheim GmbH (Magdeburg, Germany) enthielten 350 ppm CO_2, Sauerstoffkonzentrationen wie oben angegeben und Stickstoff. Nach 2 Stunden wurden die Schoten geerntet und sofort in flüssigem Stickstoff schockgefroren. Samen wurden von 13-14 Tagen alten lyophilisierten Schoten wie in Gibon et al. (2002) Plant J 30:221-235 beschrieben präpariert.

Zur Analyse der Metaboliten ATP, ADP und Milchsäure wurden Samen in einer mit flüssigem Stickstoff gekühlten Schwingmühle der Firma Retch (Haan, Germany) homogenisiert und anschließend einer Extraktion mit Trichloressigsäure unterzogen. Die Quantifizierung der Metaboliten erfolgte anschließend wir in Gibon et al. (2002) Plant J 30: 221-235 beschrieben.

Abbildung 4 zeigt den Effekt der samenspezifischen Expression von AHB2 auf das ATP/ADP Verhältnis (A) und den Milchsäuregehalt (B) in reifenden Samen die unter normalen (21 %) Sauerstoffbedingungen oder unter hyoxischen Bedingungen (4 %) kultiviert wurden. Die Ergebnisse entsprechen Mittelwerte und Standardfehler aus 6 unabhängigen Messungen. Signifikante Änderungen zum Wildtyp (basierend auf der statistischen t-test Analyse) sind durch Sternchen (*) hervorgehoben.

Die samenspezifische Überexpression von AHB2 führt unter natürliche Sauerstoffkonzentrationen in der Umgebung zu einem 2-4fach höheren ATP zu ADP Verhältnis in den Samen der transgenen Linien (4-8) als in den Wildtyp-Samen (2). Dies deutet auf eine verbesserte Energieversorgung durch die Atmungskette in den transgenen Samen auch unter den niedrigen Sauerstoffkonzentrationen innerhalb des Samens hin.

Eine Absenkung der Sauerstoffkonzentration in der Umgebung auf 4 % führt in den Wildtypsamen zu einer Reduktion des Energiestatus, welches sich in der Reduktion des ATP zu ADP Verhältnisses von 2 auf 0,4 wiederspiegelt. Die Absenkung des Energiestatus ging mit einer Akkumulation von Milchsäure in den Samen einher (20 µmol gDW^{-1} bei 21 % O_2; 50 µmol gDW^{-1}). Dies demonstriert, dass die Wildtyp-Samen fehlende Energie partiell durch energetisch ungünstigere, anaerobe Fermentation kompensieren.

Eine Absenkung der Sauerstoffkonzentration in der Umgebung auf 4 % führt in den AHB2 überexprimierenden Samen ebenfalls zu einer Reduktion des Energiestatus. Allerdings beläuft sich das ATP zu ADP Verhältnis in diesen Pflanzen auf 0,8 – 2 und ist damit signifikant höher als in den Wildtyp-Samen (0,4). Dies deutet auf eine weiterhin ausreichende aerobe Energieversorgung bei einer Sauerstoffkonzentration in der Umgebung von 4 % hin. Bestätigt wird dies dadurch, dass es in den transgenen Samen nicht zu einer Steigerung der durch aerobe Fermentation gebildeten Milchsäure kommt.

Patentansprüche

1. Verfahren zur Veränderung des ATP-ADP-Verhältnisses in mindestens einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze, dadurch gekennzeichnet, daß die Aktivität mindestens eines Hemproteins verändert wird.

2. Verfahren nach Anspruch 1 dadurch gekennzeichnet, daß die Aktivität mindestens eines Leghemoglobin verändert wird.

3. Verfahren nach einem der Ansprüche 1 oder 2 dadurch gekennzeichnet, daß die Aktivität eines Hemproteins erhöht wird.

4. Verfahren nach einem der Ansprüche 1 bis 3 dadurch gekennzeichnet, daß in die Aktivität mindestens eines Polypeptids erhöht wird, welches kodiert wird von einem Nukleinsäuremolekül umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:
a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;
b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;
c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;
d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;
e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert
f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und
g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder de-

ren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt.

5. Verfahren nach einem der Ansprüche 1 bis 4 dadurch gekennzeichnet, daß in die Aktivität mindestens eines Hemproteins durch Expression, bevorzugt Überexpression, welches kodiert wird von einem Nukleinsäuremolekül umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Amino-

säurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;
erhöht wird

6. Verfahren nach einem der Ansprüche 1 bis 5, dadurch gekennzeichnet, dass das Leghemoglobin und Hemoglobin aus Pflanzen aus der Gruppe bestehend aus Arabidopsis thaliana, Lupinus luteus, Glycine max, Medicago sativa, Medicago trunculata, Phaseolus vulgaris, Vicia faba, Pisum sativum, Vigna unguiculata, Lotus japonicus, Psophocarpus tetragonolobus, Sesbania rostrata, Casuarina glauca und Canvalaria lineata ausgewählt wird.

7. Verfahren nach einem der Ansprüche 1 bis 6, dadurch gekennzeichnet, dass das Hemprotein aus Lotus japonicus oder bevorzugt Arabidopsis thaliana stammt.

8. Verfahren nach einem der Ansprüche 1 bis 7, dadurch gekennzeichnet, dass die Pflanzen derart transformiert werden, dass sie das Hemprotein speicherorganspezifisch exprimiert.

9. Verfahren nach einem der Ansprüche 1 bis 8, dadurch gekennzeichnet, dass die Pflanzen derart transformiert werden, dass sie das Hemprotein knollenspezifisch und/oder samenspezifisch exprimiert.

10. Verfahren nach einem der Ansprüche 1 bis 9, dadurch gekennzeichnet, dass monokotyle Kulturpflanzen, insbesondere der Art Gramineae transformiert werden.

11. Verfahren nach einem der vorhergehenden Ansprüche 1 bis 10, dadurch gekennzeichnet, dass dikotyledone Kulturpflanzen, insbesondere der Familie Asteraceae, Brassicacea, Compositae, Cruciferae, Cucurbitaceae, Leguminosae, Rubiaceae, Solanaceae, Sterculiaceae, Theaceae oder Umbelliferae transformiert werden.

12. Verfahren nach einem der vorhergehenden Ansprüche 1 bis 11, dadurch gekennzeichnet, dass Kartoffeln, Arabidopsis thaliana, Soja oder Raps transformiert werden.

13. Verwendung eines Nukleinsäuremoleküls umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:
a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;
b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;
c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;
d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;
e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert
f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und
g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;
h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;
zur Herstellung eines Polypeptids mit Hemprotein-Aktivität in mindestens einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze.

14. Verwendung eines Nukleinsäuremoleküls umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:
a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;

zur Veränderung des ATP-ADP-Verhältnisses in mindestens einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze.

15. Verwendung eines Nukleinsäuremoleküls umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14,

16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;

zur Herstellung mindestens einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze mit verändertem ATP-ADP-Verhältnisses, bevorzugt erhöhtem ATP-ADP-Verhältnisses.

16. Verwendung eines Nukleinsäuremoleküls umfassend mindestens ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus:

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;

zur Herstellung mindestens einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze mit verändertem Ölgehalt, bevorzugt erhöhtem Fettsäure-Gehalt, bevorzugt erhöhtem Linolensäure-Gehalt.

17. Nukleinsäuremolekül das für ein Polypeptid kodiert welches ein Polypeptid umfasst, welches kodiert wird von einem Nukleinsäuremolekül umfassend ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt.

18. Nukleinsäuremolekül das für ein Polypeptid kodiert welches ein Polypeptid umfasst, welches kodiert wird von einem Nukleinsäuremolekül

das sich in ein, zwei, drei, vier, fünf, sechs, sieben, acht, neun, zehn oder mehreren Nukleinsäuren unterscheidet von einem Nukleinsäuremolekül umfassend ein Nukleinsäuremolekül ausgewählt aus der Gruppe bestehend aus

a) Nukleinsäuremolekül, das ein Polypeptid kodiert, umfassend die in SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 gezeigte Sequenz;

b) Nukleinsäuremolekül, das zumindest ein Polynukleotid der Sequenz gezeigt in SEQ ID NO 1 ,3 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 24, 25, 26, 27, 28, 29, 30 und/oder 31 umfasst;

c) Nukleinsäuremolekül, das ein Polypeptid kodiert, dessen Sequenz eine Identität von mindestens 40% zu den Sequenzen SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 aufweist;

d) Nukleinsäuremolekül nach (a) bis (c), das für ein Fragment der Sequenzen gemäß SEQ ID NO 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 32, 33, 34, 35, 36, 37, 38, 39 und/oder 40 kodiert;

e) Nukleinsäuremolekül das man erhält in dem man einen Nukleinsäuremolekül aus einer cDNA Datenbank oder aus einer Genom-Datenbank mittels der Primer gemäß Sequenz Nr. 41 und 42 amplifiziert

f) Nukleinsäuremolekül das ein Polypeptid mit Hemprotein - Aktivität kodiert, das unter stringenten Bedingungen mit einem Nukleinsäuremolekül gemäß (a) bis (c) hybridisiert; und

g) Nukleinsäuremolekül kodierend für eine Hemprotein, das aus einer DNA-

Bank unter Verwendung eines Nukleinsäuremoleküls gemäß (a) bis (c) oder deren Teilfragmente von mindestens 15 nt, vorzugsweise 20 nt, 30 nt, 50 nt, 100 nt, 200 nt oder 500 nt als Sonde unter stringenten Hybridisierungsbedingungen isoliert werden kann;

h) Nukleinsäuremolekül kodierend für ein Polypeptid enthaltend eine Aminosäurensequenz gemäß der Consensus-Sequenz der Hemprotein – Sequenzen, die SEQ ID NO 46 und/oder 47, bevorzugt SEQ ID NO 43 und/oder 44, besonders bevorzugt SEQ ID NO 43 und/oder 45 umfaßt;
und für ein Polypeptid mit Aktivität eines Hemproteins kodiert.

19. Protein kodiert durch das Nukleinsäuremolekül nach Anspruch 17 oder 18, wobei das Protein nicht aus der in SEQ ID. NO. 2 und 4 gezeigten Sequenz besteht.

20. DNA Expressionskassette enthaltend eine Nukleinsäuresequenz die im wesentlichen identisch ist zu einem Nukleinsäuremolekül gemäss Anspruch 17 oder 18 und für ein Protein nach Anspruch 19 kodiert.

21. Vektor enthaltend eine Expressionskassette gemäss einem der Anspruch 20.

22. Transgene Zelle, enthaltend eine Expressionskassette gemäss Anspruch 20 oder einen Vektor gemäss Anspruch 21.

Abbildung 1

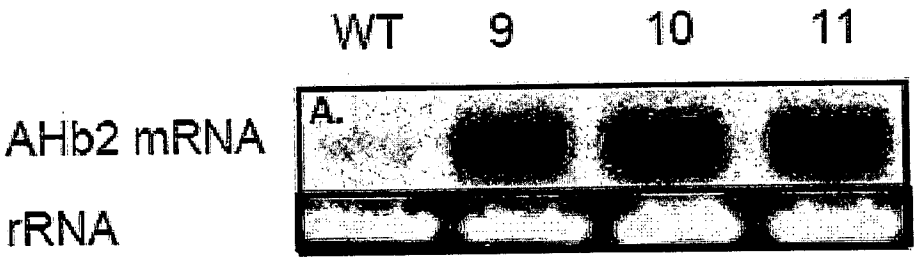


Abbildung 2

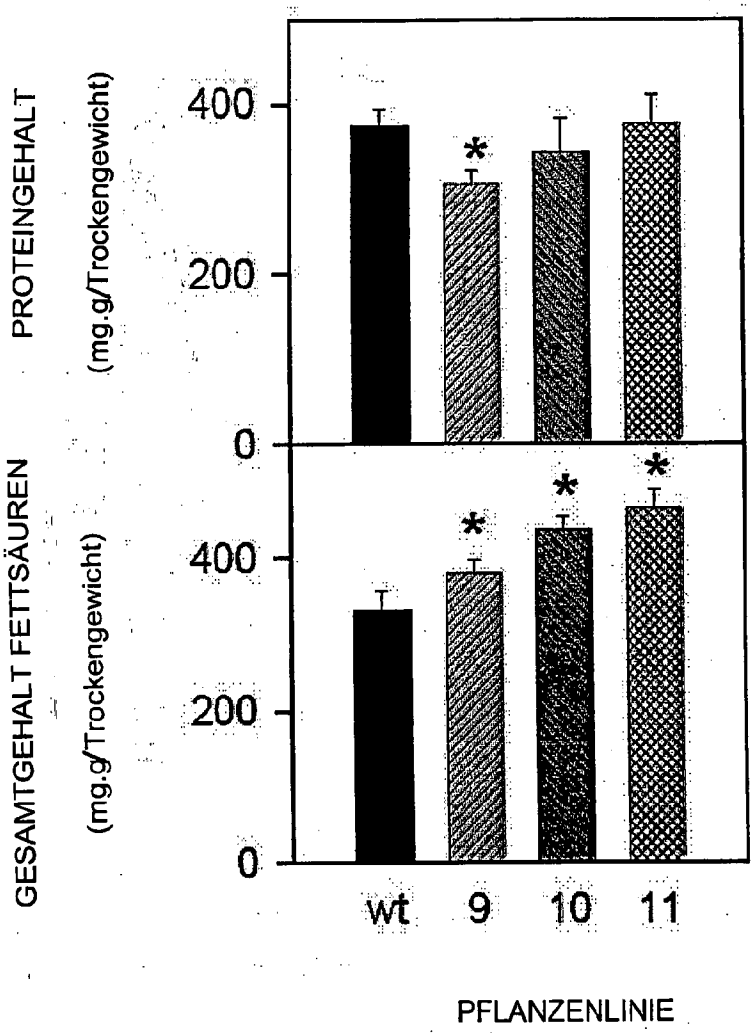

PROTEINGEHALT
(mg.g/Trockengewicht)
400
200
0
GESAMTGEHALT FETTSÄUREN
(mg.g/Trockengewicht)
400
200
0
*
wt
9
10
11
PFLANZENLINIE

Abbildung 3

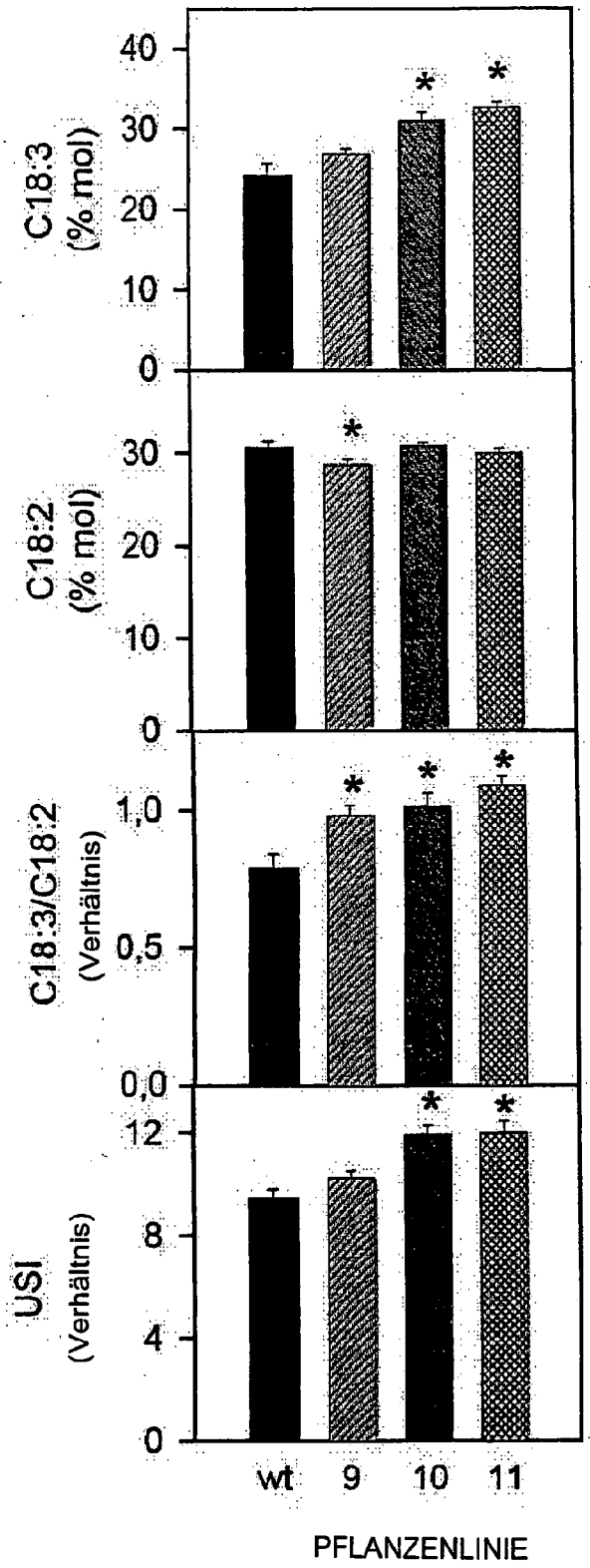
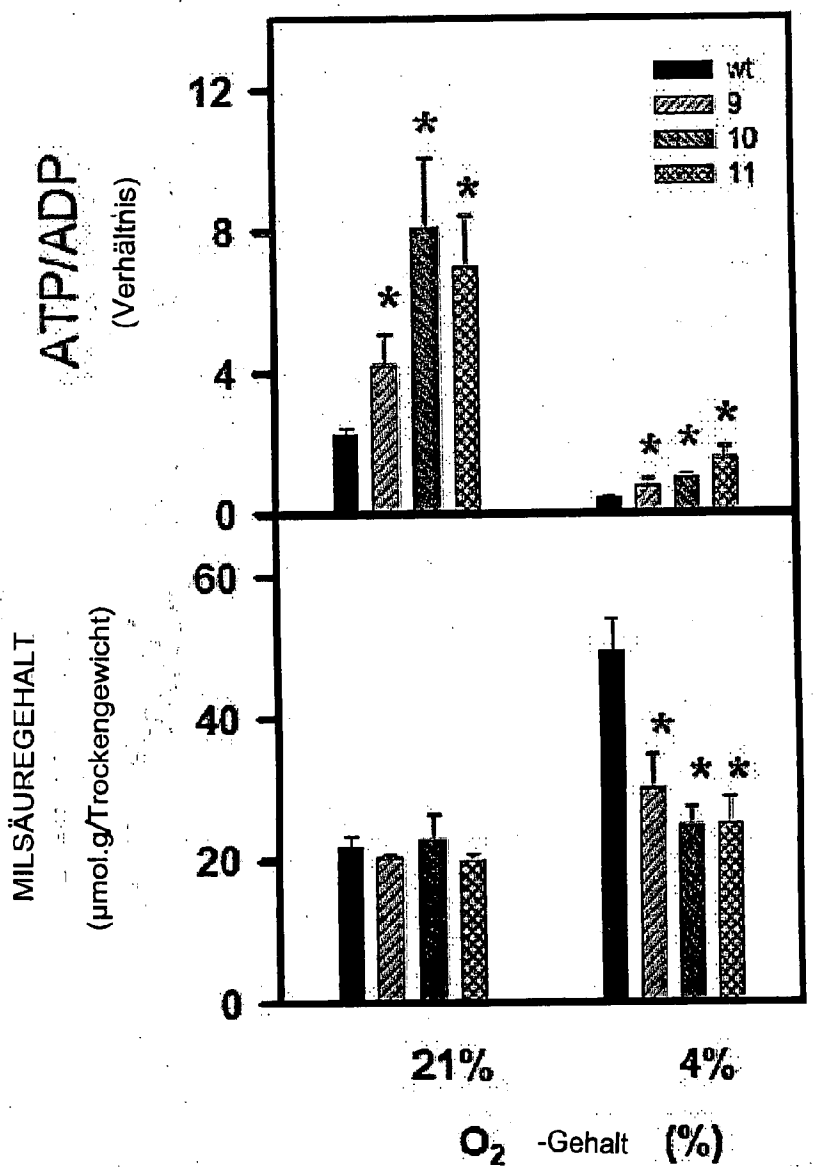

C18:3 (% mol)
40
30
20
10
0
C18:2 (% mol)
30
20
10
0
C18:3/C18:2 (Verhältnis)
1,0
0,5
0,0
USI (Verhältnis)
12
8
4
0
wt
9
10
11
PFLANZENLINIE

Abbildung 4


ATP/ADP
(Verhältnis)
12
8
4
0
wt
9
10
11
*
MILSÄUREGEHALT
(µmol.g/Trockengewicht)
60
40
20
0
21%
4%
O2 -Gehalt (%)

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro

PCT

(43) Internationales Veröffentlichungsdatum
25. Oktober 2007 (25.10.2007)

(10) Internationale Veröffentlichungsnummer
WO 2007/118751 A3

(51) Internationale Patentklassifikation:
C12N 15/82 (2006.01) *C07K 14/415* (2006.01)

(21) Internationales Aktenzeichen: PCT/EP2007/052655

(22) Internationales Anmeldedatum:
20. März 2007 (20.03.2007)

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(30) Angaben zur Priorität:
06112638.9 13. April 2006 (13.04.2006) EP

(71) Anmelder *(für alle Bestimmungsstaaten mit Ausnahme von US)*: **MAX PLANCK INSTITUT FÜR MOLEKULARE PFLANZENPHYSIOLOGIE** [DE/DE]; Wissenschaftspark Golm, Am Mühlenberg 1, 14476 Potsdam (DE).

(72) Erfinder; und

(75) Erfinder/Anmelder *(nur für US)*: **ZANK, Thorsten** [DE/DE]; Seckenheimer Str. 4-6, 68165 Mannheim (DE). **OSWALD, Oliver** [DE/DE]; Nibelungenstr. 158, 64686 Lautertal (DE). **BAUER, Jörg** [DE/DE]; Maxburgstr. 6, 67117 Limburgerhof (DE). **VIGEOLAS, Helene** [FR/DE]; Am alten Mörtelwerk 14, 14469 Potsdam (DE). **GEIGENBERGER, Peter** [DE/DE]; Quantzstr. 12, 14129 Berlin (DE). **STITT, Mark** [GB/DE]; Grosse Weinmeisterstr. 22a, 14469 Potsdam (DE).

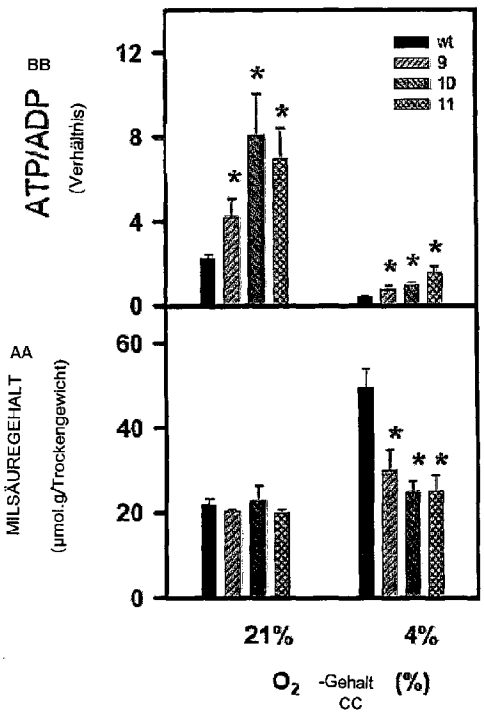
(74) Anwalt: **FITZNER, Uwe**; Hauser Ring 10, 40878 Ratingen (DE).

(81) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare nationale Schutzrechtsart)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN,

[Fortsetzung auf der nächsten Seite]

(54) Title: METHOD FOR MODIFYING THE ATP/ADP RATIO IN CELLS

(54) Bezeichnung: VERFAHREN ZUR VERÄNDERUNG DES ATP-ADP-VERHÄLTNISSES IN ZELLEN



AA LACTIC ACID CONTENT (µmol.g/dry weight)
BB ATP/ADP (ratio)
CC O2 content (%)

(57) Abstract: The invention relates to a method for modifying the ATP/ADP ratio in a cell, tissue, organ, microorganism or plant by modifying the hemoprotein activity in the cell. The invention also relates to the use of said method.

(57) Zusammenfassung: Die Erfindung betrifft ein Verfahren zur Veränderung des ATP/ADP-Verhältnisses in einer Zelle, Gewebe, Organ, Mikroorganismus oder Pflanze durch Änderung der Hemprotein Aktivität in der Zelle sowie dessen Verwendung.

WO 2007/118751 A3

WO 2007/118751 A3

IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Bestimmungsstaaten *(soweit nicht anders angegeben, für jede verfügbare regionale Schutzrechtsart)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), eurasisches (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Veröffentlicht:

— *mit internationalem Recherchenbericht*

— *vor Ablauf der für Änderungen der Ansprüche geltenden Frist; Veröffentlichung wird wiederholt, falls Änderungen eintreffen*

— *mit dem Sequenzprotokollteil der Beschreibung in elektronischer Form getrennt veröffentlicht; auf Antrag vom Internationalen Büro erhältlich*

(88) Veröffentlichungsdatum des internationalen Recherchenberichts: 21. Dezember 2007

Zur Erklärung der Zweibuchstaben-Codes und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/052655

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/82 C07K14/415

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	OTT T ET AL: "Symbiotic Leghemoglobins Are Crucial for Nitrogen Fixation in Legume Root Nodules but Not for General Plant Growth and Development" CURRENT BIOLOGY, CURRENT SCIENCE,, GB, vol. 15, no. 6, 29 March 2005 (2005-03-29), pages 531-535, XP004818992 ISSN: 0960-9822	1-3
Y	siehe gesamtes Dokument; bes. Abstrakt	4-15
X	WO 2004/057946 A (MAX PLANCK GESELLSCHAFT [DE]; GEIGENBERGER PETER [DE]; LANGER ANKE [DE]) 15 July 2004 (2004-07-15)	13, 16-18, 20-22
Y	siehe gesamtes Dokument; SEQ ID NO: 6	4-15

-/--

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 July 2007	10/10/2007

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Grosskopf, Ruediger

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/052655

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2004/087755 A (CROPDESIGN NV [BE]; MULET SALORT JOSE MIGUEL [CH]; SANZ MOLINERO ANA I) 14 October 2004 (2004-10-14)	13,17, 18,20-22
Y	siehe SEQ ID NO: 4; gesamtes Dokument	4-15
X	DORDAS C ET AL: "Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress" PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 35, 2003, pages 763-770, XP003001104 ISSN: 0960-7412	1-3
Y	siehe gesamtes Dokument; insbes. Abstrakt	4-15
X	FREY ALEXANDER D ET AL: "Dissection of central carbon metabolism of hemoglobin-expressing Escherichia coli by 13C nuclear magnetic resonance flux distribution analysis in microaerobic bioprocesses" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 67, no. 2, February 2001 (2001-02), pages 680-687, XP002440436 ISSN: 0099-2240 siehe S. 680, rechte Kolonne, Z. 1-5	1-3
X	WO 00/00597 A (UNIV MANITOBA [CA]; GUY PHILLIP [CA]; DUFF STEPHEN [CA]; XIANZHOU NIE) 6 January 2000 (2000-01-06) siehe gesamtes Dokument; insbes. Beispiel XIX	1-3
A	DOWNIE J A: "Legume Haemoglobins: Symbiotic Nitrogen Fixation Needs Bloody Nodules" CURRENT BIOLOGY, CURRENT SCIENCE,, GB, vol. 15, no. 6, 29 March 2005 (2005-03-29), pages R196-R198, XP004818980 ISSN: 0960-9822	
A	BARATA R M ET AL: "Targeting of the soybean leghemoglobin to tobacco chloroplasts: Effects on aerobic metabolism in transgenic plants" PLANT SCIENCE, LIMERICK, IE, vol. 155, no. 2, 29 June 2000 (2000-06-29), pages 193-202, XP002283115 ISSN: 0168-9452	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/052655

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see supplemental sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-22 **(partly)**

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2007/052655

PCT/ISA/210

The International Searching Authority has found that the international application contains multiple (groups of) inventions, as follows:

Invention 1: Claims 1-22 (all in part)

Method for modifying the ATP/ADP ratio in a cell, tissue, organ, microorganism or plant, characterized in that the activity of at least one hemoprotein is modified, the hemoprotein being coded by a nucleic acid molecule having SEQ ID NO:1, use of said nucleic acid molecule having SEQ ID NO:1 and nucleic acid molecule having SEQ ID NO:1 per se, as well as vectors and host cells containing said nucleic acid molecule.

Inventions 2-20: Claims 1-22 (all in part)

Method for modifying the ATP/ADP ratio in a cell, tissue, organ, microorganism or plant, characterized in that the activity of at least one hemoprotein is modified, the hemoprotein being coded by a nucleic acid molecule having SEQ ID NO:3, 5, 7, 9, 11, 13, 15, 17, 19, 21 or 23-31, use of said nucleic acid molecules having SEQ ID NOs: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 or 23-31 and nucleic acid molecules having SEQ ID NOs: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 or 23-31 per se, as well as vectors and host cells containing the same and the proteins which are coded by said nucleic acids.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2007/052655

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2004057946 A	15-07-2004	AU 2003296714 A1	22-07-2004
		BR 0317650 A	06-12-2005
		CA 2511186 A1	15-07-2004
		CN 1732265 A	08-02-2006
		EP 1578977 A2	28-09-2005
		JP 2006514547 T	11-05-2006
		US 2006053515 A1	09-03-2006
WO 2004087755 A	14-10-2004	BR PI0409079 A	18-04-2006
		CN 1768077 A	03-05-2006
		US 2006288455 A1	21-12-2006
WO 0000597 A	06-01-2000	AU 4595499 A	17-01-2000
		CA 2331772 A1	06-01-2000
		US 6936749 B1	30-08-2005

Form PCT/ISA/210 (patent family annex) (April 2005)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP2007/052655

A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES
INV. C12N15/82 C07K14/415

Nach der Internationalen Patentklassifikation (IPC) oder nach der nationalen Klassifikation und der IPC

B. RECHERCHIERTE GEBIETE

Recherchierter Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)
C12N C07K

Recherchierte, aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)
EPO-Internal, BIOSIS, WPI Data, Sequence Search

C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	OTT T ET AL: "Symbiotic Leghemoglobins Are Crucial for Nitrogen Fixation in Legume Root Nodules but Not for General Plant Growth and Development" CURRENT BIOLOGY, CURRENT SCIENCE,, GB, Bd. 15, Nr. 6, 29. März 2005 (2005-03-29), Seiten 531-535, XP004818992 ISSN: 0960-9822	1-3
Y	siehe gesamtes Dokument; bes. Abstrakt	4-15
X	WO 2004/057946 A (MAX PLANCK GESELLSCHAFT [DE]; GEIGENBERGER PETER [DE]; LANGER ANKE [DE]) 15. Juli 2004 (2004-07-15)	13, 16-18, 20-22
Y	siehe gesamtes Dokument; SEQ ID NO: 6	4-15

-/--

[X] Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen [X] Siehe Anhang Patentfamilie

* Besondere Kategorien von angegebenen Veröffentlichungen :
"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist
"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist
"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)
"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht
"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist
"T" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist
"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfinderischer Tätigkeit beruhend betrachtet werden
"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfinderischer Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist
"&" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche	Absendedatum des internationalen Recherchenberichts
3. Juli 2007	10/10/2007

Name und Postanschrift der Internationalen Recherchenbehörde	Bevollmächtigter Bediensteter
Europäisches Patentamt, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Grosskopf, Ruediger

Formblatt PCT/ISA/210 (Blatt 2) (April 2005)

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP2007/052655

C. (Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	WO 2004/087755 A (CROPDESIGN NV [BE]; MULET SALORT JOSE MIGUEL [CH]; SANZ MOLINERO ANA I) 14. Oktober 2004 (2004-10-14)	13,17, 18,20-22
Y	siehe SEQ ID NO: 4; gesamtes Dokument	4-15
X	DORDAS C ET AL: "Expression of a stress-induced hemoglobin affects NO levels produced by alfalfa root cultures under hypoxic stress" PLANT JOURNAL, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, Bd. 35, 2003, Seiten 763-770, XP003001104 ISSN: 0960-7412	1-3
Y	siehe gesamtes Dokument; insbes. Abstrakt	4-15
X	FREY ALEXANDER D ET AL: "Dissection of central carbon metabolism of hemoglobin-expressing Escherichia coli by 13C nuclear magnetic resonance flux distribution analysis in microaerobic bioprocesses" APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Bd. 67, Nr. 2, Februar 2001 (2001-02), Seiten 680-687, XP002440436 ISSN: 0099-2240 siehe S. 680, rechte Kolonne, Z. 1-5	1-3
X	WO 00/00597 A (UNIV MANITOBA [CA]; GUY PHILLIP [CA]; DUFF STEPHEN [CA]; XIANZHOU NIE) 6. Januar 2000 (2000-01-06) siehe gesamtes Dokument; insbes. Beispiel XIX	1-3
A	DOWNIE J A: "Legume Haemoglobins: Symbiotic Nitrogen Fixation Needs Bloody Nodules" CURRENT BIOLOGY, CURRENT SCIENCE,, GB, Bd. 15, Nr. 6, 29. März 2005 (2005-03-29), Seiten R196-R198, XP004818980 ISSN: 0960-9822	
A	BARATA R M ET AL: "Targeting of the soybean leghemoglobin to tobacco chloroplasts: Effects on aerobic metabolism in transgenic plants" PLANT SCIENCE, LIMERICK, IE, Bd. 155, Nr. 2, 29. Juni 2000 (2000-06-29), Seiten 193-202, XP002283115 ISSN: 0168-9452	

INTERNATIONALER RECHERCHENBERICHT

Internationales Aktenzeichen
PCT/EP2007/052655

Feld II Bemerkungen zu den Ansprüchen, die sich als nicht recherchierbar erwiesen haben (Fortsetzung von Punkt 2 auf Blatt

Gemäß Artikel 17(2)a) wurde aus folgenden Gründen für bestimmte Ansprüche kein Recherchenbericht erstellt:

1. ☐ Ansprüche Nr.
weil sie sich auf Gegenstände beziehen, zu deren Recherche die Behörde nicht verpflichtet ist, nämlich

2. ☐ Ansprüche Nr.
weil sie sich auf Teile der internationalen Anmeldung beziehen, die den vorgeschriebenen Anforderungen so wenig entsprechen, daß eine sinnvolle internationale Recherche nicht durchgeführt werden kann, nämlich

3. ☐ Ansprüche Nr.
weil es sich dabei um abhängige Ansprüche handelt, die nicht entsprechend Satz 2 und 3 der Regel 6.4 a) abgefaßt sind.

Feld III Bemerkungen bei mangelnder Einheitlichkeit der Erfindung (Fortsetzung von Punkt 3 auf Blatt 1)

Die internationale Recherchenbehörde hat festgestellt, daß diese internationale Anmeldung mehrere Erfindungen enthält:

siehe Zusatzblatt

1. ☐ Da der Anmelder alle erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht auf alle recherchierbaren Ansprüche.

2. ☐ Da für alle recherchierbaren Ansprüche die Recherche ohne einen Arbeitsaufwand durchgeführt werden konnte, der eine zusätzliche Recherchengebühr gerechtfertigt hätte, hat die Behörde nicht zur Zahlung einer solchen Gebühr aufgefordert.

3. ☐ Da der Anmelder nur einige der erforderlichen zusätzlichen Recherchengebühren rechtzeitig entrichtet hat, erstreckt sich dieser internationale Recherchenbericht nur auf die Ansprüche, für die Gebühren entrichtet worden sind, nämlich auf die Ansprüche Nr.

4. ☒ Der Anmelder hat die erforderlichen zusätzlichen Recherchengebühren nicht rechtzeitig entrichtet. Der internationale Recherchenbericht beschränkt sich daher auf die in den Ansprüchen zuerst erwähnte Erfindung; diese ist in folgenden Ansprüchen erfaßt:
1-22 (alle teilweise)

Bemerkungen hinsichtlich eines Widerspruchs

☐ Die zusätzlichen Gebühren wurden vom Anmelder unter Widerspruch gezahlt.

☐ Die Zahlung zusätzlicher Recherchengebühren erfolgte ohne Widerspruch.

WEITERE ANGABEN **PCT/ISA/** 210

Die internationale Recherchenbehörde hat festgestellt, dass diese internationale Anmeldung mehrere (Gruppen von) Erfindungen enthält, nämlich:

Erfindung 1: 1-22 (alle teilweise)

Verfahren zur Veränderung des ATP-ADP-Verhältnisses in einer Zelle, Gewebe, Organ., Mikroorganismus oder Pflanze, dadurch gekennzeichnet, dass die Aktivität mindestens eines Hemproteins verändert wird, wobei dass Hemprotein durch ein Nukleinsäuremolekül mit der SEQ ID NO: 1 kodiert wird, Verwendung des Nukleinsäuremoleküls mit der SEQ ID NO: 1 sowie das Nukleinsäuremolekül mit der SEQ ID NO: 1 selbst, sowie Vektoren und Wirtszellen, die dieses enthalten

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Erfindung 2-20: 1-22 (alle teilweise)

Verfahren zur Veränderung des ATP-ADP-Verhältnisses in einer Zelle, Gewebe, Organ., Mikroorganismus oder Pflanze, dadurch gekennzeichnet, dass die Aktivität mindestens eines Hemproteins verändert wird, wobei dass Hemprotein durch ein Nukleinsäuremolekül mit der SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 oder 23-31 2 kodiert wird, Verwendung der Nukleinsäuremoleküle mit den SEQ ID NOs: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 oder 23-31 sowie die Nukleinsäuremoleküle mit den SEQ ID NO: 3, 5, 7, 9, 11, 13, 15, 17, 19, 21 oder 23-31 selbst, sowie Vektoren und Wirtszellen, die dieses enthalten und die Proteine, die durch diese Nukleinsäuren kodiert werden

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(54) **Title:** A PROTEIN COMPOSITION AND ITS USE IN RESTRUCTURED MEAT AND FOOD PRODUCTS



(57) **Abstract:** The invention provides protein compositions containing structured protein products having protein fibers that are substantially aligned.

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A PROTEIN COMPOSITION AND ITS USE IN RESTRUCTURED MEAT AND FOOD PRODUCTS

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This is a continuation-in-part patent application of U.S. patent application Ser. No. 11/437,164, filed on May 19, 2006.

FIELD OF THE INVENTION

[0002] The present invention provides a protein composition and the use of the protein composition in vegetable products, fruit products, and in restructured meat products. The invention further provides a process for preparing the hydrated and shredded protein composition.

BACKGROUND OF THE INVENTION

[0003] An important aspect of the present invention is the development of an unstructured protein product into a structured protein product. Particularly, in one embodiment, the present invention provides a product and method for taking an unstructurized protein product with no visible grain or texture and converting it into a structurized, protein product with a definite shape having the consistency of cooked muscle meat.

[0004] The term "structure" describes a wide variety of physical properties of a food product. A product of acceptable structure is usually synonymous with the quality of a product. Structure has been defined as "the attribute of a substance resulting from a combination of physical properties and perceived by senses of touch, including kinethesia and mouth feel, sight, and hearing. Structure, as defined by the International Organization of Standardization, is "all of the rheological and structural (geometric and surface) attributes of a food product perceptible by means of mechanical, tactual and, where appropriate, visual and auditory receptors." The following terms have been used to describe product characteristics falling under the umbrella "structure":

TABLE I

ABRIDGED LIST OF FOOD STRUCTURE ADJECTIVES

Adhesive	Fleshy	Mushy	Soft
Bouncy	Fluffy	Oily	Soggy
Brittle	Foamy	Pasty	Sparkly
Bubbly	Fragile	Plastic	Splintery
Chewy	Full-bodied	Porous	Spongy
Clingy	Gooey	Powdery	Springy
Coating	Grainy	Puffy	Sticky
Cohesive	Gritty	Pulpy	Stringy
Creamy	Gummy	Rich	Syrupy
Crisp	Hard	Rough	Tender
Crumbly	Heavy	Rubbery	Thick
Crusty	Heterogeneous	Runny	Thin
Dense	Juicy	Sandy	Tingly
Doughy	Lean	Scratchy	Tough
Dry	Light	Short	Uniform
Elastic	Limp	Silky	Viscous
Fatty	Lumpy	Slippery	Watery
Firm	Moist	Slivery	Waxy
Flaky	Mouth coating	Smooth	Wiggly

[0005] Accelerated attention has been given to structure as it pertains to newer food substances including fabricated and imitation products, formed meat and fish products, where very serious efforts are made by processes to duplicate the properties of the original or other natural food substances. The use of non-traditional raw materials, synthetic flavors, fillers, stretchers, and extenders all tend to alter certain textural characteristics of the finished product. Frequently, the imitation of textural properties is of much greater difficulty in the replication of taste, odors, and colors. Numerous manipulative processes, including extrusion structurization,

have been developed to simulate natural structural properties. The processes generally find it prudent to duplicate the properties of the original substances to the extent feasible technically and economically in order to promote early market acceptance. While structure has attributes related to appearance, it also has attributes related to touch and also mouth feel or interaction of food when it comes in contact with the mouth. Frequently, these sensory perceptions involved with chewing can relate to impressions of either desirability or undesirability.

[0006] Thus, structural terms include terms relating to the behavior of the material under stress or strain and include, for example, the following: firm, hard, soft, tough, tender, chewy, rubbery, elastic, plastic, sticky, adhesive, tacky, crispy, crunchy, etc. Secondly, structure terms may be related to the structure of the material: smooth, fine, powdery, chalky, lumpy, mealy, coarse, gritty, etc. Third, structure terms may relate to the shape and arrangement of structural elements, such as: flaky, fibrous, stringy, pulpy, cellular, crystalline, glassy, spongy, etc. Last, structure terms may relate to mouth feel characteristics, including: mouth feel, body, dry, moist, wet, watery, waxy, slimy, mushy, etc.

[0007] As used herein, "unstructurized" and "structurized" describe the characteristics of the food product as set forth in Table II:

TABLE II

	Unstructurized Characteristic	Structurized Characteristic
Behavior of Material under Stress or Strain	sticky gooey plastic	firm chewy
Structure of Material	smooth	coarse
Shape and Arrangement of Structural Elements	gelatinous pulpy pasty	fibrous crusty
Mouth Feel	creamy dry	moist
mushy with body		

SUMMARY OF THE INVENTION

[0008] One aspect of the invention provides a process for producing a restructured meat composition. The process generally comprises: extruding a plant protein material under conditions of elevated temperature and pressure through a die assembly to form a structured plant protein product having protein fibers that are substantially aligned.

[0009] Another aspect of the invention provides a restructured meat composition. The restructured meat composition comprising a structured protein product having protein fibers that are substantially aligned.

FIGURE LEGENDS

[0010] **Figure 1** depicts a photographic image of a micrograph showing a structured plant protein product of the invention having protein fibers that are substantially aligned.

[0011] **Figure 2** depicts a photographic image of a micrograph showing a plant protein product not produced by the process of the present invention. The protein fibers comprising the plant protein product, as described herein, are crosshatched.

[0012] **Figure 3** depicts a perspective view of a peripheral die assembly that may be used in the extrusion process of the protein containing materials.

[0013] **Figure 4** depicts an exploded view of the peripheral die assembly of **Figure 3** showing the die insert, die sleeve and die cone.

[0014] **Figure 5** depicts a cross-sectional view taken along line 9-9 of **Figure 3** showing a flow channel defined between the die sleeve, die insert, and die cone arrangement.

[0015] **Figure 5A** depicts an enlarged cross-sectional view of **Figure 5** showing the interface between the flow channel and the outlet of the die sleeve.

DETAILED DESCRIPTION OF THE INVENTION

[0016] The present invention provides hydrated and shredded protein compositions and processes for producing each of the compositions. Typically, the protein composition will comprise animal meat and structured protein products having protein fibers that are substantially aligned. Alternatively, the protein composition will comprise a comminuted vegetable or comminuted fruit and structured protein products having protein fibers that are substantially aligned.

(I) Structured Protein Products

[0017] The protein compositions of the invention each comprise structured protein products comprising protein fibers that are substantially aligned, as described in more detail in I(e) below. In an exemplary embodiment, the structured protein products are extrudates of protein material that have been subjected to the extrusion process detailed in I(d) below. Because the structured protein products have protein fibers that are substantially aligned in a manner similar to animal meat, the protein compositions of the invention generally have the texture and eating quality characteristics of compositions comprised of one hundred percent animal meat.

(a) protein-containing materials

[0018] A variety of ingredients that contain protein may be utilized in a thermal plastic extrusion process to produce structured protein products suitable for use in the protein compositions herein. While ingredients comprising proteins derived from plants are typically used, it is also envisioned that proteins derived from other sources, such as animal sources, may be utilized without departing from the scope of the invention. For example, a dairy protein selected from the group consisting of casein, caseinates, whey protein, and mixtures thereof may be utilized. In an exemplary embodiment, the dairy protein is whey protein. By way of further example, an egg protein selected from the group consisting of ovalbumin, ovoglobulin, ovomucin, ovomucoid, ovotransferrin, ovovitella, ovovitellin, albumin globulin, and vitellin may be utilized. Further, meat proteins or protein ingredients consisting of collagen, blood, organ meat, mechanically separated meat, partially defatted tissue and blood serum proteins may be included as one or more of the ingredients of the structured protein products.

[0019] It is envisioned that other ingredient types in addition to proteins may be utilized. Non-limiting examples of such ingredients include sugars, starches, oligosaccharides, soy fiber and other dietary fibers.

[0020] It is also envisioned that the protein-containing materials may be gluten-free. Because gluten is typically used in filament formation during the extrusion process, if a gluten-free starting material is used, an edible cross-linking agent may be utilized to facilitate filament formation. Non- limiting examples of suitable cross-linking agents include Konjac glucomannan (KGM) flour, beta 1,3 glucan (Pureglucan™ or Curdlan manufactured by Takeda-Kirin Foods), transglutaminase, calcium salts, and magnesium salts. One skilled in the art can readily determine the amount of cross-linking material needed, if any, in gluten-free embodiments.

[0021] Irrespective of its source or ingredient classification, the ingredients utilized in the extrusion process are typically capable of forming extrudates having protein fibers that are substantially aligned. Suitable examples of such ingredients are detailed more fully below.

(i) plant protein materials

[0022] In an exemplary embodiment, at least one ingredient derived from a plant will be utilized to form the protein-containing materials. Generally speaking, the ingredient will comprise a protein. The amount of protein present in the ingredient(s) utilized can and will vary depending upon the application. For example, the amount of protein present in the ingredient(s)

utilized may range from about 40% to about 100% by weight. In another embodiment, the amount of protein present in the ingredient(s) utilized may range from about 50% to about 100% by weight. In an additional embodiment, the amount of protein present in the ingredient(s) utilized may range from about 60% to about 100% by weight. In a further embodiment, the amount of protein present in the ingredient(s) utilized may range from about 70% to about 100% by weight. In still another embodiment, the amount of protein present in the ingredient(s) utilized may range from about 80% to about 100% by weight. In a further embodiment, the amount of protein present in the ingredient(s) utilized may range from about 90% to about 100% by weight.

[0023] The ingredient(s) utilized in extrusion may be derived from a variety of suitable plants. By way of non-limiting example, suitable plants include legumes, corn, peas, canola, sunflowers, sorghum, rice, amaranth, potato, tapioca, arrowroot, canna, lupin, rape, wheat, oats, rye, barley, and mixtures thereof.

[0024] In one embodiment, the ingredients are isolated from wheat and soybeans. In another exemplary embodiment, the ingredients are isolated from soybeans. Suitable wheat derived protein-containing ingredients include wheat gluten, wheat flour, and mixtures thereof. Examples of commercially available wheat gluten that may be utilized in the invention include Gem of the Star Gluten, Vital Wheat Gluten (organic) each of which is available from Manildra Milling. Suitable soybean derived protein-containing ingredients ("soy protein material") include soybean protein isolate, soy protein concentrate, soy flour, and mixtures thereof, each of which are detailed below. Suitable corn derived protein-containing ingredients include corn gluten meal, for example, zein. In each of the foregoing embodiments, the soybean material may be combined with one or more ingredients selected from the group consisting of a starch, flour, gluten, a dietary fiber, and mixtures thereof.

[0025] Suitable examples of protein-containing material isolated from a variety of sources are detailed in Table III, which shows various combinations.

TABLE III Protein Combinations

First Protein Source	Second Ingredient
soybean	wheat
soybean	dairy

First Protein Source	Second Ingredient
soybean	egg
soybean	corn
soybean	rice
soybean	barley
soybean	sorghum
soybean	oat
soybean	millet
soybean	rye
soybean	triticale
soybean	buckwheat
soybean	pea
soybean	peanut
soybean	lentil
soybean	lupin
soybean	channa(garbonzo)
soybean	rapeseed (canola)
soybean	cassava
soybean	sunflower
soybean	whey
soybean	tapioca
soybean	arrowroot
soybean	amaranth
soybean	wheat and dairy
soybean	wheat and egg
soybean	wheat and corn
soybean	wheat and rice
soybean	wheat and barley
soybean	wheat and sorghum
soybean	wheat and oat

First Protein Source	Second Ingredient
soybean	wheat and millet
soybean	wheat and rye
soybean	wheat and triticale
soybean	wheat and buckwheat
soybean	wheat and pea
soybean	wheat and peanut
soybean	wheat and lentil
soybean	wheat and lupin
soybean	wheat and channa (garbonzo)
soybean	wheat and rapeseed (canola)
soybean	wheat and cassava
soybean	wheat and sunflower
soybean	wheat and potato
soybean	wheat and tapioca
soybean	wheat and arrowroot
soybean	wheat and amaranth
soybean	corn and wheat
soybean	corn and dairy
soybean	corn and egg
soybean	corn and rice
soybean	corn and barley
soybean	corn and sorghum
soybean	corn and oat
soybean	corn and millet
soybean	corn and rye
soybean	corn and triticale
soybean	corn and buckwheat
soybean	corn and pea
soybean	corn and peanut

First Protein Source	Second Ingredient
soybean	corn and lentil
soybean	corn and lupin
soybean	corn and channa (garbonzo)
soybean	corn and rapeseed (canola)
soybean	corn and cassava
soybean	corn and sunflower
soybean	corn and potato
soybean	corn and tapioca
soybean	corn and arrowroot
soybean	corn and amaranth

[0026] In each of the embodiments delineated in Table III, the combination of protein-containing materials may be combined with one or more ingredients selected from the group consisting of a starch, flour, gluten, a dietary fiber, and mixtures thereof. In one embodiment, the protein-containing material comprises protein, starch, gluten, and fiber. In an exemplary embodiment, the protein-containing material comprises from about 45% to about 65% soy protein on a dry matter basis; from about 20% to about 30% wheat gluten on a dry matter basis; from about 10% to about 15% wheat starch on a dry matter basis; and from about 1% to about 5% fiber on a dry matter basis. In each of the foregoing embodiments, the protein-containing material may comprise dicalcium phosphate, L-cysteine or combinations of both dicalcium phosphate and L-cysteine.

(ii). soy protein materials

[0027] In an exemplary embodiment, as detailed above, soy protein isolate, soy protein concentrate, soy flour, and mixtures thereof may be utilized in the extrusion process. The soy protein materials may be derived from whole soybeans in accordance with methods generally known in the art. The whole soybean may be standard soybeans (i.e., non genetically modified soybeans), commoditized soybeans, genetically modified soybeans, and combinations thereof.

[0028] Generally speaking, when soy isolate is used, an isolate is preferably selected that is not a highly hydrolyzed soy protein isolate. In certain embodiments, highly hydrolyzed soy protein isolates, however, may be used in combination with other soy protein isolates provided that the highly hydrolyzed soy protein isolate content of the combined soy protein isolates is generally less than about 40% of the combined soy protein isolates, by weight. Additionally, the soy protein isolate utilized preferably has an emulsion strength and gel strength sufficient to enable the protein in the isolate to form fibers that are substantially aligned upon extrusion. Examples of soy protein isolates that are useful in the present invention are commercially available, for example, from Solae, LLC (St. Louis, Mo.), and include SUPRO® 500E, SUPRO® EX 33, SUPRO® 620, SUPRO® 630, and SUPRO® 545. In an exemplary embodiment, a form of SUPRO® 620 is utilized as detailed in Example 3.

[0029] Alternatively, soy protein concentrate may be blended with the soy protein isolate to substitute for a portion of the soy protein isolate as a source of soy protein material. Typically, if a soy protein concentrate is substituted for a portion of the soy protein isolate, the soy protein concentrate is substituted for up to about 40% of the soy protein isolate by weight, at most, and more preferably is substituted for up to about 30% of the soy protein isolate by weight. Examples of suitable soy protein concentrates useful in the invention include Promine DSPC, Procon, Alpha 12 and Alpha 5800, which are commercially available from Solae, LLC (St. Louis, Mo.).

[0030] Soy cotyledon fiber may optionally be utilized as a fiber source. Typically, suitable soy cotyledon fiber will generally effectively bind water when the mixture of soy protein and soy cotyledon fiber is co-extruded. In this context, "effectively bind water" generally means that the soy cotyledon fiber has a water holding capacity of at least 5.0 to about 8.0 grams of water per gram of soy cotyledon fiber, and preferably the soy cotyledon fiber has a water holding capacity of at least about 6.0 to about 8.0 grams of water per gram of soy cotyledon fiber. Soy cotyledon fiber may generally be present in the soy protein material in an amount ranging from about 1% to about 20%, preferably from about 1.5% to about 20% and most preferably, at from about 2% to about 5% by weight on a moisture free basis. Suitable soy cotyledon fiber is commercially available. For example, FIBRIM® 1260 and FIBRIM® 2000 are soy cotyledon fiber materials that are commercially available from Solae, LLC (St. Louis, Mo.).

(b) additional ingredients

[0031] A variety of additional ingredients may be added to any of the protein-containing materials detailed above without departing from the scope of the invention. For example, antioxidants, antimicrobial agents, and combinations thereof may be included. Antioxidant additives include BHA, BHT, TBHQ, vitamins A, C and E and derivatives thereof. Additionally, various plant extracts such as those containing carotenoids, tocopherols or flavonoids having antioxidant properties, may be included to increase the shelf-life or nutritionally enhance the protein compositions. The antioxidants and the antimicrobial agents may have a combined presence at levels of from about 0.01% to about 10%, preferably, from about 0.05% to about 5%, and more preferably from about 0.1% to about 2%, by weight of the protein-containing materials.

(c) moisture content

[0032] As will be appreciated by the skilled artisan, the moisture content of the protein-containing materials and optional additional ingredients can and will vary. The purpose of the water is to hydrate the ingredients of the protein composition. Generally speaking, the moisture content may range from about 1% to about 80% by weight. In low moisture extrusion applications, the moisture content of the protein-containing materials may range from about 1% to about 35% by weight. Alternatively, in high moisture extrusion applications, the moisture content of the protein-containing materials may range from about 35% to about 80% by weight. In an exemplary embodiment, the extrusion application utilized to form the extrudates is low moisture. An exemplary example of a low moisture extrusion process to produce extrudates having proteins with fibers that are substantially aligned is detailed in I(d) and Example 3.

(d) extrusion of the protein-containing material

[0033] A suitable extrusion process for the preparation of structured protein products comprises introducing the protein material, and other ingredients into a mixing vessel (i.e., an ingredient blender) to combine the ingredients and form a dry blended protein material pre-mix. The dry blended protein material pre-mix may be transferred to a hopper from which the dry blended ingredients are introduced along with moisture into a pre-conditioner to form a conditioned protein material mixture. The conditioned material is then fed to an extruder in

which the mixture is heated under mechanical pressure generated by the screws of the extruder to form a molten extrusion mass. Alternatively, the dry blended protein material pre-mix may be directly fed to an extruder in which moisture and heat are introduced to form a molten extrusion mass. The molten extrusion mass exits the extruder through an extrusion die assembly forming an extrudate comprising structured protein products having protein fibers that are substantially aligned.

(i) extrusion process conditions

[0034] Among the suitable extrusion apparatuses useful in the practice of the present invention is a double barrel, twin-screw extruder as described, for example, in U.S. Pat. No. 4,600,311. Further examples of suitable commercially available extrusion apparatuses include a CLEXTRAL Model BC-72 extruder manufactured by Clextral, Inc. (Tampa, Florida); a WENGER Model TX-57 extruder, a WENGER Model TX-168 extruder, and a WENGER Model TX-52 extruder all manufactured by Wenger Manufacturing, Inc. (Sabetha, Kansas). Other conventional extruders suitable for use in this invention are described, for example, in U.S. Pat. Nos. 4,763,569, 4,118,164, and 3,117,006, which are hereby incorporated by reference in their entirety.

[0035] The screws of a twin-screw extruder can rotate within the barrel in the same or opposite directions. Rotation of the screws in the same direction is referred to as single flow whereas rotation of the screws in opposite directions is referred to as double flow. The speed of the screw or screws of the extruder may vary depending on the particular apparatus; however, it is typically from about 250 to about 400 revolutions per minute (rpm). Generally, as the screw speed increases, the density of the extrudate will decrease. The extrusion apparatus contains screws assembled from shafts and worm segments, as well as mixing lobe and ring-type shearlock elements as recommended by the extrusion apparatus manufacturer for extruding plant protein material.

[0036] The extrusion apparatus generally comprises a plurality of temperature controlled zones through which the protein mixture is conveyed under mechanical pressure prior to exiting the extrusion apparatus through an extrusion die assembly. The temperature in each successive heating zone generally exceeds the temperature of the previous heating zone by between about 10°C and about 70°C. In one embodiment, the conditioned pre-mix is transferred

through four heating zones within the extrusion apparatus, with the protein mixture heated to a temperature of from about 100°C to about 150°C such that the molten extrusion mass enters the extrusion die assembly at a temperature of from about 100°C to about 150°C.

[0037] The barrel pressure is dependent on numerous factors including, for example, the extruder screw speed, feed rate of the mixture to the barrel, feed rate of water to the barrel, and the viscosity of the molten mass within the barrel.

[0038] Water may be injected into the extruder barrel to hydrate the plant protein material mixture and promote texturization of the proteins. As an aid in forming the molten extrusion mass, the water may act as a plasticizing agent. Water may be introduced to the extruder barrel via one or more injection jets in communication with a heating zone. Typically, the mixture in the barrel contains from about 1% to about 30% by weight water. In one embodiment, the mixture in the barrel contains from about 5% to about 20% by weight water. The rate of introduction of water to any of the heating zones is generally controlled to promote production of an extrudate having desired characteristics. It has been observed that as the rate of introduction of water to the barrel decreases, the density of the extrudate decreases.

(ii) optional preconditioning

[0039] In a pre-conditioner, the protein-containing material and optional additional ingredients (protein-containing mixture) are preheated, contacted with moisture, and held under temperature and pressure conditions to allow the moisture to penetrate and soften the individual particles. The preconditioning step increases the bulk density of the particulate fibrous material mixture and improves its flow characteristics. The preconditioner contains one or more paddles to promote uniform mixing of the protein and transfer of the protein mixture through the preconditioner. The configuration and rotational speed of the paddles vary widely, depending on the capacity of the preconditioner, the extruder throughput and/or the desired residence time of the mixture in the preconditioner or extruder barrel. Generally, the speed of the paddles is from about 500 to about 1300 revolutions per minute (rpm).

[0040] Typically, the protein-containing mixture is pre-conditioned prior to introduction into the extrusion apparatus by contacting the pre-mix with moisture (i.e., steam and/or water). Preferably the protein-containing mixture is heated to a temperature of from about 20°C to about 60°C, more preferably from about 30°C to about 45°C in the preconditioner.

[0041] Typically, the protein-containing pre-mix is conditioned for a period of about 0.5 minutes to about 10.0 minutes, depending on the speed and the size of the pre-conditioner. In an exemplary embodiment, the protein-containing pre-mix is conditioned for a period of about 3.0 minutes to about 5.0 minutes. The pre-mix is contacted with steam and/or water and heated in the pre-conditioner at generally constant steam flow to achieve the desired temperatures. The water and/or steam conditions (i.e., hydrates) the pre-mix, increases its density, and facilitates the flowability of the dried mix without interference prior to introduction to the extruder barrel where the proteins are texturized. If low moisture pre-mix is desired, the conditioned pre-mix may contain from about 1% to about 35% (by weight) water. If high moisture pre-mix is desired, the conditioned pre-mix may contain from about 35% to about 80% (by weight) water.

[0042] The conditioned pre-mix typically has a bulk density of from about 0.25 g/cm^3 to about 0.60 g/cm^3. Generally, as the bulk density of the pre-conditioned protein mixture increases within this range, the protein mixture is easier to process. This is presently believed to be due to such mixtures occupying all or a majority of the space between the screws of the extruder, thereby facilitating conveying the extrusion mass through the barrel.

(iii) extrusion process

[0043] The dry pre-mix or the conditioned pre-mix is then fed into an extruder to heat, shear, and ultimately plasticize the mixture. The extruder may be selected from any commercially available extruder and may be a single screw extruder or preferably a twin-screw extruder that mechanically shears the mixture with the screw elements.

[0044] The rate at which the pre-mix is generally introduced to the extrusion apparatus will vary depending upon the particular apparatus. Generally, the pre-mix is introduced at a rate of no more than about 25 kilograms per minute. Generally, it has been observed that the density of the extrudate decreases as the feed rate of pre-mix to the extruder increases.

[0045] The pre-mix is subjected to shear and pressure by the extruder to plasticize the mixture. The screw elements of the extruder shear the mixture as well as create pressure in the extruder by forcing the mixture forwards though the extruder and through the die assembly. The screw motor speed determines the amount of shear and pressure applied to the mixture by the screw(s). Preferably, the screw motor speed is set to a speed of from about 200 rpm to about 500 rpm, and more preferably from about 300 rpm to about 400 rpm, which moves the mixture

through the extruder at a rate of at least about 20 kilograms per hour and more preferably at least about 40 kilograms per hour. Preferably the extruder generates an extruder barrel exit pressure of from about 500 to about 1500 psig, and more preferably an extruder barrel exit pressure of from about 600 to about 1000 psig is generated.

[0046] The extruder heats the mixture as it passes through the extruder denaturing the protein in the mixture. The extruder includes a means for heating and/or cooling the mixture to temperatures of from about 100°C to about 180°C. Preferably, the means for heating or cooling the mixture in the extruder comprises extruder barrel jackets into which heating or cooling media such as steam or water may be introduced to control the temperature of the mixture passing through the extruder. The extruder may also include steam injection ports for directly injecting steam into the mixture within the extruder. The extruder preferably includes multiple heating zones that can be controlled to independent temperatures, where the temperatures of the heating zones are preferably set to increase the temperature of the mixture as it proceeds through the extruder. In one embodiment, the extruder may be set in a four temperature zone arrangement, where the first zone (adjacent the extruder inlet port) is set to a temperature of from about 80°C to about 100°C, the second zone is set to a temperature of from about 100°C to 135°C, the third zone is set to a temperature of from 135°C to about 150°C, and the fourth zone (adjacent the extruder exit port) is set to a temperature of from 150°C to 180°C. The extruder may be set in other temperature zone arrangements, as desired. In another embodiment, the extruder may be set in a five temperature zone arrangement, where the first zone is set to a temperature of about 25°C, the second zone is set to a temperature of about 50°C, the third zone is set to a temperature of about 95°C, the fourth zone is set to a temperature of about 130°C, and the fifth zone is set to a temperature of about 150°C. In still another embodiment, the extruder may be set in a six temperature zone arrangement, where the first zone is set to a temperature of about 90°C, the second zone is set to a temperature of about 100°C, the third zone is set to a temperature of about 105°C, the fourth zone is set to a temperature of about 100°C, the fifth zone is set to a temperature of about 120°C, and the sixth zone is set to a temperature of about 130°C.

[0047] The mixture forms a melted plasticized mass in the extruder. A die assembly is attached to the extruder in an arrangement that permits the plasticized mixture to flow from the extruder exit port into the die assembly and produces substantial alignment of the protein fibers within the plasticized mixture as it flows through the die assembly. The die assembly may

include either a faceplate die or a peripheral die. The cutter speed is set to size the piece to no more than 5% through a 16 mesh screen, and no more than 65% on a ½ -inch screen.

[0048] One embodiment includes a peripheral die assembly as illustrated and generally indicated as 10 in FIGS. 3-5.

[0049] As shown in FIGS. 3 and 4, the peripheral die assembly 10 may include a die sleeve 12 having a cylindrical-shaped two-part sleeve die body 17. The sleeve die body 17 may include a rear portion 18 coupled to a front portion 20 that collectively define an internal chamber 31 in communication with opposing openings 72, 74. The die sleeve 12 may be adapted to receive a die insert 14 and a die cone 16 for providing the necessary structural elements to facilitate laminar flow of the plasticized mixture through the peripheral die assembly 10 during the extrusion process.

[0050] Additionally, the front portion 20 of the die sleeve 12 may be secured to a die cone 16 adapted to interface with the die insert 14 when the front portion 20 is secured to the rear portion 18 of the die sleeve 12 during assembly of the peripheral die assembly 10. As further shown, the rear portion 18 of die sleeve 12 defines a plurality of circular-shaped outlets 24 along the sleeve body 17 which are adapted to provide a conduit for the egress of the extrudate from the peripheral die assembly 10 during the extrusion process. In the alternative, the plurality of outlets 24 may have different configurations, such as square, rectangular, scalloped or irregular. As further shown, the rear portion 18 of the die sleeve 12 may include a circular flange 37 that surrounds opening 72 and defines a pair of opposing slots 82A and 82B that are used to properly align the die sleeve 12 when engaging the die sleeve 12 to the extruder.

[0051] As shown in FIG. 5, when the peripheral die assembly 10 is fully assembled the die insert 14 is disposed within the rear portion 18 of the die sleeve 12 which is secured to the front portion 20 of the die sleeve 12 such that the conical side 56 of the die cone 16 is oriented toward the chamber 31 and encased between the rear and front portions 18 and 20. In this orientation, the conical side 56 is operatively associated with the front face 27 of the die insert 14. As such, the opposing side walls 50 of each adjacent flow diverter 38, the bottom portion 64 of the die insert 14, and the conical side 56 of the die cone 16 collectively define a respective flow channel 40 in communication with a respective outlet 24. The flow channel 40 defined between the die sleeve 12, die insert 14 and die cone 16 as described above may be tapered on all

four sides of the flow channel 40. Accordingly, the flow channel 40 gradually tapers inwardly on all four sides from the entrance 84 to the outlet 24 of each flow channel 40.

[0052] Referring to FIG. 5A, an enlarged view illustrating the flow pathway "A" through flow channel 40 is shown. Specifically, flow channel 40 communicates with the outlet 24 through opening 70 defined by the die insert 14.

[0053] During the extrusion process, the peripheral die assembly 10 is operatively engaged with the extruder and produces a plasticized mixture that contacts the well 52 defined by the rear face 29 of the die insert 14 and flows into the throat 34 and enters the inner space opening 36 as indicated by flow path "A". The plasticized mixture may enter the inner space 44 defined by the die insert 14 and enter the entrance 84 of each tapered flow channel 42. The plasticized mixture then flows through each flow channel 42 and exits from a respective outlet 24 in a manner that causes the substantial alignment of the protein fibers in the extrudate produced by the peripheral die assembly 10.

[0054] The width and height dimensions of the outlet(s) 24 are selected and set prior to extrusion of the mixture to provide the fibrous material extrudate with the desired dimensions. The width of the outlet(s) 24 may be set so that the extrudate resembles from a cubic chunk of meat to a steak filet, where widening the width of the outlet(s) 24 decreases the cubic chunk-like nature of the extrudate and increases the filet-like nature of the extrudate. In an exemplary embodiment, the width of the outlet(s) 24 may be set to a width of from about 10 millimeters to about 40 millimeters.

[0055] The height dimension of the outlet(s) 24 may be set to provide the desired thickness of the extrudate. The height of the outlet(s) 24 may be set to provide a very thin extrudate or a thick extrudate. For example, the height of the outlet(s) 24 may be set to from about 1 millimeter to about 30 millimeters. In an exemplary embodiment, the height of the outlet(s) 24 may be set to from about 8 millimeters to about 16 millimeters.

[0056] It is also contemplated that the outlet(s) 24 may be round. The diameter of the outlet(s) 24 may be set to provide the desired thickness of the extrudate. The diameter of the outlet(s) 24 may be set to provide a very thin extrudate or a thick extrudate. For example, the diameter of the outlet(s) 24 may be set to from about 1 millimeter to about 30 millimeters. In an exemplary embodiment, the diameter of the outlet(s) 24 may be set to from about 8 millimeters to about 16 millimeters.

[0057] Other peripheral die assemblies suitable for use in this invention are described in U.S. Pat. App. No. 60/882,662, which is hereby incorporated by reference in its entirety.

[0058] The extrudate is cut after exiting the die assembly. Suitable apparatuses for cutting the extrudate include flexible knives manufactured by Wenger Manufacturing, Inc. (Sabetha, Kansas) and Clextral, Inc. (Tampa, Florida). Typically, the speed of the cutting apparatus is from about 1000 rpm to about 2500 rpm. In an exemplary embodiment, the speed of the cutting apparatus is about 1600 rpm.

[0059] A dryer, if one is used, generally comprises a plurality of drying zones in which the air temperature may vary. Generally, the temperature of the air within one or more of the zones will be from about 100°C to about 185°C. Typically, the extrudate is present in the dryer for a time sufficient to provide an extrudate having the desired moisture content. Generally, the extrudate is dried for at least about 5 minutes and more generally, for at least about 10 minutes. Alternatively, the extrudate may be dried at lower temperatures, such as about 70°C, for longer periods of time. Suitable dryers include those manufactured by Wolverine Proctor & Schwartz (Merrimac, Mass.), National Drying Machinery Co. (Philadelphia, Pa.), Wenger (Sabetha, Kans.), Clextral (Tampa, Fla.), and Buehler (Lake Bluff, Ill.).

[0060] The desired moisture content may vary widely depending on the intended application of the extrudate. Generally speaking, the extruded material has a moisture content of from about 6% to about 13% by weight, if dried, and needs to be hydrated in water until the water is absorbed and the fibers are separated. If the protein material is not dried or not fully dried, its moisture content is higher, generally from about 16% to about 30% by weight.

[0061] The dried extrudate may further be comminuted to reduce the average particle size of the extrudate. Suitable grinding apparatus include hammer mills such as Mikro Hammer Mills manufactured by Hosokawa Micron Ltd. (England).

(e) characterization of the structured protein products

[0062] The extrudates produced in I(d) typically comprise the structured protein products having protein fibers that are substantially aligned. In the context of this invention "substantially aligned" generally refers to the arrangement of protein fibers such that a significantly high percentage of the protein fibers forming the structured protein product are contiguous to each other at less than approximately a 45° angle when viewed in a horizontal

plane. Typically, an average of at least 55% of the protein fibers comprising the structured protein product are substantially aligned. In another embodiment, an average of at least 60% of the protein fibers comprising the structured protein product are substantially aligned. In a further embodiment, an average of at least 70% of the protein fibers comprising the structured protein product are substantially aligned. In an additional embodiment, an average of at least 80% of the protein fibers comprising the structured protein product are substantially aligned. In yet another embodiment, an average of at least 90% of the protein fibers comprising the structured protein product are substantially aligned. Methods for determining the degree of protein fiber alignment are known in the art and include visual determinations based upon micrographic images.

[0063] By way of example, **Figures 1** and **2** depict micrographic images that illustrate the difference between a structured protein product having substantially aligned protein fibers compared to a protein product having protein fibers that are significantly crosshatched. **Figure 1** depicts a structured protein product prepared according to I(a)–I(d) having protein fibers that are substantially aligned. Contrastingly, **Figure 2** depicts a protein product containing protein fibers that are significantly crosshatched and not substantially aligned. Because the protein fibers are substantially aligned, as shown in **Figure 1**, the structured protein products utilized in the invention generally have the texture and consistency of cooked muscle meat. In contrast, extrudates having protein fibers that are randomly oriented or crosshatched generally have a texture that is soft or spongy.

[0064] In addition to having protein fibers that are substantially aligned, the structured protein products also typically have shear strength substantially similar to whole meat muscle. In this context of the invention, the term "shear strength" provides one means to quantify the formation of a sufficient fibrous network to impart whole-muscle like texture and appearance to the structured protein product. Shear strength is the maximum force in grams needed to shear through a given sample. A method for measuring shear strength is described in Example 1.

[0065] Generally speaking, the structured protein products of the invention will have average shear strength of at least 1400 grams. In an additional embodiment, the structured protein products will have average shear strength of from about 1500 to about 1800 grams. In yet another embodiment, the structured protein products will have average shear strength of from about 1800 to about 2000 grams. In a further embodiment, the structured protein products will

have average shear strength of from about 2000 to about 2600 grams. In an additional embodiment, the structured protein products will have average shear strength of at least 2200 grams. In a further embodiment, the structured protein products will have average shear strength of at least 2300 grams. In yet another embodiment, the structured protein products will have average shear strength of at least 2400 grams. In still another embodiment, the structured protein products will have average shear strength of at least 2500 grams. In a further embodiment, the structured protein products will have average shear strength of at least 2600 grams.

[0066] A means to quantify the size of the protein fibers formed in the structured protein products may be done by a shred characterization test. Shred characterization is a test that generally determines the percentage of large pieces formed in the structured protein product. In an indirect manner, percentage of shred characterization provides an additional means to quantify the degree of protein fiber alignment in a structured protein product. Generally speaking, as the percentage of large pieces increases, the degree of protein fibers that are aligned within a structured protein product also typically increases. Conversely, as the percentage of large pieces decreases, the degree of protein fibers that are aligned within a structured protein product also typically decreases.

[0067] A method for determining shred characterization is detailed in Example 2. The structured protein products of the invention typically have an average shred characterization of at least 10% by weight of large pieces. In a further embodiment, the structured protein products have an average shred characterization of from about 10% to about 15% by weight of large pieces. In another embodiment, the structured protein products have an average shred characterization of from about 15% to about 20% by weight of large pieces. In yet another embodiment, the structured protein products have an average shred characterization of from about 20% to about 25% by weight of large pieces. In another embodiment, the average shred characterization is at least 20% by weight, at least 21% by weight, at least 22% by weight, at least 23% by weight, at least 24% by weight, at least 25% by weight, or at least 26% by weight large pieces.

[0068] Suitable structured protein products of the invention generally have protein fibers that are substantially aligned, have average shear strength of at least 1400 grams, and have an average shred characterization of at least 10% by weight large pieces. More typically, the structured protein products will have protein fibers that are at least 55% aligned, have average

shear strength of at least 1800 grams, and have an average shred characterization of at least 15% by weight large pieces. In exemplary embodiment, the structured protein products will have protein fibers that are at least 55% aligned, have average shear strength of at least 2000 grams, and have an average shred characterization of at least 17% by weight large pieces. In another exemplary embodiment, the structured protein products will have protein fibers that are at least 55% aligned, have average shear strength of at least 2200 grams, and have an average shred characterization of at least 20% by weight large pieces.

(II) Restructured Meat Compositions

[0069] The structured protein products are utilized in the invention as a component in restructured meat compositions. A restructured meat composition may comprise a mixture of animal meat and structured protein product, or it may comprise no meat and primarily structured protein product. The process for producing the restructured meat compositions generally comprises optionally mixing it with animal meat, coloring and hydrating the structured protein product, reducing its particle size, and further processing the composition into a food product comprising meat.

(a) optionally blend with animal meat

[0070] The structured protein product may optionally be blended with animal meat to produce animal meat compositions either before or after contacting the structured protein product with the coloring composition detailed below. In general, the structured protein product will be blended with animal meat that has a similar particle size.

[0071] It is well known in the art to produce mechanically deboned or separated raw meats using high-pressure machinery that separates bone from animal tissue, by first crushing bone and adhering animal tissue and then forcing the animal tissue, and not the bone, through a sieve or similar screening device. The animal tissue in the present invention comprises muscle tissue, organ tissue, connective tissue and skin. The process forms an unstructured, paste-like blend of soft animal tissue with a batter-like consistency and is commonly referred to as mechanically deboned meat or MDM. This paste-like blend has a particle size of from about 0.25 to about 15 millimeters, preferably up to about 5 millimeters and most preferably up to about 3 millimeters.

[0072] Although the animal tissue, also known as raw meat, is preferably provided in at least substantially frozen form so as to avoid microbial spoilage prior to processing, once the meat is ground, it is not necessary to freeze it to provide cutability into individual strips or pieces. Unlike meat meal, raw meat has a natural high moisture content with a ratio of protein to moisture of from about 1:3.6 to 1:3.7.

[0073] The raw meat used in the present invention may be any edible meat suitable for consumption. The meat may be non-rendered, non-dried, raw meat, raw meat products, raw meat by-products, and mixtures thereof. The meat or meat products are comminuted and generally supplied daily in a completely frozen or at least substantially frozen condition so as to avoid microbial spoilage. Generally the temperature of the comminuted meat is below about 40°C (104°F), preferably below about 10°C (50°F) more preferably is from about -4°C (25°F) to about 6°C (43°F) and most preferably from about -2°C (28°F) to about 2°C (36°F). While refrigerated or chilled meat may be used, it is generally impractical to store large quantities of unfrozen meat for extended periods of time at a plant site. The frozen products provide a longer lay time than do the refrigerated or chilled products.

[0074] In lieu of frozen comminuted meat, the comminuted meat may be freshly prepared for the preparation of the restructured meat product, as long as the freshly prepared comminuted meat meets the temperature conditions of not more than about 40°C (104°F).

[0075] The moisture content of the raw frozen or unfrozen meat is generally at least about 50% by weight, and most often from about 60% by weight to about 75% by weight, based upon the weight of the raw meat. In one embodiment of the invention, the fat content of the raw frozen or unfrozen meat is at least 2% by weight. Generally the fat content of the raw frozen or unfrozen meat is from about 3% by weight to about 95% by weight. In another embodiment, the fat content of the raw frozen or unfrozen meat is about 20% by weight to about 95% by weight. In other embodiments of the invention, meat products may be combined to produce a meat composition that has a fat content of from about 15% by weight to about 30% by weight. In another embodiment, the meat composition may have a fat content of less than about 10% by weight and defatted meat products may be used.

[0076] The frozen or chilled meat may be stored at a temperature of about -18°C (.4°F) to about 0°C (32°F). It is generally supplied in 20 kilogram blocks. Upon use, the blocks are permitted to thaw up to about 10°C (50°F), that is, to defrost, but in a tempered environment.

Thus, the outer layer of the blocks, for example up to a depth of about 1/4", may be defrosted or thawed but still at a temperature of about 0°C (32°F), while the remaining inner portion of the blocks, while still frozen, are continuing to thaw and thus keeping the outer portion at below about 10°C (50°F).

[0077] A variety of animal meats are suitable for use in the restructured meat composition. For example, the meat may be from a farm animal selected from the group consisting of sheep, cattle, goats, pork, bison, and horses. The animal meat may be from poultry, such as chicken, duck, goose or turkey. Alternatively, the animal meat may be from a game animal. Non-limiting examples of suitable game animals include buffalo, deer, elk, moose, reindeer, caribou, antelope, rabbit, squirrel, beaver, muskrat, opossum, raccoon, armadillo, porcupine, alligator, and snake. In a further embodiment, the animal meat may be from a fish or shellfish. Non-limiting examples of suitable fish or fish products include saltwater and freshwater fish, such as, catfish, tuna, salmon, bass, mackerel, pollack, hake, tilapia, cod, grouper, whitefish, bowfin, gar, paddlefish, sturgeon, bream, carp, trout, surimi, walleye, snakehead, and shark. In an exemplary embodiment, the animal meat is from beef, pork, or turkey.

[0078] Meat includes striated muscle which is skeletal or that which is found, for example, in the tongue, diaphragm, heart, or esophagus, with or without accompanying overlying fat and portions of the skin, sinew, nerve and blood vessels which normally accompany the meat flesh. Examples of meat by-products are organs and tissues such as lungs, spleens, kidneys, brain, liver, blood, bone, partially defatted low-temperature fatty tissues, stomachs, intestines free of their contents, and the like.

[0079] The term "meat by-products" is intended to refer to those non-rendered parts of the carcass of slaughtered animals including but not restricted to mammals, poultry and the like and including such constituents as are embraced by the term "meat by-products" in the Definitions of Feed Ingredients published by the Association of American Feed Control Officials, Incorporated.

[0080] Typically, the amount of structured protein product in relation to the amount of animal meat in the animal meat compositions can and will vary depending upon the composition's intended use. By way of example, when a significantly vegetarian composition that has a relatively small degree of animal flavor is desired, the concentration of animal meat in

the restructured meat composition may be about 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 2%, or 0% by weight. Alternatively, when a restructured meat composition having a relatively high degree of animal meat flavor is desired, the concentration of animal meat in the restructured meat composition may be about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% by weight. Consequently, the concentration of hydrated structured plant protein product in the restructured meat composition may be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% by weight. In an exemplary embodiment, the restructured meat composition will generally have from about 40% to about 60% by weight of the hydrated structured protein product and from about 40% to about 60% by weight of animal meat.

[0081] It is also envisioned that a variety of meat qualities may be utilized in the invention. For example, whole meat muscle that is either ground or in chunk or steak form may be utilized. The meat may have a fat content that varies widely.

(b) optionally blend with comminuted vegetable or comminuted fruit

[0082] The structured protein product may optionally be blended with comminuted vegetable or comminuted fruit to produce restructured meat compositions either before or after contacting the structured protein product with the coloring composition detailed below. In general, the structured protein product will be blended with comminuted vegetable or comminuted fruit that has a similar particle size.

[0083] A variety of vegetables or fruits are suitable for use in the restructured meat composition. Typically, the amount of structured protein product in relation to the amount of comminuted vegetable or comminuted fruit in the restructured meat compositions can and will vary depending upon the composition's intended use. By way of example, the concentration of comminuted vegetable or comminuted fruit in the restructured meat composition may be about 95%, 90%, 85%, 80%, 75%, 70%, 65%, 60%, 55%, 50%, 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 2%, or 0% by weight. Consequently, the concentration of hydrated structured plant protein product in the restructured meat composition may be about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% by weight. In an exemplary embodiment, the restructured meat composition will generally have

from about 40% to about 60% by weight of the hydrated structured protein product and from about 40% to about 60% by weight of comminuted vegetable or comminuted fruit.

(c) hydrating and coloring the structured protein product

[0084] The structured protein product is generally colored with a coloring composition so as to resemble raw meat and/or cooked meat. The coloring compositions of the invention may comprise thermally unstable pigments, thermally stable pigments, and browning agents. The choice of the type of pigments and the amount present in the coloring composition can and will vary depending upon the desired color of the restructured meat composition. When the restructured meat composition simulates a "pre-cooked product," the structured plant product is typically contacted with browning agents and/or thermally stable pigments. Alternatively, when the restructured meat composition simulates raw meat, the structured protein product is generally contacted with thermally unstable red pigments and also with browning agents, and/or thermally stable pigments, such that when the restructured meat composition is cooked its appearance changes from a raw meat color to fully cooked color. Suitable thermally unstable red pigments, thermally stable pigments, and browning agents are described below.

[0085] The colorant(s) may be mixed with the protein-containing material and other ingredients prior to being fed into the extruder. Alternatively, the colorant(s) may be combined with the protein-containing material and other ingredients after being fed into the extruder. In the presence of the heat or the heat and pressure utilized during the extrusion process, some combinations of colorants and protein-containing materials result in unexpected colors. As an example, when carmine (or lac) is contacted with the protein-containing material during the extrusion process, the color changes from red to violet/purple.

[0086] The colorant(s) may be a natural colorant, a combination of natural colorants, an artificial colorant, a combination of artificial colorants, or a combination of natural and artificial colorants. Suitable examples of natural colorants approved for use in food include annatto (reddish-orange), anthocyanins (red to blue, depends upon pH), beet juice, beta-carotene (orange), beta-APO 8 carotenal (orange), black currant, burnt sugar; canthaxanthin (pink-red), caramel, carmine/carminic acid (bright red), cochineal extract (red), curcumin (yellow-orange); lac (scarlet red), lutein (red-orange); lycopene (orange-red), mixed carotenoids (orange), monascus (red-purple, from fermented red rice), paprika, red cabbage juice, riboflavin (yellow),

saffron, titanium dioxide (white), and turmeric (yellow-orange). Suitable examples of artificial colorants approved for food use in the United States include FD&C Red No. 3 (Erythrosine), FD&C Red No. 40 (Allure Red), FD&C Yellow No. 5 (Tartrazine), FD&C Yellow No. 6 (Sunset Yellow FCF), FD&C Blue No. 1 (Brilliant Blue), FD&C Blue No. 2 (Indigotine). Artificial colorants that may be used in other countries include Cl Food Red 3 (Carmoisine), Cl Food Red 7 (Ponceau 4R), Cl Food Red 9 (Amaranth), Cl Food Yellow 13 (Quinoline Yellow), and Cl Food Blue 5 (Patent Blue V). Food colorants may be dyes, which are powders, granules, or liquids that are soluble in water. Alternatively, natural and artificial food colorants may be lake colors, which are combinations of dyes and insoluble materials. Lake colors are not oil soluble, but are oil dispersible; they tint by dispersion.

[0087] Suitable colorant(s) may be combined with the protein-containing materials in a variety of forms. Non-limiting examples include solid, semi-solid, powdered, liquid, and gelatin. The type and concentration of colorant(s) utilized may vary depending on the protein-containing materials used and the desired color of the colored structured protein product. Typically, the concentration of colorant(s) may range from about 0.001% to about 5.0% by weight. In one embodiment, the concentration of colorant(s) may range from about 0.01% to about 4.0% by weight. In another embodiment, the concentration of colorant(s) may range from about 0.05% to about 3.0% by weight. In still another embodiment, the concentration of colorant(s) may range from about 0.1% to about 3.0% by weight. In a further embodiment, the concentration of colorant(s) may range from about 0.5% to about 2.0% by weight. In another embodiment, the concentration of colorant(s) may range from about 0.75% to about 1.0% by weight.

[0088] A thermally unstable pigment may be used in the coloring composition to provide the red color of raw uncooked meat. The thermally unstable pigment is typically a food coloring dye or powder having a red color that resembles the red coloration of browning meat in its uncooked state (i.e., raw meat). Generally speaking, the thermally unstable pigment is a food coloring dye or powder having a structure that is degraded upon exposure to temperatures effective to cook a structured protein product. In this manner, the pigment is degraded thermally and as such, is ineffective to provide substantial coloration to the structured protein product when it is cooked. The thermally unstable pigment is typically degraded at temperatures of about 100°C or greater, more preferably at temperatures of about 75°C or greater, and most

typically at temperatures of about 50°C or greater. In one embodiment, the thermally unstable pigment is betanin, a red food coloring dye or powder having poor thermal stability. Betanin is derived from red beets and is typically prepared from red beet juice or beet powder. The thermally unstable pigment may be present in the coloring composition from about 0.005% to about 30% by dry weight of the coloring composition. When the thermally unstable pigment is betanin, the betanin preferably forms from about 0.005% to about 0.5% of the coloring composition by dry weight, and more preferably forms from about 0.01% to about 0.05% of the coloring composition by dry weight. Alternatively, a beet powder or beet extract preparation containing betanin may be present in the coloring composition from about 5% to about 30% of the composition by dry weight, and more preferably from about 10% to about 25% of the coloring composition. As an example, the coloring composition could be composed of 0.0087% annatto, 21.68% beet powder, 52.81% dextrose, and 25.43% NFE (all percentages are by weight).

[0089] A thermally stable pigment comprised of one or more thermally stable food coloring dyes may be used in the coloring composition. Suitable thermally stable pigments include those that are effective to provide a structured protein product with coloration resembling browned meat in both an uncooked state and a cooked state. Suitable thermally stable pigments include caramel food coloring material, and yellow, brown, and/or orange food-coloring agents. A variety of caramel food coloring agents are useful in the present invention and are commercially available in a powdered form or in a liquid form, including Caramel Color No. 602 (available from the Williamson Company, Louisville, Kentucky), and 5440 Caramel Powder D.S. (available from Sensient Colors, Inc., St. Louis, Missouri).

[0090] Several types of commercially available yellow/orange food colorings may be used in the thermally stable pigment. Suitable yellow/orange food colors include annatto, turmeric and artificial yellow dyes such as FD&C Yellow #5. The amount of thermally stable pigment present in the coloring composition is from about 0% to about 7% by dry weight of the coloring composition, and more preferably from about 0.1% to about 3% by dry weight of the coloring composition. The yellow/orange food coloring material, preferably annatto, may constitute from about 0% to about 2% of the coloring composition by dry weight, and preferably is present in about 0.1% to about 1%, by dry weight of the coloring composition. The caramel

food coloring material typically constitutes from about 0% to about 5% by dry weight, and preferably from about 0.5% to about 3%, by dry weight of the coloring composition.

[0091] The coloring composition may include browning agents which comprise an amine source and a reducing sugar. As detailed above, the browning agent generally causes a protein containing material in which the coloring composition is mixed to brown similarly to cook browning meat when the protein material is cooked. In an alternative embodiment, the browning agent of the coloring composition may also include an amine source. An amine compound reacts with a reducing sugar to induce browning. Suitable amine sources include a polypeptide material, a hydrolyzed protein material, or an amino acid material. Without being bound by any particular theory, a polypeptide material, hydrolyzed protein, and/or amino acid material is preferably included as an amine source in the browning agent to enhance the desired browning. In an exemplary embodiment, an isolated soy protein is the source of amine groups in the browning agent. When included in the coloring composition, the amine source is generally present in the coloring composition from about 20% to about 55% of the coloring composition by dry weight. An exemplary browning agent is a reducing sugar. Suitable reducing sugars are typically capable of undergoing a Maillard browning reaction in the presence of compounds containing amine groups to provide the desired browning when a protein containing material is cooked. Representative examples of suitable reducing sugars include xylose, arabinose, galactose, fructose, glycealdehyde, mannose, dextrose, lactose and maltose. In an exemplary embodiment, the reducing sugar is dextrose. The reducing sugar may be present in the coloring composition from about 25% to about 95% by dry weight of the coloring composition, and preferably from about 30% to about 60% by dry weight of the coloring composition.

[0092] In an exemplary embodiment, the coloring composition comprises beet pigment, annatto, caramel coloring, a reducing sugar, and an amino acid source. In one alternative of this embodiment, the amino acid source comprises peptides comprised of amino acids and secondary amino acids. In another alternative embodiment, the amino acid source is isolated soy protein.

[0093] The coloring composition may further comprise an acidity regulator to maintain the pH in the optimal range for the colorant. The acidity regulator may be an acidulent. Examples of acidulents that may be added to food include citric acid, acetic acid (vinegar), tartaric acid, malic acid, fumaric acid, lactic acid, gluconic acid, phosphoric acid, sorbic acid,

hydrochloric acid, propionic acid, and benzoic acid. The final concentration of the acidulent in a coloring composition may range from about 0.001% to about 5% by weight of the coloring composition. The acidity regulator may also be a pH-raising agent, such as disodium biphosphate, sodium carbonate, sodium bicarbonate, sodium hydroxide, and potassium hydroxide.

[0094] The coloring composition of the present invention may be prepared by combining the components using processes and procedures known to those of ordinary skill in the art. The components are typically available in either a liquid form or a powder form, and often in both forms. The components can be mixed directly to form the coloring composition, but preferably the ingredients of the coloring composition are combined in an aqueous solution at a total concentration of about 10% to about 25% by weight, where the aqueous coloring solution can be conveniently added to a quantity of water for mixing with and coloring a structured protein product.

(d) addition of optional ingredients

[0095] The restructured meat compositions may optionally include a variety of flavorings, spices, antioxidants, or other ingredients to impart a desired flavor or texture or to nutritionally enhance the final food product. As will be appreciated by a skilled artisan, the selection of ingredients added to the restructured meat composition can and will depend upon the food product to be manufactured.

[0096] The restructured meat composition may comprise from about 1% to about 30% by weight of a fat source to impart flavor. Typically, the fat source is an animal fat. Suitable animal fats include beef fat, pork fat, poultry fat and lamb fat. In an exemplary embodiment, the restructured meat composition will comprise from about 10% to about 20% by weight of a fat source. In an additional embodiment a plant derived fat source can be used, non-limiting examples include oils such as canola oil, cottonseed oil, grape oil, olive oil, peanut oil, palm oil, soybean oil, sunflower oil, vegetable oil, and combinations thereof. The percentage by weight of the plant derived fat source can be from about 10% to 20%.

[0097] The restructured meat compositions may further comprise an antioxidant. The antioxidant may prevent the oxidation of the polyunsaturated fatty acids (e.g., omega-3 fatty acids) in the animal meat, and the antioxidant may also prevent oxidative color changes in the

restructured meat composition. The antioxidant may be natural or synthetic. Suitable antioxidants include, but are not limited to, ascorbic acid and its salts, ascorbyl palmitate, ascorbyl stearate, anoxomer, N-acetylcysteine, benzyl isothiocyanate, m-aminobenzoic acid, o-aminobenzoic acid, p-aminobenzoic acid (PABA), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), caffeic acid, canthaxantin, alpha-carotene, beta-carotene, beta-caraotene, beta-apo-carotenoic acid, carnosol, carvacrol, catechins, cetyl gallate, chlorogenic acid, citric acid and its salts, clove extract, coffee bean extract, p-coumaric acid, 3,4-dihydroxybenzoic acid, N,N'-diphenyl-p-phenylenediamine (DPPD), dilauryl thiodipropionate, distearyl thiodipropionate, 2,6-di-tert-butylphenol, dodecyl gallate, edetic acid, ellagic acid, erythorbic acid, sodium erythorbate, esculetin, esculin, 6-ethoxy-1,2-dihydro-2,2,4-trimethylquinoline, ethyl gallate, ethyl maltol, ethylenediaminetetraacetic acid (EDTA), eucalyptus extract, eugenol, ferulic acid, flavonoids (e.g., catechin, epicatechin, epicatechin gallate, epigallocatechin (EGC), epigallocatechin gallate (EGCG), polyphenol epigallocatechin-3-gallate), flavones (e.g., apigenin, chrysin, luteolin), flavonols (e.g., datiscetin, myricetin, daemfero), flavanones, fraxetin, fumaric acid, gallic acid, gentian extract, gluconic acid, glycine, gum guaiacum, hesperetin, alpha-hydroxybenzyl phosphinic acid, hydroxycinammic acid, hydroxyglutaric acid, hydroquinone, N-hydroxysuccinic acid, hydroxytryrosol, hydroxyurea, rice bran extract, lactic acid and its salts, lecithin, lecithin citrate; R-alpha-lipoic acid, lutein, lycopene, malic acid, maltol, 5-methoxy tryptamine, methyl gallate, monoglyceride citrate; monoisopropyl citrate; morin, beta-naphthoflavone, nordihydroguaiaretic acid (NDGA), octyl gallate, oxalic acid, palmityl citrate, phenothiazine, phosphatidylcholine, phosphoric acid, phosphates, phytic acid, phytylubichromel, pimento extract, propyl gallate, polyphosphates, quercetin, trans-resveratrol, rosemary extract, rosmarinic acid, sage extract, sesamol, silymarin, sinapic acid, succinic acid, stearyl citrate, syringic acid, tartaric acid, thymol, tocopherols (i.e., alpha-, beta-, gamma- and delta-tocopherol), tocotrienols (i.e., alpha-, beta-, gamma- and delta-tocotrienols), tyrosol, vanilic acid, 2,6-di-tert-butyl-4-hydroxymethylphenol (i.e., Ionox 100), 2,4-(tris-3',5'-bi-tert-butyl-4'-hydroxybenzyl)-mesitylene (i.e., Ionox 330), 2,4,5-trihydroxybutyrophenone, ubiquinone, tertiary butyl hydroquinone (TBHQ), thiodipropionic acid, trihydroxy butyrophenone, tryptamine, tyramine, uric acid, vitamin K and derivates, vitamin Q10, wheat germ oil, zeaxanthin, or combinations thereof.

[0098] The concentration of an antioxidant in the restructured meat composition may range from about 0.0001% to about 20% by weight. In another embodiment, the concentration of an antioxidant in the restructured meat composition may range from about 0.001% to about 5% by weight. In yet another embodiment, the concentration of an antioxidant in the restructured meat composition may range from about 0.01% to about 1% by weight.

[0099] In an additional embodiment, the restructured meat compositions may further comprise at least one flavoring agent. The flavoring agent may be natural, or the flavoring agent may be artificial. The flavoring agent may mimic or replace constituents found in lean meat or fat tissues, such as, serum proteins, muscle proteins, hydrolyzed animal proteins, tallow, fatty acids, etc. The flavoring agent may provide an animal meat flavor, a grilled meat flavor, a rare beef flavor, etc. The flavoring agent may be an animal meat oil, oleoresins or aquaresins of spice extracts, spice oils, natural smoke solutions, natural smoke extracts, a yeast extract, or shiitake extract. Additional flavoring agents may include onion flavor, garlic flavor, or herb flavors. The restructured meat composition may further comprise a flavor enhancer. Examples of flavor enhancers that may be used include salt (sodium chloride), glutamic acid salts (e.g., monosodium glutamate), glycine salts, guanylic acid salts, inosinic acid salts, 5'-ribonucleotide salts, hydrolyzed animal proteins, and hydrolyzed vegetable proteins.

[00100] The restructured meat composition may optionally include a variety of flavorings. Suitable flavoring agents include animal meat flavor, animal meat oil, spice extracts, spice oils, natural smoke solutions, natural smoke extracts, yeast extracts, sherry, mint, brown sugar, honey. The flavors and spices may also be available in the form of oleoresins and aquaresins. Other flavoring agents include onion flavor, garlic flavor, or herb flavor. In an alternative embodiment, the flavoring agent may be nutty, sweet, or fruity. Non-limiting examples of suitable fruit flavors include apple, apricot, avocado, banana, blackberry, black cherry, blueberry, boysenberry, cantaloupe, cherry, coconut, cranberry, fig, grape, grapefruit, green apple, honeydew, kiwi, lemon, lime, mango, mixed berry, orange, peach, persimmon, pineapple, raspberry, strawberry, and watermelon. The restructured meat compositions may further include flavor enhancers. Non-limiting examples of suitable flavor enhancers include sodium chloride salt, glutamic acid salts, glycine salts, guanylic acid salts, inosinic acid salts, and 5-ribonucleotide salts, yeast extract, shiitake mushroom extract, dried bonito extract, and kelp extract. The restructured meat compositions may also utilize various sauces and marinades

which may be made by fermentation or blending flavors, spices, oils, water, flavor enhancers, antioxidants, acidulents, preservatives, and sweeteners.

[00101] In an additional embodiment, the restructured meat compositions may further comprise a thickening or a gelling agent, such as alginic acid and its salts, agar, carrageenan and its salts, processed *Eucheuma* seaweed, gums (Gum Arabic, carob bean, locust bean, guar, tragacanth, and xanthan), pectins, sodium carboxymethylcellulose, methylcellulose, and modified starches.

[00102] In a further embodiment, the restructured meat compositions may further comprise a nutrient such as a vitamin, a mineral, an antioxidant, an omega-3 fatty acid, or an herb. Suitable vitamins include Vitamins A, C, and E, which are also antioxidants, and Vitamins B and D. Examples of minerals that may be added include the salts of aluminum, ammonium, calcium, magnesium, iron, and potassium. Suitable omega-3 fatty acids include docosahexaenoic acid (DHA), EPA (eicosapentanoic acid) and ALA (alpha-linolenic acid). Herbs that may be added include bay leaves, basil, celery leaves, chervil, chives, cilantro, coriander, cumin, dill, ginger, mace, marjoram, pepper, tumeric, parsley, oregano, tarragon, and thyme.

(III) Food Products

[00103] The restructured meat compositions may be processed into a variety of food product having a variety of shapes. When the protein composition further comprises at least one ingredient selected from the group consisting of a gelling protein, an animal fat, sodium chloride, phosphates (sodium tripolyphosphate, sodium acid pyrophsphates, hexa metaphosphate, etc.), a colorant, a curing agent, an antioxidant, an antimicrobial agent, a flavorant, or mixtures thereof, the product and process are completed in a procedure similar to the product and process utilizing only the structured protein products, animal meat, and water. The protein composition is first hydrated with water and shredded to expose and separate the fibers. When hydration and shredding are complete, a colorant is added. The animal meat and water are added and the contents are mixed until a homogeneous mass is obtained. This is followed by the addition of an animal fat, a flavorant, sodium chloride, phosphates, and the gelling protein. In an additional embodiment, sodium nitrate may be added along with salt and phosphates.

[00104] The resulting homogeneous restructured meat product may be formed into strips, steaks, cutlets, patties, or generally cube-shaped for kabobs, either by hand or by machine. The restructured meat product may be formed into meat sticks. The restructured meat product may also be stuffed into permeable or impermeable casings to form sausages.

[00105] The restructured meat product once formed is either cooked, partially cooked for finishing at a later time or frozen either in an uncooked state, partially cooked state or cooked state. Cooking includes frying either as sautéing or as deep-frying, baking, smoking and impingement cooking and steaming. The fully cooked restructured meat product may be further sliced, shredded, or ground.

[00106] Further, the restructured meat product may be subjected to fermentation. Meat products are fermented by adjusting the pH of the meat product to between about 4.0 to about 5.2. Fermentation is accomplished by the addition of a lactic acid culture. Acidification may also be performed by direct acidification using citric acid, lactic acid, glucono delta lactone, and mixtures thereof.

[00107] The restructured meat product (before drying, partially dried, dried, cooked or uncooked) may be packaged as is. Further processing of the restructured meat product (before drying, partially dried, dried, cooked or uncooked) may be shock-frozen, for example in a freeze tunnel, and subsequent automatic portion packaging in containers of a suitable type, for example, plastic pouches or the like. Said type of further processing and packaging is suitable if the product is intended for fast-food outlets or for food service applications, where the product is usually deep-fried or baked before consumption.

[00108] Alternatively, after the formation of the restructured meat product (before drying, partially dried, dried, cooked or uncooked), it is also possible to spray the surface of the product with carbohydrate solutions or related substances in order to obtain uniform browning during deep frying or baking. Subsequently, the product can now be shock frozen and sold portion packed (i.e. in pouches). The restructured meat product can also be baked or processed in a convection oven by the consumer, instead of deep frying. Further, the restructured meat product also can be breaded prior to or after cooking, or coated with another type of coating. Additionally, the restructured meat product can be retort cooked in order to kill any microbes that might be present.

[00109] The restructured meat product either cooked or uncooked may also be packed and sealed in cans in a conventional manner and employing conventional sealing procedures. Normally, the cans at this stage are maintained at a temperature of between 65°C and 77°C and are carried to a retort or cooking stage as quickly as possible to prevent there being any risk of microbiological spoilage during the time between canning and sterilization during the retort or cooking stage. The restructured meat stuffed in impermeable casings designed for retort cooking may be cooked in the retort cooker to make a shelf stable sausage.

[00110] In order to ensure that the restructured meat product, once formed, has the texture of intact muscles, it is necessary that at least about 75 weight % of the protein composition contains at least about 15 weight % of large pieces comprised of vegetable protein fibers at least about 4 centimeters long, vegetable protein strands at least about 3 centimeters long, and vegetable protein chunks at least about 2 centimeters long and that at least about 75 weight % of the protein composition has a shear strength of at least about 1400 grams.

[00111] A vegetable product may be prepared by a process of combining a protein composition, preferably a hydrated and shredded soy protein composition; wherein about 75 weight % of the protein composition is comprised of at least about 15 weight % of fragments comprised of protein fibers at least about 4 centimeters long, protein strands at least about 3 centimeters long, and protein chunks at least about 2 centimeters long; and wherein at least about 75 weight % of the protein composition has a shear strength of at least about 1400 grams; with

a comminuted vegetable; and

mixing the preferred hydrated and shredded soy protein composition and the comminuted vegetable to produce a homogeneous, fibrous and structured vegetable product having protein fibers that are substantially aligned.

[00112] Examples of vegetable products prepared by the above process are vegetarian food products including vegetarian patties, vegetarian hot dogs, vegetarian sausages, and vegetarian crumbles. Another example of a vegetarian food product is cheeses that are extended with the hydrated and shredded protein composition.

[00113] A fruit product may be prepared by combining a protein composition, preferably a hydrated and shredded soy protein composition; wherein about 75 weight % of the protein composition is comprised of at least about 15 weight % of fragments comprised of protein fibers at least about 4 centimeters long, protein strands at least about 3 centimeters long,

and protein chunks at least about 2 centimeters long; and wherein at least about 75 weight % of the protein composition has a shear strength of at least about 1400 grams; with a comminuted fruit; and mixing the preferred hydrated and shredded soy protein composition and the comminuted fruit to produce a homogeneous, fibrous and structured fruit product having protein fibers that are substantially aligned.

[00114] Examples of fruit products prepared by the above process are snack food products including fruit rollups, cereals, and fruit crumbles.

DEFINITIONS

[00115] The term "animal meat" as used herein refers to the flesh, whole meat muscle, or parts thereof derived from an animal.

[00116] The term "comminuted fruit" as used herein refers to a puree of a single fruit or a mixture of a puree of more than one fruit.

[00117] The term "comminuted meat" as used herein refers to a meat paste that is recovered from an animal carcass. The meat, on or off the bone is forced through a deboning device such that meat is separated from the bone and reduced in size.

[00118] The term "comminuted vegetable" as used herein refers to a puree of a single vegetable or a mixture of a puree of more than one vegetable.

[00119] The term "extrudate" as used herein refers to the product of extrusion. In this context, the plant protein products comprising protein fibers that are substantially aligned may be extrudates in some embodiments.

[00120] The term "fiber" as used herein refers to a plant protein product having a size of approximately 4 centimeters in length and 0.2 centimeters in width after the shred characterization test detailed in Example 2 is performed. In this context, the term "fiber" does not include the nutrient class of fibers, such as soybean cotyledon fibers, and also does not refer to the structural formation of substantially aligned protein fibers comprising the plant protein products.

[00121] The term "gluten" as used herein refers to a protein fraction in cereal grain flour, such as wheat, that possesses a high content of protein as well as unique structural and adhesive properties.

[00122] The term "gluten free starch" as used herein refers to various starch products such as modified tapioca starch. Gluten free or substantially gluten free starches are made from wheat, corn, and tapioca based starches. They are gluten free because they do not contain the gluten from wheat, oats, rye or barley.

[00123] The term "hydration test" as used herein measures the amount of time in minutes necessary to hydrate a known amount of the protein composition.

[00124] The term "large piece" as used herein is the manner in which a colored or uncolored structured plant protein product's shred percentage is characterized. The determination of shred characterization is detailed in Example 2.

[00125] The term "mechanically deboned meat (MDM)" as used herein refers to a meat paste that is recovered from beef, pork and chicken bones using commercially available equipment. MDM is a comminuted product that is devoid of the natural fibrous texture found in intact muscles.

[00126] The term "moisture content" as used herein refers to the amount of moisture in a material. The moisture content of a material can be determined by A.O.C.S. (American Oil Chemists Society) Method Ba 2a-38 (1997), which is incorporated herein by reference in its entirety.

[00127] The term "protein content," as for example, soy protein content as used herein, refers to the relative protein content of a material as ascertained by A.O.C.S. (American Oil Chemists Society) Official Methods Bc 4-91(1997), Aa 5-91(1997), or Ba 4d-90(1997), each incorporated herein by reference in their entirety, which determine the total nitrogen content of a material sample as ammonia, and the protein content as 6.25 times the total nitrogen content of the sample.

[00128] The term "protein fiber" as used herein refers to the individual continuous filaments or discrete elongated pieces of varying lengths that together define the structure of the plant protein products of the invention. Additionally, because both the colored and uncolored structured plant protein products of the invention have protein fibers that are substantially aligned, the arrangement of the protein fibers impart the texture of whole meat muscle to the colored and uncolored structured plant protein products.

[00129] The term "shear strength" as used herein measures the ability of a textured protein to form a fibrous network with a strength high enough to impart meat-like texture and appearance to a formed product. Shear strength is measured in grams.

[00130] The term "simulated" as used herein refers to an animal meat composition that contains no animal meat.

[00131] The term "soy cotyledon fiber" as used herein refers to the polysaccharide portion of soy cotyledons containing at least about 70% dietary fiber. Soy cotyledon fiber typically contains some minor amounts of soy protein, but may also be 100% fiber. Soy cotyledon fiber, as used herein, does not refer to, or include, soy hull fiber. Generally, soy cotyledon fiber is formed from soybeans by removing the hull and germ of the soybean, flaking or grinding the cotyledon and removing oil from the flaked or ground cotyledon, and separating the soy cotyledon fiber from the soy material and carbohydrates of the cotyledon.

[00132] The term "soy protein concentrate" as used herein is a soy material having a protein content of from about 65% to less than about 90% soy protein on a moisture-free basis. Soy protein concentrate also contains soy cotyledon fiber, typically from about 3.5% up to about 20% soy cotyledon fiber by weight on a moisture-free basis. A soy protein concentrate is formed from soybeans by removing the hull and germ of the soybean, flaking or grinding the cotyledon and removing oil from the flaked or ground cotyledon, and separating the soy protein and soy cotyledon fiber from the soluble carbohydrates of the cotyledon.

[00133] The term "soy flour" as used herein, refers to a comminuted form of defatted soybean material, preferably containing less than about 1% oil, formed of particles having a size such that the particles can pass through a No. 100 mesh (U.S. Standard) screen. The soy cake, chips, flakes, meal, or mixture of the materials are comminuted into soy flour using conventional soy grinding processes. Soy flour has a soy protein content of about 49% to about 65% on a moisture free basis. Preferably the flour is very finely ground, most preferably so that less than about 1% of the flour is retained on a 300 mesh (U.S. Standard) screen.

[00134] The term "soy protein isolate" as used herein is a soy material having a protein content of at least about 90% soy protein on a moisture free basis. A soy protein isolate is formed from soybeans by removing the hull and germ of the soybean from the cotyledon, flaking or grinding the cotyledon and removing oil from the flaked or ground cotyledon, separating the

soy protein and carbohydrates of the cotyledon from the cotyledon fiber, and subsequently separating the soy protein from the carbohydrates.

[00135] The term "strand" as used herein refers to a plant protein product having a size of approximately 2.5 to about 4 centimeters in length and greater than approximately 0.2 centimeter in width after the shred characterization test detailed in Example 2 is performed.

[00136] The term "starch" as used herein refers to starches derived from any native source. Typically sources for starch are cereals, tubers, roots, legumes, and fruits.

[00137] The term "weight on a moisture free basis" as used herein refers to the weight of a material after it has been dried to completely remove all moisture, e.g. the moisture content of the material is 0%. Specifically, the weight on a moisture free basis of a material can be obtained by weighing the material after the material has been placed in a 45°C oven until the material reaches a constant weight.

[00138] The term "wheat flour" as used herein refers to flour obtained from the milling of wheat. Generally speaking, the particle size of wheat flour is from about 14 to about 120 μm.

[00139] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those of skill in the art that the techniques disclosed in the examples that follow represent techniques discovered by the inventors to function well in the practice of the invention. However, those of skill in the art should, in light of the present disclosure, appreciate that many changes can be made in the specific embodiments that are disclosed and still obtain a like or similar result without departing from the spirit and scope of the invention, therefore all matter set forth or shown in the accompanying drawings is to be interpreted as illustrative and not in a limiting sense.

EXAMPLES

[00140] Examples 1 - 105 illustrate various embodiments of the invention.

Example 1 Determination of Shear Strength

[00141] Shear strength of a sample is measured in grams and may be determined by the following procedure. Weigh a sample of the structured protein product and place it in a heat sealable pouch and hydrate the sample with approximately three times the sample weight of

room temperature tap water. Evacuate the pouch to a pressure of about 0.01 Bar and seal the pouch. Permit the sample to hydrate for about 12 to about 24 hours. Remove the hydrated sample and place it on the texture analyzer base plate oriented so that a knife from the texture analyzer will cut through the diameter of the sample. Further, the sample should be oriented under the texture analyzer knife such that the knife cuts perpendicular to the long axis of the textured piece. A suitable knife used to cut the extrudate is a model TA-45, incisor blade manufactured by Texture Technologies (USA). A suitable texture analyzer to perform this test is a model TA, TXT2 manufactured by Stable Micro Systems Ltd. (England) equipped with a 25, 50, or 100 kilogram load. Within the context of this test, shear strength is the maximum force in grams needed to shear through the sample.

Example 2 Determination of Shred Characterization

[00142] A procedure for determining shred characterization may be performed as follows. Weigh about 150 grams of a structured protein product using whole pieces only. Place the sample into a heat-sealable plastic bag and add about 450 grams of water at 25°C. Vacuum seal the bag at about 150 mm Hg and allow the contents to hydrate for about 60 minutes. Place the hydrated sample in the bowl of a Kitchen Aid mixer model KM14G0 equipped with a single blade paddle and mix the contents at 130 rpm for two minutes. Scrape the paddle and the sides of the bowl, returning the scrapings to the bottom of the bowl. Repeat the mixing and scraping two times. Remove ~200 g of the mixture from the bowl. Separate that mixture such that all fibers or long strands longer that 2.5 cm are segregated from the shredded mixture. Weigh the population of fibers sorted from the shredded mixture, divide this weight by the starting weight (e.g. ~200 g), and multiply this value by 100. This determines the percentage of large pieces in the sample. If the resulting value is below 15%, or above 20%, the test is complete. If the value is between 15% and 20%, then weigh out another ~200 g from the bowl, separate the fibers or long strands longer that 2.5 cm from the shredded mixture, and perform the calculations again.

Example 3 Production of Plant Protein Products

[00143] The following extrusion process may be used to prepare the colored structured plant protein products of the invention. Added to a dry blend mixing vessel are the following: 1000 kilograms (kg) Supro 620 (soy isolate), 440 kg wheat gluten, 236 kg wheat starch, 34 kg soy cotyledon fiber, 8 kg dicalcium phosphate, and 2 kg L-cysteine. The contents are mixed to form a dry blended soy protein mixture. The dry blend is then transferred to a hopper from which the dry blend is introduced into a preconditioner along with 480 kg of water to form a conditioned soy protein pre-mixture. The conditioned soy protein pre-mixture is then fed to a twin-screw extrusion apparatus at a rate of not more than 25 kg/minute. The extrusion apparatus comprises five temperature control zones, with the protein mixture being controlled to a temperature of from about 25°C in the first zone, about 50°C in the second zone, about 95°C in the third zone, about 130°C in the fourth zone, and about 150°C in the fifth zone. The extrusion mass is subjected to a pressure of at least about 400 psig in the first zone up to about 1500 psig in the fifth zone. Water, 60 kg, is injected into the extruder barrel, via one or more injection jets in communication with a heating zone.

[00144] A die assembly is attached to the extruder in an arrangement that permits the plasticized mixture to flow from the extruder exit port into the die assembly and produces substantial alignment of the protein fibers within the plasticized mixture as it flows through the die assembly.

[00145] As the extrudate containing protein fibers that are substantially aligned exits the die assembly, it is cut with knives and the cut mass is then dried to a moisture content of about 10% by weight. Once dry, a portion of the cut mass is sized into small pieces and larger pieces. A total of 25 pieces are obtained for each size. These pieces are then measured and tabulated in Table IV.

Table IV

Sample Number	**Small Pieces**				**Large Pieces**	
	L (mm)	W (mm)			L (mm)	W (mm)
1	11	10			16	11
2	10	6			16	13
3	8	8			22	11
4	11	8			19	11
5	14	9			17	11
6	10	8			20	13
7	14	4			15	10
8	8	6			21	12
9	10	8			19	12
10	11	8			12	12
11	13	8			15	10
12	19	9			17	9
13	12	9			11	10
14	14	6.5			14	10
15	10	7			14	10
16	10	7			17	12
17	10	6			15	13

18	12	8			14	14
19	10	7			19	12
20	9	6			18	10
21	10	8			14	12
22	9	5			19	12
23	10	7			12	11
24	11	9			16	10
25	9	8			16	9

Avg	**11.0**	**7.4**		Avg	**16.3**	**11.2**
Std. Dev	**2.3**	**1.4**		Std. Dev.	**2.8**	**1.3**
Max	**19.0**	**10.0**		Max	**22.0**	**14.0**
Min	**8.0**	**4.0**		Min	**11.0**	**9.0**

Examples 4 - 94 Production of Plant Protein Products

[00146] Examples 4-94 are repeats of Example 3. Table V below delineates the analyses of Examples 3 – 94.

TABLE V

Example Number#	**% Large Pieces**	**Shear Strength (g)**	**Hydration**	**Density (g/cc)**
3	30.2	2150	80	0.27
4	24.2	2366	80	0.24
5	29.4	2341	60	0.30
6	26.0	2142	70	0.29
7	27.1	2291	70	0.28
8	32.7	2442	70	0.23
9	17.4	2668	70	0.27
10	26.1	2511	90	0.26

Example Number#	% Large Pieces	Shear Strength (g)	Hydration	Density (g/cc)
11	21.1	2260	80	0.28
12	22.3	2421	80	0.24
13	21.9	2490	75	0.28
14	22.4	2438	104	0.28
15	7.8	59	81	0.30
16	7.3	675	83	0.28
17	9.3	553	100	0.24
18	7.3	226	90	0.23
19	3.5	412	72	0.24
20	0.0	055	100	0.23
21	2.6	511	75	0.25
22	2.7	168	100	0.25
23	2.0	207	102	0.25
24	7.7	247	62	0.29
25	1.2	51	73	0.28
26	0.2	164	63	0.27
27	6.6	966	68	0.28
28	4.9	164	50	0.31
29	5.0	812	58	0.28
30	9.6	108	60	0.31
31	5.8	864	70	0.27
32	6.5	473	58	0.25
33	0.7	879	65	0.28
34	5.4	688	70	0.29
35	0.3	038	74	0.26
36	9.3	074	73	0.28
37	1.5	937	70	0.39
38	2.5	462	77	0.40

Example Number#	% Large Pieces	Shear Strength (g)	Hydration	Density (g/cc)
39	0.1	051	66	0.28
40	7.9	384	54	0.31
41	8.1	064	58	0.28
42	9.2	158	60	0.27
43	0.0	834	58	0.28
44	6.8	202	58	0.28
45	2.8	363	57	0.26
46	3.9	361	57	0.28
47	6.9	293	103	0.25
48	6.3	205	73	0.28
49	9.0	286	53	0.29
50	2.6	206	63	0.25
51	0.5	125	63	0.31
52	5.5	290	55	0.29
53	8.2	274	55	0.26
54	1.5	205	42	0.33
55	1.3	185	55	0.31
56	1.8	969	40	0.30
57	9.1	028	55	0.31
58	7.2	598	63	0.37
59	8.3	869	60	0.31
60	9.7	044	50	0.29
61	7.6	216	52	0.28
62	5.0	001	53	0.28
63	8.1	096	45	0.27
64	9.0	796	53	0.27
65	0.0	924	51	0.27
66	3.7	295	51	0.28