| Example Number# | % Large Pieces | Shear Strength (g) | Hydration | Density (g/cc) |
|---|---|---|---|---|
| 67 | 7.4 | 259 | 50 | 0.29 |
| 68 | 9.2 | 204 | 43 | 0.28 |
| 69 | 5.3 | 059 | 38 | 0.31 |
| 70 | 6.1 | 284 | 70 | 0.32 |
| 71 | 3.6 | 085 | 70 | 0.30 |
| 72 | 5.6 | 279 | 44 | 0.28 |
| 73 | 3.7 | 170 | 44 | 0.32 |
| 74 | 1.2 | 128 | 49 | 0.29 |
| 75 | 2.4 | 068 | 50 | 0.29 |
| 76 | 0.1 | 939 | 40 | 0.30 |
| 77 | 8.7 | 592 | 50 | 0.30 |
| 78 | 9.6 | 812 | 68 | 0.28 |
| 79 | 5.2 | 848 | 64 | 0.28 |
| 80 | 3.6 | 973 | 70 | 0.30 |
| 81 | 3.7 | 078 | 66 | 0.36 |
| 82 | 5.6 | 940 | 44 | 0.31 |
| 83 | 8.5 | 339 | 33 | 0.29 |
| 84 | 0.2 | 366 | 50 | 0.24 |
| 85 | 8.1 | 425 | 40 | 0.29 |
| 86 | 9.6 | 122 | 59 | 0.27 |
| 87 | 7.5 | 193 | 56 | 0.16 |
| 88 | 1.1 | 186 | 56 | 0.28 |
| 89 | 2.4 | 061 | 56 | 0.27 |
| 90 | 1.3 | 143 | 50 | 0.27 |
| 91 | 4.4 | 108 | 54 | 0.26 |
| 92 | 9.9 | 101 | 53 | 0.30 |
| 93 | 2.3 | 551 | 55 | 0.25 |
| 94 | 4.3 | 164 | 57 | 0.28 |

| Example Number# | % Large Pieces | Shear Strength (g) | Hydration | Density (g/cc) |
|---|---|---|---|---|
| | | | | |
| 1st Quartile | 2.6 | 045 | 53 | 0.27 |
| Median | 6.5 | 164 | 60 | 0.28 |
| 3rdQuartile | 0.2 | 291 | 70 | 0.30 |
| Mean | 6.6 | 156 | 63 | 0.28 |

**Example 95 Production of Protein Composition**

**[00147]** Added to a mixing vessel are 3625 grams of tap water at about 10°C (50°F) and while stirring 1160 grams of a dried, low moisture (about 7% to about 12%) soy protein composition, identified as FXP MO339, available from Solae, LLC, St. Louis, MO comprising a soy protein isolate, soy cotyledon fiber, wheat gluten and starch is added until the soy protein composition is hydrated and the fibers are separated. Added to the mixer are 5216 grams of comminuted meat of mechanically deboned chicken having a moisture content of at least about 50%. The mechanically deboned chicken is at a temperature of from about 2°C (36°F) to about 4°C (39°F). The contents are mixed until a homogeneous restructured meat product is obtained. The restructured meat product is transferred to a Hollymatic forming machine where the restructured meat product is formed into steaks or cutlets that are then frozen.

**[00148]** The procedure of Example 3 is repeated, except that 1500 grams of a non-dried low moisture (about 28- about 35%) soy protein composition comprising a soy protein isolate, soy cotyledon fiber, wheat gluten and starch is hydrated with 3175 grams water. The restructured meat product is transferred to a stuffing machine where the restructured meat product is stuffed into impermeable casings, which are then frozen. Stuffing machines are available from various commercial manufacturers including, but not limited to, HITEC Food Equipment, Inc., located in Elk Grove Village, Ill., Townsend Engineering Co., located in Des Moines, Iowa, Robert Reiser & Co., Inc., located in Canton, Mass., and Handtmann, Inc., located in Buffalo Grove, Ill.

**Example 97**

**[00149]** Added to a first mixing vessel are 2127 grams of tap water at about 12°C (54°F) and while stirring 1000 grams of a dried, low moisture (about 7% to about 12%) soy protein composition is added until the soy protein composition is hydrated and the fibers are separated. Caramel coloring, 43 grams, is then added to the hydrated soy protein composition. At about 2°C (36°F), 4500 grams of a comminuted meat of mechanically deboned chicken having a moisture content of about 50% is added. Then added are 100 grams sodium chloride and 30 grams of sodium tripolyphosphate to extract/solubilize myofibriller protein in the comminuted meat for binding. As mixing is continued, 500 grams beef fat and 100 grams beef flavor are added and mixing is continued. In a second mixing vessel, a gelling protein of 600 grams of Supro® 620 is hydrated in 1000 grams water and is added to the first mixing vessel. The contents are mixed until a homogeneous restructured meat product is obtained. The restructured meat product is transferred to a Hollymatic (Hollymatic Corp., Park Forest IL) forming machine where the restructured meat product is formed into patties, which are then frozen.

**Example 98**

**[00150]** Added to a mixing vessel are 3000 grams of tap water at about 10°C (50°F) and while stirring 1500 grams of a soy protein extrudate prepared from Supro® 620 is added until the soy protein composition is hydrated and the fibers are separated by shredding. Added to the mixer are 5000 grams of a comminuted meat of mechanically deboned chicken having a moisture content of at least about 50%. The mechanically deboned chicken is at a temperature of from about 2°C (36°F) to about 4°C (39°F). The contents are mixed until a homogeneous restructured meat product is obtained. The restructured meat product is transferred to a Hollymatic forming machine where the restructured meat product is formed into steaks or cutlets that are then frozen.

**Example 99**

**[00151]** The procedure of Example 98 is repeated except that the hydrated and shredded soy protein composition comprises a soy protein isolate, rice flour, and a gluten free starch.

**Example 100**

**[00152]** The procedure of Example 98 is repeated except that the hydrated and shredded soy protein composition comprises a soy protein isolate and rice flour.

**Example 101**

**[00153]** The procedure of Example 98 is repeated except that the hydrated and shredded soy protein composition comprises a soy protein isolate and a gluten free starch.

**Example 102**

**[00154]** The procedure of Example 98 is repeated except that the hydrated and shredded soy protein composition comprises a soy protein isolate, wheat flour and starch.

**Example 103**

**[00155]** The procedure of Example 98 is repeated except that the hydrated and shredded soy protein composition comprises a soy protein isolate and soy cotyledon fiber.

**Example 104**

**[00156]** The procedure of Example 98 is repeated except that the hydrated and shredded soy protein composition comprises a soy protein isolate, soy cotyledon fiber, and wheat gluten.

**Example 105**

**[00157]** Added to a mixing vessel are 3383 grams of tap water at about 10°C (50°F) and while stirring 1208 grams of a dried, low moisture (about 7% to about 12%) soy protein extrudate, identified as SUPROMAX® 5050, is added until the soy protein extrudate is hydrated and the fibers are separated by shredding. Added to the mixer are 3340 grams of a comminuted meat of mechanically deboned chicken having a moisture content of at least about 50% and 3383 grams of beef of a ½ inch grind having a fat content of about 10%. The mechanically deboned chicken and the beef grind are at a temperature of from about 2°C (36°F) to about 4°C (39°F). Also added are various colorants and flavorants of salt, erythorbate, sodium nitrite, dextrose, cracked black pepper, nutmeg, mace, granulated garlic, coriander, red pepper, and a rehydrated

LHP starter culture. The contents are mixed until a homogeneous restructured meat product is obtained. The restructured meat product is then formed into meat sticks.

**[00158]** While the invention has been explained in relation to its preferred embodiments, it is to be understood that various modifications thereof will become apparent to those skilled in the art upon reading the description. Therefore, it is to be understood that the invention disclosed herein is intended to cover such modifications as fall within the scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. A process for producing a restructured meat composition, the process comprising: 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.

2. The process of claim 1, wherein the die assembly comprises:
   a a die sleeve having a back portion and a front portion collectively defining an interior chamber;
   b a die insert disposed inside the interior chamber, the die insert including an insert body having a front face and a rear face with the front face defining a bottom portion and a plurality of flow diverters with a tapered flow pathway being defined between the bottom portion and any two adjacent of the plurality of flow diverters; and
   c a die cone coupled to said die sleeve wherein said die cone and said flow pathway collectively define a fully tapered flow channel.
3. The process of claim 2, wherein the tapered flow pathway is tapered along three sides.
4. The process of claim 3, wherein the fully tapered flow channel is tapered along four sides.
5. The process of claim 4, wherein the fully tapered flow channel is in communication with an entrance at one end and an outlet at an opposite end thereof.
6. The process of claim 5, wherein the fully tapered flow channel is inwardly tapered from said entrance to said outlet.
7. The process of claim 6, further comprising combining the structured plant protein product with an animal meat.
8. The process of claim 6, wherein the structured plant protein product has an average shear strength of at least 2000 grams and an average shred characterization of at least 17%.
9. The process of claim 8, wherein the structured plant protein product comprises protein fibers substantially aligned in the manner depicted in the micrographic image of Figure 1.

10. The process of claim 1, wherein the plant protein material is selected from the group consisting of legumes, corn, peas, canola, sunflowers, sorghum, rice, amaranth, potato, tapioca, arrowroot, canna, lupin, rape, wheat, oats, rye, barley, and mixtures thereof.
11. The process of claim 1, further comprising combining at least one animal protein material with the plant protein material before extruding, to produce the structured plant protein product comprising protein fibers that are substantially aligned.
12. The process of claim 11, wherein the animal protein material is selected from the group consisting of casein, caseinates, whey protein, ovalbumin, ovoglobulin, ovomucin, ovomucoid, ovotransferrin, ovovitella, ovovitellin, albumin globulin, and vitellin.
13. The process of claim 1, wherein the plant protein material comprises soy protein and wheat protein.
14. The process of claim 13, further comprising whey protein.
15. The process of claim 13, wherein the plant protein material has from about 40% to about 75% protein on a dry mater basis.
16. The process of claim 15, wherein the plant protein material comprises protein, starch, gluten, and fiber.
17. The process of claim 16, wherein the plant protein material comprises:
    a from about 45% to about 65% soy protein on a dry matter basis;
    b from about 20% to about 30% wheat gluten on a dry matter basis;
    c from about 10% to about 15% wheat starch on a dry matter basis; and
    d from about 1% to about 5% fiber on a dry matter basis.
18. The process of claim 16, wherein the plant protein material further comprises dicalcium phosphate and L-cysteine.
19. The process of claim 1, wherein the extrusion temperature is from about 90° C to about 150° C and the pressure is from about 500 psig to about 1500 psig.
20. The process of claim 7, wherein the animal meat is selected from the group consisting of whole muscle pieces, comminuted meat, mechanically deboned meat, and combinations thereof.
21. The process of claim 7, wherein the animal meat is derived from an animal selected from the group consisting of pork, beef, lamb, poultry, wild game, and fish.

22. The process of claim 1, wherein the meat composition further includes an amount of water.
23. The process of claim 6, further comprising combining the structured plant protein product with a comminuted vegetable.
24. The process of claim 6, further comprising combining the structured plant protein product with a comminuted fruit.
25. A restructured meat composition, the composition comprising a structured protein product having protein fibers that are substantially aligned.
26. The restructured meat composition of claim 25, further comprising animal meat.
27. The restructured meat composition of claim 26, wherein the composition comprises from about 40% to about 60% by weight of the structured protein product, and from about 40% to about 60% by weight of meat.
28. The restructured meat composition of claim 27, further comprising a fat source in an amount ranging from about 10% to about 20% by weight of the composition.
29. The restructured meat composition of claim 28, wherein the structured protein product comprises protein fibers substantially aligned in the manner depicted in the micrographic image of Figure 1.
30. The restructured meat composition of claim 28, wherein the structured protein product comprises soy protein, starch, gluten, and fiber.
31. The restructured meat composition of claim 28, wherein the structured protein product comprises:
    a from about 45% to about 65% soy protein on a dry matter basis;
    b from about 20% to about 30% wheat gluten on a dry matter basis;
    c from about 10% to about 15% wheat starch on a dry matter basis;
    d from about 1% to about 5% fiber on a dry matter basis.
32. The restructured meat composition of claim 30, wherein the meat is selected from beef, pork, lamb, turkey, and chicken.
33. The restructured meat composition of claim 29, further comprising a color composition.
34. The restructured meat composition of claim 33, wherein the coloring composition comprises beet, annatto, caramel coloring, and an amino acid source.
35. The restructured meat composition of claim 34, further comprising isolated soy protein.

36. The restructured meat composition of claim 35, further comprising an antioxidant, water, spices and flavoring.

37. The restructured meat composition of claim 25, further comprising comminuted vegetable.

38. The restructured meat composition of claim 37, wherein the composition comprises from about 40% to about 60% by weight of the structured protein product, and from about 40% to about 60% by weight of comminuted vegetable.

39. The restructured meat composition of claim 38, wherein the structured protein product comprises protein fibers substantially aligned in the manner depicted in the micrographic image of Figure 1.

40. The restructured meat composition of claim 38, wherein the structured protein product comprises soy protein, starch, gluten, and fiber.

41. The restructured meat composition of claim 38, wherein the structured protein product comprises:

    a from about 45% to about 65% soy protein on a dry matter basis;

    b from about 20% to about 30% wheat gluten on a dry matter basis;

    c from about 10% to about 15% wheat starch on a dry matter basis;

    d from about 1% to about 5% fiber on a dry matter basis.

42. The restructured meat composition of claim 40, wherein the coloring composition comprises beet, annatto, caramel coloring, and an amino acid source.

43. The restructured meat composition of claim 42, further comprising isolated soy protein.

44. The restructured meat composition of claim 43, further comprising an antioxidant, water, spices and flavoring.

45. The restructured meat composition of claim 25, further comprising comminuted fruit.

46. The restructured meat composition of claim 45, wherein the composition comprises from about 40% to about 60% by weight of the structured protein product, and from about 40% to about 60% by weight of comminuted fruit.

47. The restructured meat composition of claim 46, wherein the structured protein product comprises protein fibers substantially aligned in the manner depicted in the micrographic image of Figure 1.

48. The restructured meat composition of claim 46, wherein the structured protein product comprises soy protein, starch, gluten, and fiber.

49. The restructured meat composition of claim 46, wherein the structured protein product comprises:

a from about 45% to about 65% soy protein on a dry matter basis;

b from about 20% to about 30% wheat gluten on a dry matter basis;

c from about 10% to about 15% wheat starch on a dry matter basis;

d from about 1% to about 5% fiber on a dry matter basis.

50. The restructured meat composition of claim 48, wherein the coloring composition comprises beet, annatto, caramel coloring, and an amino acid source.

51. The restructured meat composition of claim 50, further comprising isolated soy protein.

52. The restructured meat composition of claim 51, further comprising an antioxidant, water, spices and flavoring.

53. The restructured meat composition of claim 25, wherein the structured protein product is extruded through a die assembly resulting in a structured protein product having protein fibers that are substantially aligned.

54. The restructured meat composition of claim 37, wherein the structured protein product is extruded through a die assembly resulting in a structured protein product having protein fibers that are substantially aligned.

55. The restructured meat composition of claim 45, wherein the structured protein product is extruded through a die assembly resulting in a structured protein product having protein fibers that are substantially aligned.



FIG. 1



FIG. 2

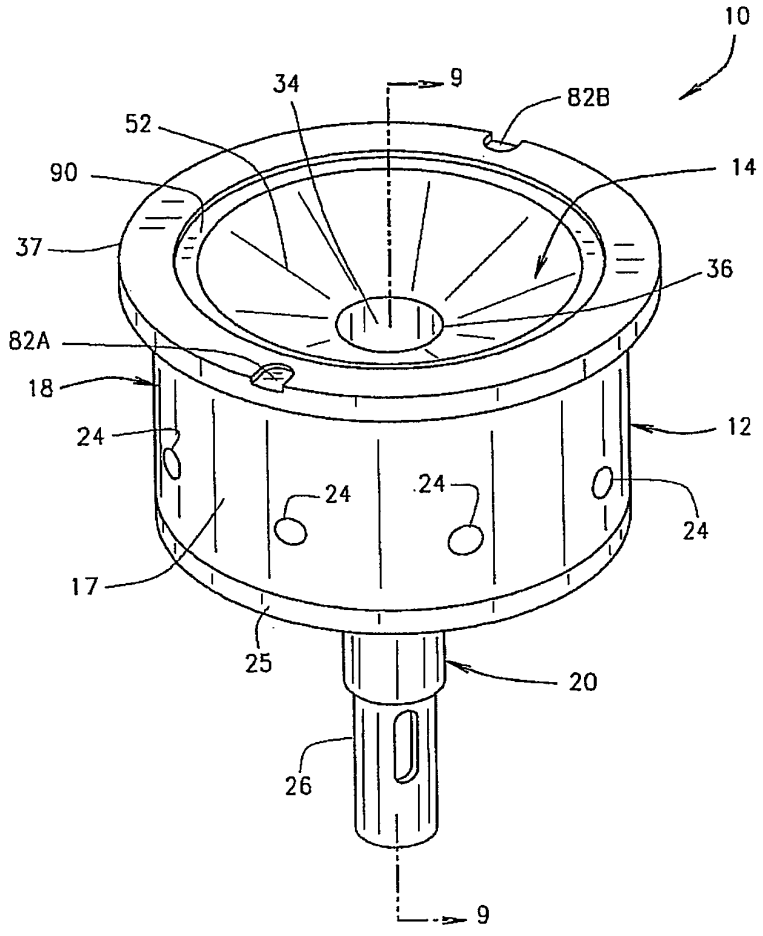



FIG. 3

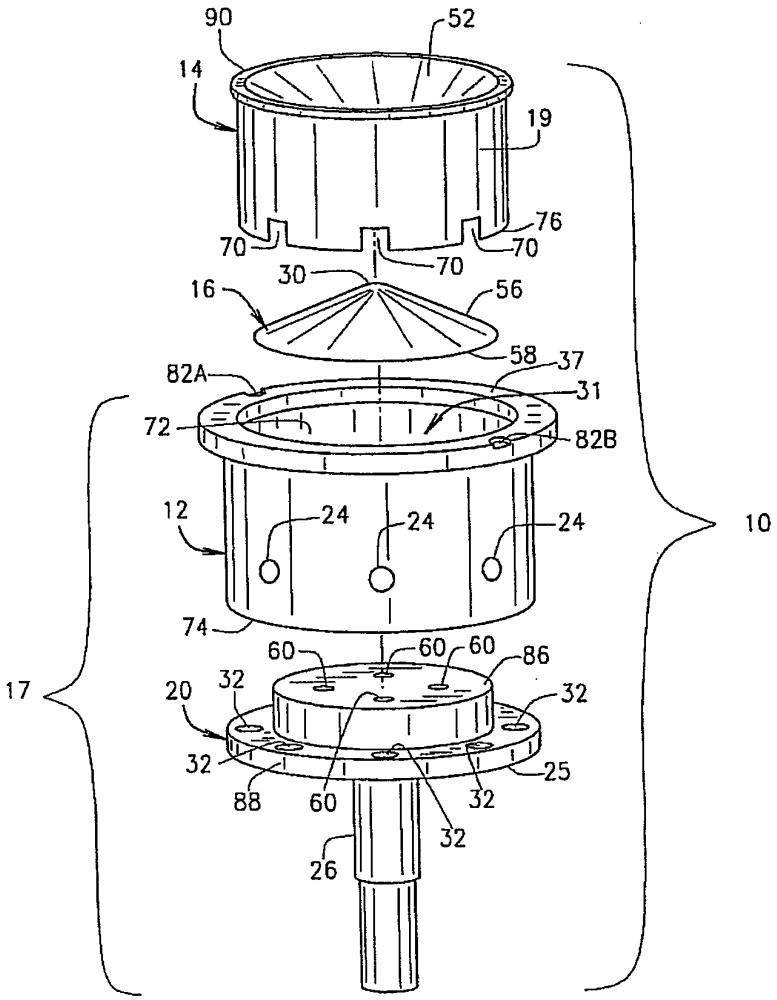

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FIG. 4

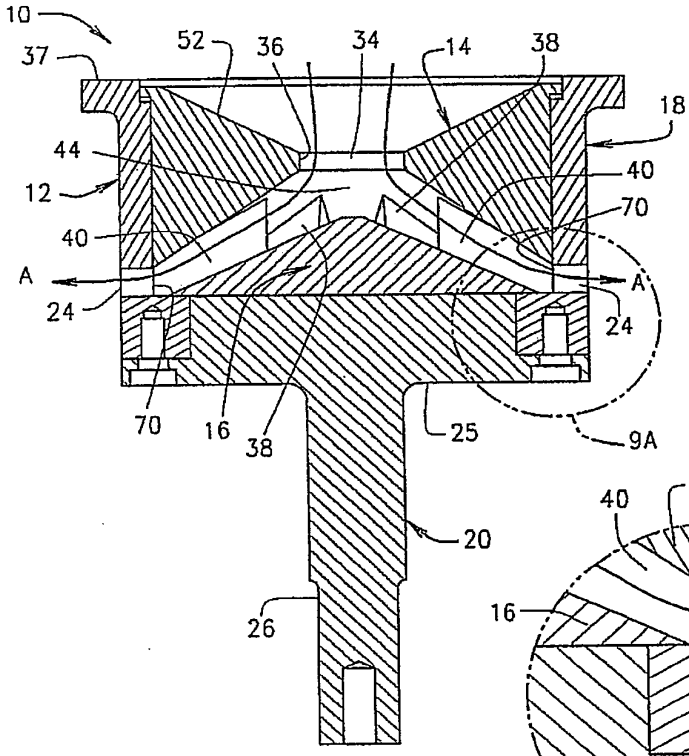

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FIG. 5

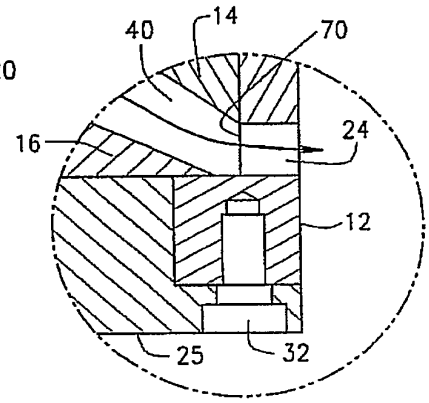

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FIG. 5A

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
29 November 2007 (29.11.2007)

PCT

(10) International Publication Number
WO 2007/137125 A3

(51) International Patent Classification:
*A23L 1/317* (2006.01) *A23J 3/14* (2006.01)
*A23J 3/26* (2006.01)

(21) International Application Number:
PCT/US2007/069157

(22) International Filing Date: 21 May 2007 (21.05.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/437,164 19 May 2006 (19.05.2006) US
11/749,540 16 May 2007 (16.05.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, 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) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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).

**Published:**
— *with international search report*

(88) Date of publication of the international search report:
24 January 2008

*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: 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.

WO 2007/137125 A3

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/069157

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23L1/317 A23J3/26 A23J3/14

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)
A23J 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, FSTA

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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☐ Further documents are listed in the continuation of Box C.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 October 2007 | 08/11/2007 |

| Name and mailing address of the ISA/ | Authorized officer |
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| European Patent Office, P.B. 5818 Patentlaan 2<br>NL – 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Saunders, Thomas |

Form PCT/ISA/210 (second sheet) (April 2005)

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Information on patent family members

International application No
PCT/US2007/069157

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
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| DD 139385 A1 | 02-01-1980 | NONE | |

Form PCT/ISA/210 (patent family annex) (April 2005)

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
14 February 2008 (14.02.2008)

PCT

(10) International Publication Number
WO 2008/017499 A1

(51) International Patent Classification:
*A23C 19/032* (2006.01) *A23C 19/068* (2006.01)
*A23C 19/05* (2006.01)

(21) International Application Number:
PCT/EP2007/007100

(22) International Filing Date: 10 August 2007 (10.08.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06016859.8 11 August 2006 (11.08.2006) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, 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) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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).

**Published:**
— *with international search report*

WO 2008/017499 A1

(54) **Title:** PREPARATION OF A FOOD STUFF FROM A PROTEIN ENRICHED SUBSTRATE UNDER THE SIMULTANEOUS USE OF TRANSGLUTAMINASE AND PROTEASE

(57) **Abstract:** The present invention relates to a method for the preparation of visco-plastic food stuff, comprising the step of adding to a liquid, protein-enriched concentrate comprising at least 5% proteins and (a) reactive protein(s) for transglutaminase, a transglutaminase and a rennet or a rennet replacement which comprises a proteolytic enzyme, whereby said transglutaminase and said rennet or said rennet replacement are added simultaneously to said protein-enriched concentrate comprising a reactive protein for transglutaminase. Also provided are means and kits for carrying out the inventive method.

**Preparation of a food stuff from a protein enriched substrate under the simultaneous use of transglutaminase and protease**

The present invention relates to a method for the preparation of visco-plastic food stuff, comprising the step of adding to a liquid, protein-enriched concentrate comprising at least 5% proteins and (a) reactive protein(s) for transglutaminase, a transglutaminase and a rennet or a rennet replacement which comprises a proteolytic enzyme, whereby said transglutaminase and said rennet or said rennet replacement are added simultaneously to said protein-enriched concentrate comprising a reactive protein for transglutaminase. Also provided are means and kits for carrying out the inventive method.

A classical protein enrichment of foods, for example matured cheeses with rennet (soft, semi hard and hard cheese) is based on the fortification of the milk constituents, mainly of casein, by the drainage of whey after a clotting process and under the use of rennet. Also the preparation of Tofu is based on a shrinking process of a protein matrix by syneresis to enrich the protein content and to achieve a certain texture.

In the 1980ties, intensive research on the amelioration of yield of cheese from milk was carried out. Trials were done to produce cheese from an ultrafiltration (UF) concentrate, in which the protein is retained completely in the retentate, without drainage of whey. This effect is achieved when an UF-membrane with a cut off of 25,000 Dalton is employed. Separation of a water phase by syneresis is called "drainage of whey" and the water phase is "whey". A separation of a water phase at an upstream process, like membrane process, is called filtrate, permeate or serum. When the ingredients of a substrate are not traditional, often the product which results from the syneresis is also called serum, e.g. at a prevention of a drainage, the term is serum binding. The disadvantage of the ultrafiltration is that in mild heat-treated milk whey proteins are present in a native state. By ultrafiltration the whey proteins are concentrated in the same ration as casein so the natural ratio of casein to whey proteins remains at approx. 80/20 and the yield may be improved. However, native whey proteins are not significantly participating in the structure formation of the resulting cheese (Schreiber, 2000. Fortschritt-

Berichte VDI Reihe 3 Verfahrenstechnik Nr. 644). The cheese from ultrafiltered milk often shows a wet surface, which easily leads to an undesired and counter-productive growth of yeasts and other disadvantages, like loosing the "skin". Due to the wet surface of the cheese this process became popular only for a few kinds of white cheeses, when this cheese is stored in brine.

With the microfiltration in the 1990ties, a membrane process was introduced to the dairy industry, which had a wider pore size (approx. 0,1 micron) than UF-membranes have and which makes it possible to separate casein and whey proteins. In said process, casein is concentrated to a greater extend than whey proteins. Whey proteins permeate into the filtrate and can be treated separately from the casein by the so-called microparticulation (SPIEGEL, 2000, Deutsche Milchwirtschaft 51 (7), S. 309-311, 2000). Microparticulation is a process wherein the whey proteins denature and aggregate due to heat and under shear conditions. Microparticulated whey proteins can be incorporated into the microfiltered concentrate. With this procedure it is assured that whey proteins are mainly present in cheese in an aggregated form. Denatured whey proteins are more relevant for the structure formation of cheese and they prevent from wet surfaces due to their serum binding capacity. Besides that the dry matter of cheese can be reduced due to the behavior of aggregated whey proteins. Furthermore, the incorporation of aggregated whey proteins leads to a more creamy structure and therefore fat reduced cheese with a pleasant consistency can be produced. The production of cheese from a micro-filtered milk concentrate is, inter alia, described in Bachmann, 2003, Agrar Forschung 10 (10), 406-410.

Generally there are several methods for milk clotting/gelation, in particular acidification and renneting. Besides that also an enzymatic, covalent cross-linking can induce a gelation of proteins (Ikura, 1992, Comments Agric & Food Chem, Vol. 2, No. 6, 389-407). An overview of possible applications of transglutaminase in milk products is given in Jaros, D., Partschefeld, C., Henle, T., Rohm, H. "Transglutaminase in dairy products: chemistry, physics, application", 2006. However, an incubation of transglutaminase in milk or milk concentrates even at high enzyme concentrations (e.g. 2 U/g protein) does not result in a gelation due to the electrostatic repulsion between the casein micelles at neutral pH-values. Therefore, transglutaminase alone is not a sufficient reagent for the preparation of a relatively firm cheese. Only by a reduction of this electrostatic repulsion through a pH-drop or through the effect of rennet at relevant protein

concentrations and relevant transglutaminase concentrations a gelation may be achieved. Yet, the knowledge of the use of transglutaminase in order to influence the behavior of a rennet gel is very limited; see, e.g. Lorenzen, 2000, Milchw 55: 433-437. The influence of the microbial transglutaminase, MTGase, (in combination with a thermal treatment of the used milk) on the gelling time at the preparation of rennet gels was here assessed. Untreated skim milk samples were treated with different temperature-time combinations (37-90 °C; 5-300 s) and were then incubated with MTGase (14.7 U /g milk protein, based on a protein content of 3.5 % in the milk) at 35 °C. After an incubation time of 0-120 minutes and the addition of 0.015 % rennet the gelling times were estimated. It was evident that with increasing pre-incubation time as well as with increasing thermal intensity of the milk either the gelling time increased or no visible gelling occurred. At a pre-heating treatment at 90 °C for more than 5 minutes and following pre-incubation of more than 5 minutes no gelling was visible. At a thermal treatment of 75 °C for a few seconds and a following pre-incubation with MTGase in the range of 0-120 minutes the gelling time increased from 13 to 35 minutes. Yet, a protein solution with 3.5 % protein and with a ratio of 80 % casein to 20 % whey proteins resulted, after a pre-incubation time of 120 min, in a non-visible flocculation. When rennet and TG was added simultaneously to a pasteurized milk (75 °C for a few seconds), a slightly extended gelling time compared to the control was detected. O`Sullivan, 2002, J Dairy Res 69: 433-442 described that the extended gelling time or the absence of gelling after an incubation with MTGase is due to a negative influence of the enzymatic phase during the renneting.

EP 1 197 152 describes an improved activity of microbial transglutaminase MTGase in pasteurized milk by the addition of glutathione rich yeast extract. It is described that an increase in yield at the preparation of Cheddar cheese through a modification of the renneting process by TG and a reducing agent such as a thiol compound is possible. However, in EP- 1 197 152, non-concentrated milk was used and the whey drainage of the curd was almost comparable to a conventionally produced cheese. The taught, modified cheese making process does not offer an advantage to the cheese making process.

The prior art has employed transglutaminase in milk products in combination with the microfiltration only in cross-linking of caseino makro peptide (CMP) in whey.

However, there is a general problem in the use of transglutaminase in particular TGs in the preparation of a rennet cheese. Such negative influence of TGs on the renneting process is also described in Lorenzen (2000), Habilitationsschrift Bundesforschungsanstalt für Milchforschung Kiel and O'Sullivan (2002), J Dairy Res 69: 433-442.

EP-A2 1 048 218 describes the incorporation of whey proteins in cheese, using non-rennet protease and TG. Transglutaminase and the non-rennet protease is used in a heat-treated dairy liquid. EP-A2 1 048 218 teaches that a cheese product containing a substantial portion of the whey proteins can be produced by sequentially contacting a heat treated dairy liquid with transglutaminase and non-rennet protease. Here, "dairy liquid" is defined as milk, milk products obtained by fractioning raw milk to provide a liquid fraction. Similarly, EP-B1 1 057 411 teaches that a cheese curd containing a substantial proportion of whey protein products can be produced by sequentially (1) contacting a dairy liquid fortified with whey protein with transglutaminase, (2) blending the modified dairy liquid with a second dairy liquid containing casein; and (3) contacting the resulting mixture with a rennet.

EP-A1 1 249 176 describes a process for a production of a whey protein stabilized fatty emulsion which is acidified and heated. After adding it to food, e.g. processed cheese, an incubation step with transglutaminase follows. In particular, EP-A1 1 249 176 teaches that whey proteins can be incorporated into foodstuffs by modifying the whey proteins so that they form whey protein-stabilized fatty emulsions. It is also described that modified whey proteins display a "modified behaviour towards the action of transglutaminase".

WO 97/01961 describes a pre-incubation of transglutaminase (10 min to 2 hours) of cheese milk with 1 to 6% protein. As potential concentrating options, evaporation, spray drying or ultrafiltration are offered. These are methods, which lead to a complete retention of all proteins, and molecules, which are larger than 20,000 Dalton.
EP 1 442 663 reports about disadvantages concerning concentrated milk when an increase of the cheese yield is intended. It is reported that a pre-incubation inhibits the rennet effect and that even a simultaneous administration inhibits the rennet effect. In EP 1 442 663, rennet is added to cold milk. After adding transglutaminase, the milk is warmed. It is concluded that the current cheese preparation technique comprising a step of concentrating the raw material milk cannot satisfy the quality of the final product as required by consumers.

EP-A1 1 232 692 discloses that a whey-less cream cheese can be produced by simultaneously treating a dairy liquid containing dairy proteins with transglutaminase and a lactic acid producing culture.

EP-A1 1 254 601 describes that a cheese product can be made by simultaneously adding a acidifying agent and transglutaminase to a dairy liquid, sufficient to crosslink at least a portion of the dairy proteins and to form a curd and a liquid whey.

US 6,767,575 teaches that a concentrate of denatured whey protein with a specific aggregate size can be produced by thermal denaturation under specific conditions.

Cozzolino (2003), Biotechnology-and-Applied-Biochemistry 38, 289-295 reports an increased incorporation of whey proteins into curd by transglutaminase. The yield increased proportional with the added amount of whey proteins. Yet, transglutaminase was added to cheese milk simultaneously or 30 min before the rennet or after cutting the curd. The disadvantage of the applied technology is a retardation of the gelling time in the case of the pre-incubation, the problem of a homogeneous mixture of TG and the curd. Furthermore, only very sensitive curd can be obtained by the technology as disclosed in Cozzolino (2003). This requires an undesired additional curd treatment in a cheese vat.

US 6,749,873 teaches that cheese yield can be enhanced by elaborately utilizing a milk whey protein or milk whey protein and transglutaminase. Therefore, a partial hydrolysate of the milk whey protein is mixed with the material milk, subjecting the resulting mixture to the milk coagulating treatment. Alternatively, transglutaminase is allowed to act on the mixture prior to subjecting the mixture to the milk coagulating treatment. Furthermore, it is taught that the partial hydrolysis of the milk whey proteins allows the transglutaminase to act on the whey proteins.

US 2006/0083822 discloses a method for making cheese, which involves combining a slurry with a cheese precursor that is subsequently processed to form the final product. It is taught that enzymes, inter alia transglutaminase, may be used to create flavors, texture, melt and/or

other functional characteristics in the final cheese product, and/or in the slurry that can be transferred to the final cheese product once the slurry and cheese have been mixed together.

Therefore, the prior art described a pre-treatment of milk or whey proteins with transglutaminase before the actual cheese making processes extensively. However, a real improvement in final yield has never been reported.
Heidebach, 2004, "Einfluss der enzymatischen Quervernetzung auf die Labgelbildung", Diploma-thesis at the Technische Universität München, has documented that the pre-incubation with transglutaminase generally leads to negative gelling effects. Even a simultaneous addition of transglutaminase together with rennet to non-concentrated cheese milk resulted in a drastically retarded gelling time. Furthermore, the simultaneous addition of TG as taught by Heidebach (2004), loc. cit. resulted in a cheese with a grainy structure and with an unpleasant water binding capacity. In addition, the yield of the final cheese product (in this case Mozzarella) was only (slightly) increased when whey proteins were added. Without whey proteins the mass of cheese was even lower than the control cheese when transglutaminase was added, i.e. even a loss compared to the standard was documented by Heidebach (2004) loc. cit.

Considering the prior art, the technical problem to be solved in accordance with the present invention is the provision of means and methods, which allow for the production of food stuff in high yield, in a particular of cheese and cheese curd.

This technical problem has been solved by the embodiments provided herein and as characterized in the appended claims.

Accordingly, the present invention relates to a method for the preparation of visco-plastic food stuff, said method comprising the step of adding to a liquid, protein-enriched concentrate comprising at least 5% proteins and (a) reactive protein(s) for transglutaminase, a transglutaminase and a rennet or a rennet replacement which comprises a proteolytic enzyme, whereby said transglutaminase and said rennet or said rennet replacement are added simultaneously to said protein-enriched concentrate comprising a reactive protein for transglutaminase.

In accordance with the present invention it was surprisingly found (as illustrated in the appended examples, that the simultaneous addition of rennet (or rennet replacements, like fruit juices comprising proteases, i.e. melon juices or papaya juices etc) and transglutaminase to a liquid, protein enriched or protein rich basic concentrate with a protein content of at least 5% (like microfiltered milk), leads to a final product of increased weight and additionally to a product which comprises gel like product wherein serum drainage was almost completely stopped. Therefore, the method of the present invention leads to a surprising elevation of the amount of resulting food product (like cheese) and said food product comprises a lower dry matter content and a higher water (or serum) content. A further, unexpected advantage of the present invention is a high stability of the curd against further treatments, like mechanical treatments (e.g. pressing, kneading, stretching or netting) or like thermal treatments. Furthermore, the efficiency of the cheese vats/vessels/machinery for the gelling process can substantially be increased, due to the higher starting protein content and the shorter processing time.

It is of note that, in accordance with this invention, a curd with a low protein content (as, inter alia provided in EP1197152), cannot represent a physically stabile food structure even if cross linking occurs, unless considerable amounts of whey are removed from the curd. The advantage of the present invention is a resulting physically stabile food structure, which forms spontaneously after the gelling process. This is, inter alia, in stark contrast to EP-1197152 wherein a regular curd treatment is carried out and the resulting whey drainage of the curd was almost comparable to a conventionally produced cheese. In contrast thereto, with the teaching of the present invention, a conventional curd treatment is not necessary and the curd can directly be filled in corresponding final forms, like perforated forms. This can, in accordance with this invention be done within (few) minutes after the gel/curd is cut. In those cases when the protein content is already adjusted to the final protein content of the said final visco-elastic foodstuff by, e.g. microfiltration, the product does not even necessarily need to be filled in perforated forms and the gel does not need to necessarily undergo a curd treatment.

Most importantly, and shown in the appended examples, with the teachings of the present invention it is now possible to influence/control the syneresis and therefore to set a limit to the serum drainage. Furthermore, a desired firmness of a final product (cheese) at a remarkable higher serum content can be obtained. In one embodiment and as a gist of the present

invention, the inventive method leads to a substantial higher yield in particular of cheese/cheese products or cheese curd. It is one important teaching of the present invention that as starting material a high-protein concentrate is used which comprises a high content of proteins that are susceptible to the enzymatic activity of transglutaminase. One important example of these proteins is casein. Accordingly, and in contrast to the prior art, whey proteins are not necessary in the present inventive method and can, if desired, optionally be added to the "protein-enriched concentrate". Therefore, in one embodiment, a desired "protein-enriched concentrate" to be employed in the inventive method is casein-rich, micro-filtered milk.

Accordingly, the method of the present invention leads to a substantial higher yield of final food product (like cheese/cheese curd) even when no whey proteins are added when the transglutaminase is used in an (acidified) MF-concentrate. The concentration of the reactive protein (susceptible to the action of transglutaminase, like casein) is in accordance with the present invention as high so that the enzyme-substrate-reaction causes an extremely improved firmness of the curd, which is not possible, when milk is first treated with transglutaminase and then concentrated.

Yet, as described herein below, it is also part of this invention that e.g. whey proteins are added to the "protein-enriched concentrate" to be employed as starting material in the method of the present invention. However, the incorporation/addition of whey proteins serves primarily the regulation of the product structure and is not relevant for a higher yield of final product. In many cases the desired product structure is achieved without the addition of (denatured) whey proteins. In relation to "cheese" as a final product of the present inventive method, the structure of said cheese/cheese product will change dependent on the amount of (denatured) whey proteins additionally added to the protein-enriched concentrate, e.g. the MF-milk concentrate. Without whey proteins the resulting cheese has a very long structure (Mozzarella-like) and with addition of (denatured) whey proteins it results in a short structure (Provolone-like). In case of additional addition of whey proteins, it is envisaged that these whey proteins are only added to a final concentration of protein reactive with transglutaminase (e.g. casein) versus whey proteins of 80% versus 20% or 70% versus 30% the most. Accordingly, if whey proteins are to be added to the protein-enriched concentrate to be employed as starting material of the inventive method, these whey proteins, in particular denatured whey proteins, are to be added up to not more than the natural casein/whey protein ratio of 80/20 or 70/30. One of the

advantages of the present invention is that an increase in final yield is not achieved by an increased incorporation of whey proteins through the use of transglutaminase, but an extremely improved effect of the cross-linking of protein susceptible to the enzymatic activity of transglutaminase (in particular casein) due to a higher density of said susceptible protein in the herein defined "protein-enriched concentrate", e.g. MF-milk concentrate. This results in a non-expected, improved incorporation of whey/serum respectively serum binding capacity. It is of note that even if the exemplified final product /exemplified visco-plastic food stuff is cheese, the present invention is not limited to the production of cheese or cheese curd.

The term "visco-plastic" means a elastic texture of the food-stuff to be obtained by the method of this invention. Visco-plastic food stuff comprises, but is not limited to cheeses, like quarg, Feta, Mozzarella, soft-cheeses, semi-hard cheeses or hard-cheeses (like Cheddar). Also "visco-plastic food stuff" are food stuffs like TOFU products or like SURIMI. However, in context of this invention also the production of other food-stuff, like thick drinks, e.g. protein drinks, are envisaged and part of this invention. Accordingly, the method of this invention is not limited to the production of cheeses and cheese-like products but also encompasses the production of other protein-enriched food-stuff and drinks.
The term "visco-plastic food stuff" also comprises curds, like cheddar curds.

The term "liquid, protein-enriched concentrate" as used herein relates to a protein solution which comprises a total protein content of at least 5%, at least 6%, at least 7%, at least 8%, at least 9%, at least 10%, at least 11%, at least 12% or at least 13% protein. All protein contents herein and in particular also in the appended examples are expressed as w/w (weight per weight). Said protein-enriched concentrate is also characterized in comprising in said at least 5% protein content, (a) protein(s) which is(are) reactive for transglutaminase in vitro. Said protein(s) reactive for transglutaminase are in particular casein, casein macro peptide, α-casein fraction, β-casein fraction, caseinate and the like. Accordingly, also other proteins susceptible to the enzymatic action of transglutaminase are envisaged. These proteins comprise but are not limited to proteins from soybean (like the non-limiting examples: globuline fraction(s), vicilin(s), legumin(s)/glycinin(s) or albumin(s)), proteins from leguminosae like peas or seed of lupins (like the non-limiting examples: globuline fraction(s), vicilin(s), legumin(s)/glycinin(s) albumin(s) or glutelin(s)). As mentioned above and in a particular embodiment, said (a)

protein(s) which is(are) reactive for transglutaminase is already comprised in said at least 5% total protein content of the liquid, protein-enriched concentrate. However, it is also envisaged that further and/or additional "protein(s) which is(are) reactive for transglutaminase" is/are added to said liquid, protein-enriched concentrate before, in accordance with this invention, the transglutaminase and the rennet/rennet replacement are added simultaneously.

A protein-enriched concentrate to be processed in accordance with this invention is most preferably microfiltered milk, but also protein-enriched other dairy products/liquids are envisaged as long as they comprise more than 5%, more than 6%, more than 7%, more than 8%, more than 9%, more than 10%, more than 11%, more than 12%, or more than 13% total protein content or at the highest a protein content of 15%, or 16%, or 17%, or 18'% or 19% or, preferably a highest protein content of 20%. As will be detailed below, protein enriched concentration can also be obtained by adding endogenous or foreign proteins to a corresponding liquid, like, e.g. casein (and the like as defined above) to food liquids, like vegetable juices, fruit juices etc.

In a further embodiment, it is also envisaged that the method of this invention comprises the addition of fat to the liquid, protein-enriched concentrate, as e.g. shown in example 3 below, said fat can, inter alia, be added in the form of natural milk fat globules, like cream, homogenised cream (reduced fat globule size) or reemulsified butterfat/oil or emulsified fat from other sources, like, e.g. palm oil or any other plant of animal fat/oil. The person skilled in the art is readily in a position to emulsify different fats or oils or fractions of fats or oils from different sources with milk proteins or many other proteins due to their behaviour to act as emulsifyers, see e.g. Dickinson, E.; Properties of emulsions stabilized with milk proteins: overview of some recent developments; J. Dairy Sci; 80:2607-2619. Due to the fact that the addition of fat reduces the total protein content the addition of fat is limited to maximal 70 % fat in dry matter. Preferably, the addition of fat is limited to 60%, more preferably 50%, more preferably 40%, more preferably 30%, more preferably 20% and most preferably 10% fat in the original liquid, protein-enriched concentrate. Again, in context of this invention, a fat content of maximal 70% fat in the final dry matter is advantageous. The addition of fat to the liquid protein concentrate leads to a reduction of the protein concentration in the resulting mixture. Therefore, in context of the invention, the maximal addition of fat to the liquid, protein-enriched concentrate as employed in the inventive method is an utmost amount that the resulting liquid, protein-enriched concentrate still comprises at least 5% proteins. The fat is

added so that the ratio of fat content to total protein content in the liquid, protein-enriched concentrate is 2.5(fat):1(protein) at the most, i.e. e.g. 2:1, 1:1 or 0:1 (no fat added). An example of a corresponding "protein-enriched concentrate" also comprising fat is given in the appended, illustrative example; i.e. in Example 3 where a fat content of (about) 8% was employed.
In this example, the addition of 20kg of cream with a fat content of 40% to 80kg of liquid, protein-enriched concentrate resulted in a total fat content of 8%. All fat contents herein and in particular also in the appended examples are expressed as w/w (weight per weight).
However, the addition of any additional ingridient results in a liquid, protein-enriched concentrate still comprising at least 5% proteins.
The term "total protein content" is known in the art (in the present case, the total protein content comprised in the protein-enriched concentrate to be employed in the inventive method is at least 5%). The total protein content of the samples can, inter alia, be assessed by the nitrogen content using a Leco FP 528 system (Leco Instrumente GmbH, Moenchengladbach, Germany) based on the Dumas method; see Wiles, 1998, Journal of AOAC International, 81, 620-632. "Total protein content" can than be calculated by multiplying the total nitrogen by 6.38. As mentioned above, the protein contents are expressed as w/w (weight per weight).

The "protein-enriched concentrate" to be employed as basic food liquid in the method of the present invention is a liquid with high protein content, i.e. comprising more than 5%, more than 6%, more than 7%, more than 8%, more than 9%, more than 10%, more than 11%, more than 12%, more than 13%, up to approximately 20%. A significant higher protein content than 20% can only be obtained by a diafiltration e.g. with drinking water, additional to the microfiltration or by addition of protein powders to a liquid protein concentrate. Accordingly, it is also envisaged that the "protein-enriched concentrate" may, exceptionally, comprise more than 20% protein content. In particular when dairy liquids (like microfiltered milk) are used, the proteins comprised in said liquids are in particular casein or casein-like proteins. Particular preferred "protein-enriched concentrates" to be used in context of the present invention are micro-filtered milk or other protein-enriched dairy liquids. Also envisaged are protein-enriched vegetable or fruit juices, or coco milk, milk permeates from ultrafiltration, microfiltration or/and nanofiltration in which added proteins or a microfiltered protein concentrate are mixed/solved. In context of this invention, it is also envisaged that the food liquids to be employed as "protein-enriched concentrates" are obtained by the addition of exogenous proteins (i.e. proteins not originally comprised in said food liquids). These proteins may in

particular comprise casein and casein like- proteins or any other protein(s) which are reactive with transglutaminase/reactive for transglutaminase. In accordance with this invention, the term "reactive with transglutaminase/reactive for transglutaminase" means that the corresponding proteins are susceptible (in particular in vitro and in corresponding food processing processes, like cheese-making) to the enzymatic activity of transglutaminase (TG) as defined herein.

As discussed above, it also envisaged that whey proteins be added to the starting "protein-enriched concentrate" before, in accordance with this invention the rennet/rennet replacement and said TG is added. Said whey proteins may, inter alia, be obtained by microparticulation, this being a process wherein the whey proteins are denatured and aggregated by heat and under shear conditions. Microparticulated whey proteins can be incorporated into, e.g. the microfiltered milk-concentrate to be employed, in one embodiment as starting "protein-enriched concentrate".
The particularly preferred "protein-enriched concentrate" comprising at least 5% protein is micro-filtered milk. The process of obtaining micro-filtered milk is well known in the art, see e.g. Bachmann, 2003 (loc. cit) or Kersten, (2000), Fortschritt-Berichte VDI Reihe 3 Verfahrenstechnik, Nr. 709, VDI Verlag and also illustrated in the appended examples. For example, it is possible to obtain said micro-filtered milk by passing normal milk (up to 3.5% protein content) through a 0.05 – 0.2 μm membrane. Preferable and employed in the appended examples is a membrane of 0.1 μm cut-off.

In one embodiment of the present invention, said at least 5% total protein content in said protein-enriched dairy liquid comprises is at least 70%, preferable about 80% casein, casein-like protein or the protein which is susceptible for transglutaminase ("reactive for transglutaminase"). As illustrated in the appended examples, an advantageous ratio between casein/casein-like proteins/proteins, which are susceptible for transglutaminase, and the remaining proteins (like whey proteins) is 80/20, i.e. 80% of casein/casein-like proteins versus rest or other proteins, which are susceptible for transglutaminase.

Therefore, the present invention is particularly useful when said protein-enriched concentrate comprises a casein/rest protein(s) ratio of at least 70/30, preferably of 80/20. In one

embodiment of the invention, said "rest protein(s)" is/are whey proteins and the main protein susceptible for TG(s) is casein.

As mentioned above, it is also envisaged in context of this invention that a "protein-enriched concentrate" is prepared prior to the inventive simultaneous addition of TG(s) and the rennet or rennet replacement as defined herein. In a preferred embodiment, the exogenous or additional protein to be added in order to prepare a "high protein concentrate" (i.e. more than 5% total protein content and up to, preferably, 20% total protein content) is a protein susceptible to the enzymatic action of transglutaminase as defined herein, e.g. casein(s). It is envisaged that said casein(s) is/are added before said transglutaminase and said rennet/rennet replacement is/are added simultaneously to said protein-enriched concentrate. However, also the addition of further proteins, like and non-limiting whey proteins (also denatured whey proteins), egg white proteins or egg yolk proteins (also denatured egg proteins), soybean protein or proteins from other leguminosae to the protein concentrate before the TG and the rennet/rennet replacement is added, is within the scope of the present invention. However, whey proteins in their native structure are less prone to the cross-linking reaction (by TG(s)) mainly due to their globular conformation stabilised by disulphide bonds. However, the cross-linking of whey proteins can be improved by a foregoing denaturation by heat treatment (Han, 1996, J. Agricultural Food Chemistry 44, 1211-1217; Sharma, 2001, International Dairy Journal, 11, 785-793). In a mixed protein system, such as milk, the caseins are preferentially cross-linked. This is in contrast to any cross-linking between whey proteins, which is less preferred (Han, 1996, loc. cit).

In accordance with the invention, the "casein", which is the most important protein reactive for/with transglutaminase, comprises, but is not limited to casein/rennin itself, casein submicellae, caseinate preparation (like, e.g. rennet caseinate, acid caseinate, $Na^+$-caseinate, $K^+$-caseinate, $Ca^{++}$-caseinate, CMP (caseino macro peptide) or glycosylated versions thereof, like GMP (glycol-caseino macro peptide). As used herein, "casein" relates to any, or all, of the phosphoproteins in milk. An important characteristic of casein is that it forms micelles in naturally occurring milk and in the dairy liquids employed in the present invention, and that clotting a dairy liquid containing casein by any suitable means provides a coagulated curd phase and a liquid whey phase that are separable from one another. Many casein components have been identified, including, but not limited to, alpha-casein (including alpha s1-casein and alpha s2-casein), beta-casein, kappa-casein, their genetic variants, and mixtures thereof.

The term "transglutaminase" is known in the art and relates, also in context of this invention, to microbial as well as non-microbial transglutaminase (TG). In context of this invention, several TGs are useful and in the appended examples the microbial TG (MTG or MTGase) from Streptoverticillium (mobaraense) is employed.

As also illustrated in EP-A2 1 048 218 transglutaminases are enzymes which catalyze the transfer of the gamma-carboxamide group of a glutaminyl residue in a protein or peptide to the epsilon-amino of a lysyl residue of the same or a different protein or peptide, thereby forming a gamma-carboxyl-epsilon-amino crosslink. Transglutaminases have a broad occurrence in living systems, and may be obtained, for example, from microorganisms such as those belonging to the genus Streptoverticillium, Bacillus subtilis, various Actinomycetes and Myxomycetes, or from plants, fish species, and mammalian sources including pig liver and the blood clotting protein activated Factor XIII. In general, transglutaminases from animal sources require calcium ions for activity. Recombinant forms of transglutaminase enzymes may be obtained by genetic engineering methods as heterologous proteins produced in bacterial, yeast, and insect or mammalian cell culture systems. The principal requirement of any transglutaminase employed in the instant invention is that it has the cross linking activity. Any enzyme having transglutaminase activity may be employed in the methods of the present invention. In a preferred embodiment, the transglutaminase is obtained from the genus Streptoverticillium. Most preferred is Activa YG®, as also employed in the appended examples. However, it is also envisaged that enzymes or enzymatic fragments are employed which show transglutaminase activity. Transglutaminase activity may be determined using known procedures. One such technique comprises colorimetric procedures, like the use of benzyloxycarbonyl-L-glutaminyl-glycine and hydroxylamine to form a gamma-carboxyl-hydroxamic acid if transglutaminase is present. An iron complex of the hydroxamic acid can be formed in the presence of ferric chloride and trichloroacetic acid. Using the absorbance at 525 nm with appropriate standards, the activity of enzyme present may be determined; see, US 5,681,598.

As illustrated in the appended examples, microbial tansglutaminases (TGs) can successfully be employed in context of this invention. Examples of such transglutaminases are TGs from Streptoverticillium (like Streptoverticillium mobaraense) or Bacillus subtilis. Yet, also other TGs, as mentioned above may be useful in context of this invention and comprise, e.g. non-

microbial transglutaminases, like TGs from fungi, from plants or from animals. Particularly, the TGs from fungi may be from actinomycetes or myxomycetes.

In context of this invention, the usage of TGs in terms of corresponding concentrations to be employed is within the skill of the artisan. However, as shown in the appended examples transglutaminase may be added to said protein-enriched concentrate in concentration of about 0.1 U/g protein to about 3 U/g protein, preferably from 1 U/g protein to 2 U/g protein of transglutaminase is employed. However, it is also within the skill of the artisan that higher amount, e.g. up to 10 U/g are employed in context of this invention. Per definition in the art, "U" describes "international units". International units are defined as the amount of enzyme which catalyses the formation of 0.5 μmol of hydroxamate per min from Nα-CBZ-L-glutaminylglycine and hydroxylamine at pH 6.0 and 37°C; see Folk, (1966). The Journal of Biological Chemistry, 241, 5518-5525. As shown in the examples, impressive results can be obtained when 1 or 2 U/g protein is used.

The term "rennet or rennet replacement" relates in accordance with this invention to a preparation with an enzymatic activity which comprises proteolytic enzymatic activity, in particular "proteases"/protease activity. Such proteases are most preferably chymosin or pepsin, in particular chymosin. For example, when milk or dairy products (with protein contents of at least 5%) are employed as "protein-enriched concentrate", the rennet to be employed is preferably natural "rennet", like the rennet obtainable from several animal species or recombinant versions thereof. Such "rennet" is, e.g., natural rennet from bovine (calf), goat, buffalo or sheep. Recombinant "rennet" is, e.g. recombinant chymosin or recombinant rennet of cattle (calf) origin produced in different microbial hosts.

Also comprised under the term "rennet or rennet replacement" are enzyme preparations comprising rennin/chymosin (EC 3.4.23.4) or even prechymosin(s). Also comprised are other enzyme preparations, which have protease activity and/or co-agulating activity. Non-limiting examples include other aspartyl proteases such as various pepsins, and a large number of proteases from nonmammalian sources, including plants, microorganisms, and marine fishes. As used herein, a "rennet protease" relates to any such protease having milk-clotting activity. Also genetically engineered mutant proteins derived from such natural proteases and having the corresponding protease activity, are available as recombinant protein products, obtained upon introducing genes encoding these proteins as heterologous genes into suitable host

organisms to produce the protein products. As used herein, all such recombinant proteases having milk-clotting activity are included in the term "rennet or rennet replacement".

Also envisaged as "rennet or rennet replacement with proteolytic enzyme activity" as to be employed in context of this invention are vegetable rennets (like rennets from fig tree bark, nettles, thistles, melon, papaya). Preferred "rennets" in this context are, e.g., thistle press juice or juice from melons or papaya.

Other "rennets or rennet replacements" are, in context of this invention, rennets of fungal origin, also known as "genetic rennet", like genetic rennet produced from Aspergillus niger, Rhizopus oryzae (Sushil-Kumar et. al Journal-of-Food-Science-and-Technology. 2004; 41(3): 279-283; 28., Fusarium subglutinans (Ghareib,-M et. al Acta-Microbiologica-Polonica. 2001; 50(2): 139-147; 22), Mucor miehei (El-Tanboly,-E et. al Milchwissenschaft. 2000; 55(11): 624-628; 24.), Mucor pusillus var. lindt (Wahba,-A et. al Alexandria-Journal-of-Agricultural-Research. 1986, recd. 1988; 31(3): 139-150; 11 ref.), Fungus Irpex lacteus (Kobayashi,-H Agricultural-and-Biological-Chemistry. 1985; 49(6): 1605-1609; 13 ref.), Rhizopus oligosporus (Krishnaswamy,-M-A et. al Journal-of-Food-Science-and-Technology,-India. 1976; 13(4): 187-191; 18 ref.), or Basidiomycetes (Kawai,-M; Mukai,-N Agricultural-and-Biological-Chemistry. 1970; 34(2): 159-63; 25

In context of this invention, it is also envisaged that chymosin/rennin is used as "rennet or rennet replacement" in the methods and means provided herein.
Accordingly, in context of this invention, the rennet or rennet replacement to be employed in the inventive method comprises a proteolytic enzyme and may be selected from the group consisting of bovine/calf rennet, buffalo rennet, sheep rennet, goat rennet, porcine rennet, microbial rennet like from Bacillus subtilis (Masek,-J Conference-Proceedings XIX International Dairy Congress. 1974; 1E, 692.), Streptomyces strains (Abdel-Fattah,-A-F Acta-Microbiologica-Polonica,-B 1974; 6(1): 27-32; 14), Streptococcus liquefaciens-108 (Srinivasan,-R-A et. al Indian-Journal-of-Dairy-Science. 1968; 21(3): 149-54; 11.), Bacillus polymyxa (Denkov,-Ts; Vasileva,-S Nauchni-Trudove,-Institut-po-Mlechna-Promishlenost. 1979; 9, 158-165; 8.) genetic rennet, recombinant rennet, chymosin preparations. Also envisaged are fruit or vegetable juices and/or pulps and the like, which have "renneting activity". Such fruit/vegetable or plant pulps or juices comprise, e.g. melon juice, papaya juice,

proteinases from Opuntia ficus-indica fruits (Garcia,-M-T; et. al Biotechnology-Progress. 2006; 22(3): 847-852; 31), extracts from berries of the plant Solanum dobium (Yousif,-B-H International-Dairy-Journal. 1996; 6(6): 637-644; 10), enzymatic complex from pineapple pulp Cattaneo,-T-M-P Milchwissenschaft. 2005; 60(4): 399-402; 14), vegetable juice, like from Ash gourd (Benincasa cerifera) (Gupta,-C-B; Eskin-N-A-M Food-Technology 1977; 31(5): 62-64, 66; 10 ref.), plant derived coagulating enzymes from Cardoon extract e.g. strain Cynara cardunculus (ALVARES, S. et. al Alimentacion-Equipos-y-Tecnologia. 2005; 24(203): 63-66; 11 ref.), Ficain and Papain (Yit-Hwei-Low et. al International-Dairy-Journal. 2006; 16(4): 335-343; 19), extract from the flowers of wild globe artichokes (Tsouli,-J Comptes-Rendus-Hebdomadaires-des-Seances-de-l'Academie-des-Sciences,-Serie-D-Science-Naturelles. 1970; 270(2): 396-99; 6 ref.), protease isolated from Dieffenbachia maculata, a stem of Dieffenbachia maculata (a plant of the Araceae family widely cultivated in India) (Sriram-Padmanabhan et. al Nahrung. 1993; 37(1): 99-101; 11). As is evident from the above, in accordance with this invention, the term "juice form plants, fruits, vegetables, is not limited to the fluids pressed from said plants, fruits or vegetables, but also comprises corresponding preparations, like pulp, extracts, isolated enzymes and the like. Further rennets or rennet replacements are also rennet from stomach of seal (US 4,526,868) or from stomach lining of Atlantic cod (Brewer,-P Canadian-Institute-of-Food-Science-and-Technology-Journal. 1984; 17(1): 38-43; 19).
As shown in the appended examples, in particular natural rennet, like calf rennet, can successfully be employed in context of this invention.

The concentration of the rennet/rennet replacement added simultaneously with TG(s) to protein concentrates with high protein content as defined herein can easily be established by the person skilled in the art. It is shown in the appended examples, that said rennet or rennet replacement may be added to said protein-enriched concentrate in concentration of 0.01 to 1.0 IMCU/g protein (International Milk Clotting Units) preferably 0.1 to 0.5 IMCU/g protein. International Milk Clotting Units are described from SAUERER, V. in dmz Deutsche Molkereizeitung 1/2001. Before the IMCU, the REMCAT method was employed. The international method "REMCAT" is the relative milk clotting activity test for which any rennet sample is compared to two standard samples. One standard rennet sample from a calf origin control powder consists of at least 98% Chymosin and to a maximum 2% bovine pepsine. The second standard rennet sample is a cattle origin control powder consisting of at least 99% bovine pepsine and to a maximum 1% chymosin. The standard is determined in reconstituted skim milk. For the

actual determination of the activity a calf and a cattle origin control powder is mixed in a ratio of the enzymes the assay consists of. The time between the addition of the rennet and the visible flocculation in the reconstituted skim milk at a pH-value of 6.5 and a temperature of 32°C is taken. Parallel, under the same conditions, the time period for the standard rennet is determined. The actual activity is calculated by comparison of the renneting substance to be assayed with standard rennet and is specified as International Milk Clotting Units (IMCU).

The method as provided herein may also comprise an additional acidification step as routinely used and employed in the food processing and food preparation methods, like cheese making. Said additional acidification of the protein-enriched concentrate may take place before or during the simultaneous addition of transglutaminase and rennet or rennet replacement. In one embodiment, said acidification of the protein-enriched concentrate comprises the adjustment of the pH a value between 5.2 and 6.8, preferably between 6.0 to 6.8. The acidification can be carried out by methods known in the art and may be obtained by the addition of a starter culture or the addition of an organic acid. Such starter culture may be from selected milk bacteria or may be from mesophilic bacteria e.g. Lactobacillus lactis subsp. lactis, Streptococcus lactis subsp. cremoris, Lactobacillus lactis subsp. lactis biovar diacetylactis, Leuconostoc mesenteroides subsp. cremoris, Leuconostoc mesenteroides subsp. dextranicum, and/or thermophilic bacteria e.g. Streptococcus thermophilus, Lactobacillus desbruekii subsp. bulgaricus (Lactobacillus bulgaricus), Lactobacillus acidophilus, Lactobacillus helveticus, Lactobacillus casei susp. casei.
Also useful, food-compatible organic acids are known and comprise, but are not limited to, citric acid, lactic acid, acetic acid, malic acid, formic acid or glucono δ lactone.

In a further embodiment of this invention, it is envisaged and may be useful that the method as provided herein comprises a further step, wherein an inhibitor of inhibitors of transglutaminase is added to said protein concentrate before said transglutaminase and said rennet or rennet replacement is added simultaneously to said protein-enriched concentrate. For example, glutathione may be added as such an inhibitor. Glutathione is well known in the art and is, inter alia, described in EP 1 197 152. In context of this invention, glutathione means the tri-peptide epsilon-L-glutamyl-L-cysteinyl-glycin, which is reversibly oxidizable due to its free thiol-groupe. Glutathione is, e.g., present in different yeast extracts in relatively high concentrations. Glutathione is a reducing agent.

The inventive method as provided herein requires that the transglutaminase (or an enzyme with transglutaminase activity or a fragment of such an enzyme with said activity) is simultaneously added to the protein-enriched concentrate with rennet/rennet replacement. Said "simultaneous addition" of said transglutaminase and said rennet/rennet replacement to said protein-enriched concentrate means that the addition of transglutaminase and rennet or rennet replacement is preferably carried out at least within 10 minutes or less, preferably within 9 minutes or less, more preferably within 8 minutes, more preferably within 7 minutes or less, more preferably within 6 minutes or less, more preferably within 5 minutes or less, more preferably within 4 minutes or less, more preferably within 3 minutes or less, more preferably within 2 minutes or less, more preferably within 1 minute or less or most preferably at the same time.
If not added at the same time, the addition of TG before the rennet/rennet replacement is preferred. However, a person skilled in the art is aware of the, less preferred, possibility of adding TG after the rennet/rennet replacement, which is also comprised as an embodiment of this invention.

A particular example of the present invention is provided in the appended experimental part. Here, the production of cheese or cheese curd is described, wherein said protein-enriched concentrate employed is micro-filtered milk with a protein content of more than 5%, and wherein said transglutaminase employed is from Streptoverticillium and wherein said rennet is calf rennet.

The obtained visco-plastic food stuff as provided by the inventive method is characterized as being a physically stabile product with high gel formability, a high water binding capacity and/or having a relatively dry surface, which is suitable for a growth of different maturing microorganisms e.g. mould like Penicillium candidum, yeast like Geotrichum candidum or mixed cheese flora like red smear culture. The product is also suitable to be packed in foil e.g. shrink-wrap foil or wax without further maturing. The food stuff is also suitable to generate a rind. At high protein content, the curd as obtainable by the inventive method is stable for example to be mixed with table salt and offered as unripened food stuff without further forming the curd (like cheddar curd).
Said visco-plastic food stuff, like curd, obtainable by the inventive method is particularly useful in the preparation of cheese or cheese products, like quarg, rennet cheese, soft-cheese, semi-hard cheese and hard-cheese. Corresponding examples for the texture are Mozzarella,

Cashcaval, Feta, Neufchatel, Petit Suisse, Queso Blanco, Camembert, Edam, Gouda, Limburger, Muenster, Provolone, Roquefort, Cheddar, Cheddar curd or Emmental.

Also provided in this invention is a kit for carrying out the inventive method for preparation of visco-plastic food stuff. Said kit comprises (a) transglutaminase(s) as characterized above and comprises (a) rennet(s) or rennet replacement(s) as characterized herein, whereby said (a) transglutaminase(s) and said rennet(s) or rennet replacement(s) are to be administered to protein-enriched concentrate as defined herein simultaneously.

The Figures show:

**Figure 1: Resistance force of MF-Gels dependent on protein content and dependent on the clotting process**

This figure shows that an increasing protein content leads to an increased gel firmness on different levels dependent on the method for gelling. The use of 1 U transglutaminase per g protein plus fermentation (acidification) leads to a resistance of 100 g at a protein content of 13.7%. The use of 2 U transglutaminase under ambient conditions leads to almost the same resistance like the use of rennet plus acidification (resistance 250 g at a protein content of 13.7%). A combination of rennet, transglutaminse and acidification resulted in doubling the firmness (approx. 500 g at a protein content of 13.7%). This synergistic effect was surprising. As rennet a natural rennet was used which had an activity of 140 IMCU/ml (international milk clotting units). Per g protein 0.3 IMCU rennet was used.

**Figure 2:** Amount of serum of MF-Gels dependent on the protein content and dependent on the clotting process

The following examples illustrate but do not limit the invention:

**Example 1: Use of TG and rennet in non-concentrated 3.3 to 3.5% milk or curd.**

Pasteurized whole milk (fat content 3.5%) from the local dairy with its natural protein content between 3.3 and 3.5% was obtained. Three cheese vessels were filled with 8 kg whole milk each and at a temperature of 10°C citric acid with a concentration of 9% was added until a pH-value of 5.9 was reached. The citric acid was prepared from 90 g crystalline technical citric acid and 910 g distilled water. After that the milk was warmed up by warm water in the double jacket wall to a temperature of 33 °C. At that temperature in all cases 0.02% natural rennet preparation (NaturenTM, Chr. Hansen, Denmark) which was diluted with 50 parts of water was used. One trial/vessel was performed as a standard, one trial/vessel was performed with 0.59 IU transglutaminase per g protein and with glutathion (Activa YG) (simultaneous with rennet) and to the third vessel transglutaminase preparation (Activa YG) was added when the curd was already prepared, i.e. after rennet was previously added.

The gelling time was estimated manual by the common method to consider the intersection of a cut in the curd with a knife or a spattle. When the curd brakes smooth the time to cut the complete curd is defined. The gelling time was 25 min for the standard and the third vessel and 28 min for the trial with simultaneous addition of transglutaminase and rennet. The curd was cut in walnut size curd cubes and was not stirred for 3 to 5 minutes. In the third vessel 0.59 Units Activa YG per g protein was added to the curd. In one case 3 Units/g protein was added. The curd was stirred carefully for 40 to 60 minutes. After that the curd was transferred to perforated forms for the drainage of whey and for an adhesion. After spending 60 to 80 min in the forms at room temperature, the weight of the adhered curd was taken (mass of the curd). The formed curd from each vessel was cut in 6 equal pieces each and then manually formed in hot water (never below 80°C). The hot water was prepared in amounts of 8 L with 1% table salt. The netting process (=stretching and kneading) of the mozzarella took about 5 min per bowl. After forming the mozzarella bowls the cheese were placed in iced water with a table salt concentration of 0.5% for approx. 30 min. Now the weight of the cheese was measured.

In further experiments denatured whey proteins was added to cheese milk. As denatured whey proteins a liquid WPC (whey protein concentrate) with a protein content of 23.6% was used. The whey was origin for a mozzarella production and was ultrafiltered and diafiltered to this protein concentration with an ultrafiltration plant with a cut off of 25.000 Dalton at a low temperature (10 °C). The WPC was heated by means of a scraped surface heat exchanger at a shear rate of 630 $s^{-1}$ and at a temperature of 85 °C for 3 minutes. The mean diameter of the denatured whey protein aggregates was approx. 2.0 micron while the particle measurement of

the concentrate (native) before the heating and shearing process showed a medium particle size of approx. 0.2 micron. The medium particle size is defined as d50.3, the volume based diameter, which means that 50% of the volume of the particles is covered by particles smaller then the mentioned size and 50% of the volume is covered by particles larger then the mentioned size. The particles were measured by a laser scattering spectroscope from the company Coulter (calculation model: protein model). At the described heating conditions the denaturation of the whey proteins is very high (> 90% denaturation of β-lactoglobulin).
These experiments were carried out as described above, yet 0.2% whey proteins (0.4%, respectively) were added to the whole milk at the begin of the procedure. Accordingly, the protein concentrate was increased to 3.7% (or 3.9%)

The yield of the curd (raw curd) and the cheese (mozzarella) was defined as Yield = kg curd or cheese/100 kg milk.
The result of this equation was defined as 100% yield for the standard and the result for the trials was calculated as relative yield.

The following results were achieved:

Table 1: Yield of curd and mozzarella cheese

| | **Rel. Yield of the curd** | **Rel. Yield of the mozzarella** |
|---|---|---|
| Standard (without TG) | 100% | 100% |
| simultaneously with rennet 0.59 U TG/g protein | 118% | 91% |
| mixed with the curd 0.59 U TG/g | 102% | 95% |
| mixed with the curd 3 U TG/g | 103% | 98% |
| Standard (without TG and without whey proteins) | 100% | 100% |
| Standard (without TG and + 0.2% whey proteins) | 116% | 105% |
| simultaneously with rennet 0.59 | >120% | 101% |

| | | |
|---|---|---|
| U TG/g protein + 0.2% whey proteins | | |
| mixed with the curd 0.59 U TG/g + 0.2% whey proteins | 117% | 108% |
| Standard (without TG and + 0.4% whey proteins) | 118% | 110% |
| simultaneously with rennet 0.59 U TG/g protein + 0.4% whey proteins | 130% | 105% (grainy product; leakage of whey) |
| mixed with the curd 0.59 U TG/g + 0.4% whey proteins | 121% | 104% |

The results provided above document that in low protein milk (3.3 to 3.9%) an intermediate increase of an intermediate product (and) could be obtained when in particular whey proteins were added in combination with TG. However, in contrast to the examples below, further processing of said card let to rather disappointing yield of finalized products. The above results document that rennet/TG administration to low protein milk (up to 3.9%) let to a final yield of 105% at the most.

In addition, it could be shown that the final product obtained in the combination of rennet/TG and a protein content in the milk of 3.9% was not a stabilized cheese product, was short-structured (grainy) and an undesired leakage of whey was observed. However, the curd obtained by the method as provided in this Example1 was sensitive against further mechanical and thermal treatments.

**Example 2: Protein combination modifications in simultaneous addition of TG and rennet to a protein enriched dairy liquid**

These following trials were carried out with pasteurized skim milk from a local dairy.

The pasteurized skim milk was microfiltered (MF) by means of a pilot plant with microfiltration moduls from the company APV Invensys, Denmark with ceramic tubular filter

elements with a declared pore size of 0.1 micron and a membrane surface of 1.68 $m^2$. With this process, microfiltered milk (MF-milk) with a protein content of at least 5% can be obtained. As shown below, even protein combinations of more than 13% can be achieved.
Even if the current experiments have been carried out in a pilot plant, microfiltation of milk in commonly used and for example described in Kersten, 2000 (Fortschritt-Berichte VDI Reihe 3 Verfahrenstechnik, Nr. 709, VDI Verlag)

In the here used pilot plant, the permeate and the retentate was circulated by centrifugal pumps in a batch process in order to adjust an equal pressure difference of 0.4 bar over the whole length of the moduls. Accordingly, uniform transmembrane pressure (UTP-principle by APV) was employed . The milk was warmed up by a plate heat exchanger to a temperature of 52 °C ± 2 °C and kept at that temperature over the whole process by cooling with water. The result was a good permeation of the whey proteins to the permeate while the casein remained in the retentate. 120 kg starting material pasteurized skim milk resulted in 25 kg retentate and 95 kg permeate. The permeate was visually absolute clear over the whole process of microfiltration. The permeation rate (flux) was approx. 50 l/$m^2$ h at the begin and approx. 25 l/$m^2$ h at the end of the filtration process.
The pasteurized skim milk and the retentate as well as the permeate was determined for the total protein content by the Dumas method (LECO FP 528). The protein content was calculated by multiplying total nitrogen by 6.38. The following protein contents were achieved: retentate: 13.7%; permeate: 0.6%

A part of the microfiltered retentate was then diluted with a permeate from the ultrafiltration (pasteurized skim milk from the local dairy; process temperature 50°C; cut off 25.000 Dalton, pilot plant from the Technical University of Munich) to different protein contents. The dilution was done with the UF-permeate to keep the milieu (minerals, lactose) constant but to keep the casein/whey protein ratio in all cheese milk preparations on the highest level of 93:7 (calculated from the protein content of the MF-permeate and the total protein content of the skim milk, under the presumption that the natural ratio of casein to whey protein in skim milk is 80:20). In the MF-permeate only whey proteins are found and in UF-permeate only peptides but no casein and no major whey proteins are present.

The samples with different protein contents were warmed up to 35 °C and 200 g of it were filled in cups. As a starter culture 2 ml of a pre-culture per kg cheese milk preparation was used. The acidified pre-culture was prepared by solving a deep frozen thermophilic, probiotic starter culture (ABT 21; CHR HANSEN) in high heated and again cooled skim milk in a ratio of 20 g starter culture to 180 g skim milk. From this amount of 200 g pre-culture 0.4 ml were added to each cup with 200 g cheese milk preparation. This procedure was done for a better dosing of the starter culture. After the starter culture was added, an amount of 0.3 IMCU of rennet per g protein (Naturen™ Premium 145; CHR HANSEN) was added. The rennet preparation has an IMCU (International Milk Clotting Unity) activity of 140 IMCU/ml (REMCAT strength), see e.g. Sauerer, dmz Deutsche Molkereizeitung (1/2001).

In accordance with the present invention, the transglutaminase was added simultaneously with the rennet. Due to the small amounts of samples the transglutaminase preparation Activa YG® from the company Ajinomoto was not solved in water before but added as a powder to the milk preparation. Activa YG® is a mixture of MTGase, Lactose, Maltodextrin, GSH, added as yeast extract and safflower oil. In other documented experiments, 1 Unit transglutaminase per g protein or 2 Units per g protein were used.
The assay samples were incubated in variation as shown in diagram 1 and 2 at a temperature of 35 °C in a pre-heated stove for approx. 9 hours. After cooling down to a temperature below 10 °C, the assay samples were measured by the texture analyser (Stevens Texure Analyser) with a cylindric probe with a diameter of 4 mm with a speed of 0.5 mm/s and a depth of 15 mm.

In the resulting samples, pH measurements showed certain variations depending on protein content in the starting material.

The following, surprising results have been obtained. Starting from high (more than 4.5%) protein content, it was surprisingly found that a final product (gel/curd) could be obtained with high firmness under the condition that the TG and the rennet was added simultaneously; see Figure 1. Furthermore, as documented in Figure 2, much less serum drainage/whey drainage could be observed when high protein content milk was employed in simultaneous addition of TG and rennet. Accordingly, a high and surprising serum/whey binding capacity could be observed. This was and is in clear contrast to the examples as provided herein above under Example 1. Accordingly, the results obtained with high protein milk (protein enriched milk

concentrate comprising more than 5% protein) are surprising, since the final product has the following unexpected advantages:

-A physically stabile product against mechanical treatments of pressing, kneading, stretching, netting;

-A high stability against thermal treatments with high gel formability;

-A high water binding capacity and a relatively dry surface which is suitable for a growth of different maturing microorganisms e.g. mould like Penicillium candidum, yeast like Geotrichum candidum or mixed cheese flora like red smear culture;

-Ability for being packed in foil e.g. shrink wrap foil or wax without further maturing;

-Ability to generate a rind under maturing conditions;

-At high protein contents the curd is stable for example to be mixed with table salt;

-Can be offered as fresh food stuff without further forming of the curd (behavior like cheddar curd);

-The curd can be filled in perforated forms only a few minutes after the gel/curd is cut;

-In those cases when the protein content is already adjusted to the final protein content of the final visco-elastic foodstuff e.g. by means of microfiltration (instead of achieving the final protein content by whey drainage), the product does not even necessarily need to be filled in perforated forms and the gel does not need to necessarily undergo a curd treatment;

-Substantially increased efficiency of the cheese vats/vessels/machinery due to the higher starting protein content and the shorter processing time;

-Low whey drainage.

This was unexpected, since the experiments documented in Example 1 led to a completely different result. In Example 1 an undesired sensitive curd was obtained and the whey drainage was basically not reduced. In accordance with the results presented in Example 1, the complete cheese making process shown in said example was not more efficient than a standard cheese making process.

Several assay samples from example 2 were further treated by a Thermomix (company Vorwerk) mixer and it resulted in granulous curd products.

Therefore, when transglutaminase was used (also in common combination with an acidification) at 1 U/g (as well as at 2 U/g), serum drainage was almost completely stopped especially at high protein contents. In contrast, rennet in combination with acidification let to

very intensive serum drainage. Simultaneous use of rennet and transglutaminase leads to a minimal serum drainage. One advantage of the present invention is that it is now possible to influence/control the syneresis and therefore to set a limit to the serum drainage. Furthermore, a desired firmness of a final product (cheese) at a remarkable higher serum content can be obtained.

When a milk gel shows a low serum drainage and still reaches a higher firmness, an increase in yield without a loss in texture firmness ca be achieved.

**Example 3: Large scale production of FETA-like cheese from concentrated milk (in contrast to example1)**

Pasteurized skim milk and pasteurized cream was obtained from the local dairy. The pasteurized skim milk was microfiltered (MF, as detailed also in example 2) and then the retentate was mixed with the cream to adjust the fat content. In particular, microfiltration was done in a pilot plant with microfiltration moduls from the company APV with ceramic tubular filter elements with a declared pore size of 0.1 micron and a membrane surface of 1.68 $m^2$. At this pilot plant the permeate was circulated as well as the retentate by centrifugal pumps in a batch process to adjust an equal pressure difference of 0.4 bar over the whole length of the moduls, i.e. it followed the principal of an uniform transmembrane pressure (UTP-principle; patented by APV). The milk was warmed up by a plate heat exchanger to a temperature of 52 °C ± 2 °C and kept at that temperature over the whole process by cooling with water. The result was a good permeation of the whey proteins to the permeate while the casein remained in the retentate. 300 kg starting material pasteurized skim milk resulted in 95 kg retentate and 205 kg Permeate. The permeate was visually absolute clear over the whole process of microfiltration. The permeation rate (flux) was approx. 50 l/$m^2$ h at the beginning and approx. 30 l/$m^2$ h at the end of the filtration process.
The pasteurized skim milk and the retentate as well as the permeate was determined for the total protein content by the Dumas method (LECO FP 528). The protein content was calculated by multiplying total nitrogen by 6.38. The following protein contents were achieved:

| Product | protein content (%) |
|---|---|
| Pasteurized skim milk | 3.5% |

| | |
|---|---|
| MF-retentate | 11.5% |
| MF-permeate | 0.55% |

In the assumption that milk has a natural casein/whey protein-ratio of 80:20 and the fact that in the permeate only whey proteins are present a casein/whey protein ratio of the retentate can be calculated by a mass balance.

300 kg skim milk = 95 kg Retentate + 205 kg Permeate

Protein mass in skim milk:

300 kg skim milk x 0.7% whey proteins = 2.1 kg whey protein
300 kg skim milk x 2.8% casein = 8.4 kg casein
300 kg skim milk x 3.5% total protein = 10.5 kg total protein

Protein mass in the permeate:

205 kg permeate x 0.55% whey proteins = 1.12 kg whey protein

Protein mass in the retentate:

95 kg retentate contains 8.4 kg casein + 2.1 kg whey protein – 1.12 kg whey protein
95 kg retentate contains
8.4 kg casein and 0.98 kg whey protein represents a casein/whey protein ratio of approx. 90:10.

The slight difference in calculation versus measured protein content in the retentate was 11.6% (calculated to 11.5%).
The total protein concentration in the liquid, protein-enriched concentrate was 11.5% as analyzed and 9.88% as calculated referring to the total protein content.

The MF retentate (11.5% analyzed protein content) was cooled down by the plate heat exchanger to a temperature of 4 °C and stored over night at that temperature.
80 kg of the MF-retentate where than mixed with 20 kg pasteurized cream with a fat content of 40% and a protein content of 2 %. The mixture considered as cheese milk preparation had a fat

content of 8% and a protein content of 9.2 kg coming from the retentate and 0.4 kg origin from the cream. The total protein content in the cheese milk preparation therefore was 9.6 %. The fat content was determined by the Gerber method.

The cheese milk preparation was warmed up in two cheese vessels with a volume of 50 kg each by warm water in a double jacked wall to 35 °C. As a starter culture 2 ml of a pre-culture per kg cheese milk preparation was used. The acidifying pre-culture was prepared by solving a deep frozen mesophilic, homofermentative starter culture (R-604; CHR HANSEN) in high heated and again cooled skim milk in a ratio of 200 g starter culture to 1800 g skim milk. From this amount of 2000 g pre-culture 100 ml were added to each cheese vessel. This procedure was chosen in order to facilitate dosing of the starter culture. After the starter culture was added in the case of the control production only an amount of 0.2 ml rennet per liter cheese milk preparation (Naturen™ Premium 145; CHR HANSEN) was added. The rennet preparation has a labeled IMCU (International Milk Clotting Unit) activity of 140 IMCU/ml (REMCAT strength). Based on the 100 kg cheese milk preparation it means per g protein 0.29 IMCU rennet were used (20 ml rennet to 100 kg cheese milk preparation with 9,600 g; 20 ml x 140 IMCU/ml = 2,800 IMCU; 2,800 IMCU/9,600 g protein = 0.29 IMCU/g protein). Here, transglutaminase was added simultaneously with rennet. In case of the use of 1 U TG/g protein an amount of 96 g of the transglutaminase preparation Activa YG® from the company Ajinomoto was dissolved in 1000 g distilled water before adding to the cheese milk (50% of the solution in each cheese vessel) together with the same amount of rennet as in the control cheese production). The preparation Activa YG® is a mixture of MTGase, Lactose, Maltodextrin, GSH, added as yeast extract and safflower oil. In the case of the use of 2 U TG/g protein, an amount of 192 g of the transglutaminase preparation Activa YG® was solved in 1000 g distilled water before adding to the cheese milk. Again, the amount of rennet (0.2 ml/l) was kept the same.
The trials were done in three following days.

The visual control of the gelling showed that the curd was ready to be cut after 26 min for the control cheese milk preparation (gelling time 26 min) whereas the cheese milk preparation with 1 U TG/g protein showed a delay in gelling of 4 min (gelling time 30 min). When the curd was cut with a spattle and the curd was lifted up with the spattle than and the cut surface showed the typical porcelain-like structure, the curd was cut with a wired frame to a size of cubes with

a length 2 cm x 2 cm x 2 cm. The cheese milk preparation with 2 U TG/g protein had a further delay in gelling time and it was possible to cut the curd 38 min after adding the enzymes. Compared to the control, the process was prolonged for 12 min. Compared to conventional cheese making with non-concentrated milk this is still an advantageous gelling time. The gelling time at conventional processes often is 40 min.

After cutting the curd the complete mass of curd and whey was circulated in the vet and was transferred to forms in between a time of some minutes and than pressed at a pressure of 0.035 $kg/cm^2$. By pressing, a smooth surface was achieved. Due to the high serum binding capacity of the curd as obtained by the method of the present invention, the pressing procedure is not related to a high loss of whey and the cheese is well formable. The weight for pressing remained over night on the surface of the cheese and the curd remained in the forms over night at room temperature so the acidification could continue. On the next day a pH of 5.0 ± 0.5 was measured in the cheese mass. The weight remained over night but after the cheese received a smooth surface the weight could have been removed earlier.

On the following day the cheese was spread with table salt (but could also be immersed in brine which is common for many kind of cheese). Due to the rather firm curd, a technology similar to the technology for the production of cheddar could have also been used. Cheddar production is, inter alia, described in Kosikowski (1997), "Cheese and Fermented Milk Foods"; Kosikowski, Westport, USA. For cheddar a curd which passes the so called cheddaring process and is milled and mixed with dry salt and then filled up in forms. If the weight of the cheese is about 200 kg the cheese does not need to be pressed in the form due to high weight of the cheese itself. It is sufficient when the form is turned 180 ° then two or more times. If the pieces of cheese are lower in weight it is pressed like rennet cheese is usually pressed. For the production of a cheese with the presented technology using a microfiltered milk and transglutaminase a similar technique like some steps for producing a cheddar can be used. One advantage of the present invention is the finding that high protein concentrate prepared by simultaneous addition of TG and rennet/rennet replacements leads to a firm food stuff even without "cheddaring process". Accordingly, the present invention avoids one additional step in (e.g.) cheese making, namely the "cheddaring process", i.e. a maturing step of curd after whey drainage. Further advantages of the method of the present invention have been described herein above.

In particular, it was surprisingly found that in accordance with the present invention, it was possible to produce 48 kg cheese from 80 kg acidified MF-concentrate (total protein content 11.5%; fat 0,2 %) + 20 kg cream (fat 40 %; total protein content 2%) = 100 kg milk preparation under the use of 1 U TG/g protein. This represents an increase in yield of approx. +12% (the control, i.e. only rennet, was only 43 kg). A doubling of the concentration of the transglutaminase to 2 U/g protein increases the yield to 53 kg cheese representing a further increase of +10% (total increase of + 23%).

Surprisingly, even if a higher weight increase of final product was obtained, the firmness of the final product remained on a similar high level. Firmness was measured by a penetration test (Stevens Texture Analyser) and is documented also in the following table.
The example also shows that fat can be added as a part of the protein enriched dairy liquid. Fat was added in the form of natural milk fat globules (cream) but the source could have also been a homogenised cream (reduced fat globule size) or reemulsified butterfat/oil or emulsified fat from other sources like palm oil or any other plant of animal fat/oil. It is state of art to emulsify different fats or oils or fractions of fats or oils from different sources with milk proteins or many other proteins due to their behaviour to act as emulsifyers. Due to the fact that the addition of fat reduces the total protein content the addition of fat is limited to 70 % fat in dry matter. It is still important that protein is a dominant ingredient of the substrate.

| **Procedure** | **Amount of cheese from 100 kg milk preparation** | **Penetration value (g) *) depth 10 mm/5 mm** | **Weight yield** |
|---|---|---|---|
| Only rennet (control) | 43 kg | 695/250 | 100% |
| + 1 U TG/g protein | 48 kg | 670/233 | 111% |
| + 2 U TG/g protein | 53 kg | 655/220 | 123% |

*) Penetration probe cylinder Ø 13 mm, penetration speed 0.2 mm/s

The variation of the penetrometer values was ± 10% of the total value. The relatively high deviation is due to the fact that the pieces of cheese cut for this test show a certain variation in size and structure. The values shown in this table were calculated as the mean value from at least 4 measurements.

The following table illustrates the dry matter balance in this experiment

Dry matter balance

| **Product** | **dry matter** | **amount** | **total amount of dry matter** |
|---|---|---|---|
| Retentate | 18.1% | 80 kg | 14.5 kg |
| Cream | 45.0% | 20 kg | 9 kg |
| | | | |
| Cheese | 44.2% | 43 kg | 19 kg |
| Whey | 8.0% | 57 kg | 4.5 kg |
| | | | |
| Cheese | 40.4% | 48 kg | 19.4 kg |
| Whey | 8.0% | 52 kg | 4.1 kg |
| | | | |
| Cheese | 37.2% | 53 kg | 19.7 kg |
| Whey | 8.0% | 47 kg | 3.8 kg |

Accordingly, the surprising elevation of the amount of resulting cheese corresponds to lower dry matter content/higher water (or serum) content.

Example 4: Simultaneous addition of TG and rennet as compared to longer incubation times

The same product- and process-parameters according to example 2 were used in the following example, where the incubation time of TG was varied before rennet was added (preincubation of TG). In all cases 2 U TG/g protein and a protein concentration of 14 % was used. After the MF-concentrate was warmed up to 35 °C the starter culture and the TG was added at the same time. The starter culture was added to have comparable conditions with example 2 but due to the relatively short incubation times the starter culture does only play a minor role in this example. Rennet was added in one case 30 min after incubation of TG and starter culture, in one case 10 min after incubation of TG and starter culture, in one case 5 min after incubation of TG and starter culture, in one case 3 min after incubation of TG and starter culture and in

one case simultaneous with TG and rennet (no preincubation time). The following results were obtained:

| Sample no. | incubation time of TG and starter culture before rennet addition | gelling time after addition of rennet |
|---|---|---|
| 1 | 0 min | 37 ± 1 min |
| 2 | 3 min | 40 ± 1 min |
| 3 | 5 min | 40,5 ± 1 min |
| 4 | 10 min | 46 ± 1 min |
| 5 | 30 min | 54 ± 1 min |

The gelling time was visually controlled. The gelling time is the time until the curd is ready to be cut after rennet addition, as described in Example 3. The gel strength was measured by the texture analyzer additionally, as described in Example 2. The resistance force was measured immediately after the gelling time was visually proofed. All samples showed a resistance force of 19 g ± 5% when the gelling point was defined visually. This means in all cases the gel has reached a comparable resistance. The results of this example document, that a simultaneous addition, i.e. addition at the same time, of TG and rennet is most preferable, since a pre-incubation of TG before rennet addition leads to remarkable and undesirable elongation of the gelling time. Therefore the rennet should be added within 10 min or less after incubation with TG.

## Claims

1. Method for the preparation of visco-plastic food stuff, comprising the step of adding to a liquid, protein-enriched concentrate comprising at least 5% proteins and (a) reactive protein(s) for transglutaminase, a transglutaminase and a rennet or a rennet replacement which comprises a proteolytic enzyme,
whereby said transglutaminase and said rennet or said rennet replacement are added simultaneously to said protein-enriched concentrate comprising a reactive protein for transglutaminase.

2. The method of claim 1, wherein said protein-enriched concentrate is selected from the group consisting of micro-filtered milk, a protein-enriched dairy liquid, protein-enriched vegetable or fruit juices.

3. The method of claim 2, wherein said micro-filtered milk is obtainable by passing milk through a 0.05 – 0.2 μm membrane.

4. The method of claim 2, wherein said protein-enriched dairy liquid comprises at least 70% casein in said at least 5% total protein content.

5. The method of any one of claims 1 to 4, wherein said transglutaminase is a microbial tansglutaminase (TG) from Streptoverticillium or Bacillus subtilis.

6. The method of claim 5, wherein said microbial transglutaminase is transglutaminase from Streptoverticillium mobaraense.

7. The method of any one of claims 1 to 6, wherein said transglutaminase is a non microbial tansglutaminase and selected from the group consisting of fungi, plants or animals.

8. The method of claim 7, wherein said non-microbial transglutaminase from fungi is selected from the group consisting of actinomycetes and myxomycetes.

9. The method of any one of claims 1 to 8, wherein said transglutaminase is added to said protein-enriched concentrate in concentration of at least 0.1 U/g protein to 10 U/g protein transglutaminase/ g protein.

10. The method of any one of claims 1 to 9, wherein said rennet or rennet replacement comprising a proteolytic enzyme is selected from the group consisting of calf rennet, buffalo rennet, sheep rennet, goat rennet, microbial rennet, genetic rennet, recombinant rennet, chymosin preparations and fruit, vegetable or plant juice with renneting activity.

11. The method of claim 10, wherein said rennet is calf rennet.

12. The method of claim 10, wherein said fruit, vegetable or plant juice with renneting activity is selected from the group consisting of melon juice, papaya juice, proteinases from Opuntia ficus-indica fruits, extracts from berries of the plant Solanum dobium, pineapple pulp extracts, juices from Ash gourd, Cardoon extracts and extract from the flowers of wild globe artichokes.

13. The method of any one of claims 1 to 12, wherein said rennet or rennet replacement is added to said protein-enriched concentrate in concentration of 0.1 to 1 IMCU.

14. The method of any one of claims 1 to 13, wherein said method comprises an additional acidification of the protein-enriched concentrate before or during the simultaneous addition of transglutaminase and rennet or rennet replacement.

15. The method of claim 14, wherein said acidification of the protein-enriched concentrate comprises the adjustment of the pH to a value between 5.2 and 6.8.

16. The method of claim 14 or 15, wherein said acidification is obtained by the addition of starter culture or the addition of an organic acid.

17. The method of claim 16, wherein said starter culture is from or are mesophilic and/or thermophilic bacteria.

18. The method of claim 16, wherein said organic acid is citric acid, lactic acid, acetic acid, malic acid, formic acid or glucono δ lactone.

19. The method of any one of claims 1 to 18, wherein said protein-enriched concentrate comprises a casein/rest protein(s) ratio of at least 70/30.

20. The method of claim 19, wherein said rest protein(s) is/are whey proteins.

21. The method of claim 19 or 20, wherein said casein is added before said transglutaminase and said rennet or rennet replacement is added simultaneously to said protein-enriched concentrate.

22. The method of any one of claims 1 to 21, further comprising the addition of whey proteins and/or casein to the protein-enriched concentrate.

23. The method of claim 22, wherein said whey proteins are denatured.

24. The method of any one of claims 1 to 23, further comprising the addition of fat.

25. The method of claim 24, wherein the fat is added so that the ratio of fat content to total protein content in the liquid, protein-enriched concentrate is 2.5(fat):1(protein) at the most.

26. The method of any one of claims 1 to 25 further comprising a step of adding glutathione before said transglutaminase and said rennet or rennet replacement is added simultaneously to said protein-enriched concentrate.

27. The method of any one of claims 1 to 26, wherein said simultaneous addition of said transglutaminase and said rennet to said protein-enriched concentrate comprises the addition of transglutaminase and rennet within 10 minutes or less.

28. The method of any one of claims 1 to 27, wherein said simultaneous addition of said transglutaminase and said rennet to said protein-enriched concentrate comprises the addition of said transglutaminase and said rennet at the same time.

29. The method of any one of claims 1 to 28 for the production of cheese or cheese curd, wherein said protein-enriched concentrate is microfiltered milk, wherein said transglutaminase is from Streproverticillim and wherein said rennet is calf rennet.

30. The method of any of the claims 1 to 29, wherein said (a) reactive protein(s) for transglutaminase is comprised in said at least 5% proteins of the liquid, protein-enriched concentrate.

31. Use of the visco-plastic food stuff as obtained by the method of any one of claims 1 to 30 for the preparation of cheese or cheese products.

32. The use of claim 18, wherein said cheese or cheese product is selected from the group consisting of quarg, rennet cheese, soft-cheese, semi-hard cheese and hard-cheese.

33. A kit for carrying out the method of any one of claims 1 to 32, said kit comprising transglutaminase as characterized in any one of claims 5 to 8 and comprising said rennet or rennet replacement as characterized in any one of claims 10 to 12.

Fig. 1

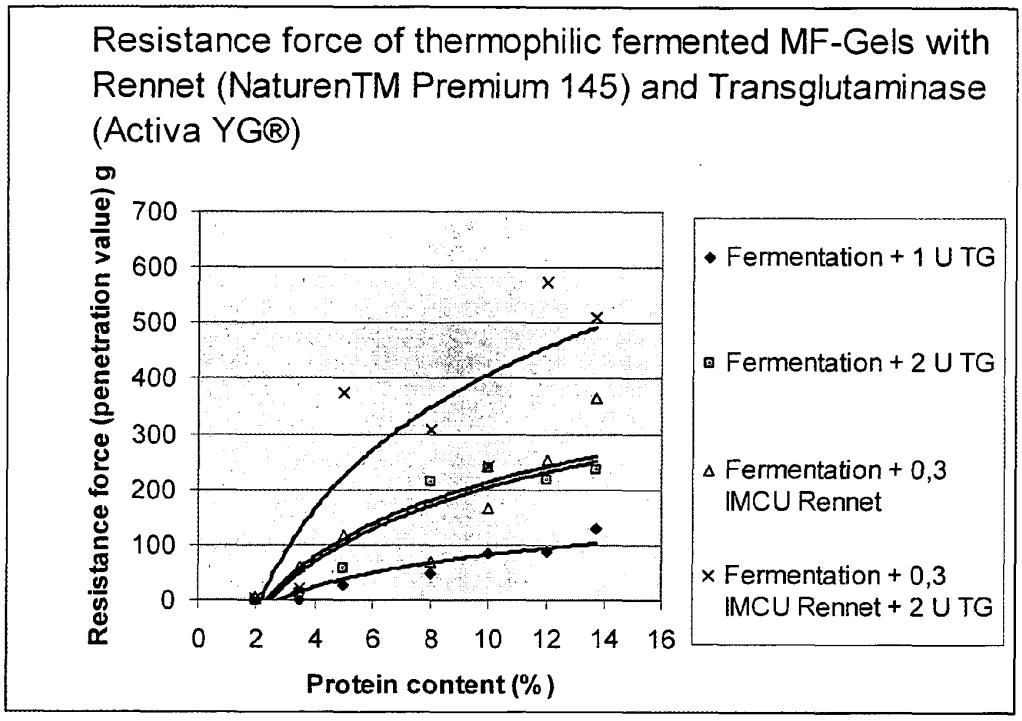
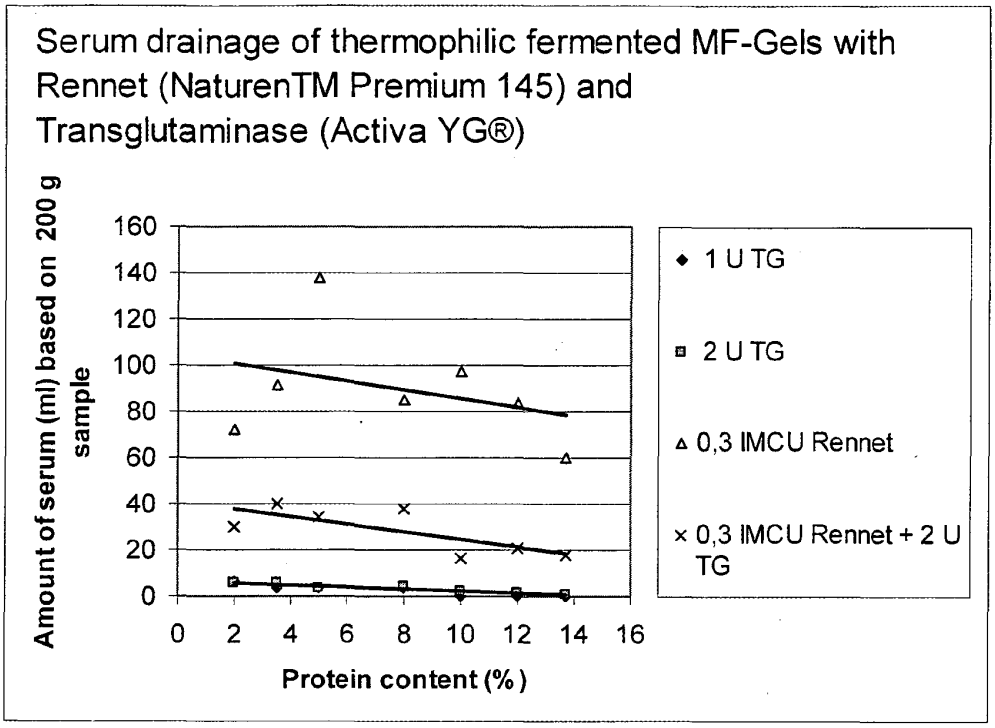

Resistance force of thermophilic fermented MF-Gels with Rennet (NaturenTM Premium 145) and Transglutaminase (Activa YG®)
Resistance force (penetration value) g
700
600
500
400
300
200
100
0
0 2 4 6 8 10 12 14 16
Protein content (%)
Fermentation + 1 U TG
Fermentation + 2 U TG
Fermentation + 0,3 IMCU Rennet
Fermentation + 0,3 IMCU Rennet + 2 U TG

Fig. 2


Serum drainage of thermophilic fermented MF-Gels with Rennet (NaturenTM Premium 145) and Transglutaminase (Activa YG®)
Amount of serum (ml) based on 200 g sample
160
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16
Protein content (%)
1 U TG
2 U TG
0,3 IMCU Rennet
0,3 IMCU Rennet + 2 U TG

# INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2007/007100

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23C19/032 A23C19/05 A23C19/068

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)
A23C

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, FSTA, BIOSIS, COMPENDEX

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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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 3 October 2007 | 11/10/2007 |

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

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Form PCT/ISA/210 (second sheet) (April 2005)

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International application No
PCT/EP2007/007100

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International application No
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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
13 March 2008 (13.03.2008)

PCT

(10) International Publication Number
WO 2008/030089 A1

(51) International Patent Classification:
*A23C 19/032* (2006.01) *A23C 9/158* (2006.01)
*A23C 9/12* (2006.01) *A23C 19/06* (2006.01)

(21) International Application Number:
PCT/NL2007/000215

(22) International Filing Date:
4 September 2007 (04.09.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
06120382.4 8 September 2006 (08.09.2006) EP

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, 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) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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).

**Published:**
— *with international search report*

WO 2008/030089 A1

(54) Title: USE OF YEAST AND BACTERIA FOR MAKING DAIRY PRODUCTS WITH IMPROVED FLAVOUR AND/OR TEXTURE QUALITY CHARACTERISTICS

(57) Abstract: The invention relates to the field of food production methods, whereby at least two micro-organisms are used, preferably a yeast and a bacterial strain.

Use of yeast and bacteria for making dairy products with improved flavour and/or texture quality characteristics

FIELD OF THE INVENTION

The present invention relates to the field of dairy production. Provided are methods for making fermented dairy products such as cheeses or cheese-derived products, like quarks, wherein mixtures of live, food-grade micro-organisms are used, especially a yeast strain, which is responsible for flavour formation, and at least one bacterial strain which is capable of controlling, especially reducing, the growth (or growth rate) of said yeast strain in such a way that flavour and/or texture quality characteristics are optimized. Also dairy products obtainable by using such micro-organisms are provided, as well as adjunct- or starter-cultures (in fresh, deep frozen or freeze dried formulation) or mixtures of adjunct cultures and starter cultures comprising such micro-organisms.

BACKGROUND OF THE INVENTION

Cheese production involves the clotting ("curdling") and acidification of milk to produce curd, by adding rennet, fermented produced chymosin (FPC) or coagulants from other sources (e.g. fungal) and starter cultures to the milk, respectively. The curd is then processed, for example by pressing to remove excess liquid (whey), before the ripening process is started during which the flavours and texture properties develop. Micro-organisms other then lactic acid bacteria (LAB) are also used in cheese production, such as Emmentaler, smear cheeses and soft cheeses. An example hereof is the production of Blue-veined cheeses like Roquefort and Danablu with yeasts and fungi. The yeasts are assumed to assist the development of fungi by gas production,, leading to curd openness in which the fungus is then able to grow. Both the yeast and the fungus are responsible for the flavour production of the soft cheese (Coghill, D. (1979). The Australian Journal of Dairy Technology, 34, 72-75; Fox, P.F. & Law,. J. (1991). Food Biotechnology, 5, 239-262.).

Up to now the use of fast $CO_2$ producing yeasts in semi-hard and hard cheeses is not feasible because it leads during ripening (especially during stages when the cheese texture becomes less flexible) to unwanted textural defects, such as crack formation, irregular eye formation and/or irregular eye distribution. In addition, some micro-

organisms cannot be used because their uncontrolled growth may result in a too high lipolytic activity, whereby "off-flavours" and/or "off-odors" are produced. The product is then said to be "rancid" and/or "soapy". Such rancidity and/or soapy taste is a result of the micro-organism producing too much lipase activity, whereby short chain fatty acids such as butyric acid are produced. Lipolysis and excessive lipolytic activity can be measured by determining the presence of free fatty acids.

Therefore, use of food grade micro-organisms for flavour development and/or flavour diversity in semi-hard and hard cheeses is currently restricted to strains which do not give undesirable side effects, such as undesirable textural characteristics (cracking, irregular eye formation and/or irregular eye distribution) and/or undesirable flavour/odor characteristics. The use of yeast species and other $CO_2$ producing micro-organisms, which are capable of producing interesting flavours, is therefore limited by the negative side effects of (i) excessive gas ($CO_2$) production which results in e.g. crack formation and therefore undesired textural properties and/or (ii) excessive lipolytic activity (Jakobsen, M. & Narvhus, J. (1996). International Dairy Journal, 6, 755-768). It is, therefore, desirable to provide methods for controlling the growth of such micro-organisms and the present invention provides methods and means for doing so.

Lourens-Hattingh and Viljoen (Food Microbiology 2002, 19: 597-604) found that the only solution to excessive gas formation in yoghurt production using yeasts *K. marxianus* and *I. orientalis* was to avoid using such yeast species and instead only rely on the inclusion of other yeast species in yoghurt production.

WO01/30172 describes the use of attenuated (at least 80% of the cells are killed) yeast cells (*Kluyveromyces lactis*), which are unable to grow but which deliver significant amount of enzymes, as adjunct culture for the production of cheeses and especially for the shortening of the ripening time and for enhancing the rate and diversity of cheese flavour. The document teaches that the addition of extra amounts of enzymes, either as such or within non-lysed dead or non-viable yeast cells, can be used to enhance the rate of flavour and texture formation in cheeses.

WO01/30171 describes methods for preparing attenuated adjunct cultures using microwave radiation.

GENERAL DEFINITIONS

"Dairy product" refers to milk-based or milk derived food products, such as yoghurt, soft, hard and semi-hard cheeses, butter, quark, buttermilk, etc.

"Dairy base" refers to the dairy base (especially milk) used as a basic ingredient during dairy food production.

"Cheese" refers to soft (Camembert, Brie, etc.), hard (Gruyere, Cheddar, Parmesan, etc.) and semi-hard cheeses (Gouda, Edam, Maasdam etc.).

"Cracking" refers to the appearance of cracks in cheeses due to gas formation.

"Off-flavour" or "rancidity" or soapy taste refers to undesired tastes and/or odors developed as a result of too much lipolytic activity of a micro-organism.

"Ripening" or "maturation" are used herein interchangeably and, with reference to cheese production methods, refers to the phase wherein the cheese is matured for days, weeks, months or even years (depending on the cheese).

"Micro-organism" refers herein to food grade micro-organisms, having a GRAS status (Generally Recognized As Safe) by the relevant national authority, such as the US Food and Drug Administration and being bacteria or fungi (e.g. yeasts, such as the unicellular yeast *Kluyveromyces lactis*). When referring to "a micro-organism" it is understood that generally a plurality of cells of a certain genus, species or strain are referred to. Food grade micro-organisms is understood to exclude food spoilage organisms and micro-organisms pathogenic to humans.

"Live" or "viable" refers to living micro-organisms, capable of growth under suitable conditions. Attenuated micro-organisms (80% or more of the cells being dead) are not included herein. However, viable frozen or freeze-dried micro-organisms are included.

"Growth" or "growth rate" of a micro-organism refers herein to either the multiplication of the cells (as determined by the cell count at one or more time points) and/or to the survival (or survival rate) of the cells (as determined by the cell count at one or more time points). When reference is made to one first micro-organism "controlling the growth of" another second micro-organism it is meant that the first micro-organism (or one or more inhibitory compounds produced by it) is capable of significantly influencing, especially reducing, the growth or growth rate (or survival or

survival rate) of the second micro-organism when compared to the growth/survival of the second micro-organism cultured under the same conditions but in the absence of the first micro-organism.

"Significantly influencing" or "significantly reducing" refers to a statistically significant effect, preferably a reduction of at least 2%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or even 100% more relative to a suitable control. A significant reduction may be determined for one or more characteristics measured or assessed during the production of a dairy product, such as (but not limited to) growth / growth rate of a micro-organism; as survival / survival rate of a micro-organism; one or more textural characteristics, such as gas formation, cracking, eye formation and distribution; development of rancidity or soapy taste; lipolytic activity, etc. Generally standard tests can be used to assess and/or quantify one or more of such characteristics. Such tests may be microbiological tests, molecular tests and/or human visual/tactile/organoleptic tests carried out by e.g. expert panels.

"Starter cultures" are cultures of one ("simple") or more ("mixed" or "compound") food-grade micro-organisms (especially LABs), each in a suitable concentration (optionally highly concentrated), suitable for being used in the production of dairy products. Starter cultures (or other live cultures according to the invention) may be fresh or frozen or freeze-dried (and regenerated). When cultures are frozen or regenerated, the viability after thawing or regeneration preferably result in a viability which is comparable to a fresh culture. Starter cultures are responsible for the acidification, flavour formation and textural properties (solid products like cheese, ed.) or consistency (liquid products like yoghurt, buttermilk ed.) properties of the dairy product.

"Solid dairy products" are herein products which have a ratio of water to protein (water/protein) which is smaller than 10.0. For example quark has a water:protein ratio of about 5.0, Feta of about 3.0; Gouda of about 1.5; and Gruyère of about 1.0. In contrast, yoghurts have a ratio of water to protein of around 16.0. (Walstra, P., Wouters, J.T.M. & Geurts, T.J., 2006. Dairy Science and Technology. 2nd ed. In: CRC Press, Taylor & Francis Group, Boca Raton. pp. 694-701).

"Adjunct cultures" are one or more food grade micro-organisms which are used in dairy products for special features, e.g. flavour diversification or bioconservation. They do not have the primary task of acidifying the milk.

"Fresh cultures" refers herein to freshly grown, live inoculum cultures of one or more micro-organisms.

"Frozen cultures" refers herein to deep frozen live cultures which become viable by defrosting them. Similarly "freeze-dried" cultures are cultures which become viable by reconstitution.

"Defined mixed strain culture" refers to both a physical mixture comprising e.g. at least two micro-organisms or at least two adjunct and/or starter cultures to generate a mixture, i.e. where each component comprises at least one strain and is still separate but will be admixed by either adding one component to the other or adding both at the same time or consecutively to a third component. A "defined mixed strain culture" may, thus, refer to a composition comprising at least two micro-organisms or to two compositions, to be admixed, each comprising at least one micro-organism.

"Direct Vat Inoculation" (DVI) is a starter culture or adjunct culture which is directly used for the inoculation of the milk

"Bulkstarter" is a deep frozen starter culture and used by dairy industry as inoculum for the preparation of a fresh starter culture.

"Pellets" are deep frozen droplets of micro-organisms.

In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

DETAILED DESCRIPTION

Cheese produced with a live *K. lactis* resulted in gas production and textural deficiencies, see Example 1. The present inventors found that this problem could be overcome by using mixtures of at least two species or strains of live, food-grade micro-organisms, wherein one micro-organism has as an inhibitory effect on the growth (and/or survival and/or metabolism) of the second micro-organism so that negative side effects (such as textural deficiencies and/or the development of off-flavours) of second micro-organism are significantly reduced or prevented in the dairy product.

Especially a mixture of at least one yeast and one bacterial strain, which controls the growth of the yeast strain, is suitably used in the production of dairy products according to the invention. When making cheeses, it was found that the addition of both an acid producing bacterium, such as *Propionibacterium*, and a flavour producing yeast strain resulted in good flavour/aroma characteristics (due to the yeast) and significantly enhanced texture characteristics (due to the *Propionibacterium* controlling the growth of the yeast) of the final cheese product (see Example 2). Especially irregular eye formation and distribution could be significantly reduced and the normal "cracking" caused by fast yeast growth and consequently a fast $CO_2$ production was not found, while the typical fruity flavour resulting from the production of flavour compounds, such as phenyl ethyl alcohol, produced by the yeast was only slightly reduced (without having an effect on the taste of the final product). This finding, that weak acid producing bacteria, such as *Propionibacteria*, can influence the growth of live yeasts during cheese production methods without reducing the taste of the end-product enables for the first time a much wider variety of viable yeast strains to be used, especially in the production of semi-hard and hard cheeses and a range of new flavour profiles may be generated by these yeasts in dairy products. In addition or alternatively, controlled growth of the yeast during various ripening stages enables not only a significant reduction (or elimination) of textural deficiencies, but can also be used to significantly reduce lipolytic activity of the yeast and the development of off-flavours. For example, certain yeast strains (e.g. of the species *Yarrowia lipolytica*) produce excessive lipase and their uncontrolled growth results in rancidity of the dairy product.

In the instant invention the outgrowth of viable yeast cells is controlled by inhibition by weak acids produced by the *Propionibacterium*. The undissociated weak acids diffuse into the yeast cell and decrease the internal pH. This leads to acidification of the cytosol and inhibition of metabolic pathways (Thomas, Hynes & Ingledew, Applied and Environmental Microbiology, April, 2002, 1616-1623). Holyoak *et al.* (Applied and Environmental Microbiology, September, 1996, 3158-3164) demonstrated that sorbic acid stimulates the plasma membrane $H^+$-ATPase activity of *Saccharomyces cerevisiae*. The synthesis of ATP appeared not to be influenced by the weak acid because oxygen consumption and $CO_2$ production were not affected significantly. It was suggested that cells cope with weak acid stress by using a larger part of the

generated ATP to export the protons instead of biomass formation. These results are in accordance with the study of Quintas, Leyva, Sotoca, Loureiro-Dias & Peinado (International Journal of Food Microbiology, 100, 2005, 125-130) which showed that the maintenance energy of the yeast *Zygosaccharomyces baillii* increased in the presence of benzoic acid. Lind, Jonsson & Schnürer (International Journal of Food Microbiology, 98, 2005, 157-165) studied the inhibition of *K. marxianus* by propionic acid, acetic acid and lactic acid and found that minimal inhibitory concentrations were 60, 80 and > 500 mM, respectively. In cheese lactic acid is the most abundant weak acid with a concentration of around 300 mM. To increase weak acid stress *Propionibacterium freudenrechii* can be used according to the invention to convert lactic acid into propionic acid and acetic acid. Experiments on synthetic media (Example 3) confirm that low levels of propionic acid, such as less than 18.7 mmol/kg already dramatically reduced the growth rate of *K. lactis* by at least 20% (≥20% and ≥50%, and ≥70% for 1.9 mmol/kg, 7.5 mmol/kg and 15.0 mmol/kg, respectively) and thereby also reduced gas formation significantly (Example 3).

Cheese trials (Example 2) have shown that after one week of cheese ripening at 13 °C propionic acid levels were just below 5 mmol per kg cheese moisture and acetate levels just below 10 mmol per kg cheese moisture in cheese comprising *Propionibacterium* and *K. lactis*. After two weeks levels of propionic acid were around 10 mmol per kg cheese moisture and acetate levels were around 15 mmol per kg cheese moisture. At a ripening temperature of 17 °C similar results were found, with levels of propionic acid being around 15 mmol per kg cheese moisture and levels of acetate around 20 mmol per kg cheese moisture after two weeks (see Fig. 4 and 5). The outgrowth of *K. lactis* in cheese produced with *Propionibacterium* (propionic acid and acetate) reduce the growth of the yeast strain to such an extent that cell count is reduced sufficiently (e.g. about a 10-fold reduction of yeast cell count in cheese ripened at 13 or 17 °C, especially during the first few weeks of ripening) in order to avoid loss of texture quality (such as the development of cracks and/or irregular eye formation and/or distribution) while at the same time the production of desired flavour compounds by the yeast cells is not reduced significantly (e.g. phenyl ethyl alcohol production is reduced by 50 % or less, such as 40, 30, 20, 10% or less compared to the amounts produced by the yeast cells in the absence of *Propionibacteria*). Importantly, the reduction in beneficial flavour compounds did not reduce the taste of the final product.

*K. lactis* strains do generally not have excessive lipolytic activity, so that their controlled growth does not influence the development of off-flavours. However, the bacteria used herein can effectively be combined with yeast species which do have excessive lipolytic activity. One can thereby significantly reduce the development of off-flavours/odors of such yeasts.

Thus, in one embodiment of the invention a method for producing dairy products is provided wherein a mixture of two live, food-grade micro-organisms is used in the production process:

(a) at least one viable, flavour-compound producing, food-grade micro-organism, preferably a yeast strain (referred to herein below as "micro-organism (a)"); and

(b) at least one viable, inhibitory (or inhibitory-component producing), food-grade micro-organism, which is capable of controlling (especially reducing) the growth of said first micro-organism by producing an inhibitory amount of one or more food-grade inhibitory compounds, preferably weak acids; whereby the concomitant culture of strain (b) with strain (a) results in strain (b) (or its acid fermentation products) being capable of controlling the growth or growth rate of strain (a) (referred to herein below as "micro-organism (b)").

Preferably the micro-organism (b) is a live and viable bacterial strain, such as *Propionibacterium* or *Acetobacter*, and preferably the addition of this micro-organism does not contribute in any significant way to flavour and/or textural properties of the dairy product as such, but does this only via influencing micro-organism (a) This can for example be tested by using organism (b) in the normal production of a dairy product and comparing the textural and flavour properties of this product to the reference product made without organism (b).

By controlling the growth of micro-organisms (a) (through addition of micro-organism b) during the production process of the dairy product, one or more of the following undesired negative properties resulting from organisms (a) can be significantly reduced or prevented:

i) the development of textural deficiencies, such as cracking of solid dairy products (such as cheeses, etc.); and/or (irregular) eye formation and/or distribution;

ii) gas ($CO_2$) production by micro-organism (a);

iii) the development of off-flavours and/or odors due to outgrowth (uncontrolled growth) of organism (a).

A "significant reduction" refers herein to a reduction of at least about 2, 5, 10, 15, 20, 30,40,50, 60, 70, 80, 90 or 100% compared to the reference dairy product (which is made using organism (a) without the use of micro-organism (b).

"During the production process" does herein not encompass post-production storage properties, such as for example acidification of yoghurt during storage. Also, the growth of the microorganism (a) is preferably not completely prevented, as during the production process growth does take place, but outgrowth (uncontrolled growth) is prevented and controlled by microorganism (b), through fermentation products such as weak acids being produced by (b). Thus, the growth rate of microorganism (a), rather than its growth as such, is controlled.

The micro-organism under (a) may be any food-grade (GRAS) micro-organisms producing flavour compounds, and is preferably a yeast species / strain. In a preferred embodiment it is a yeast strain of the genus *Kluyveromyces* (e.g. *K. lactis*; *K. marxianus*), preferably *K. lactis*, and/or of the genus *Yarrowia*, preferably *Y. lipolytica*. However, the strain may also be of the genus *Debaryomyces* (e.g. *Debaryomyces hansenii*), *Candida* (e.g. *Candida versatilis*), *Cryptococcus, Rhodotorula, Pichia, Trichosporon* (e.g. *Trichosporon beigelii*), *Torulaspora, Issatchenkia* (e.g. *Issatchenkia orientalis*), *Geotrichum*, *Saccharomyces* or *Zygosaccharomyces*. Such strains are available in the art and can be either obtained from deposit institutions or they can be isolated from food products. Examples of food grade yeasts are for example described in Lourens-Hattingh and Viljoen (2002, Food Microbiology 19: 597-604). Micro-organism (a) may, thus, be any live, food-grade micro-organism which is suitable for use in dairy products and which results in one or more of the above negative side effects when used in normal fermentation (without a micro-organism b). In a preferred embodiment only one species of microorganism (a) is comprised in the production process, e.g. only one yeast species, preferably only *K. lactis*.

The micro-organism under (b) may be any food-grade (GRAS) weak acid producing micro-organism, preferably a bacterial strain, most preferably of the genus *Propionibacterium* (e.g. *P. freudenreichii*), which produces propionic acid and acetate, or of the genus *Acetobacter*. Examples of *Propionibacteria* which may be used are strains of the species *Propionibacterium freudenreichii, Propionibacterium acidipropioni, Propionibacterium arabinosum, Propionibacterium thoenii* and/or *P. jensenii*, and others. Such bacterial strains are available in the art and can be either obtained from deposit institutions or they can be isolated from food products.

The second micro-organism (b) (e.g. bacterium) is capable of (significantly) controlling the growth/survival of micro-organism (a) and preferably produces one or more growth-inhibitory compounds, such as weak acids, to control the growth of the first micro-organism under (a) and to prevent its outgrowth and one or more undesired negative properties (see above) which would normally result when using the micro-organisms of (a) in the absence of the second micro-organism (b) in dairy production methods. The micro-organism (e.g. bacterium) preferably produces weak acids, such as propionic acid, acetate, and/or free fatty acids.

Obviously, the amount of weak acid produced depends on a large number of factors, such as the strains' species, strains' concentration used for production, production method of dairy product and ripening conditions (temperature etc.). However, routine experimentation can be used to determine whether a micro-organism (e.g. bacterium) is suitable for controlling (especially for reducing) the growth of the yeast strain under (a) during dairy production methods. Preferably, the micro-organism produces during the production process propionic acid at a level of at least 2 mmol per kg cheese moisture, preferably at least 4 mmol, 10 mmol, 15 mmol or 20 mmol per kg. The micro-oroganism need not produce more than 25 mmol propionic acid per kg cheese moisture, e.g. a production of about 20 mmol or less, is sufficient.

For example, one can compare the cell counts in a dairy product comprising both micro-organisms at various time points (e.g. during cheese ripening, see Examples) to the cell count of a suitable control dairy product, such as a cheese, lacking the micro-organism of (b). If the cell count of the micro-organism under (a) decreases faster or is reduced after a certain period in the presence of the micro-organism under (b)

compared to the control culture, then (b) has a significant effect on the growth of (a). For example it was found that during cheese ripening at 13°C or at 17°C, the presence of *Propionibacterium freudenreichii*, had the effect that the cell count of *K. lactis* decreased about 5 to 10-fold faster (and cell counts of yeast were 5x to 10x lower) compared to the control cultures lacking *Propionibacterium freudenreichii*. This effect was especially seen during the first weeks of ripening and is responsible for the absence of cracking in the cheeses.

The term "to control the growth of" refers herein to the capability of one micro-organism to reduce the growth / growth rate of another (measured e.g. as cfu/gram or cfu/ml of dairy product over at least two time points) by at least 2% or 5%, preferably by at least 10%, 20%, 30%, 40%, 50% or more, compared to a suitable reference dairy product.

The method comprises adding at least two micro-organism, at least one of (a) and one of (b) to base of the dairy product, such as the cheese milk. The production process is carried out using known methods. The two micro-organisms may be added together (as or to generate a defined mixed strain culture) or consecutively, within a short time period, such as several minutes or hours. The most suitable concentration of each of the strains may be determined experimentally, depending on the production method which is to be used and the growth characteristics of the micro-organisms. For example in the making of hard and semi-hard dairy products, such as cheeses, a mixture of about 6 x $10^5$ cfu per ml milk *K. lactis* and 1.5 x $10^6$ cfu per ml milk *Propionibacterium freudenreichii* was found to be suitable. However, other suitable amounts may range from about 1 x $10^4$ cfu/ml to about 1 x $10^{10}$ cfu/ml of each of micro-organism (a) and (b), such as at least about 1 x $10^5$ cfu/ml, 1 x $10^6$ cfu/ml, 1 x $10^7$ cfu/ml, 1 x $10^8$ cfu/ml, 1 x $10^9$ cfu/ml or more. The two micro-organisms may thus be present in ratios of 1 : 100 to 100 : 1, including values in between, e.g. 1 : 1, 2 : 1 or 1 : 2, 1: 5, 5: 1, 1: 10 or 10 : 1, etc.

The mixture of the two micro-organisms may conveniently be added to the dairy base at about the same time as the starter culture. For hard and semi-hard diary products, such as cheese production methods, it may be added to the dairy base (e.g. milk)

together with a commercial LAB starter culture (comprising e.g. *Lactococci, Lactobacilli , Leuconostoc* and/or *Strepococci*).

The two micro-organisms (a) and (b) can, therefore, conveniently be added to the dairy base at the beginning of the normal (known) production process of the dairy product, generally to the milk (e.g. the cheesemilk). Thus, other micro-organisms will normally be added also, especially LAB and/or other adjunct cultures (e.g. protective cultures; probiotic cultures), etc. In addition it is feasible to add more complex mixtures of micro-organisms, such as two or more species or strains of (a) and/or of (b).

The basic steps of the method thus comprises:

i) producing or providing an adjunct culture comprising micro-organisms (a) and/or (b); and/or a starter culture comprising micro-organism (a) and/or (b);

ii) adding the adjunct culture(s) and/or starter culture(s) to a dairy base so that a dairy base is provided which comprises at least organism (a) and (b);

iii) producing a final dairy product therefrom using known methods;

iv) optionally processing and/or packaging the final product further.

Adjunct cultures or starter cultures can be produced using known microbiological methods, whereby the micro-organism(s) is/are grown to a suitable concentration, is optionally harvested (e.g. by centrifugation) and suspended in appropriate medium and is then optionally deep frozen at an appropriate concentration and in an appropriate medium or freeze-dried.

In a preferred embodiment the method is a method for producing fermented dairy products such as cheese, preferably semi-hard or hard cheese, although a method for producing soft cheese is also encompassed herein. The starter culture is often prepared fresh in the dairy plant by using a Bulkstarter. In that case the fresh starter culture and the adjunct culture can be added to the milk at about the same time. The adjunct cultures, comprising strain (a) and/or (b), can be added as two single adjunct cultures or as a mixture containing both strains. Both micro-organisms will thus eventually be comprised within the same composition, even if they are added separately. Preferably,

both micro-organisms are present in one adjunct culture which can be used conveniently to add the two micro-organisms during the production process.

In case a DVI (Direct Vat Inoculation) starter culture is used, the starter and adjunct culture can still be used separately at the same time, or a mixture of the starter culture and adjunct culture can be use. Such a mixture of adjunct culture and/or starter culture is also an embodiment of the invention. Preferably it comprises the two micro-organism in suitable amounts to be used directly or in highly concentrated form which are diluted or split into portions prior to use as inoculum. A deep frozen adjunct culture or mixture of adjunct cultures may for example comprise one or more cartons with a weight between 50 to 500 or more grams pellets.

The dairy base comprising both micro-organisms is processed further to make the final product, such as cheese, and the further process may include one or more production steps, removal of whey and a maturation stage. The temperature used during maturation may vary, e.g. from 5 °C to 20 °C for semi-hard and hard cheeses. In cheese production, the method is especially suitable at cheese ripening temperatures below about 20°C, as at higher temperatures the yeast strain may show reduced growth and survival and the bacterial strain (e.g. *Propionibacterium*) would significantly contribute to flavour and texture development, which is undesirable). In general, the method is particularly useful at temperatures which would otherwise result in uncontrolled growth and/or $CO_2$ production and/or high lipolytic activity of the yeast strain used (and textural deficiencies and off-flavours and/or odors of the cheese), which may vary for different yeast genera and species or even strains.

The effect of strain (b) on the cell count of strain (a) is preferably determined at one or more time points during the maturation (ripening) stage. This stage may range from days to weeks or months or even years. Preferably the effect is determined during the phase where the yeast strain normally grows significantly if un-inhibited by the bacterium.

Defined mixed strain cultures, such as starter cultures and/or adjunct cultures, comprising a suitable amount of live or viable micro-organisms of (a) and (b), as

described above, (either as a mixture or in separate containers or compartments) are also an embodiment of the invention. Such cultures may be sold for the production of dairy products having new flavors and/or improved quality characteristics. The cultures may be provided in any form and volume, as described.

In another embodiments a dairy base, as well as intermediate and final products made according to the above methods are provided herein, which comprise an organism of (a) and (b). The presence of the micro-organisms can be determined using known methods, such as DNA based methods (PCR, nucleic acid hybridization, etc.) or microbiological methods. For example, specific strains can be distinguished and identified from other strains in this way.

The final dairy product can have a fruity flavour, at least equal to or better than the flavour of a suitable reference product, and is free of (or is reduced in) one or more negative side effects of micro-organisms (a), such as textural defects (e.g. cracks), off-flavours, etc. which reduce product quality. The amount of desired flavour compounds (e.g. phenylethylalcohol), acids produced by the bacterium (e.g. propionic acid and acetate) can also be quantified to define the product, see Fig. 4-6. The absence of off-flavours and/or odors can be determined using organoleptic evaluations by expert panels.

Dairy products include all solid fermented milk products with a protein to water ratio of at least 10.

The following non-limiting Examples describe the methods and products according to the invention. Unless stated otherwise, the practice of the invention will employ standard conventional methods of molecular biology, virology, microbiology or biochemistry.

EXAMPLES

EXAMPLE 1 – texture problems during cheese production using yeast

1.1 Method of cheese production

Semi-hard cheese was produced according standard production process with the non gas-producing mesofilic Bulkstarter containing *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* (commercially available under Code 61 at CSK Food Enrichment P.O. Box 225 8901 BA Leeuwarden, The Netherlands), either without the yeast strain *K. lactis CSK 1407* (0.3 %-v/v fresh inoculation) or with *K. lactis CSK* 1407 as adjunct culture, as described also in Example 2. The influence of gasformation on the cheese texture was evaluated after 6 and 13 weeks of ripening and graded by eye formation, eye distribution and eye shape.

1.2 Results

As shown below, cheese produced with a live *K. lactis* was found to lead to gas production and textural defects. Reference cheese produced with the non gas-producing mesofilic Bulkstarter without *K. lactis*. The experimental cheese was produced with fresh *K. lactis* cells as adjunct culture, resulting in gas formation and textural deficiencies, see Table 1 and 2 below.

The texture of the 6 and 13 week old cheeses was graded by an expert panel, whereby eye formation, eye distribution and eye shape, etc. were evaluated, indicating gas formation. It was found that the reference cheese did not produce any gas. The cheese produced with the live *K. lactis* had textural defects at both sampling times.

Table 1

| 6 weeks | reference | live *K. lactis* 0.3% |
|---|---|---|
| eye formation | 0 | 2.0 |
| irregular eye distribution | 0 | 0.4 |
| irregular eye shape | 0 | 0.6 |
| many small eyes | 0 | 0 |
| open texture | 0 | 0 |

score from 0 – 4; 0 = none (=good), 4 = a lot (=bad)

Table 2

| 13 weeks | reference | live *K.lactis* 0.3% |
|---|---|---|
| eye formation | 0.4 | 3.2 |

| | | |
|---|---|---|
| irregular eye distribution | 0 | 0 |
| irregular eye shape | 0 | 1.1 |
| many small eyes | 0 | 0 |
| open texture | 0 | 0 |

The activity of *K. lactis* was also reflected in the viable cell count. Viable cell count was carried out using standard procedure. Cells are extracted from the cheese by disolving 5 gram of cheese in 45 gram of 2 w/v-% sodium citrate and Stomachered for 5 minutes at 45 °C. The yeast cell count is determined on OGY agar (Oxytetracyclin Glucose Yeast) 5 days at 25 °C. Cell counts of *K. lactis* in the cheese milk and of 24 hours and 14 weeks old cheese are given in Table 3. It was found that cell count of *K. lactis* increases 10-fold from cheese milk to 24 hours old cheese which is partly due by the concentration of the cells in the curd.

Table 3

| cell count yeast *$10^5$ cfu/ml or cfu/gr | reference | live *K.lactis* 0.3% |
|---|---|---|
| cheese milk | <10 | 4.9 |
| 24 hour old cheese | <10 | 46 |
| 14 days in cheese | <10 | 47 |

EXAMPLE 2 – Controlled growth of yeast during cheese production

2.1. Material and Methods

Semi-hard cheeses (1.500 liter cheese milk) were produced with the commercial mesofilic L-starter containing *Lactococcus lactis* and *L. cremoris* and *Leuconostoc* sp. (commercially available under Code 19 at CSK Food Enrichment P.O. Box 225 8901 BA Leeuwarden, The Netherlands. The reference, vat 1, is inoculated with only the freshly prepared Bulkstarter, other vats are produced with the Bulkstarter and *Propionibacterium freudenreichii* (commercially available under Code 08 from CSK Food Enrichment P.O. Box 225 8901 BA Leeuwarden, The Netherlands) and the yeast strain *Kluyveromyces lactis CSK 1407*, see Table 4.

Table 4 – inoculum for cheese milk

| Vat | reference | 2 | 3 | 4 |
|---|---|---|---|---|
| Bulkstarter | 0.7 % | 0.5 % | 0.5 % | 0.5 % |
| *K. lactis* | - | - | $6.1*10^5$ cfu/ml | $6.1*10^5$ cfu/ml |
| *Propionibacterium* | - | $1.5*10^6$ cfu/ml | - | $1.5*10^6$ cfu/ml |

Cfu = colony forming units, cfu/ml = cfu per ml cheesemilk

The cheeses were ripened the first 6 weeks at 13 °C, 17°C or 20 °C and from weeks 6 to 13 at 13°C. Cell counts of *K. lactis* and *Propionibacterium* were performed on frozen cheese samples taken at 1, 2, 4, 6 and 13 weeks of ripening. The *K. lactis* and *Propionibacterium* cell count is determined on OGY agar (Oxytetracyclin Glucose Yeast) 5 days at 25 °C and LA (Lactate Agar, 7 days anaerobically at 30 °C), respectively. Phenylethylalcohol, propionic acid and acetate were determined at various stages during ripening.

2. 2 Results

2.2.1 Cheese production data

Production data are shown in Table 5. The pH decrease in all four cheeses was comparable. The vitality of the *K. lactis* is not reflected in the pH decrease because it does not produce acids. The pH decrease is the result of the conversion of lactose to lactate by the LABs of the Bulkstarter.

No remarkable differences between the vats was found and the chemical composition of the four cheeses was comparable.

Table 5 – cheese production data

| **cheese vat** | **vat 1** | **vat 2** | **vat 3** | **vat 4** |
|---|---|---|---|---|
| Bulkstarter | 0.7 % C19 | 0.5 % C19 | 0.5 % C19 | 0.5 % C19 |
| Adjunct-culture | - | *Propionibacterium* | *K. lactis* | ***Propionibacterium*** + ***K. lactis*** |
| pH after 4 hrs | 6,06 | 6,01 | 6,02 | 6,01 |
| pH after 5.5 hrs | 5,77 | 5,71 | 5,67 | 5,60 |

| | | | | |
|---|---|---|---|---|
| pH after 6 hrs | 5,64 | 5,60 | | 5,47 |
| pH after 6.5 hrs | 5,53 | 5,45 | 5,42 | |
| pH of the cheese after 24 hrs | 5,26 | 5,21 | 5,27 | 5,25 |
| Composition after 14 days: | | | | |
| Moisture content % | 41,8 | 42,4 | 41,6 | 42,1 |
| Fat content in dry matter % | 51,0 | 51,0 | 51,1 | 50,8 |
| Salt content in dry matter % | 3,29 | 3,31 | 3,03 | 3,40 |
| pH | 5,27 | 5,25 | 5,30 | 5,28 |

2.2.2 Cell counts of *K. lactis* and *Propionibacterium* during cheese ripening

The cell count development of *Propionibacterium* and *K. lactis* are given in Figures 1 to 3. Figure 1 shows the cell count at 13 °C ripening temperature (the number after the species name indicates the vat). The cheese milk was inoculated with 1.5 x $10^6$ cfu of *Propionibacterium* per ml. Assuming that all the cells are incorporated in the curd a cell count of 1.5 x $10^7$ cfu/gr per cheese is expected at the start of the ripening. The *Propionibacterium* cell count with and without *K. lactis* shows a stable level of 2 to 7 x $10^7$ cfu per gram cheese. The minimal growth of *Propionibacterium* is expected due to the low ripening temperature of 13 °C. After more then 2 weeks of ripening the cell count of *K. lactis* decreased. In the presence of *Propionibacterium* (lactis-4) the decrease of *K. lactis* cell count was found to be around 10-fold faster compared to the absence of *Propionibacterium* (lactis-3) and the decrease started at least about one week earlier.

This Example shows that *Propionibacterium* controls the growth of the yeast *K. lactis* and is capable of reducing the yeast cell count / growth rate by a factor 10 at a ripening temperature of 13 °C and leads to a decrease of *K. lactis* cell count at an earlier moment in ripening.

Figure 2 shows the cell count at 17 °C ripening temperature (the number after the species name indicates the vat). The *Propionibacterium* cell count in cheese ripened at 17 °C shows a similar trend as when ripened at 13 °C, see Figure 2. Also the effect of *Propionibacterium* on the cell count of *K. lactis* at 17 °C is comparable with that found at 13 °C ripening temperature above.

Figure 3 shows the cell count at 20 °C ripening temperature (the number after the species name indicates the vat). The *Propionibacterium* cell count of the cheese ripened at 20 °C increased during the first weeks to around 5 x $10^8$ cfu/gr. This 5- to 10-fold growth was not seen at a ripening temperature of 13 or 17 °C. The cell count of *K. lactis* was relatively low, both with and without *Propionibacterium*, with a maximum of around 1 x $10^6$ cfu per gram cheese.

This Example shows that the effect of *Propionibacterium* on the yeast growth is especially effective when ripening temperatures and conditions used are such that the yeast strain has a relatively good survival under the conditions used. When cheese was ripened at 20 °C, no outgrowth of the *K. lactis* strain used herein occurred and consequently no significant impact of *Propionibacterium* on *K. lactis* was therefore observed.

2.2.3 Propionic acid concentration in cheese

Fat and free fatty acids are extracted from the cheese samples and are isolated with capilar colum. The propionic acid concentration was determined using Gas Chromatography (GC).

Figure 4 shows the propionic acid concentration after 1, 7 and 14 days of ripening in the different cheeses, expressed as mmol per kg cheese moisture.

The propionic acid concentration increased during the ripening and is higher when the cheeses are ripened at evaluated temperatures. No influence of *K. lactis* was found on the propionic acid production of *Propionibacterium*.

Propionic acid was produced in the vats with *Priopionibacteria*, although the concentrations which were found were much lower than the spoilage micro-organism-inhibiting concentrations described in the literature (Lind et al., 2005. International Journal of Food Microbiology 98, 175-165). Therefore the minimal inhibitory level of propionic acid for controlling yeast growth in cheese appears to be below 20mM or

below 10mM after two weeks or one week ripening (respectively) at temperatures below 20°C (see Fig. 4).

2.2.4 Acetate concentration in cheese

Fat and free fatty acids are extracted from the cheese samples and are isolated with capilar colum. Acetate concentration was determined using Gas Chromatography (GC).

Figure 5 shows the amount of actetate produced in mmol per kg cheese moisture. The amount was found to be below 20mM after 2 and 1 week of ripening at temperature below 20°C (see Fig. 5).

2.2.5 Production of Phenyl ethyl alcohol in cheeses

The phenyl ethyl aclohol concentration was determined using Purge&Trap – Thermal Desorption-Gas Chromatographic- Mass Spectronomy (PT-TD-GC-MS). Cheese samples are disolved in water with the Stomacher, volatiles are released by extraction with Helium of 42 °C and are captured on Tenax column. The captured volatiles are subsequently desorbated at high temperatures, separated with a capillary colom and identified and quantified with mass spectronomy.

Figure 6 shows the amount of the flavour compound phenyl ethyl alcohol produced by the yeast and expressed as mg per kg in the cheese moisture.

In Figure 6 the phenyl ethyl alcohol concentration in 13 week old cheese ripened at 13, 17 and 20 °C is given. As expected, no phenyl ethyl alcohol is found in the cheeses produced without *K. lactis*. Further is show in Figure 6 that higher ripening temperatures results in a lower phenyl ethyl alcohol concentration. The phenyl ethyl alcohol concentration in the cheese with *Propionibacterium* ripened at 13 and 17 °C was around 25 and 50 % lower compared with the cheeses ripen at 20 °C, respectively. The influence of the ripening temperature on the phenyl ethyl alcohol concentration is comparable with the effect on cell count development.

The reduction of phenyl ethyl alcohol due to the presence of *Propionibacterium* did not significantly reduce the flavour of the cheeses ripened at 13 or 17°C, as shown herein below.

2.2.6 Organoleptic evaluation of the cheeses

The cheese matured at 13 °C was organoleptic evaluated by an expert panel after 13 weeks of ripening. A fruity and yeasty flavour was found in the cheeses produced with *K. lactis* alone and *K. lactis* and *Propionibacterium* ripened at 13 or 17 °C. The aroma profile of the cheeses ripened at 13 and 17°C was not influenced by *Propionibacterium*. Only at 20 °C *Propionibacterium* had an impact of the flavour.

2.2.7 Texture evaluation of the cheese

The cheeses made with only *K. lactis* have significant textural deficiencies, while the cheeses made with *K. lactis* and *Propionibacterium* ripened at 13 and 17°C have good textural characteristics (no cracking).

Example 3 – Propionic acid reduces the growth rate and gas production rate of *K. lactis*.

3.1 Materials and methods

The effect of propionic acid (0, 1.9, 7.5, 15 and 30 mmol/kg medium) on the growth rate of *K. lactis* CSK 1407 was determined in M17 (Oxoid) with 3 % glucose. After addition of sodium propionate (BDH, England) the pH of M17 was adjusted to 5.2 with 5 M HCl. The medium was inoculated with $1.5*10^6$ cfu $mL^{-1}$ *K. lactis* cells and incubated at 30 °C. The culture was kept homogenous by stirring (100 rpm). The growth was followed by measuring the optical density ($OD_{600}$) in quadruple during the incubation. The maximum growth rate ($\mu$, expressed as $h^{-1}$) of *K. lactis* was calculated ($\mu = \ln(OD_{t=t}/OD_{t=0})/\Delta t$) during the exponential growth phase under the said different propionic acid concentrations. The effect of propionic acid (0, 1.9, 7.5 and 15 mmol/kg medium) on the gas production rate ($r_{S,gas}$) by *K. lactis* was determined in Nilac (Nizo food research, The Netherlands) milk. Bottles (270 mL) were filled with 200 mL Nilac milk after the addition of sodium propionate (BDH, England) the pH was adjusted to 5.4 with 4 M lactic acid (Boom, The Netherlands). Gas production was followed by measuring the pressure development (P, expressed as N $m^{-2}$) in the headspace with

Oxitop OC110 system (WTW, Germany) during incubation at 13 °C. The maximum gas production rate was calculated as $r_{S,gas} = \Delta P/\Delta t$ and expressed as N $m^{-2}$ $h^{-1}$.

3.2 Results

Figure 7 shows the influence of propionic acid on the growth rate [1/h] (♦) of *K. lactis* grown in M17 + 3% glucose (pH 5.2) at 30 °C and on the gas production rate [$10^{-3}$ N $m^{-2}$ $h^{-1}$] (■) development by *K. lactis* in Nilac milk at 13 °C.

The influence of propionic acid on the growth rate of *K. lactis* was studied in synthetic media under low oxygen tension. It was found that under optimal conditions, so without inhibition by propionic acid *K .lactis* had growth rate of 1.4 $h^{-1}$. In the presence of 1.9 mM and 7.5 mmol $kg^{-1}$ propionic acid the growth decreased with 21 and 50 % to a growth rate of 1.1 and 0.7 $h^{-1}$, respectively (Fig. 7). A further reduction of the growth rate to 0.3 $h^{-1}$ is found at a concentration of 30 mmol $kg^{-1}$.

The impact of propionic acid on the gas production rate was studied in milk incubations at 13 °C, which is a normal ripening temperature for semi-hard cheese. Without propionic acid the gas production rate is $13*10^{-3}$ N $m^{-2}$ $h^{-1}$. In the presence of 1.9 mmol $kg^{-1}$ and 15 mmol $kg^{-1}$ propionic acid the gas production rate decreases with 15 and 39 % to $11*10^{2}$ and $8*10^{2}$ N $m^{-2}$ $h^{-1}$, respectively (Fig. 7).

The results show that both the growth rate and gas production rate decreases dramatically already at low propionic acid concentrations under conditions comparable to ripening.

# CLAIMS

1. A method for making a solid dairy product comprising adding at least two live food grade micro-organisms to a dairy base, wherein said two micro-organisms are at least one a yeast strain selected from the genus *Kluyveromyces* and at least one bacterial strain selected from the genus *Propionibacterium* and wherein said bacterial strain controls the growth and/or survival of said yeast strain during the production process of said dairy product.

2. The method according to claim 1, wherein said dairy product develops a significantly reduced amount of one or more negative side effects selected from the group consisting of: development of $CO_2$, development of textural deficiencies such as cracks, development of off-flavours and/or off-odors.

3. The method according to claim 1 or 2 wherein said yeast strain belongs to the species *Kluyveromyces lactis*.

4. The method according to any one of claims 1 to 3, wherein said bacterial strain belongs to the species *Propionibacterium freudenreichii*.

5. The method according to any one of the preceding claims, wherein not more than one yeast species is added.

6. The method according to any one of the preceding claims, wherein the ratio of said two micro-organisms is equal to or between 100 : 1 to 1 : 100.

7. The method according any one of the preceding claims, wherein each of said micro-organisms is added in an amount equal to or between $1x10^4$ to $1x10^{10}$ cfu/ml.

8. The method according to any one of the preceding claims wherein the growth of said yeast strain is reduced at least 2% compared to the growth of said yeast strain in a reference dairy product lacking said bacterium.

9. The method according to any one of the preceding claims, wherein said dairy product is semi-hard or hard cheese.

10. A solid dairy product obtainable according to any one of the preceding claims or a part thereof.

11. A dairy base, preferably milk, comprising a live food grade bacterial strain and a live food grade yeast strain, wherein the bacterial strain is of the genus *Propionibacterium* and the yeast strain is of the genus *Kluyveromyces*.

12. An adjunct culture or starter culture for the production of fermented dairy products comprising a mixture of at least one live food grade yeast strain selected from the genus *Kluyveromyces* and at least one live food grade bacterium selected from the genus *Propionibacterium*, wherein said bacterium is capable of reducing the growth and/or survival of said yeast strain during the production process of said fermented dairy product.

13. The adjunct culture or starter culture according to claim 12, wherein the yeast strain and bacterium strain are separated in two different compartments or containers.

14. Use of a food grade *Propionibacterium* or *Acetobacter* bacterium for controlling, preferably reducing, the growth and/or survival of a live, food grade yeast strain during the production process of a solid, fermented dairy product, preferably cheese.

15. Use according to claim 14, wherein said solid dairy product is semi-hard or hard cheese, such as Gruyere, Cheddar, Parmesan, or Gouda.

Fig 1

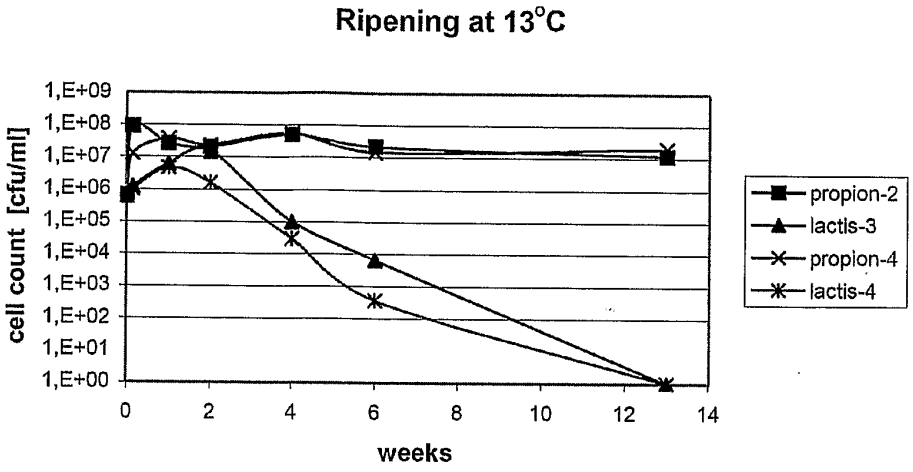

Ripening at 13°C
cell count [cfu/ml]
1,E+09
1,E+08
1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
0
2
4
6
8
10
12
14
weeks
propion-2
lactis-3
propion-4
lactis-4

Fig 2

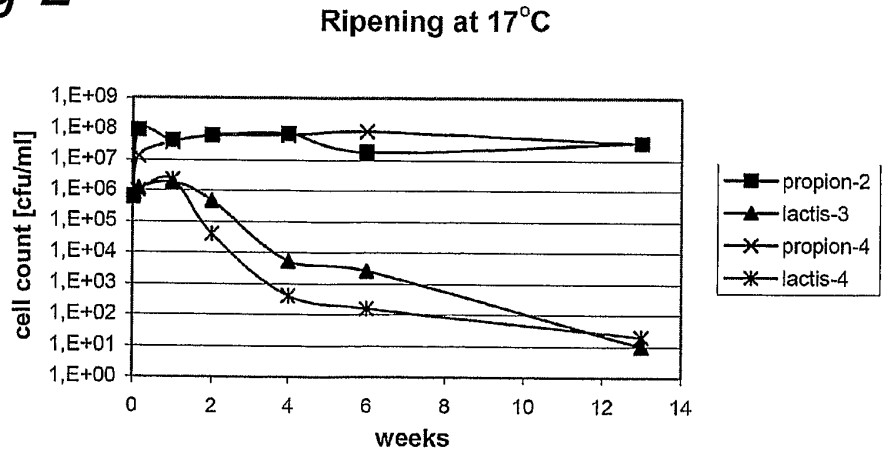

Ripening at 17°C
cell count [cfu/ml]
1,E+09
1,E+08
1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
0
2
4
6
8
10
12
14
weeks
propion-2
lactis-3
propion-4
lactis-4

Fig 3

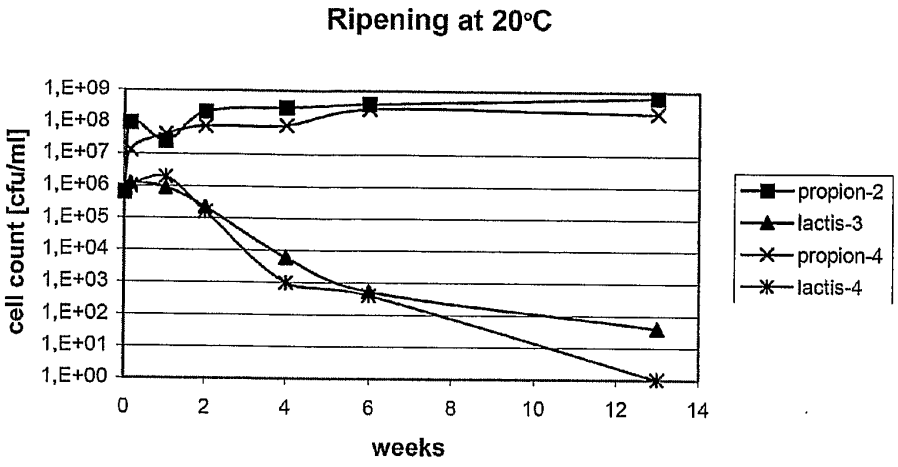

Ripening at 20°C
cell count [cfu/ml]
1,E+09
1,E+08
1,E+07
1,E+06
1,E+05
1,E+04
1,E+03
1,E+02
1,E+01
1,E+00
0
2
4
6
8
10
12
14
weeks
propion-2
lactis-3
propion-4
lactis-4

Fig 4

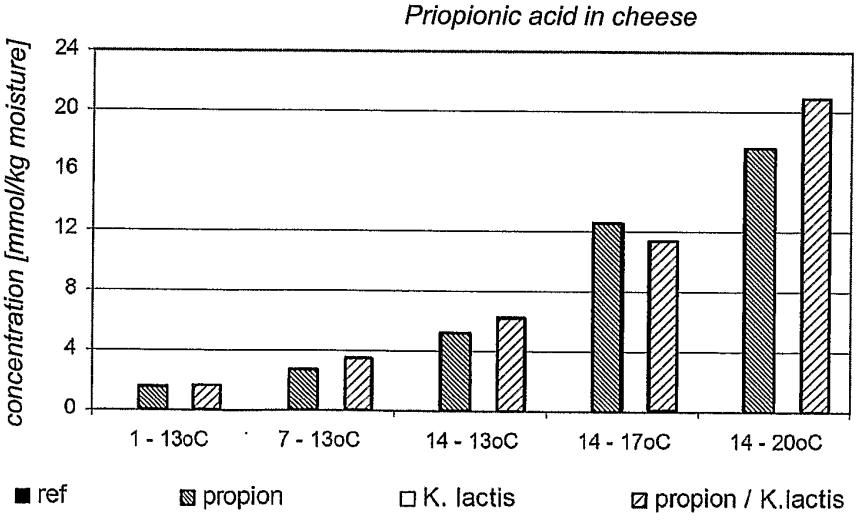

Priopionic acid in cheese
concentration [mmol/kg moisture]
24
20
16
12
8
4
0
1 - 13oC
7 - 13oC
14 - 13oC
14 - 17oC
14 - 20oC
ref
propion
K. lactis
propion / K.lactis

Fig 5

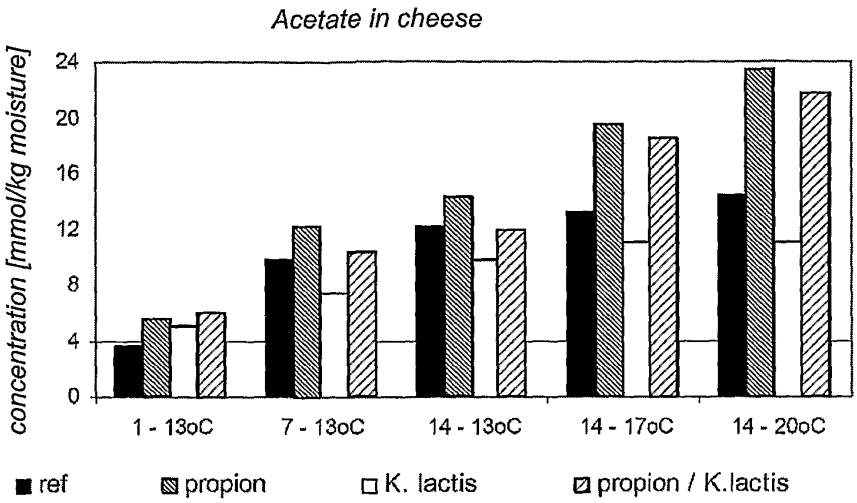

Acetate in cheese
concentration [mmol/kg moisture]
24
20
16
12
8
4
0
1 - 13oC
7 - 13oC
14 - 13oC
14 - 17oC
14 - 20oC
ref
propion
K. lactis
propion / K.lactis

Fig 6

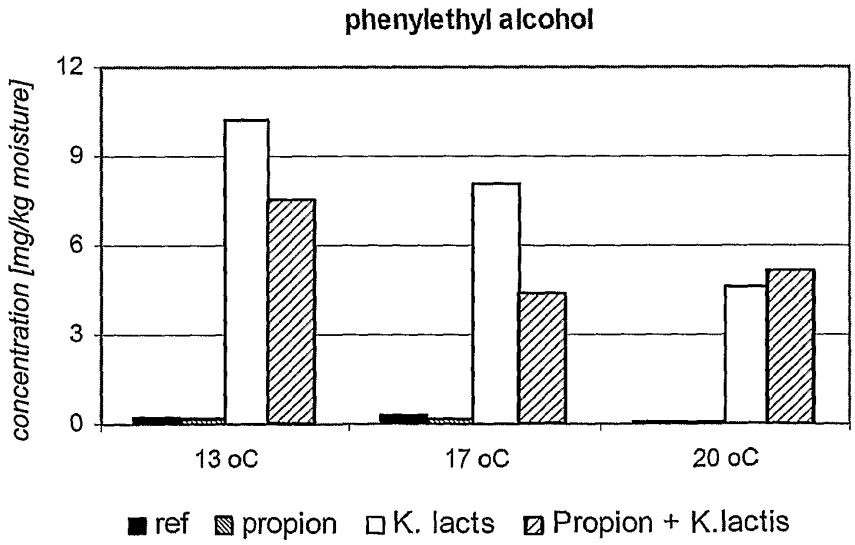
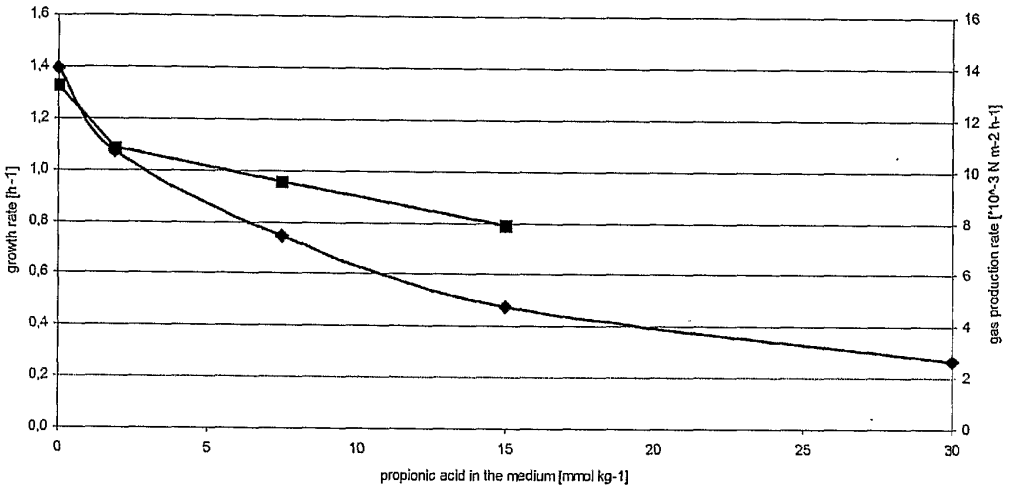

phenylethyl alcohol
concentration [mg/kg moisture]
12
9
6
3
0
13 oC
17 oC
20 oC
ref
propion
K. lacts
Propion + K.lactis

Fig 7


1,6
1,4
1,2
1,0
0,8
0,6
0,4
0,2
0,0
growth rate [h-1]
16
14
12
10
8
6
4
2
0
gas production rate [*10^-3 N m-2 h-1]
0
5
10
15
20
25
30
propionic acid in the medium [mmol kg-1]

# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2007/000215

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23C19/032 A23C9/12 A23C9/158 A23C19/06

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)
A23C

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, FSTA, EMBASE, 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 FSTA [Online]<br>INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANKFURT-MAIN, DE; 2001,<br>EFFAT B A ET AL: "Comparison of antifungal activity of metabolites from Lactobacillus rhamnosus, Pediococcus acidilactici and Propionibacterium thoenii."<br>XP002456558<br>Database accession no. 2002-00-p0700<br>abstract<br>& EFFAT BA ET AL.: EGYPTIAN JOURNAL OF DAIRY SCIENCE,<br>vol. 29, no. 2, 2001, pages 251-262,<br>FOOD & DAIRY SCI. DEP., NAT. RES. CENT., DOKKI, CAIRO, EGYPT<br>-----<br>-/-- | 1-15 |

[X] Further documents are listed in the continuation of Box C. [ ] 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 |
|---|---|
| 25 October 2007 | 12/11/2007 |

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

Form PCT/ISA/210 (second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No
PCT/NL2007/000215

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]<br>INTERNATIONAL FOOD INFORMATION SERVICE (IFIS), FRANKFURT-MAIN, DE; 1996,<br>ABD-EL-GHANI S ET AL: "Effects of Propionibacterium freudenreichii subsp. shermanii on the growth and viability of yeasts in yoghurt like products."<br>XP002412259<br>Database accession no. 97-1-07-p0158<br>abstract<br>& EGYPTIAN JOURNAL OF DAIRY SCIENCE 1996<br>DEP. OF FOOD TECH. & DAIRYING,,<br>vol. 24, no. 2, 1996, page 247,<br>DOKKI, CAIRO, EGYPT | 1-15 |
| Y | DAS S ET AL: "Effect of yeast and bacterial adjuncts on the CLA content and flavour of a washed-curd, dry-salted cheese"<br>INTERNATIONAL DAIRY JOURNAL, ELSEVIER APPLIED SCIENCE, BARKING,, GB,<br>vol. 15, no. 6-9, June 2005 (2005-06), pages 807-815, XP004978605<br>ISSN: 0958-6946<br>page 808, right-hand column, paragraph 2.3<br>page 811, left-hand column, paragraph 3.4 | 10-13 |
| Y | MARTIN N ET AL: "FLAVOR GENERATION IN CHEESE CURD BY COCULTURING WITH SELECTED YEAST, MOLD AND BACTERIA"<br>JOURNAL OF DAIRY SCIENCE, AMERICAN DAIRY SCIENCE ASSOCIATION, SAVOY, IL, US,<br>vol. 82, no. 6, June 1999 (1999-06), pages 1072-1080, XP000831306<br>ISSN: 0022-0302<br>page 1072; figures 1,4 | 10-13 |

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL2007/000215

**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 additional sheet

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

2. ☒ As all searchable claims could be searched without effort justifying an 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 reportcovers 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 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)

**FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210**

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-10

   Method of making solid dairy products using a strain selected from the genus Kluyveromyces and a strain selected from the genus Propionibacterium.

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2. claims: 11-13

   A dairy base or an adjunct culture comprising a strain selected of the genus Kluyveromyces and a strain selected from the genus Propionibacterium.

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3. claims: 14, 15

   Use of Propionibacterium or Acetobacter bacterium for controlling growth and/or survival of a live food grade yeast strain during the production of a solid fermented dairy product.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 July 2008 (10.07.2008)

PCT

(10) International Publication Number
WO 2008/083117 A2

(51) International Patent Classification:
*A23J 1/14* (2006.01) *A23J 3/26* (2006.01)
*A23J 1/12* (2006.01) *A23L 1/00* (2006.01)
*A23J 3/14* (2006.01) *A23L 1/272* (2006.01)

(21) International Application Number:
PCT/US2007/088696

(22) International Filing Date:
21 December 2007 (21.12.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/882,662 28 December 2006 (28.12.2006) US
11/963,375 21 December 2007 (21.12.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, 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) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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,

*[Continued on next page]*

(54) Title: GROUND MEAT AND MEAT ANALOG COMPOSITIONS HAVING IMPROVED NUTRITIONAL PROPERTIES



(57) Abstract: The invention provides ground meat and meat analog compositions having reduced fat and cholesterol. The ground meat compositions comprise a structured plant protein product and optionally meat.

WO 2008/083117 A2

PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— *without international search report and to be republished upon receipt of that report*

# GROUND MEAT AND MEAT ANALOG COMPOSITIONS HAVING IMPROVED NUTRITIONAL PROPERTIES

## CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority from U.S. Provisional Application Serial No. 60/882,662, filed on December 28, 2006, which is hereby incorporated by reference in its entirety.

## FIELD OF THE INVENTION

[0002] The present invention provides ground meat compositions and meat analog compositions having improved nutritional properties. In particular, the ground meat compositions and meat analog compositions comprise a structured protein product and may optionally include meat.

## BACKGROUND OF THE INVENTION

[0003] Given the link between red meat and heart disease and colon cancer, the consumption of red meat has declined over the past thirty years. Despite this decline, however, beef remains the second highest source of protein in the US diet (chicken being the top source). In 2005, Americans on average consumed about 67 pounds of beef per person, with males in general consuming the most ground beef and male teenagers consuming about 95 pounds of beef per person (Davis and Lin 2005). Given the affinity that Americans (and increasingly, others around the world) have for beef patties, there is a need for healthy, reduced-fat beef patty products having the sensory properties (e.g., appearance, flavor, and texture) characteristic of all beef patties.

[0004] There have been many attempts to make a healthier beef patty, ranging from all vegetable protein patties to mixtures of beef and vegetable and/or dairy proteins. Many of these, however, lack the proper moisture, flavor, and texture to be accepted by most consumers. What is needed, therefore, is a healthy beef patty with lower levels of cholesterol and fat that not only has the taste and texture of an all beef patty, but also looks like an all beef patty. That is, the healthier beef patty should have a reddish color in the raw state and a brownish color in the cooked state, in addition to great flavor and texture characteristics.

## SUMMARY OF THE INVENTION

[0005] One aspect of the invention encompasses a ground meat composition. The ground meat composition comprises structured protein product, the product having protein fibers that are substantially aligned; meat; and an optional color composition having coloring agents selected from the group consisting of a thermally unstable pigment, a thermally stable pigment, and a reducing sugar.

[0006] Another aspect of the invention encompasses a meat analog composition. The meat analog composition comprises a structured plant protein product, the product having protein fibers that are substantially aligned, and an optional coloring composition having coloring agents as described above.

[0007] Another aspect of the invention provides a process for coloring a ground meat composition or meat analog composition. The process comprises contacting a mixture comprising structured protein product that optionally may include meat, with a coloring composition comprising beet, annatto, carmel coloring, dextrose, and an amino acid source.

[0008] A further aspect of the invention encompasses food products comprising ground meat compositions.

[0009] Other aspects and features of the invention are described in more detail below.

## REFERENCE TO COLOR FIGURES

[0010] The application contains at least one photograph executed in color. Copies of this patent application publication with color photographs will be provided by the Office upon request and payment of the necessary fee.

## FIGURE LEGENDS

[0011] **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.

[0012] **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.

[0013] **Figure 3** is a bar graph and table presenting the mean overall liking scores for two different beef/structured vegetable protein patty formulations (T5 and T6) and all beef control patties. The patties were precooked, frozen, and then warmed prior to analysis.

[0001] **Figure 4** is a bar graph depicting the mean "similarity to beef" scores for two different beef/structured vegetable protein patty formulations (T5 and T6) and all beef control patties.

[0002] **Figure 5** is a bar graph and table presenting the mean overall liking scores for 80% lean beef, 90% lean beef, beef/SVP 1/8" grind, and beef/SVP 3/16" grind patties. The patties were frozen in the raw state and then cooked prior to analysis.

[0003] **Figure 6** depicts photographic images of 80% lean all beef patties (left) and beef/SVP patties comprising 40% meat replacement (right). Panel A presents a surface view of raw patties. Panel B presents a surface view and Panel C present a cross-sectional view of patties cooked to an internal temperature of 165°F.

## DETAILED DESCRIPTION OF THE INVENTION

[0004] The present invention provides ground meat compositions or simulated ground meat compositions (meat analog compositions) and processes for producing each of the ground meat compositions. Typically, the ground meat composition will comprise animal meat and structured plant protein products having protein fibers that are substantially aligned. Alternatively, the simulated ground meat composition will comprise structured plant protein products having protein fibers that are substantially aligned. Advantageously, as illustrated in the examples, the ground meat compositions of the invention have improved nutritional properties, such as reduced fat and cholesterol, without sacrificing the flavor, texture, mouth feel, and aroma of ground animal meat.

### *(I) Structured Protein Products*

[0005] The ground meat compositions and simulated ground meat compositions of the invention each comprise structured protein products comprising protein fibers that are substantially aligned, as described in more detail in I (f) 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(e) below. Because the structured protein products have protein fibers that are substantially aligned in a manner similar to animal meat, the ground meat compositions of the invention generally have the texture, mouthfeel, and eating quality characteristics of compositions comprised of one hundred percent ground animal meat. The resulting products have the meat-like texture consumers desire in a meat or meat substitute product.

**(a) protein-containing starting materials**

[0006] 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 ground meat simulated meat compositions. 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.

[0007] 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.

[0008] While in some embodiments gluten may be used as a protein, it is also envisioned that the protein-containing starting 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, BetaGlucan manufactured by Takeda (USA), 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.

[0009] 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

[0010] 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.

[0011] 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.

[0012] 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 Manildra Gem of the West 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. 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.

[0013] Suitable examples of protein-containing material isolated from a variety of sources are detailed in Table A, which shows various combinations.

**Table A Protein Combinations**

| First protein source | second ingredient |
|---|---|
| soybean | wheat |
| soybean | dairy |
| 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 |

| | |
|---|---|
| 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 |
| 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 |

[0014] In each of the embodiments delineated in Table A, 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% starch 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

[0015] 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.

[0016] 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. It is also possible to use membrane filtered soy isolates. 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, SUPRO® EX45, SUPRO® 595, and SUPRO® 545. In an exemplary embodiment, a form of SUPRO® 620 is utilized as detailed in Example 8.

[0017] 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 55% of the

soy protein isolate by weight. The soy protein concentrate can be substituted for up to about 50% of the soy protein isolate by weight. It is also possible in an embodiment to substitute 40% by weight of the soy protein concentrate for the soy protein isolate. In another embodiment, the amount of soy protein concentrate substituted is up to about 30% of the soy protein isolate by weight. Examples of suitable soy protein concentrates useful in the invention include PROCON, ALPHA 12, and ALPHA 5800, which are commercially available from Solae, LLC (St. Louis, Mo.). If soy flour is substituted for a portion of the soy protein isolate, the soy flour is substituted for up to about 35% of the soy protein isolate by weight. The soy flour should be a high protein dispersidility index (PDI) soy flour.

[0018] Any fiber known in the art can be used as the fiber source in the application. 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 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. When present in the soy protein material, 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) reducing sugar**

[0019] The protein-containing material detailed in I(a) may optionally be combined with at least one reducing sugar and co-extruded. Alternatively, the reducing sugar may be combined with the structured protein product after its extrusion. Generally speaking, when the mixture of protein-containing material and reducing sugar is subjected to an elevated temperature, the mixture undergoes a Maillard reaction, which typically produces a product having a dark color (e.g., brown or tan) and savory

flavor. Without being bound by any particular theory, it is believed that the Maillard reaction is typically initiated by a non-enzymatic condensation of the reducing sugar, with a primary amine group that is present within the protein-containing material, to form a Schiff base; which then undergoes an Amadori rearrangement to regenerate carbonyl activity (see, e.g., Smith et al. (1993) Proc. Natl. Acad. Sci. USA 91, 5710-5714).

[0020] A variety of reducing sugars are suitable for use in the present invention to the extent the reducing sugar is capable of undergoing a Maillard reaction with protein-containing material when the combination is subjected to elevated temperature. The reducing sugar may be a monosaccharide, a disaccharide or a polysaccharide. Exemplary monosaccharide reducing sugars include pentoses and hexoses. Other suitable reducing sugars include ribose, xylose, arabinose, lactose, glyceraldehyde, fructose, maltose, and dextrose (glucose).

[0021] As will be appreciated by the skilled artisan the amount of reducing sugar combined with the protein-containing material can and will vary depending upon the desired color of the resulting product. For example, the amount of reducing sugar may range from about 0.001% to about 15% on a dry matter basis of the protein-containing materials. In another embodiment, the amount of reducing sugar may range from 0.05% to about 10% by weight on a dry matter basis of the protein-containing materials. In yet another embodiment, the amount of reducing sugar may range from about 0.05% to about 2% by weight on a dry matter basis of the protein-containing materials.

**(c) additional ingredients**

[0022] A variety of additional ingredients may be added to any of the combinations of protein-containing materials and reducing sugars 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 ground meat (animal meat) or simulated meat 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 that will be extruded.

**(d) moisture content**

[0023] As will be appreciated by the skilled artisan, the moisture content of the protein-containing materials and optional additional ingredients can and will vary depending on the thermal process the combination is subjected to e.g. retort cooking, microwave cooking, and extrusion. Generally speaking in extrusion applications, 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(e) and Example 8.

**(e) extrusion of the protein-containing material**

[0024] A suitable extrusion process for the preparation of structured protein products comprises introducing the protein material which includes plant protein material and optionally other 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 from a molten extrusion mass. The molten extrusion mass exits the extruder through an extrusion die forming an extrudate

comprising structured protein products having protein fibers that are substantially aligned.

(i) extrusion process conditions

[0025] 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.

[0026] A single-screw extruder could also be used in the present invention. Examples of suitable, commercially available single-screw extrusion apparatuses include the WENGER Model X-175, the WENGER Model X-165, and the WENGER Model X-85, all of which are available from Wenger Manufacturing, Inc.

[0027] 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 or counter rotating. 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 450 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.

[0028] The extrusion apparatus generally comprises a plurality of heating zones through which the protein mixture is conveyed under mechanical pressure prior to exiting the extrusion apparatus through an extrusion die. 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 at a temperature of from about 100°C to about 150°C. One skilled in the art could adjust the temperature either heating or cooling to achieve the desired properties. Typically, temperature changes are due to work input and can happen suddenly.

[0029] The pressure within the extruder barrel is typically between about 50 psig to about 500 psig preferably between about 75 psig to about 200 psig. Generally, the pressure within the last two heating zones is from about 100 psig to about 3000 psig preferably between about 150 psig to about 500 psig. 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.

[0030] Water is 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 15% to about 35% 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. Typically, less than about 1 kg of water per kg of protein is introduced to the barrel. Preferably, from about 0.1 kg to about 1 kg of water per kg of protein are introduced to the barrel.

(ii) optional preconditioning

[0031] In a pre-conditioner, the protein-containing material, reducing sugar and other ingredients (protein-containing mixture) are preheated, contacted with moisture, and held under controlled 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 100 to about 1300 revolutions per minute (rpm). Agitation must be high enough t to obtain even hydration and good mixing.

[0032] 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 25°C to about 80°C, more preferably from about 30°C to about 40°C in the preconditioner using appropriate water temperature.

[0033] Typically, the protein-containing pre-mix is conditioned for a period of about 30 to about 60 seconds, depending on the speed and the size of the pre-conditioner. 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.

[0034] The conditioned pre-mix typically has a bulk density of from about 0.25 $g/cm^3$ to about 0.6 $g/cm^3$. 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

[0035] 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.

[0036] Whatever extruder is used, it should be run in excess of about 50% motor load. The rate at which the pre-mix is generally introduced to the extrusion apparatus will vary depending upon the particular apparatus. Typically, the conditioned pre-mix is introduced to the extrusion apparatus at a rate of between about 16 kilograms per minute to about 60 kilograms per minute. In another embodiment, the conditioned pre-mix is introduced to the extrusion apparatus at a rate between 20 kilograms per minute to about 40 kilograms per minute. the conditioned pre-mix is introduced to the extrusion apparatus at a rate of between about 26 kilograms per minute to about 32 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.

[0037] 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. 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 450 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 50 to about 3000 psig, and more preferably an extruder barrel exit pressure of from about 600 to about 1000 psig is generated.

[0038] The extruder controls the temperature of the mixture as it passes through the extruder denaturing the protein in the mixture. The extruder includes a means for heating the mixture to temperatures of from about 100°C to about 180°C. Preferably the means for heating 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. For example, 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. For example, 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.

[0039] 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, wherein the die assembly consists of a die and a back plate. The back plate is attached to the inner face of the die for the purpose of directing the flow of material entering the die towards the die aperture(s). Additionally, the die assembly produces substantial alignment of the protein fibers within the plasticized mixture as it flows through the die assembly. The back plate in combination with the die creates a central chamber that receives the melted plasticized mass from the extruder through a central opening. From at least one central chamber, the melted plasticized mass is directed by flow directors into at least one elongated tapered channel. Each elongated tapered channel leads directly to an individual die aperture. The extrudate exits the die through at least one aperture in the periphery or side of the die assembly at which point the protein fibers contained within are substantially aligned. It is also contemplated that the extrudate may exit the die assembly through at least one aperture in the die face, which may be a die plate affixed to the die.

[0040] The width and height dimensions of the die aperture(s) are selected and set prior to extrusion of the mixture to provide the fibrous material extrudate with the desired dimensions. The width of the die aperture(s) may be set so that the extrudate resembles from a cubic chunk of meat to a steak filet, where widening the width of the

die aperture(s) decreases the cubic chunk-like nature of the extrudate and increases the filet-like nature of the extrudate. Preferably the width of the die aperture(s) is/are set to a width of from about 5 millimeters to about 40 millimeters.

[0041] The height dimension of the die aperture(s) may be set to provide the desired thickness of the extrudate. The height of the aperture(s) may be set to provide a very thin extrudate or a thick extrudate. Preferably, the height of the die aperture(s) may be set to from about 1 millimeter to about 30 millimeters, and more preferably from about 8 millimeters to about 16 millimeters.

[0042] It is also contemplated that the die aperture(s) may be round. The diameter of the die aperture(s) may be set to provide the desired thickness of the extrudate. The diameter of the aperture(s) may be set to provide a very thin extrudate or a thick extrudate. Preferably, the diameter of the die aperture(s) may be set to from about 1 millimeter to about 30 millimeters, and more preferably from about 8 millimeters to about 16 millimeters.

[0043] The extrudate can be 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). A delayed cut can also be done to the extrudate. One such example of a delayed cut device is a guillotine device.

[0044] The dryer, if one is used, generally comprises a plurality of drying zones in which the air temperature may vary. Examples known in the art include convection dryers. The extrudate will be present in the dryer for a time sufficient to produce an extrudate having the desired moisture content. Thus, the temperature of the air is not important; if a lower temperature is used (such as 50°C) longer drying times will be required than if a higher temperature is used. Generally, the temperature of the air within one or more of the zones will be from about 100°C to about 185°C. At such temperatures the extrudate is generally dried for at least about 45 minutes and more generally, for at least about 65 minutes. Suitable dryers include those manufactured by Wolverine Proctor & Schwartz (Merrimac, MA.), National Drying Machinery Co. (Philadelphia, PA), Wenger (Sabetha, KS), Clextral (Tampa, FL), and Buehler (Lake Bluff, IL).

[0045] Another option is to use microwave assisted drying. In this embodiment, a combination of convective and microwave heating is used to dry the product to the desired moisture. Microwave assisted drying is accomplished by simultaneously using forced-air convective heating and drying to the surface of the product while at the same time exposing the product to microwave heating that forces the moisture that remains in the product to the surface whereby the convective heating and drying continues to dry the product. The convective dryer parameters are the same as discussed previously. The addition is the microwave-heating element, with the power of the microwave being adjusted dependent on the product to be dried as well as the desired final product moisture. As an example the product can be conveyed through an oven that contains a tunnel that is equipped with wave-guides to feed the microwave energy to the product and chokes designed to prevent the microwaves from leaving the oven. As the product is conveyed through the tunnel the convective and microwave heating simultaneously work to lower the moisture content of the product whereby drying. Typically, the air temperature is 50°C to about 80°C, and the microwave power is varied dependent on the product, the time the oven is in the oven, and the final moisture content desired.

[0046] 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 less than 10% moisture and typically from about 5% to about 11% by weight, if dried. Although not required in order to separate the fibers, hydrating in water until the water is absorbed is one way to separate the fibers. If the protein material is not dried or not fully dried and is to be used immediately, its moisture content can be higher, generally from about 16% to about 30% by weight. If a protein material with high moisture content is produced, the protein material may require immediate use or refrigeration to ensure product freshness, and minimize spoilage.

[0047] 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), Fitzmill® manufactured by the Fitzpatrick Company (Elmhurst, IL), Comitrol® processors made by Urschel Laboratories, Inc. (Valparaiso, IN), and roller mills such as RossKamp Roller Mills manufactured by RossKamp Champion (Waterloo, IL).

**(f) characterization of the structured protein products**

[0048] The extrudates produced in I(e) 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. 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(e) 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.

[0049] In certain embodiments where the protein material is co-extruded with a reducing sugar, a Maillard reaction may occur, and the resulting structured protein products generally have a dark color. Depending upon the reaction conditions, the color can be optimized to match the color of a desired ground animal meat product. In some embodiments, the color may be a shade of brown, e.g., light brown, medium brown, and

dark brown. In other embodiments, the color may be a shade of tan, e.g., light tan, medium tan, and dark tan.

[0050] 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 6. 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.

[0051] 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. A method for determining shred characterization is

detailed in Example 7. 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.

[0052] 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 an 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. In a further embodiment, the structured protein products will have protein fibers that are at least 55% aligned, have average shear strength of at least 2400 grams, and have an average shred characterization of at least 20% by weight large pieces.

### *(II) Ground Meat and Meat Analog Compositions*

[0053] The structured protein products are utilized in the invention as a component in ground meat and meat analog compositions. A ground meat composition may comprise a mixture of animal meat and structured plant protein product, or it may comprise no animal meat and primarily structured plant protein product. The process

for producing the ground 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 ground meat.

**(a) optionally blend with animal meat**

[0054] The structured protein product may 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.

(i) animal meat

[0055] The animal meat compositions, in addition to structured plant protein product, also comprise animal meat. By way of example, meat and meat ingredients defined specifically for the various structured vegetable protein patents include intact or ground beef, pork, lamb, mutton, horsemeat, goat meat, meat, fat and skin of poultry (domestic fowl such as chicken, duck, goose or turkey) and more specifically flesh tissues from any fowl (any bird species), fish flesh derived from both fresh and salt water fish such as catfish, tuna, sturgeon, salmon, bass, muskie, pike, bowfin, gar, paddlefish, bream, carp, trout, walleye, snakehead and crappie, animal flesh of shellfish and crustacean origin, animal flesh trim and animal tissues derived from processing such as frozen residue from sawing frozen fish, chicken, beef, pork etc., chicken skin, pork skin, fish skin, animal fats such as beef fat, pork fat, lamb fat, chicken fat , turkey fat, rendered animal fat such as lard and tallow, flavor enhanced animal fats, fractionated or further processed animal fat tissue, finely textured beef, finely textured pork, finely textured lamb, finely textured chicken, low temperature rendered animal tissues such as low temperature rendered beef and low temperature rendered pork, mechanically separated meat or mechanically deboned meat (MDM) (meat flesh removed from bone by various mechanical means)such as mechanically separated beef, mechanically pork, mechanically separated fish including surimi, mechanically separated chicken, mechanically separated turkey, any cooked animal flesh and organ meats derived from any animal species. Meat flesh should be extended to include muscle protein fractions derived from salt fractionation of the animal tissues, protein

ingredients derived from isoelectric fractionation and precipitation of animal muscle or meat and hot boned meat as well as mechanically prepared collagen tissues and gelatin. Additionally, meat, fat, connective tissue and organ meats of game animals such as buffalo, deer, elk, moose, reindeer, caribou, antelope, rabbit, bear, squirrel, beaver, muskrat, opossum, raccoon, armadillo and porcupine as well as well as reptilian creatures such as snakes, turtles and lizards should be considered meat.

[0056] It is also envisioned that a variety of meat qualities may be utilized in the invention depending upon the product's intended use. For example, intact muscle or whole muscle meat that are either ground or in chunk or steak form may be utilized. In an additional embodiment, mechanically deboned meat (MDM) may be utilized. In the context of the present invention, "MDM" is a meat paste that is recovered from a variety of animal bones, such as, beef, pork and chicken bones, using commercially available equipment. MDM is generally a comminuted product that is devoid of the natural fibrous texture found in intact muscles. In other embodiments, a combination of MDM and whole meat muscle may be utilized.

[0057] Typically, the amount of structured plant 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 predominantly structured vegetable or plant protein composition that has a relatively small degree of animal flavor is desired, the concentration of animal meat in the ground meat composition may be about 45%, 40%, 35%, 30%, 25%, 20%, 15%, 10%, 5%, 2%, or 0% by weight. Alternatively, when an animal meat composition having a relatively high degree of animal meat flavor is desired, the concentration of animal meat in the animal meat composition may be about 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95% by weight. Consequently, the concentration of structured plant protein product in the ground 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 ground meat composition will generally have from about 40% to about 60% by weight of the structured protein product and from about 40% to about 60% by weight of animal meat.

[0058] Depending upon the food product, the animal meat is typically pre-cooked to partially dehydrate the flesh and prevent the release of those fluids during

further processing applications (e.g., such as retort cooking), to remove natural liquids or oils that may have strong flavors, to coagulate the animal protein and loosen the meat from the skeleton, or to develop desirable and textural flavor properties. The pre-cooking process may be carried out in steam, water, oil, hot air, smoke, or a combination thereof. The animal meat is generally heated until the internal temperature is between 60°C and 85°C. In one embodiment, the animal meat composition is mixed with the hydrated structured plant protein at an elevated temperature corresponding to the temperature of the meat product.

**(III) Process for Producing Food Products Comprising Animal Meat and Simulated Meat Compositions**

[0059] Another aspect of the invention provides a process for producing food products comprising animal meat compositions. An animal meat composition may comprise a mixture of animal meat and structured plant protein product, or it may comprise structured plant protein product. The process generally comprises hydrating the structured plant protein product, reducing its particle size if necessary, optionally flavoring and coloring the structured plant protein product, optionally mixing it with animal meat, and further processing the composition into a food product.

**(b) hydrating and coloring the structured protein product**

[0060] The structured plant protein product may be mixed with water to rehydrate it. The amount of water added to the structured plant protein product can and will vary. The ratio of water to structured plant protein product may range from about 1.5:1 to about 4:1. In a preferred embodiment, the ration of water to structured plant protein product may be about 2.5:1.

[0061] The structured protein product is generally colored with a coloring composition so as to resemble raw ground meat and/or cooked ground meat. The coloring compositions of the invention may comprise a thermally unstable pigment, a thermally stable pigment, and/or a browning agent. 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 ground meat composition. When the ground meat composition simulates a "pre-cooked product," the structured plant product is typically contacted with a browning agent and/or a thermally stable pigment. Alternatively, when the ground

meat composition simulates raw meat, the structured protein product is generally contacted with a thermally unstable red pigment and also with a browning agent and/or a thermally stable pigment, such that when the ground 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.

[0062] A thermally unstable pigment may be used in the coloring composition to provide the red color of raw uncooked ground 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 20% of the coloring composition.

[0063] 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 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 D. D. Williamson Company, Louisville, Kentucky), and 5438 Caramel Powder D.S. (available from Sensient Colors, St. Louis, Missouri).

[0064] 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, cumin, saffron, yellow #6, and carotene. 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 0.01% 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 1% to about 3%, by dry weight of the coloring composition.

[0065] The coloring composition may include a browning agent. As detailed in I(b), 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. 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, 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 35% to about 45% by dry weight of the coloring composition.

[0066] In an alternative embodiment, the browning agent of the coloring composition may also include an amine source. Suitable amine sources include a

polypeptide material, a hydrolyzed protein material, or an amino acid material. The polypeptide material, hydrolyzed protein, and/or amino acid material are preferably included as an amine source in the browning agent to enhance the desired browning of the meat composition. In an exemplary embodiment, a hydrolyzed soy protein is the amino source in the browning agent. When included in the coloring composition, the amine source is generally present in the coloring composition from about .001% to about 55% of the coloring composition by dry weight.

[0067] 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 reducing sugar is dextrose, and 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.

[0068] 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, phosphoric acid, sorbic 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, as known in the industry, such as disodium biphosphate, sodium tripolyphosphate, and/or sodium hydroxide.

[0069] 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 1% 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.

**(c) optional blend with animal meat**

[0070] The structured protein product may be blended with animal meat as described in II above, 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.

**(d) reducing particle size**

[0071] Because the meat compositions are used in ground meat applications, the particle size of the structured plant protein product and animal meat, if present, is typically reduced to a relatively small particle size by passing the composition through though a meat grinder. The particle size can and will vary. In one embodiment, the particle size may be from about 1/16 of an inch to about 5/32 of an inch. In an exemplary embodiment, the particle size is from about 1/8 of an inch to about 1/4 of an inch.

**(e) addition of optional ingredients**

[0072] The ground meat compositions including simulated meat compositions or the compositions blended with animal meat, 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 ground meat composition can and will depend upon the food product to be manufactured.

[0073] The ground 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 ground meat composition will comprise from about 10% to about 20% by weight of a fat source.

[0074] The ground meat composition may also comprise an isolated soy protein. Typically, the isolated soy protein is added in an amount that is sufficient to impart improved texture to the ground meat composition. Methods for determining "texture improvement" are detailed in the Examples.

[0075] The ground 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 ground 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. The concentration of an antioxidant in the ground meat composition may range from about 0.0001% to about 20% by weight. In another embodiment, the concentration of an antioxidant in the ground meat composition may range from about 0.001% to about 5% by weight. In yet another embodiment, the concentration of an antioxidant in the ground meat composition may range from about 0.01% to about 1% by weight.

[0076] In an additional embodiment, the ground 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, spice extracts, spice oils, natural smoke solutions, or natural smoke extracts. Additional flavoring agents may include onion flavor, garlic flavor, or herb flavors. The ground 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, yeast extracts, Shiitake extracts, and hydrolyzed vegetable proteins. Examples of exemplary flavoring agents are described in the Examples.

[0077] In a further embodiment, the ground meat composition may be flavored through the addition of a flavored emulsion base, vegetable gum, and gelatin (flavored). Any known method may be used to produce the flavored emulsion base, for example U.S. patent 7,070,827 and U.S. published patent application 2006/0204644, hereby fully incorporated by reference, discloses a method for creating and including a flavor emulsion base.

[0078] In an additional embodiment, the ground 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 (carob bean, guar, tragacanth, and xanthan), pectins, sodium carboxymethylcellulose, and modified starches.

[0079] In a further embodiment, the ground meat compositions may further comprise a nutrient such as a vitamin, a mineral, an antioxidant, an omega-3 fatty acid, an autolysed yeast flavoring, 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, and potassium. Suitable omega-3 fatty acids include docosahexaenoic acid (DHA) and eicosapentanoic acid (EPA). Herbs that may be added include basil, celery leaves, chervil, chives, cilantro, parsley, oregano, tarragon, and thyme.

[0080] The ground meat compositions can be fortified with nutrients, such as vitamins, minerals, antioxidants, omega-3 fatty acids, or other nutrients typically found in animal meat products, to produce a food product with the desired nutrient value. The nutrients added to the ground meat and simulated meat are provided to create a product with a nutrient composition comparable to animal meat products. In an additional embodiment, the ground meat and simulated ground meat produced can be a nutraceutical. If a nutraceutical product is desired the type and amount of nutrients added will be such that the food product produced has a higher nutrient value than typical animal meat products. The types and amounts of nutrients added will depend on the desired end food product.

### *(IV) Food Products*

[0081] The ground meat compositions may be processed into a variety of food products having a variety of shapes. For example, the ground meat composition may be formed into a link, a patty, or into bulk packaging (i.e., chub and tube). In one exemplary embodiment, the ground meat composition is formed into a patty utilizing technology generally known in the art, such as a Formax F-6 fitted with a "Tenderform" forming plate. The patties may be pre-cooked fresh patties, pre-cooked frozen patties, raw frozen patties, and raw fresh patties. The patties may simulate the flavor and taste of a wide variety of all meat ground animal patties. Suitable patties may include beef patties (e.g., hamburger-like products), pork patties (i.e., sausage), lamb patties, and turkey patties.

[0082] In an exemplary embodiment, the ground meat composition will simulate ground beef. In one alternative of this embodiment, the ground beef product

will comprise beef meat, structured plant protein product, water, isolated soy protein, antioxidant, spices and flavoring. In another alternative of this embodiment, the ground beef product will comprise beef meat, structured plant protein product, water, antioxidant, spices and flavoring. In yet another alternative of this embodiment, the ground beef product will comprise beef meat, structured plant protein product, water, caramel coloring, antioxidant, spices and flavoring. In a further alternative of this embodiment, the ground beef product will comprise beef meat, structured plant protein product, water, isolated soy protein, antioxidant, spices, flavoring and a coloring composition comprising beet powder, annatto, caramel coloring reducing sugar, and an amino acid source. In still another alternative of this embodiment, the ground beef product will comprise beef meat, structured plant protein product, beef broth, isolated soy protein, antioxidant, spices, and flavoring. In an additional alternative of this embodiment, the ground beef product will comprise beef meat, structured plant protein product, beef broth, water, isolated soy protein, antioxidant, spices, and flavoring. In each of the foregoing embodiments, the beef composition comprises from about 40% to about 60% by weight beef, from about 40% to about 60% by weight hydrated structured plant protein product, and from about 1% to about 20% beef fat.

[0083] The invention also encompasses a variety of food products comprising the ground meat compositions. For example, the ground meat compositions may be utilized in meatloaf, meatballs, batter-breaded products, and restructured products. The invention also encompasses ground meat analog compositions comprising primarily structured protein product, flavorings, and colorings such that the composition will simulate ground meat.

## DEFINITIONS

[0084] 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.

[0085] 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 7 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.

[0086] The term "animal meat" as used herein refers to the flesh, whole meat muscle, or parts thereof derived from an animal including beef, pork, poultry, wild game, fish and combinations thereof.

[0087] 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.

[0088] 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, barley, corn gluten, or distillers grain products.

[0089] 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 7.

[0090] The term "protein fiber" as used herein refers 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.

[0091] The term "simulated" as used herein refers to an animal meat composition that contains no animal meat.

[0092] 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 protein material and soluble carbohydrates of the cotyledon.

[0093] 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.

[0094] 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.

[0095] 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.

[0096] 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 7 is performed.

[0097] 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.

[0098] 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.

[0099] The following examples are included to demonstrate preferred embodiments of the invention. It should be appreciated by those skilled 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

[00100] Examples 1-8 illustrate various embodiments of the invention.

### Example 1. Healthier Beef Patties comprising 40% Meat Replacement and Flavoring Agents.

[00101] One goal of this research was to develop a healthier beef patty in which part of the beef was replaced with hydrated structured vegetable protein (SVP) ingredient, such that a patty containing as little as 10% fat would be considered to taste like an all beef patty with a much higher fat content. Flavor development objectives included: (1) develop and optimize flavoring systems that mask the slight cereal and soy flavors inherent to the SVP and possibly isolated soy protein (ISP) ingredients; (2) enhance meat flavors of the meat remaining in the formulation; and (3) add meat flavors to replace flavor components lost through meat replacement.

[00102] Healthier beef patties were prepared in which 40% of the beef was replaced with hydrated SVP and ISP ingredients, such that the patties were 90% lean. Table 1 present the nutritional profile of beef/SVP patties and traditional beef patties. The beef/SVP patties had 30% less calories, 50% less fat, and 40% less cholesterol than 80% lean beef patties.

[00103] Two different types of beef/SVP patties were prepared, each having a different combination of flavoring agents provided by International Flavor & Fragrances, Inc. (IFF) that impart various aspects of beef flavor. Flavoring agents IFF #711300 and 711303 were added to one (T5), and flavoring agents IFF #711300, 711302, 711303, and 711304 were added to the other (T6). The sensory profiles of T5, T6, and 80% lean all beef patties were compared using several different sensory evaluations as described in Examples 2 and 3.

| Table 1. Nutrition Facts | | |
|---|---|---|
| | Raw Beef Patty | Raw Beef/SVP Patty |
| Serving Size | 114 g | 114 g |
| Calories | 90 | 210 |
| Protein | 19 g | 21 g |
| Total Fat | 23 g | 12 g |
| Cholesterol | 80 mg | 45 mg |
| Total Carbohydrate | 0 g | 3 g |
| Sodium | 250 mg | 400 mg |

**Example 2. Sensory Analysis of Precooked Patties Using a Hedonic Acceptance Scale.**

[00104] The three types of patties prepared in Example 1 were evaluated by 69 sensory panelists who regularly consumed beef patties. The three types of raw patties were precooked to an internal temperature of 160°F and frozen. Tempered patties were reheated to an internal temperature of 150°F in a 350°F convection oven. Each panelist received a whole beef/SVP or all beef patty on a 6" white styrofoam disposable plate with a unique 3-digit code for identification purposes. The patties were presented sequential monadically (one at a time), and the serving order was rotated so the each that each type of patty was seen first an equal number of times.

[00105] The sensory characteristics of each patty were evaluated using 9-point hedonic acceptance scale, where 1= extremely dislike, 5 = neither like nor dislike, and 9 = extremely like. The following sensory attributes were rated:

Liking of Overall Product

Liking of Flavor

Liking of Texture

Liking of Juiciness

The scores were tabulated; the mean, median, and standard deviation were calculated. The data were further analyzed using an analysis of variance, accounting for panelist and sample effects, with means separations using Tukey's significant difference (HSD) test.

[00106] Figure 3 presents the mean liking scores for each sensory characteristic, and the data are summarized in Tables 2-5. The T5 patty had the highest mean score for each attribute evaluated. In terms of "overall liking," T5 had a mean rating of 6.47, which was significantly different from both T6 (5.78) and all beef patties (5.49) (Table 2).

Table 2. Summary of Overall Liking Scores

| *Sample* | *Count* | *Median* | *Mean*[1] | *Standard Deviation* |
|---|---|---|---|---|
| **All Beef** | 68 | 6.00 | 5.49 b | 2.189 |
| **T5** | 68 | 7.00 | 6.47 a | 1.569 |
| **T6** | 68 | 6.00 | 5.78 b | 1.674 |

[1] Means sharing a common letter were not different (P > 0.05).

[00107] In terms "liking of flavor," T5 had the highest mean liking score (6.47), which was significantly different from T6 (5.79). The mean score for the all beef control (6.04) fell between the two test samples (Table 3).

Table 3. Summary of Liking of Flavor Scores

| *Sample* | *Count* | *Median* | *Mean*[1] | *Standard Deviation* |
|---|---|---|---|---|
| **All Beef** | 68 | 7.00 | 6.04 ab | 2.147 |
| **T5** | 68 | 7.00 | 6.47 a | 1.643 |
| **T6** | 68 | 6.00 | 5.79 b | 1.715 |

[1] Means sharing a common letter were not different (P > 0.05).

[00108] With regard to "liking of texture," again T5 had the highest mean liking score (6.44), which was significantly different from both T5 (5.94) and the all beef control (5.53) (Table 4).

Table 4. Summary of Liking of Texture Scores

| Sample | Count | Median | Mean[1] | Standard Deviation |
|---|---|---|---|---|
| **All Beef** | 68 | 6.00 | 5.53 b | 2.216 |
| **T5** | 68 | 7.00 | 6.44 a | 1.782 |
| **T6** | 68 | 6.00 | 5.94 ab | 1.674 |

[1] Means sharing a common letter were not different (P > 0.05).

[00109] Lastly, T5 had the highest average "liking of juiciness" score (6.44), with T6 (6.13) scoring nearly as high. Both of these were significantly different from the all beef control (4.88) (Table 5).

Table 5. Summary of Liking of Juiciness Scores

| Sample | Count | Median | Mean[1] | Standard Deviation |
|---|---|---|---|---|
| **All Beef** | 68 | 5.00 | 4.88 b | 2.236 |
| **T5** | 68 | 7.00 | 6.44 a | 1.633 |
| **T6** | 68 | 6.00 | 6.13 a | 1.573 |

[1] Means sharing a common letter were not different (P > 0.05).

[00110] The panelists also rated the patties according to their similarity to beef patties using a 5-point scale, where 1 = not at all like beef patties and 5 = exactly like beef patties. Figure 4 presents the mean scores for the different patties, and Table 6 summarizes the data. T5 had the highest mean similarity score (3.51), which differed from T6 (3.06) (Table 6). The all beef control (3.31) fell between the two test samples.

Table 6. Summary of Similarity to Beef Patties Scores

| Sample | Count | Median | Mean[1] | Standard Deviation |
|---|---|---|---|---|
| **All Beef** | 68 | 3.00 | 3.31 ab | 1.284 |
| **T5** | 68 | 4.00 | 3.51 a | 1.000 |
| **T6** | 68 | 3.00 | 3.06 b | 1.020 |

[1] Means sharing a common letter were not different (P > 0.05).

**Example 3. Sensory Analysis Of Precooked Patties Using the Sensory Spectrum Descriptive Profiling Method.**

[00111] The three types of patties prepared in Example 1 were also rated by 11 panelists that were trained in the Sensory Spectrum Descriptive Profiling Method. Sixteen flavor or sensory attributes were evaluated on a 15-points scale, with 0 = none/not applicable and 15 = very strong/high in the sample. The attributes and their definitions are presented in Table 7. The intensity scores were based upon the following references for flavor attributes:

2.5 Baking soda note in a saltine cracker

5.0 Cooked apple note in Motts Applesauce

7.5 Cooked orange note in MinuteMaid Orange Juice

10.0 Cooked note in Welch's Concord Grape Juice

12.0 Cinnamon note in Big Red Gum

Table 7. Meat Patty Flavor Lexicon

| Attribute | Preparation | Reference |
|---|---|---|
| **AROMATICS** | | |
| Overall Flavor Impact | The overall intensity of the product aromas, an amalgamation of all perceived aromatics, basic tastes and chemical feeling factors. | |
| Meat Complex | The general category used to describe the total meat flavor impact of the product | |
| -Beef | The aromatic associated with lean red meat | Cooked (boiled) lean ground beef |
| -Pork | Aromatic associated with cooked/cured lean pork | Ground pork, Pork trimmed of visible fat. |
| -Chicken | The aromatics associated with freshly cooked chicken. | Ground Chicken, Baked/broiled chicken breasts/thighs. |
| -Fat | Aromatic reminiscent of dairy lipid products, melted vegetable shortening cooked chicken skin, and beef tallow | Melted butter, Crisco, boiled chicken skins, beef tallow. |
| Browned/ Caramelized/ Roasted | The aromatic associated with the outside of grilled or broiled meat. | Broiled meat, roasted chicken breast |
| TVP/Vegetative | The aromatic associated with texturized vegetable protein (TVP) | Hydrated TVP |
| Onion/Garlic | The aromatics associated with dehydrated onion and garlic powders | Onion, garlic and celery powder solutions. Garlic Oil Capsules |
| White/Black Pepper | The aromatic associated with white and black pepper | White pepper and black pepper solutions |

| BASIC TASTES | | |
|---|---|---|
| Sweet | The taste on the tongue stimulated by sucrose and other sugars, such as fructose, glucose, etc., and by other sweet substances, such as Aspartame, and Acesulfame-K. | Sucrose solution:<br>2% 2.0<br>5% 5.0 |
| Sour | The taste on the tongue stimulated by acid, such as citric, malic, phosphoric, etc. | Citric acid solution:<br>0.05% 2.0<br>0.08% 5.0 |
| Salt | The taste on the tongue associated with sodium salts. | Sodium chloride solution:<br>0.2% 2.0<br>0.35% 5.0 |
| Bitter | The taste on the tongue associated with caffeine and other bitter substances, such as quinine and hop bitters. | Caffeine solution:<br>0.05% 2.0<br>0.08% 5.0 |
| Umami | The taste on the tongue associated with monosodium glutamate. Savory. | MSG solution:<br>6% 5.0 |
| **CHEMICAL FEELING FACTOR** | | |
| Astringent | The shrinking or puckering of the tongue surface caused by substances such as tannins or alum. | Alum solution:<br>0.005% 3.0<br>0.0066% 5.0 |

[00112] Patties were heated in a standard oven maintained at 300°F. Foil was used to maintain moisture in the samples during reconstitution. The patties were brought to an internal temperature to 175°F before serving. Panelists were given four quarters from different patties per evaluation. The samples were presented monadically in duplicate.

[00113] Table 8 presents the mean scores for flavor attributes for the three types of patties. Analysis of variance (ANOVA) was performed to test product and replication effects. When the ANOVA result was significant, multiple comparisons of means were performed using the Tukey's HSD t-test. All differences were significant at a 95% confidence level unless otherwise noted. For flavor attributes, mean values < 1.0 indicate that not all panelists perceived the attribute in the sample. A value of 2.0 is threshold for all flavor attributes, which is the minimum level that the panelist can detect and still identify the attribute. The attributes at threshold or lower are in gray font, and attributes above thresholds are in black font in Table 8.

Table 8. Mean[1] Scores for Flavor Attributes.

| | All Beef | 75 | 50 | P |
|---|---|---|---|---|

| | | | | p-value |
|---|---|---|---|---|
| *Aromatics* | | | | |
| Overall Flavor Impact | 6.4 a | 6.2 b | 6.4 a | ** |
| Meat Complex | 5.3 a | 3.0 b | 2.5 c | *** |
| Beef | 5.0 a | 2.9 b | 2.5 c | *** |
| Pork | 0.0 a | 0.0 a | 0.0 a | n/a |
| Chicken | 0.0 a | 0.0 a | 0.0 a | n/a |
| Fat | 2.3 a | 1.5 b | 1.6 b | *** |
| Browned/Roasted/ Caramelized | 2.9 c | 3.7 a | 3.3 b | *** |
| TVP/Vegetative | 0.0 c | 3.1 b | 3.8 a | *** |
| Onion/Garlic | 2.4 a | 2.6 a | 2.6 a | * |
| White/Black Pepper | 2.2 a | 2.2 a | 2.2 a | NS |
| *Basic Tastes and Feeling Factors* | | | | |
| Sweet | 0.2 b | 0.6 a | 0.6 a | ** |
| Sour | 2.1 b | 2.1 ab | 2.2 a | *** |
| Salt | 4.3 a | 4.4 a | 4.4 a | NS |
| Bitter | 2.1 b | 2.4 a | 2.5 a | *** |
| Umami | 2.7 b | 3.2 a | 3.1 a | *** |
| Astringent | 2.1 b | 2.2 a | 2.3 a | *** |
| Other: Metallic | 2.0 (9%) | 0.0 | 0.0 | |

[1] Means in the same row sharing a common letter were not different (P > 0.05).

***-95% Confidence, **-90% Confidence, *-80% Confidence, NS-Not Significant

The attributes at threshold or lower are gray. The attributes above threshold are black. For other attributes, % score is the percentage of times the attribute was perceived.

[00114] The major flavor differences between T5 and the all beef control were that T5 scored slightly lower in "overall flavor impact" and significantly lower in "meat complex," "beef," and "meat fat" aromatics. T5 scored significantly higher in "TVP/vegetative" and "browned/roasted/caramelized" aromatics, and slightly higher in the "onion/garlic," "bitter," "umami," and "astringent" attributes than the all beef control. T5 and the control were similar in the "black pepper" and "salty" attributes.

[00115] A comparison of T6 and the all beef control revealed that T6 scored significantly lower in "meat complex," "beef," and "meat fat" aromatics. Similar to T5, T6 also scored significantly higher in "TVP/vegetative" and "browned/roasted/caramelized" aromatics, and slightly higher in the "onion/garlic," "bitter," "umami," and "astringent" attributes than the all beef control. T6 and the all beef control were similar in "overall

flavor impact" and "black pepper" and "salty" attributes. In summary, this sensory analysis revealed that T5 scored very close to the all beef control patties in terms of "meaty" and "beefy' aromatics.

**Example 4. Sensory Analysis of Raw Prefrozen Patties Using the Hedonic Acceptance Scale.**

[00116] A series of beef/SVP and all beef patties that were frozen before cooking were also evaluated for several sensory characteristics. Healthier beef patties were prepared that included 40% SVP and 1% ISP, and the beef/SVP mixture was ground through 1/8$^{th}$ inch or 3/16$^{th}$ inch grinder plates. All beef patties that were 80% lean or 90% lean were ground through 1/8$^{th}$ inch grinder plates.

[00117] The four different patties were evaluated by 60 sensory panelists who regularly consumed beef patties. Patties were grilled from a frozen state to an internal temperature of 161°F and held warm in a food-service water bath unit to maintain temperature. Each panelist received half of a patty on 6" white styrofoam disposable plate with a unique 3-digit code for identification purposes. The patties were presented sequential monadically (one at a time), and the serving order was rotated so the each that each type of patty was seen first an equal number of times.

[00118] The sensory characteristics of each patty were evaluated using the 9-point hedonic acceptance scale, where 1= extremely dislike, 5 = neither like nor dislike, and 9 = extremely like. The following sensory attributes were rated:

Liking of Overall Product

Liking of Flavor

Liking of Texture

Liking of Juiciness

[00119] The mean overall liking scores are presented in Figure 5 and summarized in Tables 9-12. In general, the 80% lean beef patty scored highest in all attributes, with the 1/8$^{th}$ inch grind beef/SVP patty scoring nearly as high.

Table 9. Summary of Liking of Overall Product Scores

| *Sample* | *Count* | *Median* | *Mean*[1] | *Standard Deviation* |
|---|---|---|---|---|

| | | | | |
|---|---|---|---|---|
| **80% Beef** | 60 | 7.00 | 6.88 a | 1.678 |
| **90% Beef** | 60 | 6.50 | 6.05 b | 1.978 |
| **Beef/SVP 1/8"** | 60 | 6.00 | 5.83 b | 1.906 |
| **Beef/SVP 3/16"** | 60 | 6.00 | 5.43 b | 1.899 |

[1] Means sharing a common letter were not different (P > 0.05).

Table 10. Summary of Liking of Appearance Scores

| *Sample* | *Count* | *Median* | *Mean[1]* | *Standard Deviation* |
|---|---|---|---|---|
| **80% Beef** | 60 | 7.00 | 6.50 a | 1.546 |
| **90% Beef** | 60 | 6.00 | 6.03 a | 2.025 |
| **Beef/SVP 1/8"** | 60 | 7.00 | 6.37 a | 1.573 |
| **Beef/SVP 3/16"** | 60 | 6.00 | 6.22 a | 1.738 |

[1] Means sharing a common letter were not different (P > 0.05).

Table 11. Summary of Liking of Flavor Scores

| *Sample* | *Count* | *Median* | *Mean[1]* | *Standard Deviation* |
|---|---|---|---|---|
| **80% Beef** | 60 | 7.00 | 6.95 a | 1.641 |
| **90% Beef** | 60 | 7.00 | 6.52 a | 1.970 |
| **Beef/SVP 1/8"** | 60 | 6.00 | 5.50 b | 2.175 |
| **Beef/SVP 3/16"** | 60 | 5.00 | 5.03 b | 2.170 |

[1] Means sharing a common letter were not different (P > 0.05).

Table 12. Summary of Liking of Texture Scores

| *Sample* | *Count* | *Median* | *Mean[1]* | *Standard Deviation* |
|---|---|---|---|---|
| **80% Beef** | 60 | 7.00 | 6.72 a | 1.823 |
| **90% Beef** | 60 | 6.00 | 5.85 b | 2.073 |
| **Beef/SVP 1/8"** | 60 | 6.00 | 5.88 b | 1.833 |
| **Beef/SVP 3/16"** | 60 | 5.00 | 5.37 b | 2.099 |

[1] Means sharing a common letter were not different (P > 0.05).

**Example 5. Color Analysis of Raw and Cooked Patties**

[00120] Another goal was to develop a healthier beef patty comprising beef and structured vegetable protein (SVP) whose color resembled that of raw all-beef patties. Prior to cooking, the beef/SVP patty should resemble fresh red meat containing about 10-30% fat, and the red beef/SVP patty should turn brown during cooking. The coloring system (see Table 13) comprised unstable red pigment and other pigments,

natural flavor enhancer (source of amino acids), and reducing sugar. With this system, when the product was subjected to heat, the unstable red color pigment faded while reducing sugars reacted with amino acids in the natural flavor enhancer to develop brown color. The SVP was hydrated in the colored hydration solution; the formulations of healthier beef patty and traditional beef patty are presented in Table 14.

| Table 13. SVP Hydration and Coloring Formulation | |
|---|---|
| Ingredient | Formulation Content (%) |
| Annatto | 0.0020 |
| Beet Powder | 0.5500 |
| Dextrose | 1.3397 |
| Natural Flavor Enhancer (Kikkoman) (amino group) | 0.6450 |
| Water | 97.4633 |
| Total | 100.0000 |

| Table 14. Patty Formulations | | |
|---|---|---|
| Ingredient | Beef/SVP (%) | Beef Patty (%) |
| Beef (lean) | 45.38 | 77.70 |
| Beef Fat | 10.02 | 21.50 |
| SVP (SUPROMAX 5050) | 10.00 | |
| Hydration and Coloring Solution (see Table 13) | 30.00 | |
| ISP | 1.00 | |
| Water | 2.00 | |
| Flavors | 1.25 | |
| Salt | 0.15 | 0.60 |
| Herb/Spice | 0.20 | 0.20 |
| Total | 100.00 | 100.00 |

[00121] The raw beef/SVP patty was similar in color and appearance to an 80% lean all beef patty (Figure 6A). All of the patties were cooked to an internal temperature of 165°F. Again the cooked beef/SVP patty was similar in color and appearance to the all beef patty (Figure 6B and C). The color of the different raw and cooked patties was analyzed using a numerical system. One system is Hunter Lab Color Scale that describes color three dimensionally for utilizing L-, a- and b-values.

The L-Value describes brightness or darkness; with zero equivalent to black and 100 equivalent to white. The a- and b- axes have no specific numerical limits. On the a-axis, a positive value is red and negative value is green. Similarly on the b-axis, a positive value is yellow and a negative value is blue.

[00122] The surface color of each raw patty was analyzed, and the internal color of each cooked patty was analyzed. The L-, a-, and b- values are presented in Table 15. Similar to the visual images presented in Figure 6, all of the color values were quite similar between the healthier beef/SVP patty and the corresponding the all beef patty.

Table 15. Color Values of Beef/SVP and All Beef Patties

| | L-value | a-value | b-value |
|---|---|---|---|
| All Beef – raw, surface color | 46.28 | 20.45 | 13.55 |
| Beef/SVP – raw, surface color | 51.17 | 20.15 | 15.24 |
| | | | |
| All Beef – cooked, internal color | 50.82 | 8.16 | 13.36 |
| Beef/SVP - cooked, internal color | 51.65 | 9.80 | 15.40 |

**Example 6. Determination of Shear Strength**

[00123] Shear strength of a sample is measured in grams and may be determined by the following procedure. Weigh a sample of the structured plant 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 7. Determination of Shred Characterization**

[00124] A procedure for determining shred characterization may be performed as follows. Weigh about 150 grams of a structured plant 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 ~200g of the mixture from the bowl. Separate the ~200g of mixture into one of two groups. Group 1 is the portion of the sample having fibers at least 4 centimeters in length and at least 0.2 centimeters wide. Group 2 is the portion of the sample having strands between 2.5 cm and 4.0 cm long, and which are ≥ 0.2 cm wide. Weigh each group, and record the weight. Add the weight of each group together, and divide by the starting weight (e.g. ~200g). 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 ~200g from the bowl, separate the mixture into groups one and two, and perform the calculations again.

**Example 8. Production of Structured Plant Protein Products**

[00125] The following extrusion process may be used to prepare the structured plant protein products of the invention, such as the soy structured plant protein products utilized in Examples 6 and 7. Added to a dry blend mixing tank are the following: 1000 kilograms (kg) Supro® 620 (soy isolate), 440 kg wheat gluten, 171 kg wheat starch, 34 kg soy cotyledon fiber, 9 kg dicalcium phosphate, and 1 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 (Wenger Model TX-168 extruder by Wenger Manufacturing, Inc. (Sabetha, KS))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 per hour, is injected into the extruder barrel, via one or more injection jets in communication with a heating zone. The molten extruder mass exits the extruder barrel through a die assembly consisting of a die and a backplate. As the mass flows through the die assembly the protein fibers contained within are substantially aligned with one another forming a fibrous extrudate. As the fibrous extrudate exits the die assembly, it is cut with flexible knives and the cut mass is then dried to a moisture content of about 10% by weight.

[00126] While the invention has been explained in relation to exemplary embodiments, it is to be understood that various modifications thereof will become apparent to those skilled in the art upon reading the description. Therefore, it is to be understood that the invention disclosed herein is intended to cover such modifications as fall within the scope of the following claims.

## CLAIMS

WHAT IS CLAIMED IS:

1. A ground meat composition, the composition comprising:
    a. structured plant protein product, the product having protein fibers that are substantially aligned;
    b. animal meat; and
    c. a color composition having coloring agents selected from the group consisting of a thermally unstable pigment, a thermally stable pigment, a reducing sugar, and combinations thereof.
2. The ground meat composition of claim 1, wherein the composition comprises from about 40% to about 60% by weight of the structured plant protein product, and from about 40% to about 60% by weight of animal meat.
3. The ground meat composition of claim 2, further comprising a fat source in an amount ranging from about 10% to about 20% by weight of the composition.
4. The ground meat composition of claim 1, wherein the structured plant protein product comprises protein fibers substantially aligned in the manner depicted in the micrographic image of Figure 1.
5. The ground meat composition of claim 1, wherein the structured plant protein product has an average shear strength of at least 1400 grams and an average shred characterization of at least 10%
6. The ground meat composition of claim 3, wherein the structured plant protein product is selected form the group consisting of soy protein, starch, gluten, and fiber.
7. The ground meat composition of claim 3, wherein the structured plant protein product comprises:
    a. from about 35% to about 65% soy protein on a dry matter basis;
    b. from about 20% to about 30% wheat gluten on a dry matter basis;
    c. from about 10% to about 15% wheat starch on a dry matter basis;
    d. from about 1% to about 5% starch on a dry matter basis.
8. The ground meat composition of claim 5, wherein the animal meat is selected from beef, pork, lamb, poultry, wild game, fish, and mixtures thereof.

9. The ground meat composition of claim 7, wherein the coloring composition is selected from the group consisting of beet, annatto, carmel coloring, a reducing sugar, an amino acid source, and combinations thereof.
10. The ground meat composition of claim 8, further comprising isolated soy protein.
11. The ground meat composition of claim 9, further comprising an antioxidant water, spices and flavoring.
12. The ground meat composition of claim 10, wherein the animal meat is beef, and reducing sugar is dextrose, and the particle size of the composition is from about 1/8 of an inch to about ¼ of an inch.
13. A simulated ground meat composition, the simulated ground meat composition comprising:
    (a) A structured plant protein product comprising protein fibers that are substantially aligned, the structured plant protein product comprising an extrudate of plant protein material; and,
    (b) A color composition having coloring agents selected from the group consisting of a thermally unstable pigment, a thermally stable pigment, a reducing sugar, and combinations thereof
14. A process for coloring a ground meat composition, the process comprising contacting a mixture comprising structured plant protein product and animal meat with a coloring composition comprising beet, annatto, carmel coloring, dextrose, and an amino acid source.
15. The process of claim 14, wherein the structured plant protein product comprises:
    a. from about 35% to about 65% soy protein on a dry matter basis;
    b. from about 20% to about 30% wheat gluten on a dry matter basis;
    c. from about 10% to about 15% wheat starch on a dry matter basis;
    d. from about 1% to about 5% starch on a dry matter basis.
16. The process of claim 15, wherein the mixture comprises from about 5% to about 95% by weight of the structured plant protein product, and from about 5% to about 95% by weight of animal meat.

17. A food product comprising the ground meat composition of claim 1.

18. The food product of claim 17, wherein the food product is formed into a patty or link.

19. The food product of claim 18, wherein the patty is a beef patty or a sausage patty.

20. The food product of claim 17, comprising a product selected from the group consisting of meatballs, meat loaf, batter-breaded products, and restructured meat products.

21. A beef patty comprising the ground meat composition of claim 12.

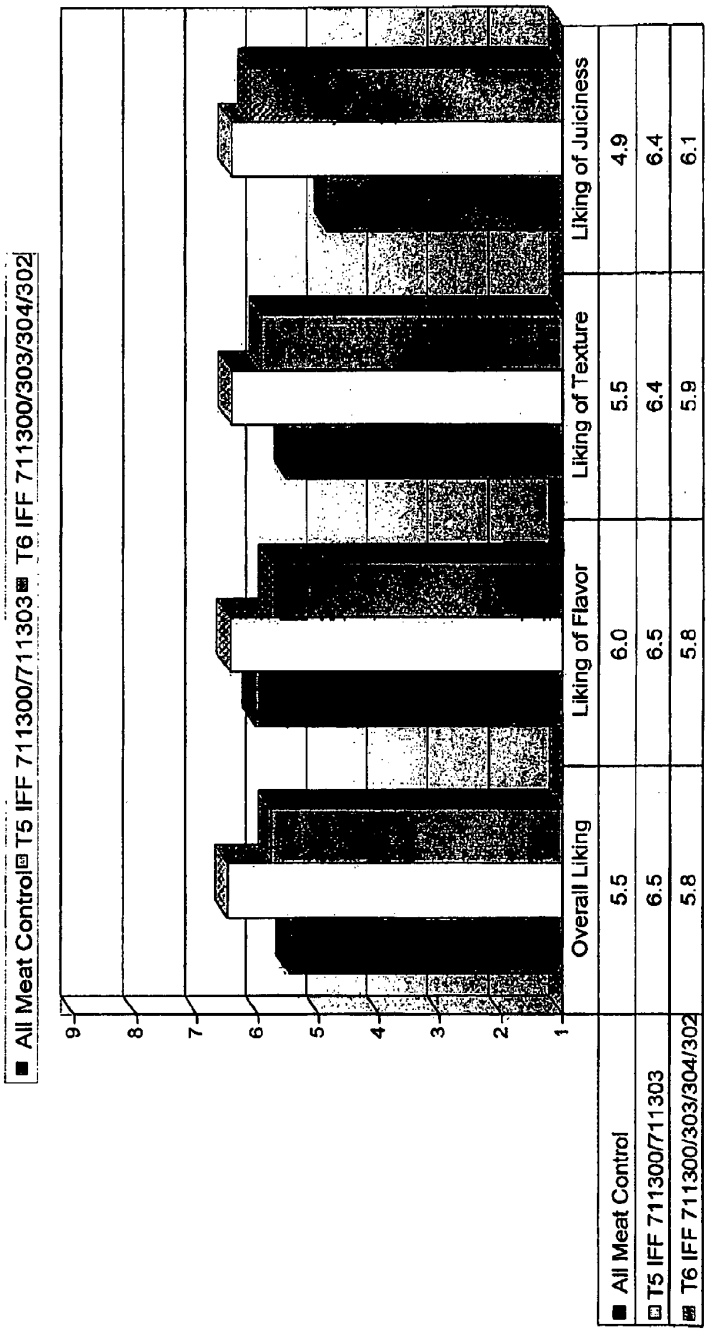


FIG. 1



FIG. 2

FIG. 3




| | Overall Liking | Liking of Flavor | Liking of Texture | Liking of Juiciness |
|---|---|---|---|---|
| All Meat Control | 5.5 | 6.0 | 5.5 | 4.9 |
| T5 IFF 711300/711303 | 6.5 | 6.5 | 6.4 | 6.4 |
| T6 IFF 711300/303/304/302 | 5.8 | 5.8 | 5.9 | 6.1 |

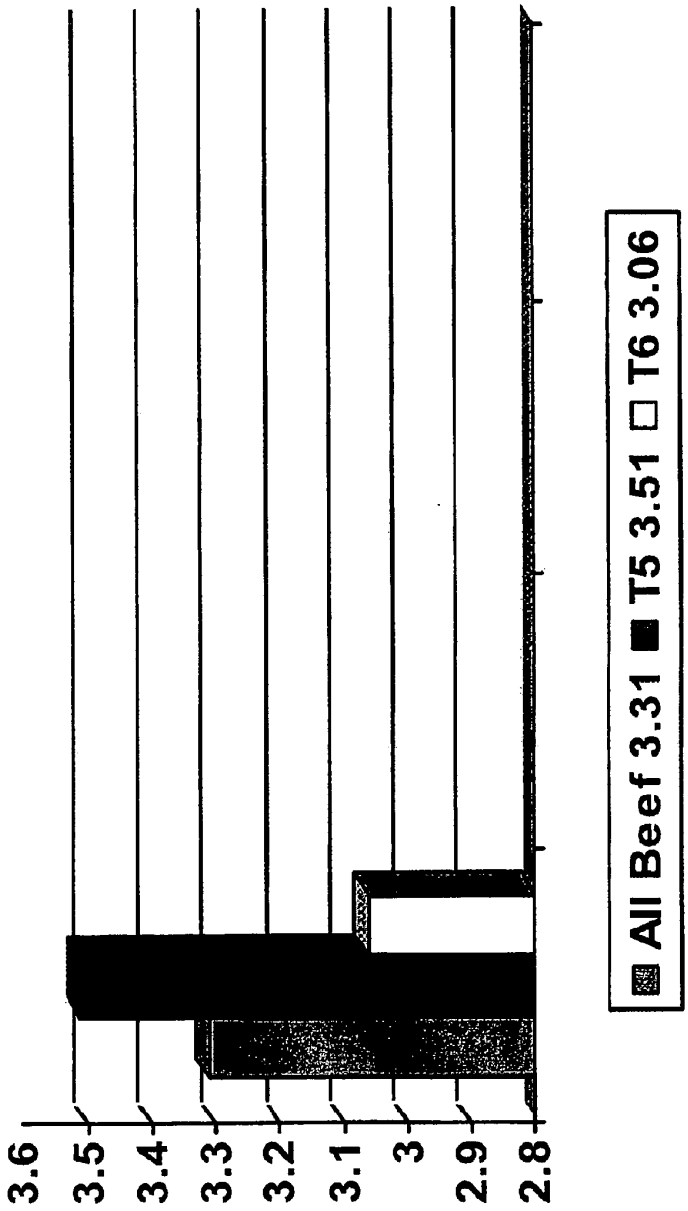
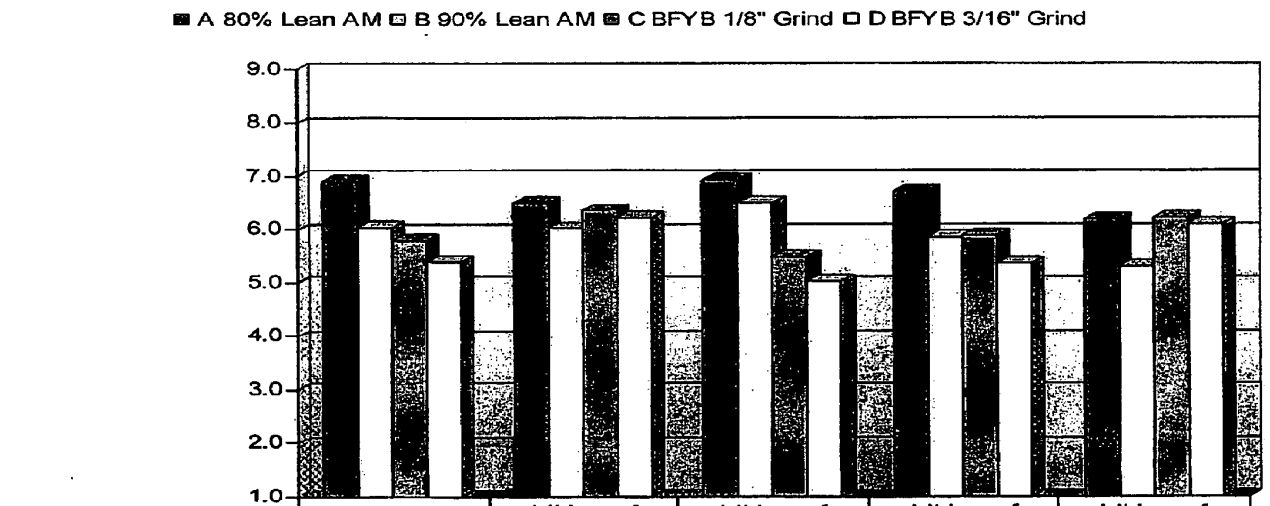



FIG. 4

# FIG. 5




| | Overall Liking | Liking of Apperance | Liking of Flavor | Liking of Texture | Liking of Juiciness |
|---|---|---|---|---|---|
| A 80% Lean AM | 6.9 | 6.5 | 7.0 | 6.7 | 6.2 |
| B 90% Lean AM | 6.1 | 6.0 | 6.5 | 5.9 | 5.3 |
| C BFYB 1/8" Grind | 5.8 | 6.4 | 5.5 | 5.9 | 6.2 |
| D BFYB 3/16" Grind | 5.4 | 6.2 | 5.0 | 5.4 | 6.1 |



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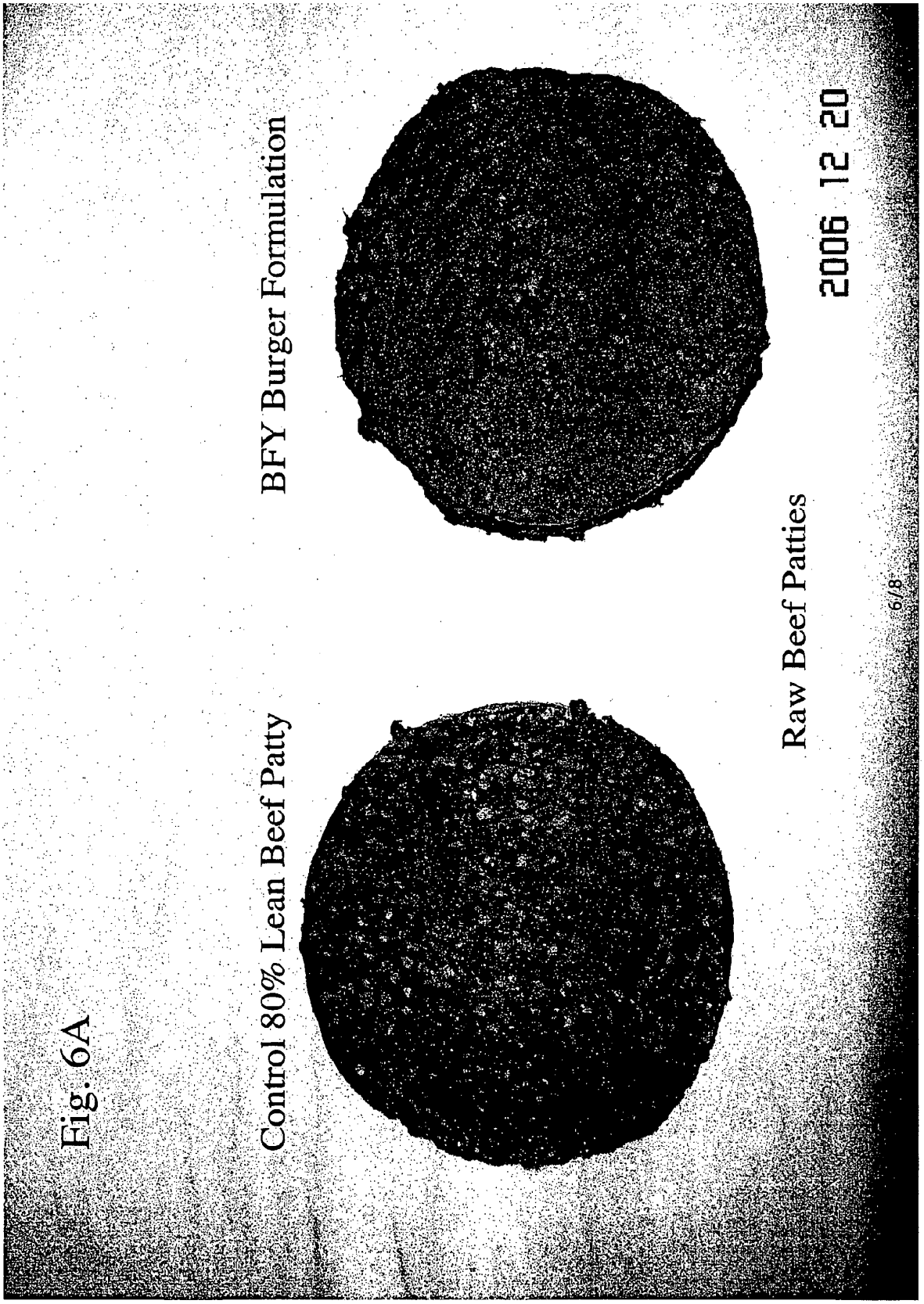

Control 80% Lean Beef Patty
BFY Burger Formulation
Raw Beef Patties
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6/8

Fig. 6A

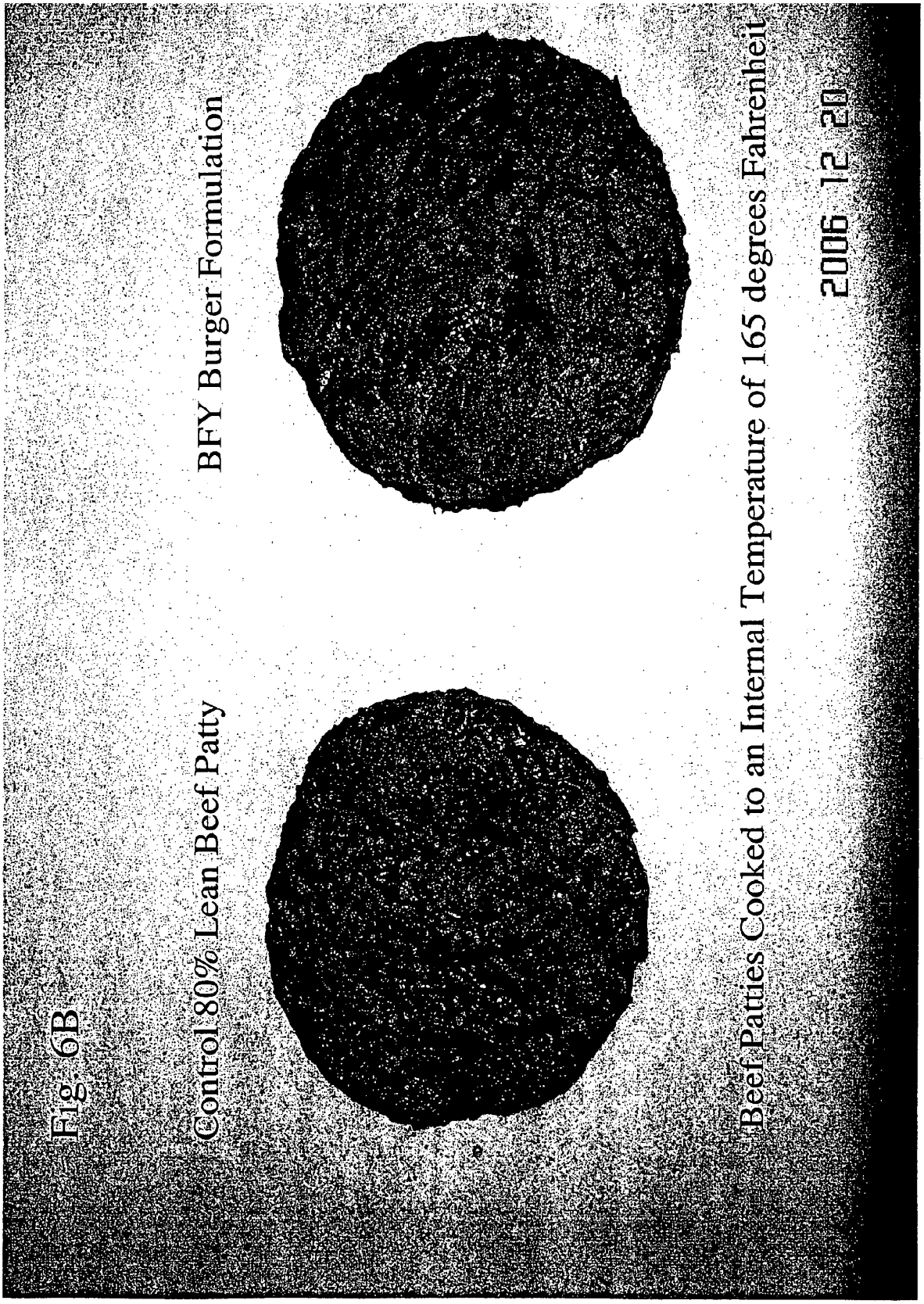

Control 80% Lean Beef Patty
BFY Burger Formulation
Beef Patties Cooked to an Internal Temperature of 165 degrees Fahrenheit
2006 12 20

Fig. 6B

# FIG. 6C

Control 80% Lean Beef Patty



Beef/SVP Patty - 40% Meat Replacemer



Beef Patties Cooked to an Internal Temperature of 165 degrees Fahrenheit

2006 12 20



Case No.: IPR2023-00208
U.S. Patent No. 10,039,306

Motif Exhibit 1148, Page 1188 of 3341

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 July 2008 (10.07.2008)

PCT

(10) International Publication Number
WO 2008/083117 A3

(51) International Patent Classification:
*A23J 1/14* (2006.01) *A23J 3/26* (2006.01)
*A23J 1/12* (2006.01) *A23L 1/00* (2006.01)
*A23J 3/14* (2006.01) *A23L 1/272* (2006.01)

(21) International Application Number:
PCT/US2007/088696

(22) International Filing Date:
21 December 2007 (21.12.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/882,662 28 December 2006 (28.12.2006) US
11/963,375 21 December 2007 (21.12.2007) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, 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) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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,

*[Continued on next page]*

(54) Title: GROUND MEAT AND MEAT ANALOG COMPOSITIONS HAVING IMPROVED NUTRITIONAL PROPERTIES

FIG. 1



(57) Abstract: The invention provides ground meat and meat analog compositions having reduce fat and cholesterol. The ground meat compositions comprise a structured plant protein product, optionally meat, and a color composition having coloring agents selected from the group consisting of a thermally unstable pigment, a thermally stable pigment, a reducing sugar and combinations thereof

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WO 2008/083117 A3



PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, 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*

**(88) Date of publication of the international search report:**
21 August 2008

# INTERNATIONAL SEARCH REPORT

International application No
PCT/US2007/088696

A. CLASSIFICATION OF SUBJECT MATTER
INV. A23J1/14 A23J1/12 A23J3/14 A23J3/26 A23L1/00
A23L1/272

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

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A23J A23L

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Form PCT/ISA/210 (patent family annex) (April 2005)

Espacenet

# Bibliographic data: WO2009060678 (A1) — 2009-05-14

## SOY PROTEIN GEL AND METHOD OF PRODUCING THE SAME

No documents available for this priority number.

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**Classification:** **- international:** ***A23J3/16; A23L1/00***
**- cooperative:** **A23J3/16**; **A23L1/05**; **A23L1/2006**; **A23L1/3255**; A23V2002/00more

**Application number:** WO2008JP68146 20081006

**Priority number(s):** JP20070291384 20071108

**Also published as:** US2010311950 (A1) US8304522 (B2) CN101909460 (A)

## Abstract of WO2009060678 (A1)

It is intended to provide a soy protein gel which has such gel properties that have never been obtained hitherto and is excellent in taste and color tone, and a soy protein food taking advantage of the gel properties. It has been found out that gel properties can be modified at an extremely elevated level and thus the desired soy protein gel can be obtained by preparing a gel by using a soy protein material in which the lipophilic protein content is reduced and conducting the gelation with the utilization of not only heat but also the action of a protein crosslinking enzyme.

Last updated: 11.12.2013 Worldwide Database 5.8.15; 92p

(12) 特許協力条約に基づいて公開された国際出願

(19) 世界知的所有権機関
国際事務局

(43) 国際公開日
2009 年5 月14 日 (14.05.2009)

PCT

(10) 国際公開番号
WO 2009/060678 A1

(51) 国際特許分類:
*A23J 3/16* (2006.01) *A23L 1/00* (2006.01)

(21) 国際出願番号: PCT/JP2008/068146

(22) 国際出願日: 2008 年10 月6 日 (06.10.2008)

(25) 国際出願の言語: 日本語

(26) 国際公開の言語: 日本語

(30) 優先権データ:
特願2007-291384 2007 年11 月8 日 (08.11.2007) JP

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(81) 指定国 (表示のない限り、全ての種類の国内保護が可能): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, 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, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) 指定国 (表示のない限り、全ての種類の広域保護が可能): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), ユーラシア (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), ヨーロッパ (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

添付公開書類:
— 国際調査報告書

WO 2009/060678 A1

(54) **Title:** SOY PROTEIN GEL AND METHOD OF PRODUCING THE SAME

(54) 発明の名称: 大豆蛋白ゲル及びその製造法

(57) **Abstract:** It is intended to provide a soy protein gel which has such gel properties that have never been obtained hitherto and is excellent in taste and color tone, and a soy protein food taking advantage of the gel properties. It has been found out that gel properties can be modified at an extremely elevated level and thus the desired soy protein gel can be obtained by preparing a gel by using a soy protein material in which the lipophilic protein content is reduced and conducting the gelation with the utilization of not only heat but also the action of a protein crosslinking enzyme.

(57) 要約: 本発明は、従来は得ることができなかったゲル物性を有する大豆蛋白ゲルであってさらに風味や色調にも優れたものを提供すること、そしてこのゲル物性を利用した大豆蛋白食品を提供することを課題とする。 脂質親和性蛋白質を低減した大豆蛋白素材を使用してゲルを調製すること、さらにゲル化には加熱のほか、蛋白質架橋酵素の作用を寄与させることによって、ゲルの物性改変の程度が著しく増大し、目的とする大豆蛋白ゲルが得られることを見出した。

# 明 細 書

## 大豆蛋白ゲル及びその製造法

## 技術分野

[0001] 本発明は、大豆蛋白ゲル及びその製造法に関する。

## 背景技術

[0002] 大豆は重要な食糧資源であり、古くから、煮豆、豆乳、豆腐、がんも、油揚げ、凍り豆腐、納豆、テンペ、味噌、醤油などの大豆食品として利用されてきた。その蛋白質は良質の食用蛋白質であって、ゲル物性、乳化性、保水性などをはじめとする様々な物理特性も有しており、従来より大豆から大豆蛋白質を分離して食品への利用が推し進められてきた。更に近年においては、大豆蛋白を主体とした組立食品のような新しいソイ・フードも提案され定着してきている。

[0003] 特に最近では健康志向の高まりから、大豆を主体とした食品のニーズの高まりがあり、美味しさと栄養健康といった価値に加えて、携帯性、簡便性、ゲルテクスチャー改良による食べやすさ・飲み込みやすさといったような価値の創出が求められているところである。

[0004] 大豆の貯蔵蛋白はpH4．5付近で等電点沈澱させることにより、比較的簡単に沈殿画分として回収することができ、食品工業において多くはこの画分、すなわち「分離大豆蛋白」の形態で利用されている。例えば、この分離大豆蛋白は、そのまま、あるいは必要によりさらに加工して他の食材に添加するなどして、大豆蛋白主体食品や畜肉製品や水産練り製品などの惣菜をはじめ、スナック・栄養バー・菓子類・飲料などの嗜好品、純植物性食材、乳・卵アレルギー患者用食品、嚥下・咀嚼困難者用食品、高蛋白食品、栄養バランス食品などの特殊用途食品などへ利用されている。

[0005] この大豆の貯蔵蛋白質は超遠心分析による沈降定数から、2S、7S、11S、15Sの各グロブリンに分類され、このうち、7Sグロブリンと11Sグロブリンはグロブリン画分の主要な構成蛋白質成分である。一方、大豆由来の蛋白質には、細胞膜をはじめプロテインボディーやオイルボディー等の膜を構成する極性脂質との親和力の高い蛋白質群（脂質親和性蛋白質）が存在し、工業的に生産する分離大豆蛋白の約35％をも

占めていることが佐本らにより報告されている(非特許文献1)。

この脂質親和性蛋白質は膜蛋白質を主体とする蛋白群の総称で、特にSDS－ポリアクリルアミド電気泳動による推定分子量において主に34kDa、24kDa、18kDaを示す蛋白質を含み、クロロホルム:エタノール＝2:1の極性溶媒により抽出される極性脂質を10～12重量%程度含有することが知られている。

[0006] このように、広く利用されてきた分離大豆蛋白を構成する蛋白質には、7Sグロブリンと11Sグロブリンの他にも脂質親和性蛋白質が存在することがわかってきた。そして各々の蛋白質は大豆から食品工業的に分画できるようになってきた(特許文献1、特許文献2、非特許文献2)。

しかしながら、個々の蛋白質の物理特性、特に脂質親和性蛋白質の物理特性は未だ充分に明らかにはなっていなかった。

[0007] 一方、トランスグルタミナーゼを利用して食用蛋白質を架橋して改質するなどの方法が知られている。この酵素は、ペプチド鎖内にあるグルタミン残基のγ－カルボキシアミド基のアシル転移反応を触媒する酵素である。このトランスグルタミナーゼは、アシル受容体としてのタンパク質中のリジン残基のε－アミノ基に作用し、タンパク質分子の分子内において及び分子間においてε－(γ－Glu)－Lys架橋結合を形成する。また、水がアシル受容体として機能するときは、グルタミン残基が脱アミド化されてグルタミン酸残基になる反応を進行させる。トランスグルタミナーゼを大豆蛋白に作用させる例に関しては、特許文献3～5にみられるように、大豆蛋白含有スラリーにトランスグルタミナーゼを添加してゲル化させる手法が開示されている。

[0008] (参考文献)

特許文献1:国際公開WO2002／028198号公報

特許文献2:国際公開WO2006／129647号公報

特許文献3:特開昭58－149645号号公報

特許文献4:特開昭64－27471号公報

特許文献5:国際公開WO2005－94608号公報

非特許文献1:M Samoto etc, Biosci. Biotechnol. Biochem., 62(5), 935-940, 1998.

非特許文献2:M Samoto etc, Food Chemistry, 102, 317-322, 2007.

## 発明の開示

### 発明が解決しようとする課題

[0009] 大豆蛋白素材を加熱しゲル化させた大豆蛋白ゲルの場合、ゲルの硬さや崩れやすさなどの物性の相違は食品への適性や嗜好性に大きく影響する。

例えば比較的柔い食感の大豆蛋白ゲルにする場合、つるりと飲み込みやすくて豆腐のように崩れすぎない物性を求めるニーズがある(ちなみに寒天などの多糖類やゼラチンなどの他の原料が主体のゲルは大豆蛋白ゲルではない)。一方、比較的硬い食感の大豆蛋白ゲルにする場合、子供や老人にも好まれる食感のコンニャクのような、たわみのある硬い食感の物性を求めるニーズがある。

上記の文献では、従来の分離大豆蛋白を構成している分画大豆蛋白の画分の違いや加熱変性の有無によって、発現するゲル物性が異なることが明らかとなってきた。また沈殿大豆蛋白(分離大豆蛋白)、7Sグロブリン、11Sグロブリンのトランスグルタミナーゼによるゲル化の状態には差がないということなどが教示されている。

しかしながら大豆蛋白ゲルでいかにして上述の物性を得るかについて、従来の研究だけでは有効な知見が得られていなかった。

そこで本発明は、従来は得ることができなかったゲル物性を有する大豆蛋白ゲルであってさらに風味や色調にも優れたものを提供すること、そしてこのゲル物性を利用した大豆蛋白食品を提供することを課題とする。具体的には、硬くたわみのあるコンニャク様の食感、或いは、柔くつるりとして飲み込みやすく豆腐のように崩れすぎない食感を有する大豆蛋白ゲルを提供し、これらのゲルの食品への利用用途を提供することを課題とする。

### 課題を解決するための手段

[0010] 本発明者らは上記課題に鑑み、分画大豆蛋白の物性挙動や、各種酵素や各種金属塩による物性改変効果を鋭意研究する中で、意外にも、脂質親和性蛋白を低減した大豆蛋白素材を使用してゲルを調製すること、さらにゲル化には加熱のほか、蛋白質架橋酵素の作用を寄与させることによって、ゲルの物性改変の程度が著しく増大し、目的とする大豆蛋白ゲルが得られることを見出した。

さらに、上記知見を応用し、脂質親和性蛋白質が低減された大豆蛋白素材を含む

材料に蛋白質架橋酵素を作用させることによって引き出されるゲル化能を利用することで、食品などの物理特性を望ましい方向へと改変したり、食品などの物理特性を殆ど変化させることなく他素材から大豆蛋白へ代替できることも見出した。

[0011] すなわち、本発明は、

1. 大豆蛋白素材を含む材料を加熱して得られるゲルであって、該大豆蛋白素材は脂質親和性蛋白質が低減されたものであり、かつ、該ゲルは蛋白質架橋酵素が作用したものであることを特徴とする大豆蛋白ゲル、

2. 大豆蛋白素材が全脂豆乳、脱脂豆乳、分離大豆蛋白、7S大豆蛋白、11S大豆蛋白である前記1. 記載の大豆蛋白ゲル、

3. 破断応力が50～1000（×1000N／$m^2$）であって、かつ破断歪率が50～100％である前記1. 記載の大豆蛋白ゲル、

4. 破断応力が5～50（×1000N／$m^2$）であって、かつ破断歪率が40～80％である前記1. 記載の大豆蛋白ゲル、

5. 前記1. 記載の大豆蛋白ゲルを利用した食品、

6. 脂質親和性蛋白質を低減した大豆蛋白素材を原料とし、該大豆蛋白素材を含む材料に蛋白質架橋酵素を作用させた後、加熱することを特徴とする大豆蛋白ゲルの製造法、

7. 蛋白質架橋酵素を作用させるときの該大豆蛋白素材を含む材料のpHが6～9である前記6. 記載の人豆蛋白ゲルの製造法、

8. 蛋白質架橋酵素を作用させるときの該大豆蛋白素材への食塩添加量が大豆蛋白を含む材料中1重量％以下である前記6. 記載の大豆蛋白ゲルの製造法、

9. 脂質親和性蛋白質を低減した大豆蛋白素材が予め変性温度以上の予備加熱を施されておらず、該大豆蛋白素材を含む材料に蛋白架橋酵素を作用させた後、該大豆蛋白素材の変性温度以上で加熱を施すことを特徴とする前記6. 記載の方法、

10. 脂質親和性蛋白質を低減した大豆蛋白素材が予め変性温度以上の予備加熱を施されていて、該大豆蛋白素材を含む材料に蛋白架橋酵素を作用させた後、該酵素が失活するに充分な加熱を施す加熱である前記6. 記載の方法、

11. 脂質親和性蛋白質が低減されており、蛋白質架橋酵素が作用したものであって

、かつ、大豆蛋白ゲル調製用であることを特徴とする大豆蛋白素材、

12．脂質親和性蛋白質が低減されており、蛋白質架橋酵素を作用させて使用するものであって、かつ、大豆蛋白ゲル調製用であることを特徴とする大豆蛋白素材、

13．大豆蛋白ゲルを予め調製し、これを混合してなるものである前記5．記載の食品、

14．食品原料中に脂質親和性蛋白質を低減した大豆蛋白素材及び蛋白質架橋酵素が配合され、製造過程で大豆蛋白ゲルが形成されてなるものである前記5．記載の食品、

である。

## 発明の効果

[0012] 本発明により、従来の大豆蛋白ゲルでは体現しえなかった、つるりとした柔い食感や、たわみのある硬い食感などの新規な物性を有する大豆蛋白ゲルを得ることができる。これにより、新規な大豆蛋白ゲル化食品を創出することが可能である。

また本発明の大豆蛋白ゲルは大豆由来の雑味を感じにくく風味にも優れたものであるので、従来にはない薄い味付けや、爽やかなデザート風味のような大豆食品とは異なる風味付けを行うことができ、新しい大豆食品（ニューソイフード）のバリエーションを増やすことができる。

さらに、本発明による大豆蛋白ゲルを利用することにより、食品の物理特性を望ましい方向へと改良したり、既存食品の物理特性を殆ど変化させることなくその主原料を大豆蛋白へ代替することが可能となる。

## 発明を実施するための最良の形態

[0013] 以下、本発明の実施形態について詳細に説明する。

本発明の大豆蛋白ゲルは、大豆蛋白素材を含む材料を加熱して得られるゲルであって、該大豆蛋白素材は脂質親和性蛋白質が低減されたものであり、かつ、該ゲルは蛋白質架橋酵素が作用したものであることを特徴とする。また本発明の大豆蛋白ゲルの製造法は、脂質親和性蛋白質を低減した大豆蛋白素材を原料とし、該大豆蛋白素材を含む材料に蛋白質架橋酵素を作用させた後、加熱することを特徴とする。

[0014] （大豆蛋白素材）

本発明において「大豆蛋白素材」は、大豆由来の蛋白質が抽出された素材を意味し、具体的には丸大豆や脱脂大豆などから蛋白質を抽出して必要により精製して得られる抽出大豆蛋白素材である。例えば、全脂豆乳、脱脂豆乳、分離大豆蛋白、7S大豆蛋白、11S大豆蛋白などが該当し、これらの混合物も含まれる。ただし、蛋白質が抽出されている限り、不溶性のオカラ分が含まれることを除外するものではない。

[0015] なお、特許文献2に記載の通り、11S大豆蛋白とは、11Sグロブリン（グリシニンとも呼ばれる）の純度を高めた大豆蛋白素材であり、大豆蛋白質あたりの含量が少なくとも45%以上のものをいう。特に75%以上、さらには85%以上、さらには90%以上であるのが好ましい。

また7S大豆蛋白は7Sグロブリン（β－コングリシニンとも呼ばれる）の純度を高めた大豆蛋白素材であり、大豆蛋白質あたりの含量が少なくとも25%以上であるのが好ましい。特に40%以上、さらには50%以上、さらには60%以上であるのが好ましい。

一方、7Sと11Sの分画を行っていない一般の分離大豆蛋白の大豆蛋白質中の11S蛋白質含量はおよそ30～40%程度であり、7S蛋白質含量はおよそ13～22%程度である。

[0016] 大豆蛋白素材中の7Sグロブリンと11Sグロブリンの大豆蛋白質あたりの含量はSDS－ポリアクリルアミド電気泳動法（SDS-PAGE）により測定することができる。Laemmli(Nature, 227, 680, 1970 )の方法に基づき、ゲル濃度10～20%のグラジエントゲルで分析し、クマシーブリリアントブルー（CBB）にて染色した後、得られた泳動パターンをデンシトメーターで測定し、その全体に対する該当蛋白質の面積比率を純度とする。7Sグロブリンはα，α’，βサブユニットの総量とし、11S蛋白質の含量は酸性ポリペプチド（A）と塩基性ポリペプチド（B）の総量とする。

[0017] 上記に例示される大豆蛋白素材は、いずれも公知の方法によって得ることができる。11S大豆蛋白や7S大豆蛋白の場合、例えば、一般的な組成を有する大豆から調製した豆乳や分離大豆蛋白などから7S蛋白質と11S蛋白質を分画する技術を用いて上記の組成に調製した大豆蛋白質素材を得ることができる。11S蛋白質を分画除去する技術としては、従来公知の方法を用いることができる。例えば、工業的規模で

の製造を可能とする国際公開WO2000／58492号、国際公開 WO2002／028198号、国際公開WO2004／043160号、国際公開WO2006／129647号等に開示されるような技術を利用することができる。また、予め7S蛋白質や11S蛋白質の一部もしくは全部を遺伝子操作や育種技術により欠失させた大豆（Breeding Science, 46, 11, 1996など）から通常の方法を用いて、上記の組成に調製した大豆蛋白質素材を得ることもできる。また上記の分画技術と欠失大豆を使用する技術の両方を用いることもできる。

[0018] （大豆蛋白素材における脂質親和性蛋白質の低減）

本発明の大豆蛋白ゲルに使用される大豆蛋白素材は、特に「脂質親和性蛋白質」（Lipophilic Proteins、以下「LP」と称することがある。）を低減したもの、ないしは除去したものであることが必須である。

[0019] LPは大豆の酸沈殿性大豆蛋白質の内、7Sグロブリンと11Sグロブリン以外のマイナーな酸沈殿性大豆蛋白質群をいい、レシチンや糖脂質などの極性脂質を多く随伴するものである。以下、単に「LP」と略記することがある。このLP中にはSDS-PAGEによる推定分子量において主に34kDa、24kDa、18kDaを示す蛋白質、リポキシゲナーゼ、γ－コングリシニンや、その他多くの雑多な蛋白質が含まれる。

[0020] LPの低減化は7S大豆蛋白や11S大豆蛋白の場合、これらを上記の方法、特に国際公開WO2002／028198号や国際公開WO2004／043160号や国際公開WO2006／129647号の方法により調製することによって達成される。またこれらの分画蛋白質や豆乳や分離大豆蛋白は、蛋白質の水溶液を比較的強力な遠心力によって遠心分離し、沈殿物を除去することによっても達成される。LPの低減の程度はLP推定含量（Lipophilic proteins Content Index、以下LCIと略する。）を算出することによって確認できる。これは7S、11S、LPの各蛋白質中の主要な蛋白質を選択し、それらのSDS-PAGEでのCBB染色比率を求め、これらの比率からLP含量を簡易的に推定したものである。LCI値の算出方法は、本出願人による特許文献2（WO2006/129647号公報）に記載の[LP含量の推定方法](a)～(d)及び（表1）に記載の方法に従い、（数1）の計算式により算出するものとする。詳細の方法は以下に説明する。本発明の大豆蛋白素材のLCI値は38％以下、好ましくは35％以下、より好ましくは30％以下

、さらに好ましくは25％以下である。

[0021] 〔LP含量の推定方法〕

(a) 各蛋白質中の主要な蛋白質として、7Sはαサブユニット及びα'サブユニット(α＋α')、11Sは酸性サブユニット(AS)、LPは34kDa蛋白質及びリポキシゲナーゼ(P34＋Lx)を選択し、SDS－PAGEにより選択された各蛋白質の染色比率を求める。電気泳動は下表の条件で行うことが出来る。

(b) X(％)＝(P34＋Lx)／{(P34＋Lx)＋(α＋α')＋AS}×100(％)を求める。

(c) 低変性脱脂大豆から調製された分離大豆蛋白のLP含量を加熱殺菌前に上記方法1，2の分画法により測定すると凡そ38％となることから、X＝38(％)となるよう(P34＋Lx)に補正係数k*＝6を掛ける。

(d) すなわち、以下の(式1)によりLP推定含量(Lipophilic Proteins Content Index、以下「LCI」と略する。)を算出する。

[0022] 〈電気泳動条件〉

| | |
|---|---|
| アプライ量 | 蛋白質 0．1％サンプル溶液を各ウェルに 10μl |
| ウェル幅 | 5mm |
| ウェル容積 | 30μl |
| 染色液 | クマシーブリリアントブルー（CBB）1g、メタノール 500ml、氷酢酸 70ml（CBB をメタノールに完全に溶解させた後、酢酸と水を加えて 1L にする。） |
| 染色時間 | 15 時間 |
| 脱色時間 | 6 時間 |
| デンシトメーター | GS-710 Calibrated Imaging Densitometer / Quantity One Software Ver. 4.2.3 (Bio Rad Japan Co.Ltd) スキャン幅：5.3mm、感度：30 |

[0023] (式1)

$$LCI\ (\%) = \frac{k^{*} \times (\mathrm{P34} + \mathrm{Lx})}{k^{*} \times (\mathrm{P34} + \mathrm{Lx}) + (\alpha + \alpha') + \mathrm{AS}} \times 100$$

k* ：補正係数（6）
P34：LP 主要成分、34kDa 蛋白質
Lx ：LP 主要成分、リポキシゲナーゼ
α ：7S 主要成分、αサブユニット
α' ：7S 主要成分、α'サブユニット
AS ：11S 主要成分、酸性サブユニット

[0024] LPが低減されていない大豆蛋白素材は低減された大豆蛋白素材と比較すると大豆の雑味を感じ、風味の面で劣る。また、後述のトランスグルタミナーゼを作用させたときのゲル物性は破断応力が低下し不十分なものとなってしまう。

[0025] 本発明の大豆蛋白素材は粉末状のものを使用することができるが、液状のものもそのまま使用することができる。ただしゲル化させる工程がかなり離れている場合やゲル化させる前に一旦大豆蛋白素材を含む材料として保存しておきたい場合などは、後述するトランスグルタミナーゼを添加する前又は後において、濃縮、冷凍又は粉末化しておくことができる。

[0026] (大豆蛋白素材を含む材料)

この大豆蛋白素材を適当な濃度に水と必要により他の原料に分散させ、大豆蛋白ゲルの調製に供する。この大豆蛋白素材を含む材料は粘土状、ペースト状、スラリー状、液状などの所望の性状のものとすることができる。

大豆蛋白素材を含む材料に必要により含まれる他の材料としては、動物・植物・微生物・鉱物・有機物・無機物などを由来とする、天然物又はその抽出物もしくは加工物、あるいはそれらの混合物からなる群から選択することができる。例えば、畜肉の薄切り・ミンチ・ペースト、魚肉のフィレ・ミンチ・すり身、卵類・乳類の加工品などの動物由来のものや、とうもろこし・小麦等の穀物、大豆・小豆等の豆類、大根・人参等の根菜類などの植物由来のものや、油脂・炭水化物・糖類・アミノ酸・ペプチドなどの有機物や、塩類などの無機物などが挙げられる。

[0027] (蛋白質架橋酵素の作用)

本発明の大豆蛋白ゲルは、上記の大豆蛋白素材が使用されることに加え、蛋白質架橋酵素が作用したものであることが重要である。蛋白質架橋酵素を作用させることによって大豆蛋白ゲルはつるりとした柔い食感のゲルや、たわみのある硬い食感のゲルに改質することが可能となる。蛋白質架橋酵素は蛋白質分子同士の架橋を触媒する酵素であり、例えばアミノ基が関与するグルタミン残基[－(CH2)2－CO－NH2]とリジン残基[NH2－(CH2)4－]の縮合反応、アスパラギン残基[－CH2－CO－NH2]とリジン残基の縮合反応などのε－アミノ基が関与する反応を触媒するものが挙げられる。代表的にはグルタミン残基とリジン残基の縮合を触媒するトランスグルタミナ

ーゼ(EC2.3.2.13)を例示することができる。トランスグルタミナーゼの起源は特に限定されず、動物由来、微生物由来、植物由来のものをいずれも使用できる。また精製した酵素を使用しても良いし、市販の「アクティバ」シリーズ(味の素(株))などの酵素製剤を使用することができる。

トランスグルタミナーゼの添加量は使用する大豆蛋白素材やこれを含む材料の固形分によっても変動するが、酵素反応後に大豆蛋白素材を含む材料がサクい寒天様のゲルを形成する程度が好ましく、この量は当業者がゲルの状態を見つつ適宜設定することができる。具体的には該材料中の大豆蛋白素材の粗蛋白質1gあたり、トランスグルタミナーゼ製剤は0．1～5ユニット程度添加すればよい。該材料の固形分が低いほどユニット量の下限は大きくするのが好ましく、0．75ユニット以上、さらに1．5ユニット以上とすることができる。また、該材料の固形分が高いほどユニット量の上限は小さくすることができ、3．2ユニット以下、さらに1．6ユニット以下とすることができる。

[0028] 蛋白質架橋酵素を作用させるpHや温度は当業者が適宜選択でき、その酵素の基質に対する至適pHや至適温度を参考にすることができ、さらに大豆蛋白のゲル物性の側面から作用pHを選択することが好ましい。例えば、市販のトランスグルタミナーゼ剤の至適pHはpH5～8で、好ましくはpH6～7であって、そのようなpH域で架橋反応による高分子化は進みやすい。

ただし、大豆蛋白のゲル形成(特に11S大豆蛋白)にとっては、必ずしもそのようなpH域が望ましいわけではない。例えば、11S大豆蛋白を12%濃度含む材料にトランスグルタミナーゼを作用させて加熱し、大豆蛋白ゲルを得ようとした場合、pH5．5程度ではボソボソして離水の多いゲルとなり、pH6．5程度ではたわんで途中でシャキっと切れるゲルとなり、pH7．5～8．5程度ではたわんで最後にプチっと切れるコンニャク的な好ましいゲルとなる。このように、架橋させるための酵素化学的な反応至適pHと、好ましい物性となることを考慮した反応至適pHとは、若干のずれがある。すなわち、物性向上のための反応pHは、大豆蛋白質の等電点である4．5からより遠いpH域であって、かつトランスグルタミナーゼが作用し得るpH域から選択する必要があり、pH6～9が好ましい。

[0029] （食塩の添加による影響）

大豆蛋白ゲルに食塩を添加する場合には、大豆蛋白を含む材料中の食塩添加量はゲル物性に影響を与えるので重要である。特に大豆蛋白の溶解性が低くなる食塩添加量域では、ゲルネットワークが水を保持しにくくなるためか、好ましい物性には至らない。例えば、12%程度濃度の11S大豆蛋白を含む材料（pH7．5）に、食塩を添加しない場合には、たわんで最後にプチっと切れるコンニャク的な好ましいゲルとなる。これに対して、該材料に食塩を添加して、大豆蛋白ゲルを得ようとした場合、該材料中の食塩添加量が0．6重量%程度では、ややボソボソしてやや離水を伴うゲルとなり、該材料中の食塩添加量が1．2重量%程度では、ボソボソして離水の多いゲルとなる。更に該材料中の食塩添加量を1．8重量%程度まで高めて溶解性を向上させた場合は、塩味が生じてくるものの物性的には再びゲル化能を発揮してたわんで途中でシャキっと切れるゲルとなる。このように、大豆蛋白を含む材料への食塩添加量は、求める味や物性に応じて選択することができ、食塩添加量は、該材料あたり1重量%以下、又は1．6重量%以上が好ましい。

[0030] （加熱による大豆蛋白ゲルの改質）

本発明の大豆蛋白ゲルを得るには上述の大豆蛋白素材を含む材料を加熱することが必須である。加熱によって酵素を失活させると共に、蛋白質架橋酵素を作用させて得られるゲルを本発明が目的とする物性に改質することができる。例えば、LPを低減した大豆蛋白素材を12%含有する材料に蛋白質架橋酵素を作用させるのみでは、ゲルは形成されるものの寒天のような脆くてたわみにくいゲルにしかならない。そして酵素反応後に加熱を行うことによって、たわみがあって最後にプチンと切れる、コンニャク様の特徴的な食感のゲルを得ることができる。

[0031] 加熱の方式は、公知の方法を用いることができ、具体的な加熱条件の例を挙げると、LPが低減された大豆蛋白素材がその固有の変性温度域以上の加熱変性を予め受けていない場合は、蛋白質架橋酵素を作用させた後に、その固有の変性温度域又はそれを超える温度域での加熱を施すことで、そのゲルの破断応力は極大となる。

なお、示唆熱熱量計による11Sグロブリンの吸熱ピークは80～92℃の間に、7Sグ

ロブリンの吸熱ピークは67～78℃の間に現れる。

したがって例えば、破断応力を高める目的においては、未変性の11S大豆蛋白に蛋白質架橋酵素を作用させた後に加熱する場合、80～120℃、好ましくは85～100℃で、1～60分、好ましくは10～40分の範囲での加熱を行うことが適当であり、90℃30分の加熱でゲルの破断応力は極大となる。

また未変性の7S大豆蛋白に蛋白質架橋酵素を作用させた後に加熱する場合、70～120℃、好ましくは75～100℃で、1～60分、好ましくは10～40分の範囲での加熱を行うことが適当であり、80℃30分の加熱でゲルの破断応力は極大となる。なお、加熱なしでは破断応力が低く、120℃10分の加熱を行う場合も過度の加熱履歴となるためか破断応力が低くなる傾向にある。その他の7Sグロブリンと11Sグロブリンを両方含む大豆蛋白素材については、吸熱ピークの高い11Sグロブリンの条件に準ずれは良い。

[0032] 一方、LPを低下させた大豆蛋白素材が直接蒸気吹込方式や間接加熱やジュール加熱やマイクロ波加熱等の加熱手段によって、予め含有する蛋白質に固有の変性温度域又はそれ以上の加熱変性を受けている場合は、蛋白質架橋酵素を作用させることで、冷却ゲル形成能が顕著に引き出され、上述のレベルの加熱を行わなくとも破断応力が高いものとすることが可能である。そして、蛋白質架橋酵素を失活させるに足る加熱を施すことで、本発明の大豆蛋白ゲルを得ることができる。酵素を失活させるに足る加熱条件としては、酵素剤の性質にもよるが例えば80℃以上で30分程度である。ただし120℃を超える加熱を行うと過度の加熱履歴によるためか破断応力は低下する傾向となるため、120℃以下が好ましい。

[0033] （大豆蛋白ゲルの物性）

以上述べたように、本発明の大豆蛋白ゲルは、大豆蛋白素材、加熱条件、酵素反応条件などの選択によって、使用用途に合わせて多様な物性を有するゲルに適宜調製することができる。そして、本発明の大豆蛋白ゲルの物性は、数値的には破断応力と破断歪率の組合せによって表現することができる。

例えば、たわみのある硬い物性のゲルを調製したい場合、破断応力が50～1000（×1000N／$m^2$）、好ましくは100～800（×1000N／$m^2$）であって、かつ破断歪率

が50～100％となるようにすることが好ましい。特に、コンニャク的なゲルを調製したい場合、破断応力が150～250（×1000N／$m^2$）となるようにすることが好ましい。また、蒲鉾的なゲルを調製したい場合、破断応力が250～600（×1000N／$m^2$）となるようにすることが好ましい。この際の大豆蛋白を含む材料中の大豆蛋白素材の濃度は1～50重量％、好ましくは11～30重量％、より好ましくは11～20重量％の範囲から選ぶことができる。

また、つるりとした柔い物性のゲルを調製したい場合、破断応力が5～50（×1000N／$m^2$）であって、かつ破断歪率が40～80％となるようにすることが好ましい。この際の大豆蛋白素材を含む材料中の大豆蛋白素材の濃度は1～10重量％、好ましくは5～10重量％、より好ましくは7～10重量％の範囲から選ぶことができる。

[0034] なお、これらの物性値は室温において大豆蛋白ゲルをレオメーター（山電(株)製など）を用いて破断強度解析を行うことによって求める。測定条件として、サンプル高は20mm、進入速度は1mm／秒とし、プランジャーは硬い物性のゲルの場合はφ5mm球を用い、柔い物性のゲルの場合はφ15mm球を用いることとする。破断強度解析にて得られた破断荷重に（式2）を適用して破断応力を求める。また、破断強度解析にて得られた破断変形に（式3）を適用して破断歪率を求める。

[0035] （式2）破断応力Y＝F×9．8÷（r×r×3．14）×1000

式中、F：破断荷重（g）、r：プランジャー半径

[0036] （式3）破断歪率X＝T÷h×100

式中、T：破断変形（mm）、h：サンプル高さ（mm）とした。

[0037] なお、ゲルの硬さの程度は破断応力Yでほぼ表現することができ、ゲルのたわみの程度は破断歪率Xでほぼ表現することができるが、更にゲルのしなやかさの程度を客観的に表現できる指標として、「破断指標Z」を用いることができる。破断指標Zは、破断強度解析で得られた波形データを、縦軸が破断応力Yで横軸が破断歪率Xで表した座標上に描いて、その破断点と原点を通る完全弾性体を仮想し、原点O（0，0）から破断点P（a，ma）までの曲線の軌跡がどの程度その仮想曲線に沿っているかを表したもので、（式4）を用いて求める。

[0038] （式4）

$$\text{破断指標}\quad Z=\int_0^a\{f(x)-mx\}d(x)\Big/\int_0^a\{mx\}d(x)\times 100$$

[0039] 破断指標Zの値が正の値や0%に近い値である場合、そのゲルはしなやかではなく、破断指標Zが負の値であってZ＝－40%のように低い数値であれば、そのゲルは非常にしなやかなゲルであることが、市販ゲル食品の物性評価結果からわかっている。

例えば、コンニャクや蒲鉾などのようにたわんでしなやかなゲルを調製したい場合、破断指標Zが－10%～－80%、好ましくは－15%～－60%、更に好ましくは－20%～－40%となるように調製することが適当である。

[0040] （大豆蛋白ゲルの風味・色調）

本発明で得られる大豆蛋白ゲルは従来の大豆蛋白ゲルよりも風味と色調に優れることも特徴である。

LPが低減化されていない大豆蛋白ゲルでは大豆の雑味を感じやすくて風味が劣る。また大豆蛋白素材の中でも11S大豆蛋白を使用したゲルが最も大豆の雑味を感じにくく風味が優れている。また、11S大豆蛋白のゲルが最もくすみや黄色みが少なく、そのような色調が求められる用途において有利である。

一方、7S大豆蛋白のゲルは黄色みがあって透明感のあるゲルである。また、7S大豆蛋白及び11S大豆蛋白の両方からなるゲルも、くすみや黄色みが少ないゲルである。このようにLPを除去することで、物性・風味以外にも色調面においても改善効果がある。

[0041] （大豆蛋白ゲルの食品への応用）

本発明の大豆蛋白ゲルはその特徴的な物性や色調を利点として様々なゲルが利用される食品へ応用することが可能である。大豆蛋白ゲルを食品へ利用する態様としては、予め大豆蛋白ゲルを調製しておき、これをそのまま食品へ加工したり、食品中に混合する場合は無論、食品の製造過程においてLPを低減した大豆蛋白素材と蛋白質架橋酵素を他の原料と共に配合しておき、ゲルを形成させる場合を含む。後者においては公知の食品の物理特性を改良したり、逆に食品の物理特性を殆ど変

化させることなくその原料である他素材を大豆蛋白に代替させたりすることができる。

[0042] 予め大豆蛋白ゲルを調製し、これを利用する例として、新食感の大豆食品などや低糖・低脂質で高蛋白質のニーズを満たす食品などに利用できる他、大豆由来の栄養分を摂取できるたわむ食感のグミ菓子・栄養バーや栄養ゼリーなどに利用でき、おでんの具材や炊き込みご飯などの惣菜用途として利用するコンニャク代替物などとしても利用できる。

[0043] また、豚のゼラチン質や牛筋肉のようなプルプルした食感を楽しむような用途の代替品などとして使用したり、すり身などの魚肉やミンチなどの畜肉を使用しない純植物性の食品に使用したり、必要に応じてゲルに切れ目を入れるなどしてアワビ風・貝柱風のような魚介類風のゲルなどとしたり、刻んでハンバーグ・ソーセージ・餃子・しゅうまいなどに混ぜ込んでヘテロな食感を与えたり、適当な大きさにカットしてグラタンやシチューなどの具材として利用したり、薄くスライスするなどしてピザトッピングなどにしたりすることができる。

[0044] また、ゲルを形成させる際に、薄く膜状に成型するなどして、生春巻のたわむ皮などの代替や、寿司の海苔などの代わりとして具を巻く用途に用いたり、湯葉の代替としたり、刻んで海藻の代替としたり、サラダトッピングなどとしたりすることができる。

[0045] また、シート状に成型して細長く切れ目を入れて束ねるなどして蟹足風などに加工したり、麺帯のような形状に成型してたわみある栄養麺などとして使用したり、餃子の皮やラザニアなどのようにして利用したり、サラダや寿司などを包むための薄焼き卵のフィルムなどの代替として用いたりすることができる。

[0046] また、球状に成型して魚卵代替などとして用いたり、栄養ドリンク中に球状ゲルを浮遊させるなど、視覚に訴えるような製品にも用いることもできる。また、甘みと鮮やかな色を付けるなどして、和菓子や洋菓子のトッピングなどに用いることもでき、プラスチック成型を参考にして金型を選択することで、金魚などのような形に成型して食を楽しむこともできる。また、ゲルを味噌や醤油やタレやダシなどに付けて、味噌漬や佃煮などといった惣菜として利用することもできる他、味噌汁やスープの具材などや、咀嚼・嚥下困難者の食事用などに使用することができる。

[0047] 次に、食品の製造過程においてLPを低減した大豆蛋白素材と蛋白質架橋酵素を

配合しておき、ゲルを形成させる例を挙げると、畜肉製品（ハンバーグ、ソーセージ、ハムなど），魚肉製品（カニ、ホタテ、アワビ風等のイミテーション蒲鉾・揚蒲鉾、蒸蒲鉾、焼蒲鉾などの蒲鉾類・ちくわ類・フィッシュボールなど），乳製品（チーズ・ヨーグルトなど），卵製品（玉子焼き・茶碗蒸しなど），穀物製品（餅・麺など），肉や魚介などのイミテーションフード，低炭水化物食・高蛋白食・嚥下食・大豆たん白主体食品などの機能性食品などの製造過程において原料中にLPを低減した大豆蛋白素材と蛋白質架橋酵素を配合しておき、酵素を作用させてゲルを形成させることができる。

また、LPが低減された大豆蛋白素材に蛋白質架橋酵素を作用できる水系であれば、非食品であってもそのゲルを利用することができる（例えばペットフード、飼料、肥料などの他、砂漠の緑化を行う為の保水剤や水系塗料など）。

より具体的には、本発明の大豆蛋白ゲルと食品原料から生成するゲルの物理特性を踏まえつつ、大豆蛋白素材の配合量、蛋白質架橋酵素の添加量、生地のpH、塩濃度、油脂添加量、糖添加量、ミネラル（Ca、Mg等）添加量などのファクターを適宜変更することで上記の食品等に利用することができる。

例えば、ミンチ肉の一部をLPの低減された大豆蛋白に置換し、これに蛋白質架橋酵素を作用させることで、やや脆いソーセージの物性をたわみある物性へと改変することができる。また、すり身の一部をLPの低減された大豆蛋白に置換し、これに架橋酵素を作用させることで、たわみある物理特性が特徴である蒲鉾の物性を殆ど損うことなく、すり身と大豆蛋白のハイブリッド蒲鉾を製造できる。

[0048] （大豆蛋白ゲル調製用大豆蛋白素材）

以上の知見から、本発明は特徴的な大豆蛋白ゲル及びそれを利用した食品を提供するほか、このような大豆蛋白ゲルを消費者や製造メーカーにおいても容易に調製でき、種々の食品へ利用されることを可能とするため、大豆蛋白ゲル調製用大豆蛋白素材を提供する。この大豆蛋白素材はLPが低減され、かつ、蛋白質架橋酵素が作用したものであることを特徴とするものである。

また本発明は製造メーカー自身が蛋白質架橋酵素を作用させて本発明の大豆蛋白ゲルを容易に調製することを可能とする大豆蛋白素材も提供する。この大豆蛋白素材はLPが低減されていることを特徴し、食品の製造において蛋白質架橋酵素を

作用させて使用するものである。

[0049] これらの大豆蛋白素材は、求められる用途・粘度・色調・物性などに応じて、7S大豆蛋白や11S大豆蛋白、分離大豆蛋白など前述した種々のタイプの大豆蛋白素材とすることが可能である。これらの素材は凍結品・乾燥品などの形態で流通させることが可能である。

実施例

[0050] (LPが低減された大豆蛋白素材の調製)

非特許文献2(M Samoto etc, Food Chemistry, 102, 317-322, 2007.)に記載の方法に準じて以下の通り、LPが低減されたタイプの大豆蛋白素材を調製した。

1)低変性脱脂大豆に加熱処理を施してNSI(水溶性窒素指数)を低下させた脱脂大豆(NSI70%)の温水抽出スラリーを遠心分離機にてオカラ画分を除き脱脂豆乳とした。

2)脱脂豆乳を更に遠心分離機にかけ、沈殿してくる難溶性の画分を除いた。この画分は比較的親水性の低い脂質親和性蛋白であり、以下「難溶性LP」と称する。次に残りの上清のpHを5.8に調整して遠心分離機にて沈殿カード画分を回収した。この画分が11S大豆蛋白であり、これを「11S蛋白」と称することとする。

3)次に、残りの上清のpHを5.0に調整し、55℃で10分間放置後、次いでpH5.5に調整後、遠心分離機にて沈殿カード画分を回収した。この画分も上記難溶性LP画分と同様に、脂質親和性蛋白であり、これを「LP」と称することとする。

4)次に、残りの上清のpHを4.5に調整し、遠心分離機にて沈殿カード画分を回収した。この画分が7S大豆蛋白であり、これを「7S蛋白」と称することとする。

5)得られた各画分(11S蛋白, 7S蛋白, LP)より、中和後加熱処理を行わずに噴霧乾燥した未変性大豆蛋白素材(A)と、中和後140℃×10秒の加熱変性処理後、噴霧乾燥した変性大豆蛋白素材(B)の2タイプを調製した。

6)本操作による乾物収率比は、11S蛋白:LP:7S蛋白の比はおよそ2:2:1であった。この11S蛋白(A)、LP(A)及び7S蛋白(A)を2:2:1の比率で混合したタイプの大豆蛋白素材を得た。これは上記の難溶性LP画分のみを除去したものであるので、「脱難溶性LP蛋白」と称することとする。

7）11S蛋白（A）及び7S蛋白（A）を2：1の比率で混合したタイプの大豆蛋白素材を得た。これは上記の難溶性LP画分と3）のLP画分を両方とも除去したものであり、「脱LP蛋白」と称することとする。

[0051] （LPが低減されていない大豆蛋白素材の調製）

別途、特許文献5に準じて以下の通り、LPが低減されていないタイプの大豆蛋白素材、すなわち分離大豆蛋白を調製した。

1）低変性脱脂大豆の温水抽出スラリーを遠心分離機にてオカラ画分を除き脱脂豆乳とした。

2）得られた脱脂豆乳をpHを4．5に調整して等電点沈殿せしめ、遠心分離機にて酸沈殿カードを得て中和した。

3）次にこの中和液を140℃10秒の予備加熱を行った上で、噴霧乾燥して分離大豆蛋白（SPI）として調製した。一方で、この中和液を140℃10秒の予備加熱を行った上で、トランスグルタミナーゼ製剤「アクティバ」（味の素（株）製、以下「TGase」と称する。）を粗蛋白質1gあたり0．5ユニット添加し、50℃で30分反応させて、140℃10秒の加熱を施し噴霧乾燥させたものをTGase処理分離大豆蛋白「TG－SPI」として調製した。

[0052] 表1に、得られた各種大豆蛋白素材のリストと、各素材のLPの推定含量であるLCI値の測定結果を示した。LCI値の測定方法は前述の通りである。

[0053] （表1）

| 大豆蛋白素材 | 内　　容 | ＬＣＩ値 |
|---|---|---|
| １１Ｓ蛋白（Ａ） | 未加熱タイプ | 21 |
| １１Ｓ蛋白（Ｂ） | 加熱変性タイプ | |
| ＬＰ（Ａ） | 未加熱タイプ | 55 |
| ＬＰ（Ｂ） | 加熱変性タイプ | |
| ７Ｓ蛋白（Ａ） | 未加熱タイプ | 16 |
| ７Ｓ蛋白（Ｂ） | 加熱変性タイプ | |
| 脱難溶性ＬＰ蛋白 | １１Ｓ蛋白（Ａ）、ＬＰ（Ａ）及び７Ｓ蛋白（Ａ）の２：２：１混合品 | 34 |
| 脱ＬＰ蛋白 | １１Ｓ蛋白（Ａ）及び７Ｓ蛋白（Ａ）の２：１混合品 | 19 |
| ＳＰＩ | 分離大豆蛋白 | 39 |
| ＴＧ－ＳＰＩ | TGase処理分離大豆蛋白 | － |

[0054] (試験例1)硬い大豆蛋白ゲルの調製とゲル物性・風味の評価

表1の各種大豆蛋白素材を12%濃度となるよう水に溶解してpH7．5に調整し、遠心脱泡してスラリー状とした。TGaseを添加するものについては、大豆蛋白素材の粗蛋白質1gあたり3．2ユニットとなるよう添加後、ケーシングチューブに充填し、55℃×30分の酵素反応を行った。引き続き90℃×30分の加熱を行った後、一晩冷蔵し、室温に戻して、物性評価用の大豆蛋白ゲルとした。大豆蛋白ゲルはケーシング剥離し、山電(株)製レオメーターにて測定を行い、破断強度と破断変形の解析を行った(測定条件:サンプル高20mm、φ5mm球プランジャー、1mm／秒)。得られた破断荷重及び破断変形に(式2)及び(式3)を適用して破断応力(×1000N／$m^2$)及び破断歪率(%)を求めた。また、ゲルを試食し、風味の評価を10点満点で点数で表し、食感について評価を行った。結果を表2及び表3に示した。

[0055] (表2)変性大豆蛋白素材のゲルに対するTGaseの物性への効果

| | 大豆蛋白素材 | TGase添加 | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|
| T－1 | 11S蛋白(B) | 有 | 172 | 83 | 10 | 蒟蒻的たわみ |
| T－2 | 7S蛋白(B) | 有 | 221 | 87 | 9 | 蒟蒻的たわみ |
| T－3 | LP(B) | 有 | 67 | 69 | 4 | 柔蒟蒻的たわみ |
| T－4 | SPI(B) | 有 | 142 | 80 | 5 | 蒟蒻的たわみ |
| T－5 | TG-SPI(B) | 無 | 27 | 70 | 5 | べたつく |
| T－6 | 11S蛋白(B) | 無 | 4 | 42 | 9 | ドロリ |
| T－7 | 7S蛋白(B) | 無 | 44 | 71 | 7 | 蕨餅的たわみ |
| T－8 | LP(B) | 無 | 4 | 31 | 2 | ドロリ |
| T－9 | SPI(B) | 無 | 17 | 50 | 4 | べたつく |

[0056] 表2の結果の通り、大豆蛋白素材が変性タイプの場合、TGase無添加区と比較して、TGase添加区では、破断応力と破断歪率が大きくなり、いずれもたわみのある硬い食感の方向へと改質されていた。同じ濃度での比較において、7S(B)、11S(B)が最もたわみのある硬い食感となり風味も良好であった。一方、LP(B)はたわみと硬さが不十分で風味が悪く、SPI(B)は、たわみがあって硬い食感であったものの、風味面では7S(B)や11S(B)の方が顕著に優れていた。

[0057] 本発明の課題の一つである「たわみのある硬い食感の大豆蛋白ゲル」について、我々は、この評価法において満足できる目標レベルとして、風味8～10点、破断応力50～1000(×1000N／$m^2$)、破断歪率50～100%と設定した。従い、本実験例に

おいて、課題を解決しうるものとしては、T－1の11S蛋白(B)(TGase処理)及びT－2の7S蛋白(B)(TGase処理)であった。すなわち、従来の分離大豆蛋白から、LP画分を低減させることが重要であった。

[0058] (表3)未変性大豆蛋白素材のゲルに対するTGaseの物性への効果

| | 大豆蛋白素材 | TGase添加 | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|
| T－10 | 11S蛋白(A) | 有 | 119 | 79 | 10 | 柔蒟蒻的たわみ |
| T－11 | 7S蛋白(A) | 有 | 136 | 84 | 8 | 蒟蒻的たわみ |
| T－12 | LP(A) | 有 | 9 | 23 | 3 | ドロリ |
| T－13 | 11S蛋白(A) | 無 | 20 | 38 | 8 | やや豆腐的 |
| T－14 | 7S蛋白(A) | 無 | 55 | 73 | 6 | 蕨餅的たわみ |
| T－15 | LP(A) | 無 | 3 | 6 | 1 | 液状 |

[0059] 表3の結果の通り、大豆蛋白素材が未変性タイプの場合でも、TGase無添加区と比較し、TGase添加区では、破断応力と破断歪率が大きくなり、いずれもたわみのある硬い食感の方向へと改質されていた。しかし、LP(A)についてはTGaseの添加によっても殆ど改質されなかった。したがって、風味がよくてたわみのある硬い食感の大豆蛋白ゲルを得るには、LP画分を低減化する工程が必須であることが示された。

[0060] (試験例2)柔い大豆蛋白ゲルの調製とゲル物性の評価

表4に示したように各種大豆蛋白素材を材料中の濃度が所定の濃度(重量%)となるよう水に溶解してpH7．5に調整し遠心脱泡してスラリー状の材料とした。TGaseを大豆蛋白素材の粗蛋白質1gあたり3．2ユニットとなるよう添加後、亀甲容器に充填して蓋をし、55℃×30分の酵素反応を行った。引き続き90℃×30分の加熱を行った後、一晩冷蔵し、室温に戻して、物性評価用の大豆蛋白ゲルとした。大豆蛋白ゲルについて試験例1と同様にして物性、風味、食感の評価を行った。ただし柔い食感のゲル物性を評価できるように、レオメーターのプランジャーは大きなプランジャー(φ15mm円柱プランジャー)を用いた。

[0061] (表4)各大豆蛋白素材濃度におけるTGase作用ゲルの物性

| | 大豆蛋白素材 | 濃度 | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|
| T－16 | 脱難溶性LP蛋白 | 6.6 | 0.4 | 69 | 8 | 液状 |
| T－17 | 脱難溶性LP蛋白 | 8.3 | 3 | 60 | 8 | ドロリ |
| T－18 | 脱難溶性LP蛋白 | 9.9 | 13 | 63 | 8 | ややつるり |
| T－19 | 脱LP蛋白 | 6.6 | 0.4 | 52 | 9 | 液状 |
| T－20 | 脱LP蛋白 | 8.3 | 7 | 50 | 9 | ややつるり |
| T－21 | 脱LP蛋白 | 9.9 | 24 | 55 | 9 | つるり |
| T－22 | 11S蛋白（A） | 6.6 | 0.9 | 85 | 10 | 液状 |
| T－23 | 11S蛋白（A） | 8.3 | 4 | 48 | 10 | ドロリ |
| T－24 | 11S蛋白（A） | 9.9 | 13 | 50 | 10 | つるり |

[0062] 本発明の課題の一つである「つるりとした柔い食感の大豆蛋白ゲル」について、我々は、この評価法において満足できる目標レベルとして、風味8～10点、破断応力5～50（×1000N／$m^2$）、破断歪率40～80％と設定した。従い、表4において課題を解決しうる条件はT－18、T－20、T－21、T－24であった。すなわち、従来型の分離大豆蛋白から、LP画分を低減することによって、初めて課題を解決しうるものである。

[0063] （試験例3）大豆蛋白ゲルの物性に与える油脂・塩・pHの影響

11S蛋白（A）の濃度が12％となるよう水に溶解し、パーム油「パームエース10」（不二製油（株）製）、食塩、砂糖を溶解・分散させ、表5の所定のpH（pH5．5～8．5）に調整して遠心脱泡してスラリー状の材料とした。TGaseを大豆蛋白素材の粗蛋白質1gあたり3．2ユニットとなるよう添加後、ケーシングチューブに充填し、55℃×30分の酵素反応を行った。引き続き95℃×30分の加熱を行った後、一晩冷蔵し、室温に戻して、物性評価用のゲルとした。大豆蛋白ゲルについて試験例1と同様にして物性、風味、食感の評価を行った。風味は点数が10点満点中8点以上であったものに○を付した。

[0064] （表5）大豆蛋白素材を含む材料に加える他の原料による物性への影響

| | 油脂(%) | 食塩(%) | pH | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|---|
| T－25 | 0 | 0 | 7.5 | 148 | 88 | ○ | 蒟蒻的たわみ |
| T－26 | 5 | 0 | 7.5 | 97 | 87 | ○ | 柔蒟蒻的たわみ |
| T－27 | 10 | 0 | 7.5 | 97 | 83 | ○ | 柔蒟蒻的たわみ |
| T－28 | 17 | 0 | 7.5 | 104 | 82 | ○ | 柔蒟蒻的たわみ |
| T－29 | 0 | 0.6 | 7.5 | 55 | 50 | ○ | ややボソつく |
| T－30 | 0 | 1.2 | 7.5 | 37 | 35 | ○ | ボソボソ |
| T－31 | 0 | 1.8 | 7.5 | 148 | 67 | 塩味強 | たわんでシャキ |
| T－32 | 0 | 0 | 5.5 | 22 | 32 | 酸味強 | ボソボソ |
| T－33 | 0 | 0 | 6.5 | 104 | 65 | ○ | たわんでシャキ |
| T－34 | 0 | 0 | 8.5 | 197 | 93 | ○ | 蒟蒻的たわみ |

[0065] 大豆蛋白素材を含む材料に加える他の原料やpHによって、大豆蛋白ゲルの風味・物性は影響を受ける場合があった。具体的には、pH5．5(T－32)では酸味が強くてボソボソした食感となり、大豆蛋白素材を含む材料への食塩添加量が1．2重量%(T－30)ではボソボソした食感となっていた。なお、各々のゲルをSDS－PAGE(電気泳動)に供し、TGaseによって生じた分子間架橋の程度(高分子の量)を調べたところ、いずれのゲルも同程度に分子間架橋を生じていたことから、これらの現象は、TGaseが反応しにくくなり生じた結果ではなく、各々の条件下での大豆蛋白の溶解性や保水性やゲル形成能と関連していると考えられた。従い、ある程度の食塩量が必要になってくるような惣菜用途に応用したい場合は、大豆蛋白素材濃度を少し高めにして、pHをやや高めにするなどの工夫を施すことができそうであると期待でき、実際、以降の実施例にて工夫できることを実証する。

[0066] なお、油脂によって破断応力はやや低下するが破断歪率は低下せず、たわみのある食感を維持した。この理由としては、LPを低減することによって、油脂が、大豆蛋白素材の液に対して相分離しやすくなっているからではないかと考えられる。また、データには示していないが、大豆蛋白素材を含む材料中に砂糖を5%添加しても物性の変化はさほど大きくはなかったことから、甘味系のデザート的なものへの応用が可能であると示唆され、以降の応用例にて説明する。

[0067] (応用例1)大豆蛋白ゲルを利用した食品(生貝柱風の大豆蛋白ゲル)

グルタミン酸Na:0．05重量部、グリシン:0．5重量部、アラニン:0．2重量部、食塩:0．1重量部、コハク酸Na:0．3重量部、砂糖:0．2重量部、ホタテエキス「TC－F2」(焼津水産化学工業(株)製):0．05重量部を、冷水:8．6重量部に溶解して調味

液(H)とした。

11S蛋白(A)の粉末12．9重量部を73．3重量部の冷水に溶解し、上記の調味液(H)を10重量部添加し、pH8．0に調整して遠心脱泡してスラリー状の材料とした。TGaseを10重量%となるよう冷水に溶解させた液を、TGase1．6重量%(対大豆蛋白素材固形分重量)となるようスラリー状の材料に添加後、折径6．4cmのケーシングチューブに充填(大豆蛋白素材の濃度は12重量%)し、55℃×30分の酵素反応を行った。引き続き95℃×30分の加熱を行った後、一晩冷蔵し、室温に戻して、大豆蛋白ゲルとした。大豆蛋白ゲルは試験例1と同様にして物性について評価した。一方で、大豆蛋白ゲルを厚さ1．5cmの輪切りにし、得られた円柱ゲルの両面について、幅1mm前後の鹿の子模様(細かい斜めの格子状の模様)となるよう深さ5mmの切り込みを入れて、貝柱の繊維風に調理し、風味を確認した。

[0068] (表6)

| | 調理内容 | 大豆蛋白素材濃度 | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|
| T－35 | 鹿の子切り無 | 12 | 123 | 67 | 貝柱味 | 柔蒟蒻的 |
| T－36 | 鹿の子切り有 | 12 | 測定無 | 測定無 | 貝柱味 | 生貝柱的 |

[0069] T－35では生貝柱的食感にはならなかったが、切り込みを適度に入れることで、ゲルの束からなる繊維感を舌で感じうるものとなった。このゲルは比較的つるりとした食感を有していた。加熱済の貝柱は繊維感が目立ちすぎ、一方、生貝柱は繊維感が少ないが、このゲルは比較的つるりとした食感であって適度な繊維感も有していた。すなわち、本発明で得られる大豆蛋白ゲルに切り込みなどの公知の調理加工を施すことで、更にテクスチャーの幅が広がるものとなる。なお、鹿の子状に切り込みを入れたT－36の「生貝柱風の大豆蛋白ゲル」を寿司の具とし、甘しょうゆダレをつけると、鹿の子状の切れ目が美しく映え、食欲をそそるものとなり、更に美味しく食せるものであった。

[0070] (応用例2)大豆蛋白ゲルを利用した食品2(杏仁風味の大豆蛋白ゲル)

市販の甜杏仁粉20重量部を60重量部の温水に分散後、遠心分離して得た上清から40重量部を採取して冷却し、砂糖5重量部を加えて調味液(N)とした。なお、表7のT－40では、砂糖5重量部の代わりに、ステビア甘味料製剤(大日本インキ化学

工業製)を0．05重量部加えて調味液(N)の代わりとした。

先に調製した脱難溶性LP蛋白、脱LP蛋白、11S蛋白(A)の粉末24重量部を、各々136重量部の冷水に溶解し、上記の調味液(N)を40重量部添加し、pH7．5に調整して遠心脱泡してスラリー状の材料とした。TGaseを10重量%となるよう冷水に溶解させた液を、TGaseの添加量が大豆蛋白素材の粗蛋白質1gあたり3．2ユニットとなるよう該材料に添加後、蓋のできるプラスチック容器に60重量部充填し(大豆蛋白素材の濃度9．9%)、55℃の温浴中にセットして、毎分2℃上昇するように徐々に90℃まで湯温を昇温させた後、90℃20分の加熱を行った後、一晩冷蔵し、室温に戻して大豆蛋白ゲルとした。これらの大豆蛋白ゲルは容器の蓋を外し、試験例1と同様にして物性、風味、食感を評価した。

[0071] (表7)

| | 大豆蛋白素材 | 大豆蛋白素材濃度 | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|
| T－37 | 脱難溶性ＬＰ蛋白 | 9.9 | 6 | 48 | やや雑味 | ややつるり |
| T－38 | 脱ＬＰ蛋白 | 9.9 | 9 | 45 | 杏仁味○ | つるり |
| T－39 | １１Ｓ蛋白（Ａ） | 9.9 | 6 | 40 | 杏仁味○ | ややつるり |
| T－40 | 脱ＬＰ蛋白 | 9.9 | 10 | 44 | 爽杏仁味○ | つるり |

[0072] 脱難溶LP蛋白を使用したT－37のゲルで感じられた大豆由来の雑味が、脱LP蛋白を使用したT－38や11S(A)を使用したT－39のゲルでは感じられず、雑味のない杏仁味となった。食感はT－38のゲルの方がT－39のゲルよりもつるりとしていて好ましく、砂糖の代わりにステビア甘味料製剤を用いたT－40のゲルは爽やかな甘みを呈し好ましかった。T－40の「大豆でできた杏仁ゼリー」を角切りにしてシロップ漬し、レトルト袋に充填して加熱殺菌後、冷蔵して食しても、型崩れなく、美味しく食せるものであった。

[0073] (応用例3)大豆蛋白ゲルを利用した食品3(蒲鉾風の大豆蛋白ゲル)

グルタミン酸Na:0．02重量部、食塩:0．7重量部、砂糖:0．7重量部、「サカナエキスHN－55」(仙味エキス(株)製):1．5重量部を、冷水:7．1重量部に溶解して調味液(T)とした。

先に調製した11S蛋白(A)の粉末15．7重量部を、69．6重量部の冷水に溶解し、上記の調味液(T)を10重量部添加し、pH8．0に調整してスラリー状の材料とした。

TGaseを10重量%となるよう冷水に溶解させた液を、TGaseの添加量が大豆蛋白素材の粗蛋白質1gあたり3．2ユニットとなるように添加後、逆さ蒲鉾型の容器に充填（大豆蛋白素材の濃度15重量%）し、蒲鉾板を容器上に浮かせ、55℃のコンビオーブン中にセットして30分後、90℃まで昇温させ30分の加熱を行った後、容器から外して蒲鉾板ごとラッピングし、一晩冷蔵し、室温に戻して大豆蛋白ゲルとした。得られたゲルはラッピングを外し、試験例1と同様にして物性、風味、食感について評価した。

[0074] （表8）

| | 大豆蛋白素材 | 大豆蛋白素材濃度 | 破断応力 | 破断歪率 | 風味 | 食感 |
|---|---|---|---|---|---|---|
| T－41 | 11S（A） | 15 | 201 | 62 | 蒲鉾味○ | 蒲鉾的 |

[0075] 該材料中に食塩が1%程度存在すると、表5のT－30のように、大豆蛋白素材濃度が12%程度（pH7．5）では、保水力のあるゲルネットワークを構築しにくく、ボソついた食感になってしまう。そこでT－41のように大豆蛋白素材の濃度を15%と高くすると、食塩濃度が0．7%以上であっても、蒲鉾的なたわみのある食感にすることが可能であった。おそらく、大豆蛋白素材濃度を高めにすることでネットワークの材料が増え、該材料のpHを8．0とやや高めにして大豆蛋白素材の等電点から遠ざけることでゲルネットワークの保水力が高まったと考えられる。

このように、本発明の大豆蛋白ゲルは惣菜用途においても利用することができ、例えば、近年高騰している魚肉のすり身を使用しなくとも、蒲鉾的な食感・風味の「蒲鉾風の大豆蛋白ゲル」を製造することができる。

なお、塩の存在下で11S蛋白を含有するスラリー状の材料を加熱した場合、そのゲルは明るい白色に白濁する傾向にあるので、色調の面からも蒲鉾に近くなった。また、T－41で得られた大豆蛋白ゲルを、おでんの煮込み液にて煮込むことで、おでんの煮込み液がゲルの内部へ速やかに浸透するため味浸みが良く、型崩れもなく、美味しく食せるものであった。

[0076] （応用例4）大豆蛋白ゲルを利用した食品4（他素材代替）

冷凍すり身（スケソウFA：水分75%）67重量部、食塩2．1重量部を塩擂りし、冷水

33重量部を加え、T－42のゲル調製用の生地を調製した。

冷凍すり身45重量部、LPが低減された大豆蛋白素材液（11S蛋白（A）ペースト（水分80％））を55重量部、食塩2．1重量部、TGaseを生地中蛋白1gあたり8ユニットとなるよう加えて塩ズリし、T－43のゲル調製用の生地を調整した。

各生地は、真空脱泡してケーシングチューブに充填し、30℃×30分加温後、90℃×30分で加熱し、一晩冷蔵後、室温に戻した後、ケーシング剥離し、試験例1と同様にして物性の評価を行った。ただし、プランジャーはφ3mm円柱プランジャーを用いた。φ3mm円柱プランジャーでの測定は、T－41のゲルについても実施し（T－44）、市販の蒲鉾についても実施した（T－45、46）。表9の結果は、φ3mm円柱プランジャーで測定したためφ5mm球プランジャーの場合よりもT－41の破断応力が高い数値となっているが、表9のデータにおける相対比較は可能である。なお、ゲルのたわみの程度は破断歪率Xでほぼ表現することができるが、更にゲルのしなやかさの程度を客観的に表現できる指標として、破断指標Z（式4）を用いて物性を評価した。

[0077] （表9）

| | 内容 | 破断応力Y（φ3mm） | 破断歪率X（φ3mm） | 破断指標Z（φ3mm） | 食感 |
|---|---|---|---|---|---|
| T－42 | 無置換 | 424 | 60 | －22 | 蒲鉾的 |
| T－43 | 乾物半量代替 | 483 | 51 | －31 | 蒲鉾的 |
| T－44 | 乾物全量代替 | 516 | 65 | －41 | 蒲鉾的 |
| T－45 | 市販の板付蒲鉾 | 540 | 58 | －38 | 蒲鉾的 |
| T－46 | 市販の成型蒲鉾 | 276 | 50 | －31 | 蒲鉾的 |

[0078] すり身100％品や市販板付蒲鉾や市販成型蒲鉾（T－42、T－45、T－46）では、破断変形XがX＞50％で、破断指標ZはZ＜－20％であって、食感はたわみある蒲鉾的でしなやかさを有するものあった。なお、T－45は、硬さとたわみとしなやかさに優れた比較的高級な蒲鉾であり、T－46は、硬さとたわみとしなやかさを有した中級の蒲鉾であった。T－42は、硬さとたわみに優れしなやかさにやや欠けるものであったが、蒲鉾的食感の範疇であった。従い、T－42、T－45、T－46の各々の食感は、硬さやたわみやしなやかさの程度は異なってはいたが、いずれも蒲鉾的な食感として認識できるものであった。

本応用例における測定値によれば、破断応力Yが250～600（×1000N／$m^2$）、破断歪率Xが50％以上、破断指標Zが－20％以下であれば、蒲鉾的食感の範疇に

あることが期待できた。

[0079] そこでT－43を評価すると、大豆蛋白をすり身と半量置換してもなお、蒲鉾的食感の範疇にあって、たわみのある蒲鉾の物性を殆ど損うことなくすり身と大豆蛋白素材のハイブリッド蒲鉾にすることができた。これはすり身が不足したり、高騰した場合などに有効に利用することができるものであった。更にT－44では、すり身を全く使用しなくても蒲鉾的食感にすることができるものであった。

すなわち、LPを低減させた大豆たん白素材を用いることにより、風味・色調は良好なものとなっており、蒲鉾のような薄味の食品への利用にも適していることが示された。

また、LPが低減された大豆蛋白素材液100重量部に、なたね油を10重量部加えて乳化させておくことで、破断応力Yが0．8倍となったが、破断歪率Xや破断指標Zは殆ど変化させることなく、蒲鉾的なたわむ食感を作り上げることができ、T－42よりも白っぽい食品に仕上げることも可能であった。

# 請求の範囲

[1] 大豆蛋白素材を含む材料を加熱して得られるゲルであって、該大豆蛋白素材は脂質親和性蛋白質が低減されたものであり、かつ、該ゲルは蛋白質架橋酵素が作用したものであることを特徴とする大豆蛋白ゲル。

[2] 大豆蛋白素材が全脂豆乳、脱脂豆乳、分離大豆蛋白、7S大豆蛋白、11S大豆蛋白である請求項1記載の大豆蛋白ゲル。

[3] 破断応力が50～1000（×1000N／$m^2$）であって、かつ破断歪率が50～100％である請求項1記載の大豆蛋白ゲル。

[4] 破断応力が5～50（×1000N／$m^2$）であって、かつ破断歪率が40～80％である請求項1記載の大豆蛋白ゲル。

[5] 請求項1記載の大豆蛋白ゲルを利用した食品。

[6] 脂質親和性蛋白質を低減した大豆蛋白素材を原料とし、該大豆蛋白素材を含む材料に蛋白質架橋酵素を作用させた後、加熱することを特徴とする大豆蛋白ゲルの製造法。

[7] 蛋白質架橋酵素を作用させるときの該大豆蛋白素材を含む材料のpHが6～9である請求項6記載の大豆蛋白ゲルの製造法。

[8] 蛋白質架橋酵素を作用させるときの該大豆蛋白素材への食塩添加量が大豆蛋白を含む材料中1重量％以下である請求項6記載の大豆蛋白ゲルの製造法。

[9] 脂質親和性蛋白質を低減した大豆蛋白素材が予め変性温度以上の予備加熱を施されておらず、該大豆蛋白素材を含む材料に蛋白架橋酵素を作用させた後、該大豆蛋白素材の変性温度以上で加熱を施すことを特徴とする請求項6記載の方法。

[10] 脂質親和性蛋白質を低減した大豆蛋白素材が予め変性温度以上の予備加熱を施されていて、該大豆蛋白素材を含む材料に蛋白架橋酵素を作用させた後、該酵素が失活するに充分な加熱を施す加熱である請求項6記載の方法。

[11] 脂質親和性蛋白質が低減されており、蛋白質架橋酵素が作用したものであって、かつ、大豆蛋白ゲル調製用であることを特徴とする大豆蛋白素材。

[12] 脂質親和性蛋白質が低減されており、蛋白質架橋酵素を作用させて使用するものであって、かつ、大豆蛋白ゲル調製用であることを特徴とする大豆蛋白素材。

[13] 大豆蛋白ゲルを予め調製し、これを混合してなるものである請求項5記載の食品。

[14] 食品原料中に脂質親和性蛋白質を低減した大豆蛋白素材及び蛋白質架橋酵素が配合され、製造過程で大豆蛋白ゲルが形成されてなるものである請求項5記載の食品。

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP2008/068146

A. CLASSIFICATION OF SUBJECT MATTER
*A23J3/16*(2006.01)i, *A23L1/00*(2006.01)i

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)
A23J3/16, A23L1/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Jitsuyo Shinan Koho 1922-1996 Jitsuyo Shinan Toroku Koho 1996-2008
Kokai Jitsuyo Shinan Koho 1971-2008 Toroku Jitsuyo Shinan Koho 1994-2008

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
JSTPlus(JDreamII), JST7580(JDreamII)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X/Y | JP 58-149645 A (Ajinomoto Co., Inc.), 06 September, 1983 (06.09.83), Examples 3, 7; page 3, upper right column, lines 15 to 19 (Family: none) | 1-8,11-13/ 9-10,14 |
| Y/A | WO 2002/028198 A1 (Fuji Oil Co., Ltd.), 11 April, 2002 (11.04.02), Page 10, line 5 to page 11, line 5 & EP 1323353 A1 & WO 2002/028198 A1 & AU 9229101 A & CN 1466420 A | 9-10/1-8, 11-14 |

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

* Special categories of cited documents:
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"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 |
|---|---|
| 17 October, 2008 (17.10.08) | 28 October, 2008 (28.10.08) |
| Name and mailing address of the ISA/ Japanese Patent Office<br>Facsimile No. | Authorized officer<br>Telephone No. |

Form PCT/ISA/210 (second sheet) (April 2007)

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/JP2008/068146

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y/A | JP 10-056976 A (Ajinomoto Co., Inc.), 03 March, 1998 (03.03.98), Examples (Family: none) | 14/1-13 |
| Y/A | Samoto M. et al., Improvement of the off-flavor of soy protein isolate by removing oil-body associated proteins and polar lipids., Biosci. Biotechnol. Biochem., 1998, 62(5), p.935-940 | 14/1-13 |
| A | WO 2005/094608 A1 (Fuji Oil Co., Ltd.), 13 October, 2005 (13.10.05), Full text & EP 1731041 A1 & CN 1960641 A | 1-14 |
| A | JP 01-027471 A (Ajinomoto Co., Inc.), 30 January, 1989 (30.01.89), Full text & US 5156956 A & EP 379606 A1 & DE 68917582 C | 1-14 |
| A | Chanyongvorakul Y. et al., Physical properties of soy bean and broad bean 11S globulin gels formed by transglutaminase reaction., Journal of Food Science, 1995, 60(3), p.483-488, 493 | 1-14 |

| 国際調査報告 | 国際出願番号　ＰＣＴ／ＪＰ２００８／０６８１４６ |
|---|---|

Ａ．　発明の属する分野の分類（国際特許分類（ＩＰＣ））

Int.Cl.　A23J3/16(2006.01)i, A23L1/00(2006.01)i

Ｂ．　調査を行った分野

調査を行った最小限資料（国際特許分類（ＩＰＣ））

Int.Cl.　A23J3/16, A23L1/00

最小限資料以外の資料で調査を行った分野に含まれるもの

| | |
|---|---|
| 日本国実用新案公報 | １９２２－１９９６年 |
| 日本国公開実用新案公報 | １９７１－２００８年 |
| 日本国実用新案登録公報 | １９９６－２００８年 |
| 日本国登録実用新案公報 | １９９４－２００８年 |

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

JSTPlus(JDreamII), JST7580(JDreamII)

Ｃ．　関連すると認められる文献

| 引用文献のカテゴリー＊ | 引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示 | 関連する請求の範囲の番号 |
|---|---|---|
| X/Y | JP 58-149645 A（味の素株式会社）1983.09.06, 実施例３、実施例７、第３頁右上欄第１５－１９行（ファミリーなし） | 1-8, 11-13/9-10, 14 |
| Y/A | WO 2002/028198 A1（不二製油株式会社）2002.04.11, 第１０頁第５行－第１１頁第５行 & EP 1323353 A1 & WO 2002/028198 A1 & AU 9229101 A & CN 1466420 A | 9-10/1-8, 11-14 |

☑　Ｃ欄の続きにも文献が列挙されている。　　☐　パテントファミリーに関する別紙を参照。

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|---|---|
| １７．１０．２００８ | ２８．１０．２００８ |

| 国際調査機関の名称及びあて先 | 特許庁審査官（権限のある職員） | ４Ｎ | ３８４６ |
|---|---|---|---|
| 日本国特許庁（ＩＳＡ／ＪＰ）<br>郵便番号１００－８９１５<br>東京都千代田区霞が関三丁目４番３号 | 三原　健治<br>電話番号　０３－３５８１－１１０１　内線　３４８８ | | |

国際調査報告　　国際出願番号　ＰＣＴ／ＪＰ２００８／０６８１４６

Ｃ（続き）．　関連すると認められる文献

| 引用文献のカテゴリー＊ | 引用文献名　及び一部の箇所が関連するときは、その関連する箇所の表示 | 関連する請求の範囲の番号 |
|---|---|---|
| Y/<br>A | JP 10-056976 A (味の素株式会社) 1998.03.03, 実施例<br>(ファミリーなし) | 14/<br>1-13 |
| Y/<br>A | Samoto M. et al., Improvement of the off-flavor of soy protein isolate by removing oil-body associated proteins and polar lipids.,<br>Biosci. Biotechnol. Biochem., 1998, 62(5), p.935-940 | 14/<br>1-13 |
| A | WO 2005/094608 A1 (不二製油株式会社) 2005.10.13, 全文<br>& EP 1731041 A1 & CN 1960641 A | 1-14 |
| A | JP 01-027471 A (味の素株式会社) 1989.01.30, 全文<br>& US 5156956 A & EP 379606 A1 & DE 68917582 C | 1-14 |
| A | Chanyongvorakul Y. et al., Physical properties of soy bean and broad bean 11S globulin gels formed by transglutaminase reaction.,<br>Journal of Food Science, 1995, 60(3), p.483-488, 493 | 1-14 |

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
10 September 2010 (10.09.2010)

PCT

(10) International Publication Number
WO 2010/101625 A2

(51) International Patent Classification:
*A61B 5/15* (2006.01) *G01N 33/49* (2006.01)
*A61B 5/00* (2006.01) *G01N 33/72* (2006.01)

(21) International Application Number:
PCT/US2010/000630

(22) International Filing Date:
2 March 2010 (02.03.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

| | | |
|---|---|---|
| 61/156,632 | 2 March 2009 (02.03.2009) | US |
| 61/163,710 | 26 March 2009 (26.03.2009) | US |
| 61/269,436 | 24 June 2009 (24.06.2009) | US |
| 61/257,731 | 3 November 2009 (03.11.2009) | US |

(71) Applicant *(for all designated States except US)*: **SEVENTH SENSE BIOSYSTEMS, INC.** [US/US]; 101 Binney Street, Cambridge, MA 02142 (US).

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(75) Inventors/Applicants *(for US only)*: **LEVINSON, Douglas, A.** [US/US]; 91 Lake Street, Sherborn, MA 01770 (US). **BERNSTEIN, Howard** [US/US]; 33 A Trowbridge Street, Cambridge, MA 02138 (US).

(74) Agent: **OYER, Timothy, J.**; Wolf, Greenfield & Sacks, P.c., 600 Atlantic Avenue, Boston, MA 02210-2206 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published**:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) Title: OXYGEN SENSOR

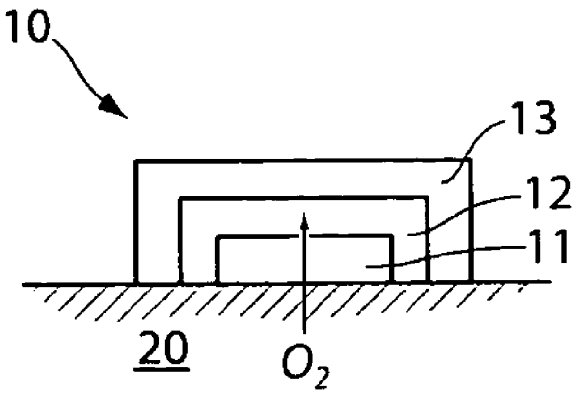



Fig. 1

(57) Abstract: The present invention generally relates to systems and methods for determining oxygen in a sample, or in a subject. In one aspect, the present invention is generally directed to an article exhibiting a determinable feature responsive to oxygen, such as oxygen-sensitive particles. The particles may exhibit a determinable change with a change in oxygen concentration, and such particles can accordingly be used to determine oxygen. For example, in one set of embodiments, the particles may be at least partially coated with a protein, such as hemoglobin, that is able to interact with oxygen. In some cases, the protein may aggregate under certain conditions (e.g., under relatively low oxygen concentrations), and such protein aggregation may be used, for example, to cause the particles to become aggregated, which can be determined in some way. In some cases, such aggregation may be irreversible; i.e., the degree of aggregation corresponds to the most extreme oxygen concentrations that the proteins were exposed to. Such articles may be used, for example, to determine oxygen within a sample, or within a subject, such as a human subject. For instance, the article may be formed as a skin patch, or administered to the skin of a subject, e.g., on the surface of the skin, within the dermis or epidermis, etc., to determine oxygen within the subject.

WO 2010/101625 A2

# OXYGEN SENSOR

## RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application Serial No. 61/163,710, filed March 26, 2009, entitled "Systems and Methods for Creating and Using Suction Blisters or Other Pooled Regions of Fluid within the Skin," by Levinson, *et al.*; U.S. Provisional Patent Application Serial No. 61/156,632, filed March 2, 2009, entitled "Oxygen Sensor," by Levinson, *et al.*; U.S. Provisional Patent Application Serial No. 61/269,436, filed June 24, 2009, entitled "Devices and Techniques associated with Diagnostics, Therapies, and Other Applications, Including Skin-Associated Applications," by Levinson, *et al.*; and U.S. Provisional Patent Application Serial No. 61/257,731, filed November 3, 2009, entitled "Devices and Techniques associated with Diagnostics, Therapies, and Other Applications, Including Skin-Associated Applications," by Bernstein, *et al.* Each of the above is incorporated herein by reference.

## FIELD OF INVENTION

The present invention generally relates to systems and methods for determining oxygen in a sample, or in a subject.

## BACKGROUND

The blood delivers vital oxygen from the lungs to the cells of the body. However, various medical conditions are characterized by low levels of oxygen within the blood (hypoxemia) or within the body (hypoxia). Under such conditions, tissues within the body are deprived of adequate oxygen supply, and such tissues may be temporarily or permanently harmed as a result. Such conditions may arise, for example, due to sleep apnea, pressure ulcers or blisters, bed sores, or in certain infants. Systems and methods for the determination of oxygen within a subject are thus of great importance.

## SUMMARY OF THE INVENTION

The present invention generally relates to systems and methods for determining oxygen in a sample, or in a subject. The subject matter of the present invention involves, in some cases, interrelated products, alternative solutions to a particular problem, and/or a plurality of different uses of one or more systems and/or articles.

In one aspect, the present invention is directed to an article. According to one set of embodiments, the article includes a skin delivery device able to selectively determine

localized oxygen within one of the dermis, the epidermis, the interstitial fluid, or the blood within the skin when the skin delivery device is applied to a subject.

In another set of embodiments, the article includes a device at least partially insertable into the skin of a subject. In some cases, the device is able to determine oxygen concentration of or proximate at least a portion of the skin of the subject.

The article, in yet another set of embodiments, includes a skin patch exhibiting a determinable feature responsive to oxygen when the skin patch is applied to a subject. In still another set of embodiments, the article includes a skin delivery device containing a plurality of agents that exhibit increasing aggregation with decreasing oxygen concentration.

In accordance with yet another set of embodiments, the article includes a plurality of particles at least partially coated with sickle-cell hemoglobin. The article, in another set of embodiments, includes a skin delivery device containing a plurality of particles at least partially coated with a hemoglobin.

In one set of embodiments, the article includes a liquid containing a plurality of agents that are able to aggregate when the concentration of oxygen within the liquid is less than about 90% of the saturation oxygen concentration of the liquid, but are not able to substantially aggregate when the liquid is saturated with oxygen.

The article, in accordance with another set of embodiments, includes a liquid containing a plurality of particles coated with a polymer that exhibits at least about 10% polymerization when the concentration of oxygen within the liquid is less than about 90% of the saturation oxygen concentration of the liquid, but is not able to substantially polymerize when the liquid is saturated with oxygen.

In still another set of embodiments, the article includes a plurality of particles coated with a polymer that exhibits at least about 10% polymerization when exposed to blood containing a concentration of oxygen less than about 90% of the saturation oxygen concentration of the blood.

In another aspect, the present invention is directed to a device able to determine localized oxygen proximate the skin when the device is applied to the skin of a subject.

The invention, in yet another aspect, is directed to a method. In one set of embodiments, the method includes an act of determining blood oxygen in a subject by administering an oxygen-sensitive agent to the subject.

According to another set of embodiments, the method includes an act of determining blood oxygen in a subject by applying a skin patch to the subject.

The method, in yet another set of embodiments, includes an act of administering a plurality of particles at least partially coated with hemoglobin to the skin of a subject.

According to still another set of embodiments, the method includes an act of determining a region on the skin of a subject having low oxygen, relative to the oxygen of the blood of the subject, by applying a skin patch to the subject. In yet another set of embodiments, the method includes an act of determining a region on the skin of a subject having low oxygen, relative to the blood of the subject, by administering a plurality of particles to the skin of the subject.

In one set of embodiments, the method includes an act of diagnosing a subject suspected or at risk of having sleep apnea by determining a determinable feature of a skin patch applied to the subject prior to the subject sleeping. In another set of embodiments, the method includes an act of diagnosing a subject suspected or at risk of having sleep apnea by determining a determinable feature of a plurality of particles applied to the skin of the subject.

The method, in still another set of embodiments, includes an act of applying an oxygen-sensitive agent to a tissue.

In another aspect, the present invention is directed to a method of making one or more of the embodiments described herein, for example, an article exhibiting a determinable feature responsive to oxygen. In another aspect, the present invention is directed to a method of using one or more of the embodiments described herein, for example, an article exhibiting a determinable feature responsive to oxygen.

Other advantages and novel features of the present invention will become apparent from the following detailed description of various non-limiting embodiments of the invention when considered in conjunction with the accompanying figures. In cases where the present specification and a document incorporated by reference include conflicting and/or inconsistent disclosure, the present specification shall control. If two or more documents incorporated by reference include conflicting and/or inconsistent disclosure with respect to each other, then the document having the later effective date shall control.

## BRIEF DESCRIPTION OF THE DRAWINGS

Non-limiting embodiments of the present invention will be described by way of example with reference to the accompanying figures, which are schematic and are not intended to be drawn to scale. In the figures, each identical or nearly identical component illustrated is typically represented by a single numeral. For purposes of clarity, not every component is labeled in every figure, nor is every component of each embodiment of the invention shown where illustration is not necessary to allow those of ordinary skill in the art to understand the invention. In the figures:

Fig. 1 illustrates one embodiment of the invention comprising a patch applied to the skin of a subject;

Fig. 2 illustrates another embodiment of the invention in which particles are administered into the skin of a subject;

Figs. 3A-3B illustrate the aggregation of particles having first and second regions, according to yet another embodiment of the invention;

Figs. 4A-4B illustrate devices according to certain embodiments of the invention;

Figs. 5A-5C illustrate devices according to various embodiments of the invention;

Fig. 5D illustrates a kit containing more than one device, in yet another embodiment of the invention; and

Fig. 5E illustrates a device according to still another embodiment of the invention.

## DETAILED DESCRIPTION

The present invention generally relates to systems and methods for determining oxygen in a sample, or in a subject. The invention provides, in some embodiments, compositions and devices to be positioned on, in, or proximate the skin of a subject, which can determine oxygen levels associated with skin, blood, and/or interstitial fluid, and/or which delivers a signal indicative of oxygen level. As described more fully below, the signal can be visual, or another sensory signal such as a stimulus affecting feel, smell, taste, or the like, or a signal readable by an instrument. The signal can be readable by the subject in which oxygen level determination is being made, or by another person or machine, or other entity. In several embodiments, devices of the invention function by including one or more agents which can react with or otherwise be affected by oxygen, or by another species in a subject that can be used to determine oxygen level. In most of the description herein, the agents are particles that are functionalized so as to

interact with the species in the subject, for example by clustering and providing a different visual appearance relative to a non-clustered state in the absence of the species. Other particle behaviors and signaling techniques are provided below. It is to be understood that wherever particles are described as useful in devices and to techniques of the invention, other agents, as described generally below, can be substituted.

In one aspect, devices of the invention are provided that can monitor oxygen level within a subject either continuously, or at any discrete point in time, or both. For example, a device can be constructed to provide constant, real-time signal production indicative of oxygen level, or as another example, a device that changes color, or color intensity, reversibly and/or essentially instantaneously in reaction to skin oxygen contents. Or, a device of the invention can be constructed to measure oxygen at a particular point in time and "hold" the signal indicative of that oxygen content at that point in time, for example, a point in time during the night when a subject is sleeping. Or, a device can be constructed to measure and report a highest-level and/or lowest-level oxygen content; a device can be applied to the skin and at the end of a determination period (for example, overnight) report the lowest oxygen level of the subject during that measuring period of time. As a non-limiting example, in some embodiments, the device may include agents such as particles that interact with oxygen, and can exhibit aggregation as a function oxygen concentration, reversibly and/or irreversibly; this aggregation may be determined to determine oxygen levels within a subject.

Species that can interact with agents (e.g., particles) and devices of the invention to measure and report oxygen content can be natural bodily-occurring species, non-naturally occurring species that are added to a subject, or the like. In some cases, devices and compositions of the invention can be provided in the form of skin-adhesive patches, implants, devices otherwise held proximate the skin (on or in, for example, lotion, clothes, and/or other personally proximate objects such as bandages, jewelry, stocking, etc.).

The above introductory description outlines, generally, various aspects of the invention. More details of various aspects and embodiments are provided below.

In one aspect, the present invention is generally directed to an article exhibiting a determinable feature responsive to oxygen, such as oxygen-sensitive particles. The particles may exhibit a determinable change with a change in oxygen concentration, and such particles can accordingly be used to determine oxygen. For example, in one set of

embodiments, the particles may be at least partially coated with a protein, such as hemoglobin, that is able to interact with oxygen. In some cases, the protein may aggregate under certain conditions (e.g., under relatively low oxygen concentrations), and such protein aggregation may be used, for example, to cause the particles to become aggregated, which can be determined in some way. In some cases, such aggregation may be irreversible; i.e., the degree of aggregation corresponds to the most extreme oxygen concentrations that the proteins were exposed to. Such articles may be used, for example, to determine oxygen within a sample, or within a subject, such as a human subject. For instance, the article may be formed as a skin patch, or administered to the skin of a subject, e.g., on the surface of the skin, within the dermis or epidermis, etc., to determine oxygen within the subject.

Thus, various aspects of the present invention are generally directed to systems and methods for determining oxygen amounts or concentrations in a sample, or in a subject. Such determinations of oxygen may be quantitative, and/or qualitative in some cases, e.g., the determination may be that the amount or concentration of oxygen within a sample has increased or decreased in some fashion, or that there are sufficient or insufficient levels of oxygen present. "Determine," in this context, generally refers to the analysis of a species such as oxygen, for example, quantitatively or qualitatively, and/or the detection of the presence or absence of the species. The species may be, for example, a bodily fluid and/or an analyte suspected of being present in the bodily fluid. For instance, the concentration or the amount of oxygen may be determined. "Determining" may also refer to the analysis of an interaction between two or more species, for example, quantitatively or qualitatively, and/or by detecting the presence or absence of the interaction, e.g. determination of the binding between oxygen and another species, such as is discussed below.

Certain embodiments are directed to the determination of amounts or concentrations of oxygen in a subject, such as a human subject. In some cases, however, the subject may be a non-human animal. Examples of such subjects include, but are not limited to, a mammal such as a dog, a cat, a horse, a rabbit, a cow, a pig, a sheep, a goat, a rat (e.g., *Rattus Norvegicus*), a mouse (e.g., *Mus musculus*), a guinea pig, a hamster, a primate (e.g., a monkey, a chimpanzee, a baboon, an ape, a gorilla, etc.), a bird, a reptile, a fish, or the like. In certain cases, as discussed below, the oxygen concentration within the skin of the subject is determined in some fashion. For example, a skin delivery

device or a skin patch may be applied to the skin of a subject, and used to determine oxygen within the subject, e.g., within the skin or within the blood of the subject, depending on placement.

However, in other embodiments, the amount or concentration of oxygen within a sample may be determined, for example, a biological sample or a chemical sample such as a solution or a liquid. As non-limiting examples, in one embodiment, the amount or concentration of oxygen within a tissue sample or an isolated organ may be determined. In another example, the amount or concentration of oxygen within a reactor may be determined. In yet another example, the amount or concentration of oxygen within a food, a drug or a pharmaceutical preparation, or a consumer item may be determined.

As a non-limiting example, an embodiment of the invention, as used to determine the oxygen concentration within the skin of a subject, is now described with reference to the schematic diagram shown in Fig. 1 (not to scale). In this figure, skin patch 10 is disposed on the surface of the skin 20 of a subject. Typically, a skin patch includes one or more layers of material that are adhered to the surface of the skin. For instance, in this example, skin patch 10 includes a first adhesive layer 11, which is used to affix the patch to the surface of the skin; a detection layer 12 for determining oxygen concentrations or amounts; and a selectively impermeable layer 13 to prevent the detection layer from being exposed to the external environment (e.g., containing atmospheric oxygen, water, etc.) surrounding the patch. In this example, oxygen from skin 20 is able to pass through adhesive layer 11 into detection layer 12; thus, adhesive layer 11 may be formed from an oxygen-permeable material, or adhesive layer 11 may be sufficiently porous to allow oxygen transport to occur therethrough, etc. In contrast, selectively impermeable layer 13 may be sufficiently impermeable to oxygen such that detection layer 12 is not significantly affected by the concentration of oxygen outside of selectively impermeable layer 13 (although in some cases, selectively impermeable layer 13 may be permeable to species other than oxygen, i.e., the impermeability of layer 13 is determined with reference to oxygen, not to other species). Thus, "selectively impermeable" layer, as used herein, means that the layer that is essentially impermeable to a species that would affect the technique adversely (e.g., oxygen), but which may or may not be permeable to other species, e.g., to other species that do not adversely affect the assay or other technique of the invention. "Essentially" impermeable, in this context, means that the

layer resists permeability to species that would adversely affect the assay or other technique, e.g., within the time frame of the assay or other technique.

Detection layer 12, in this example, includes an agent that exhibits a determinable change when exposed to different concentrations or amounts of oxygen, i.e., the agent is one that is "oxygen-sensitive." For instance, in one set of embodiments, detection layer 12 may contain a plurality of particles that are at least partially coated with a protein, such as hemoglobin, that is able to interact with oxygen. For example, the protein may be one that can aggregate or polymerize under certain conditions, such as under relatively low oxygen concentrations. In one embodiment, the hemoglobin may be a sickle cell hemoglobin or other modified hemoglobin that shows increased sensitivity to oxygen, relative to unmodified hemoglobin. By controlling the amount of protein coated on the particle, e.g., by controlling the location and/or concentration of protein on the surface of the particle, the amount of sensitivity of the particles to oxygen may be controlled. Thus, for example, the particles may exhibit substantially no aggregation when exposed to normal concentrations of oxygen (for example, normal concentrations of atmospheric oxygen, normal concentrations of dissolved oxygen within the blood), but the particles may exhibit some aggregation as the concentration of oxygen decreases, e.g., below a certain threshold concentration. As discussed below, the detection of such particles, e.g., within detection layer 12, may be achieved due to aggregation of the particles (e.g., causing a difference in appearance, color, light scattering, etc.), or in some cases, the particles, when aggregated may produce a determinable signal, e.g., a change in temperature, or color. It should be noted that detection layer 12, in this example, is able to detect oxygen in or proximate the skin, as oxygen diffusing across the skin into the device may be determined as outlined above.

As another non-limiting example, Fig. 2 illustrates another embodiment of the invention where oxygen-sensitive particles 30, such as those discussed above, are administered or delivered directly to the skin 20 of a subject. In some cases, the particles may be administered to any suitable location within the skin of the subject, e.g., to the epidermis, dermis, or below the dermis in some cases. In one embodiment the particles are administered to a suction blister, as discussed below. Such particles may, for example, exhibit relatively little or no aggregation when exposed to normal concentrations of oxygen (e.g., within the skin or blood of the subject), but exhibit some

aggregation at lower concentrations of oxygen. By determining the degree of aggregation, the concentration of oxygen may be determined.

As discussed, several aspects of the present invention are directed to varying agents that exhibits a determinable change when exposed to different concentrations or amounts of oxygen. Such agents may, in some cases, be contained within suitable articles, which can be delivered to a sample, or to a subject. Non-limiting examples of an oxygen-sensitive agent include particles, such as anisotropic particles, that exhibit oxygen sensitivity. Other examples of oxygen-sensitive agents include, but are not limited to, polymers that exhibit different degrees of polymerization when exposed to different oxygen concentrations, dyes or other entities sensitive to oxygen, methelyne blue, or 2,6-dichlorophindophenol. Methylene blue or 2,6-dichlorophindophenol are generally colorless until oxidized in the presence of $O_2$. For instance, under exposure to at least 30 mmHg $O_2$, methylene blue or 2,6-dichlorophindophenol may change colors, indicating the presence of oxygen. The response time for such dyes may be between 20-30 s to 1 hour in some cases.

Accordingly, certain embodiments of the invention are directed to oxygen-sensitive particles that may be delivered to a sample, or to a subject. The particles may include microparticles and/or nanoparticles in some cases. The particles may be chosen, in some embodiments, to be relatively non-toxic or non-reactive, i.e., to the sample or to the subject, depending on the application, and examples of compositions of such particles are discussed in detail below.

In one set of embodiments, the particles may be rendered oxygen-sensitive by using an agent that exhibits a determinable change when exposed to different concentrations or amounts of oxygen, for example, a change in aggregation, polymerization, or the like. The agent may be formed as part of the particle or homogenously contained within the particle, or in some cases, the agent may be one that is coated on at least a portion of the surface of the particle, for instance, covalently attached to the surface of the particle.

For example, at least a portion of the particle may be functionalized, i.e. comprising surface functional moieties, to which a protein or other agent may be bound to, thereby coating at least a portion of the surface of the particle with the protein or other agent. The functional moieties may include simple groups, selected from the groups including, but not limited to, -OH, -CHO, -COOH, $-SO_3H$, -CN, $-NH_2$, -SH, -COSH, -

COOR, or halide; biomolecular entities including, but not limited to, amino acids, proteins, sugars, DNA, antibodies, antigens, and enzymes; grafted polymer chains, selected from a group of polymers including, but not limited to, polyamide, polyester, polyimide, polyacrylic; a thin coating covering the surface of the particle, including, but not limited to, the following groups of materials: metals, semiconductors, and insulators, which may be a metallic element, an oxide, an sulfide, a nitride, a selenide, a polymer, a polymer gel, or the like. The protein or other agent can then be reacted with the functional moiety, e.g., using a suitable cross-linking reagent, such as glutaradehyde.

In one set of embodiments, the agent is a protein that is able to interact with oxygen, and in some cases, the protein may bind to oxygen, e.g., through coordination chemistry. Non-limiting examples of such proteins include hemoglobin, myoglobin, hemocyanin, hemerythrin, chlorocruorin, vanabin, erythrocruorin, pinnaglobin, leghemoglobin, etc. The protein may be human or from another species. If the protein is hemoglobin, the hemoglobin may be adult hemoglobin (e.g. human hemoglobin A) or fetal hemoglobin (e.g., human hemoglobin F). Upon interaction with oxygen, the protein may exhibit a conformational change, and/or a change in a physical property, that alters the ability of the protein to interact with other species, e.g., with other proteins or ligands. Thus, as an example, oxyhemoglobin (hemoglobin bound to oxygen) may exhibit a first affinity to a surface (e.g., to another particle), while deoxyhemoglobin (hemoglobin free of oxygen) may exhibit a second affinity to the surface, and thus, the surface may have different concentrations of particles adsorbed thereon, depending on the concentration of oxygen.

In one set of embodiments, the protein may be sickle cell hemoglobin, where the hemoglobin contains one or more mutations that causes the hemoglobin to agglomerate or polymerize in solution, in some cases forming fibers or other aggregates. For instance, one form of sickle cell hemoglobin has a sequence that is the same as that of normal (wild-type) hemoglobin except that glutamic acid in position 6 (in the beta chain) has been mutated to valine. This mutation allows the deoxygenated form of the sickle cell hemoglobin to polymerize, and the degree of polymerization is dependent, at least in part, on the concentration of oxygen. Other mutations to hemoglobin, not necessarily in patients having sickle cell anemia, may also have exhibit similar polymerization or aggregation tendencies. Accordingly, one embodiment of the invention comprises a plurality of particles containing a sickle-cell hemoglobin, for example, such that the

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 August 2012 (16.08.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/106751 A1

(51) International Patent Classification:
*A61K 8/97* (2006.01) *A61K 9/107* (2006.01)
*A61K 8/06* (2006.01) *A61K 47/42* (2006.01)
*A61K 8/64* (2006.01) *A61K 47/44* (2006.01)
*A61K 8/98* (2006.01)

(21) International Application Number:
PCT/AU2012/000103

(22) International Filing Date:
7 February 2012 (07.02.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2011900383 7 February 2011 (07.02.2011) AU

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published**:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

WO 2012/106751 A1

(54) Title: ARTIFICIAL OIL BODIES

(57) Abstract: The present invention relates to artificial oil bodies comprising oleosin (which, as presently defined, also encompasses caleosin, steroleosin and polyoleosin), a surfactant such as a phospholipid, and an oil comprising fatty acids, such as polyunsaturated fatty acids having four or more double bonds. The present invention also relates to methods of preparing said artificial oil bodies. These AOBs may further comprise other molecules such as bioactive molecules, used in a wide variety of products, and are particularly useful for producing oxidatively stable oil-in-water emulsions in the absence of added antioxidants. The present invention further encompasses a method for the partial purification of oleosin from a plant extract.

# ARTIFICIAL OIL BODIES

## FIELD OF THE INVENTION

The present invention relates to artificial oil bodies comprising fatty acids, and methods of preparing said artificial oil bodies. The artificial oil bodies can be used in a wide variety of products, and are particularly useful for producing oxidatively stable oil-in-water emulsions in the absence of antioxidants.

## BACKGROUND OF THE INVENTION

Fat has a high calorific content and makes a major contribution to the human dietary energy intake. Certain types of fat such as saturated fats and *trans* fats have been implicated in a range of disease conditions including cardiovascular disease. However, fat also plays a positive role in human health and nutrition, and international dietary guidelines allow a certain proportion of dietary energy to be derived from fat as long as it is 'good fat'. The 'good fats' are monounsaturated and polyunsaturated oils, and there is increasing interest in replacement of saturated fats in food products with healthier unsaturated oils. Moreover, for many years now, a particular group of polyunsaturated oils, namely long-chain omega-3 oils, have received considerable attention due to a multitude of health benefits including protection against cardiovascular disease which have been attributed to them. Spurred by the growing list of health benefits of omega-3 oils, the food industry worldwide is striving to produce food products fortified with omega-3 oils.

One of the major impediments to the expansion of omega-3 fortified foods and similar healthy foods containing highly unsaturated oil is the extreme susceptibility of these oils to oxidative deterioration during storage. Oxidation generates products some of which can have highly undesirable flavour characteristics thereby reducing the palatability and shelf-life of the product. It is important therefore to adequately protect these oils from oxidation. Whilst microencapsulation and addition of anti-oxidants have successfully been used as strategies to control oxidation of highly unsaturated oils in food products, these technologies have several drawbacks in relation to cost, naturalness, and general applicability.

Oil in plants and oilseeds occurs in the form of discrete deposits referred to as oil bodies (OBs) which are similar in structure irrespective of the plant species they are present in. A remarkable feature of natural OBs is their exceptional physical stability both inside the cells and in isolated preparations. In both situations, OBs occur as individual entities, and do not aggregate or coalesce when they are pressed against one

another even after prolonged storage; *in vivo* due to desiccation, and *in vitro* after flotation centrifugation (Tzen and Huang, 1992). The exceptional physical stability of OBs has been attributed to unique amphipathic proteins called oleosins occurring in the oil body surface (Huang, 1994). It has been suggested that the entire surface of the oil body is covered by oleosin such that the compressed OBs never coalesce or aggregate in the cells of a mature seed (Tzen and Huang, 1992). The content of oleosin ranges from 1-4% of the total oil bodies; the greatest amount occurring in species in which the OBs are the smallest, e.g. rape / canola (Huang, 1992; Tzen, 1993).

In addition to good physical stability, OBs within oilseeds have natural protection against oxidation. Recent studies have shown that this oxidative stability is extended to aqueous dispersions oil body isolates. Fisk and co-workers (2008) showed intact OBs extracted from sunflower seed and dispersed in continuous aqueous phase were more resistant to oxidation than equivalent emulsions prepared from sunflower oil and emulsifier. More recently, similar results have been obtained for echium oil body isolates containing the highly unsaturated stearidonic acid (Gray et al, 2010). Superior oxidative stability of aqueous dispersions of intact canola OBs over equivalent canola oil-in-water emulsions prepared using Tween40 as the emulsifier has also been reported (Shen and Wijesundera, 2009).

Limited attempts have been made to reassemble OBs from their constituents. Murphy and Cummins (1989) reported that incubation of TAG extracted from rapeseed oil or purified triolein with rapeseed oil body membrane material resulted in the appearance of oil stable droplets of similar dimensions to native oil bodies. Later, Tzen and Huang (1992) successfully prepared physically stable artificial (reconstituted) oil bodies (AOBs) by combining a TAG mixture of triolein and trilinolein (1:2 molar) with dioleoyl phosphatidylcholine and oleosin isolated from wheat, rice, rapeseed, soybean or jojoba. However, the oxidative stability of such artificial OBs does not seem to have been investigated.

There is a need for means to protect fatty acids from oxidation which do not rely on the use of added antioxidants.

## SUMMARY OF THE INVENTION

Oils containing high levels of polyunsaturated fatty acids with four or more double bonds, such as marine oils rich in ω3 fatty acids, are chemically distinct from oils produced in plants such as oilseeds. However, the present inventors were surprised to find that oleosin extracted from plant meal, such as commercial canola meal, can be used to construct artificial oil bodies with highly polyunsaturated oils, such as ω3 fatty acid rich marine and/or fish oils. The present inventors where also surprised to find that the phospholipid component of oil bodies could be substituted with a wide variety of surfactants without adversely affecting oxidative stability.

Thus, in a first aspect the present invention provides an artificial oil body comprising oleosin, surfactant, and oil comprising polyunsaturated fatty acids with three or more double bonds, more preferably four or more double bonds.

In an embodiment, the surfactant is phospholipid.

In an embodiment, at least 5%, at least 10%, at least 15%, at least 20% or at least 25%, of the polyunsaturated fatty acids have four or more double bonds.

In another aspect, the present invention provides an artificial oil body comprising oleosin, surfactant, and oil comprising fatty acids, wherein at least some of the surfactant is not phospholipid.

Whilst the oleosin can be obtained from any source, in a preferred embodiment the oleosin is an oilseed oleosin.

The oil may also be obtained from any source. In a preferred embodiment, the oil is marine oil and/or fish oil.

In a further embodiment, at least 50%, at least 75%, or at least 90%, of the fatty acids are in the form of glycerides.

In an embodiment, at least 80%, at least 90%, or at least 95%, of the dry weight of artificial oil body is oil.

The oil bodies of the invention can be used as a carrier for, and hence may further comprise, one or more other molecules. Examples such other molecules include, but are not limited to, a probiotic, a preservative, a therapeutic agent, a diagnostic agent, a delivery agent or a food colouring agent.

The size of the oil body can be varied by manipulating the relative concentration of oil to oleosin, with lower relative amounts of oleosin promoting larger sizes. In an embodiment, the oil body has a size of between about 0.1 and about 100 μm, or at least between about 0.5 and about 50 μm, or at least between about 0.5 and about 10 μm, or at least between about 0.5 and about 2 μm.

In yet a further aspect, the present invention provides a composition comprising one or more artificial oil bodies of the invention, and a carrier. In an embodiment, the carrier is deionized water.

In an embodiment, the composition is an oil-in-water emulsion. In particular, an oil-in-water emulsion of the invention is more resistant to oxidation than an oil-in-water emulsion comprising fatty acids produced using as an emulsifier such as Tween40 in the absence of oleosin.

The present inventors have found that the artificial oil bodies of the invention are generally resistant to oxidation. Thus, in an embodiment, the artificial oil bodies and/or composition does not comprise a synthetic antioxidant. Furthermore, in a preferred embodiment the artificial oil bodies of the invention are oxidatively stable.

In an embodiment, the artificial oil body consists of oleosin, surfactant, and oil comprising fatty acids such as polyunsaturated fatty acids with four or more double bonds.

Apart from being useful in the delivery of a therapeutic agent, artificial oil bodies of the invention can be used to treat or prevent diseases due to the well known benefits of the fatty acids, particularly polyunsaturated fatty acids, of the oil bodies. Thus, in a further aspect, the present invention provides a method of treating or preventing a condition which would benefit from fatty acids, the method comprising administering to a subject with the condition one or more oil bodies of the invention and/or a composition of the invention.

Also provided is the use of one or more oil bodies of the invention and/or a composition of the invention for the manufacture of a medicament for treating or preventing a condition which would benefit from fatty acids.

Furthermore, provided is the use of one or more oil bodies of the invention and/or a composition of the invention as a medicament for treating or preventing a condition which would benefit from fatty acids.

In an embodiment of the above method and uses, the condition would benefit from polyunsaturated fatty acids with four or more double bonds.

The oil bodies can be used in a wide variety of products. Accordingly, in another aspect the present invention provides a product comprising one or more artificial oil bodies, and/or a composition, of the invention. Examples of such products include, but are limited to, a food or feed product, a drink product, a personal care product, a pharmaceutical product or an industrial product.

In a preferred embodiment, the product is, or comprises, an oil-in-water emulsion.

Also provided is the use of one or more artificial oil bodies of the invention, and/or a composition of the invention, for the preparation of a product.

In a further aspect, the present invention provides a method of preparing a feed, food or drink, the method comprising admixing one or more artificial oil bodies of the invention, and/or a composition of the invention, with one or more other edible ingredients.

In a further aspect, the present invention provides a method of producing artificial oil bodies, the method comprising

i) obtaining oleosin, surfactant, and oil comprising polyunsaturated fatty acids with four or more double bonds, and

ii) mixing the oleosin, the surfactant and the oil to produce the artificial oil bodies.

In another aspect, the present invention provides a method of producing artificial oil bodies, the method comprising

i) obtaining oleosin, surfactant, and oil comprising fatty acids, wherein at least some of the surfactant is not phospholipid, and

ii) mixing the oleosin, the surfactant and the oil to produce the artificial oil bodies.

In an embodiment, the surfactant is mixed and dissolved in the oil before mixing in the olesoin.

In a further embodiment, the method further comprises,

iii) selecting artificial oil bodies.

The present inventors have also identified methods of obtaining oleosin for use in the artificial oil bodies of the invention. The present inventors were particularly surprised to find that a protein extract comprising a highly heterogeneous population of proteins with relatively low quantities of oleosin (for example about 10% of the total protein) could be used, with the natural affinity of the oil, surfactant and oleosin seemingly being highly efficient at forming the artificial oil bodies. This is particularly advantageous because it avoids the need to conduct extensive purification procedures and provides a relatively cheap source of the oleosin. Thus, in a further embodiment, step i) comprises

a) obtaining an extract of a plant comprising oleosin, and

b) at least partially purifying protein from the extract, wherein the protein comprises oleosin.

Preferably, step b) comprises

1) adjusting the pH of the extract to a pH of at least about 11.5,

2) separating 1) to produce a solid and liquid phase and selecting the liquid phase,

3) reducing the pH of the liquid phase to precipitate the proteins,

4) separating 3) to produce a solid and liquid phase and selecting the solid phase, and

5) dispersing the solid phase in a carrier.

The present invention also provides an artificial oil body produced by a method of the invention.

In a further aspect, the present invention provides a method of partially purifying oleosin from a plant extract, the method comprising

1) adjusting the pH of the extract to a pH of at least about 11.5,

2) separating 1) to produce a solid and liquid phase and selecting the liquid phase,

3) reducing the pH of the liquid phase to precipitate the proteins,

4) separating 3) to produce a solid and liquid phase and selecting the solid phase, and

5) dispersing the solid phase in a carrier.

In an embodiment, step 3) comprises reducing the pH of the liquid phase to less than about 7, to about pH 4 to about 7, or about pH 5.5 to about 7.

Preferably, the method of the two above aspects does not use an organic solvent.

The extract can be from any plant such as plant meal. However, in a preferred embodiment the plant is an oilseed.

The present inventors have also found that meal resulting from the extraction of oils from plant material, such as the seeds of oilseeds, can be used as a source for oleosin. As an added benefit of the claimed invention, following the partial purification of the oleosin the remaining plant extract can be used as an animal feed. Thus, in a preferred embodiment the extract is meal produced following the extraction of oil.

The present invention also provides at least partially purified oleosin produced by a method of the invention.

Any embodiment herein shall be taken to apply *mutatis mutandis* to any other embodiment unless specifically stated otherwise.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended for the purpose of exemplification only. Functionally-equivalent products, compositions and methods are clearly within the scope of the invention, as described herein.

Throughout this specification, unless specifically stated otherwise or the context requires otherwise, reference to a single step, composition of matter, group of steps or group of compositions of matter shall be taken to encompass one and a plurality (i.e. one or more) of those steps, compositions of matter, groups of steps or group of compositions of matter.

The invention is hereinafter described by way of the following non-limiting Examples and with reference to the accompanying figures.

**BRIEF DESCRIPTION OF THE ACCOMPANYING DRAWINGS**

**Figure 1:** Procedure for the extraction of oleosin from canola meal.

**Figure 2:** Electrophoretic anaylysis of oleosin extract from canola meal.

**Figure 3:** Effect of pH of the extracting medium on oleosin recoveries from canola meal.

**Figure 4:** Electrophoretic analysis of oleosin extracted at different pH values. Lane 2 and 3 – pH 10.5; Lane 4 and 5 – pH 11.0; Lane 6 and 7 – pH 11.5; Lane 8 and 9 – pH 12.0.

**Figure 5:** Change in particle size (μm) of D[3,2} and d (0.5) for Tween 40 and olesoin emulsions subjected to accelerated auto oxidation (60°C). Average data from duplicate measuremnts.

**Figure 6:** Electrophoretic analysis of surface proteins of artificial tuna oil bodies. Lanes: M – markers, Raw pH12 –pH12 soluble crude protein extract from canola meal, CP1 – protein extracted from artificial canola oil bodies made from canola oil and re-solubilised pH 6.5-precipitated canola meal protein, CP1+PLs - protein extracted from artificial canola oil bodies made from canola oil, re-solubilised pH 6.5-precipitated canola meal protein, and phospholipids.

**Figure 7:** Development of trans,trans,2,4,heptadienal during accelerated oxidation of tuna oil emulsions prepared with Tween40 and Oleosin extract, repectively. Average data from duplicate measuremnts. 7A – Oxidation at 60 °C; 7B – Oxidation at 40 °C.

**Figure 8:** Depletion of EPA and DHA in tuna oil emulsions made with Tween40 and Oleosin extract (respectively) during storage at 60°C. Average data from duplicate measuremnts. 8A – Oxidation at 60 °C; 8B – Oxidation at 40 °C.

**Figure 9:** Peroxide value (PV) of oils extracted from tuna oil artificial oil body emulsions and equivalent Tween40 emulsions subjected to accelerated oxidation at 40 °C.

**Figure 10:** Development of trans, trans, 2,4-heptadienal during accelerated storage (40° C, air) of tuna oil emulsions stabilised by oleosin, Isolexx and sodium caseinate, respectively.

**Figure 11:** Development of trans, trans, 2,4-heptadienal during accelerated storage (40° C, air) of tuna oil emulsions prepared using oleosin in combination with monoacylglycerol (MAG), Tween40 (Tween), and sodium stearoyl lactylate (SSL), repectivley as the secondary surfactant.

**KEY TO THE SEQUENCE LISTING**

SEQ ID NO 1: *Brassica napus* oleosin (CAA57545.1)
SEQ ID NO 2: *Brassica napus* oleosin S1-1 (ACG69504.1)
SEQ ID NO 3: *Brassica napus* oleosin S2-1 (ACG69503.1)
SEQ ID NO 4: *Brassica napus* oleosin S3-1 (ACG69513.1)
SEQ ID NO 5: *Brassica napus* oleosin S4-1 (ACG69507.1)
SEQ ID NO 6: *Brassica napus* oleosin S5-1 (ACG69511.1)
SEQ ID NO 7: *Arachis hypogaea* oleosin 1 (AAZ20276.1)
SEQ ID NO 8: *Arachis hypogaea* oleosin 2 (AAU21500.1)
SEQ ID NO 9: *Arachis hypogaea* oleosin 3 (AAU21501.1)
SEQ ID NO 10: *Arachis hypogaea* oleosin 5 (ABC96763.1)
SEQ ID NO 11: *Ricinus communis* oleosin 1 (EEF40948.1)
SEQ ID NO 12: *Ricinus communis* oleosin 2 (EEF51616.1)
SEQ ID NO 13: *Glycine max* oleosin isoform a (P29530.2)
SEQ ID NO 14: *Glycine max* oleosin isoform b (P29531.1)
SEQ ID NO 15: *Linum usitatissimum* oleosin low molecular weight isoform (ABB01622.1)
SEQ ID NO 16: *Linum usitatissimum* oleosin high molecular weight isoform (ABB01624.1)
SEQ ID NO 17: *Helianthus annuus* oleosin (CAA44224.1)
SEQ ID NO 18: *Zea mays* oleosin (NP_001105338.1)

SEQ ID NO 19: *Brassica napus* steroleosin (ABM30178.1)
SEQ ID NO 20: *Brassica napus* steroleosin SLO1-1 (ACG69522.1)
SEQ ID NO 21: *Brassica napus* steroleosin SLO2-1 (ACG69525.1)
SEQ ID NO 22: *Sesamum indicum* steroleosin (AAL13315.1)
SEQ ID NO 23: *Zea mays* steroleosin (NP_001152614.1)
SEQ ID NO 24: *Brassica napus* caleosin CLO-1 (ACG69529.1)
SEQ ID NO 25: *Brassica napus* caleosin CLO-3 (ACG69527.1)
SEQ ID NO 26: *Sesamum indicum* caleosin (AAF13743.1)
SEQ ID NO 27: *Zea mays* caleosin (NP_001151906.1)

## DETAILED DESCRIPTION OF THE INVENTION

General Techniques and Definitions

Unless specifically defined otherwise, all technical and scientific terms used herein shall be taken to have the same meaning as commonly understood by one of ordinary skill in the art (e.g., in lipid chemistry, molecular genetics, chemistry, and biochemistry).

Unless otherwise indicated, the recombinant protein, cell culture, and immunological techniques utilized in the present invention are standard procedures, well known to those skilled in the art. Such techniques are described and explained throughout the literature in sources such as, J. Perbal, A Practical Guide to Molecular Cloning, John Wiley and Sons (1984), J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory Press (1989), T.A. Brown (editor), Essential Molecular Biology: A Practical Approach, Volumes 1 and 2, IRL Press (1991), D.M. Glover and B.D. Hames (editors), DNA Cloning: A Practical Approach, Volumes 1-4, IRL Press (1995 and 1996), and F.M. Ausubel et al. (editors), Current Protocols in Molecular Biology, Greene Pub. Associates and Wiley-Interscience (1988, including all updates until present).

The term "and/or", e.g., "X and/or Y" shall be understood to mean either "X and Y" or "X or Y" and shall be taken to provide explicit support for both meanings or for either meaning.

As used herein, the term about, unless stated to the contrary, refers to +/- 20%, more preferably +/- 10%, even more preferably +/- 5%, of the designated value.

Throughout this specification the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

Oils and Fatty Acids

As used herein, the term "fatty acid" refers to a carboxylic acid with a long aliphatic tail of at least 8 carbon atoms in length, either saturated or unsaturated. Typically, fatty acids have a carbon-carbon bonded chain of at least 12 carbons in length. Most naturally occurring fatty acids have an even number of carbon atoms because their biosynthesis involves acetate which has two carbon atoms. The fatty acids may be in a free state (non-esterified) or in an esterified form such as part of a TAG, DAG, MAG, acyl-CoA (thio-ester) bound, or other covalently bound form. When covalently bound in an esterified form, the fatty acid is referred to herein as an "acyl" group. Saturated fatty acids do not contain any double bonds or other functional groups along the chain. The term "saturated" refers to hydrogen, in that all carbons (apart from the carboxylic acid [-COOH] group) contain as many hydrogens as possible. In other words, the omega (ω) end contains 3 hydrogens (CH3-) and each carbon within the chain contains 2 hydrogens (-CH2-). Unsaturated fatty acids are of similar form to saturated fatty acids, except that one or more alkene functional groups exist along the chain, with each alkene substituting a singly-bonded "-CH2-CH2-" part of the chain with a doubly-bonded "-CH=CH-" portion (that is, a carbon double bonded to another carbon). The two next carbon atoms in the chain that are bound to either side of the double bond can occur in a *cis* or *trans* configuration. Apart from double bonds, the acyl chain of the fatty acid may have, triple bonds, acyl side chains such a methyl or ethyl groups, hydroxyl groups or other modifications known in the art. Artificial oil bodies of the invention may include fatty acids such as, but not limited to, stearic acid, palmitic acid, oleic acid, linoleic acid, α-linolenic acid, or a combination of two or more thereof.

As used herein, the terms "polyunsaturated fatty acid" or "PUFA" refer to a fatty acid which comprises at least 18 carbon atoms in its carbon chain and at least three, more preferably at least four, alkene groups (carbon-carbon double bonds). One aspect of the invention relates to artificial oil bodies comprising polyunsaturated fatty acids (PUFAs) with four or more double bonds. Examples of such PUFAs include, but are not limited to, stearidonic acid (SDA, 18:4Δ6,9,12,15, ω3), arachidonic acid (ARA, 20:4Δ5,8,11,14; ω6), eicosatetraenoic acid (ETA, 20:4Δ8,11,14,17, ω3), eicosapentaenoic acid (EPA, 20:5Δ5,8,11,14,17; ω3), docosapentaenoic acid (DPA, 22:5Δ7,10,13,16,19, ω3), docosahexaenoic acid (DHA, 22:6Δ4,7,10,13,16,19, ω3), as well as mixtures of two or more thereof. The artificial oil bodies may further comprise other fatty acids such as those described above with, for example, two or less double

bonds, triple bonds, side chains such as a methyl group, a hydroxyl group and/or an epoxide group.

The fatty acid may be in a free state (non-esterified) or in an esterified form such as part of a triglyceride, diacylglyceride, monoacylglyceride, acyl-CoA bound or other bound form, or mixtures of two or more thereof. The fatty acid may be esterified as a phospholipid such as a phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol forms.

The term "glyceride" as used herein encompasses lipids selected from the group consisting of triglycerides, diglycerides, monoglycerides, phosphoglycerides and combinations thereof. More preferably, the glycerides are selected from the group consisting of triglycerides and diglycerides. Most preferably, the glyceride is triglyceride oil.

"Triacylglycerol" or (TAG) is glyceride in which the glycerol is esterified with three fatty acids. In the Kennedy pathway of TAG synthesis, the precursor *sn*-glycerol-3-phosphate is esterified by a fatty acid coenzyme A ester in a reaction catalysed by a glycerol-3-phosphate acyltransferase at position *sn*-1 to form lysophosphatidic acid (LPA), and this is in turn acylated by an acylglycerophosphate acyltransferase in position *sn*-2 to form phosphatidic acid. The phosphate group is removed by the enzyme phosphatidic phosphohydrolase, and the resultant 1,2-diacyl-sn-glycerol is acylated to form the triacyl-sn-glycerol.

In one embodiment, the glyceride (preferably, TAG) content of the oil comprises essentially the all (for example at least 75%, or at least 90%, or at least 95%) of the fatty acids.

The oil comprising fatty acids can be obtained from a wide variety of sources including micro-organisms including yeast, crusteceans, fish, animals and plants. The organism may or may not be genetically modified.

In an embodiment, seedoil is present in the artificial oil bodies. As used herein, the term "seedoil" refers to a composition obtained from the seed/grain of a plant which comprises at least 60% (w/w) lipid, or obtainable from the seed/grain if the seedoil is still present in the seed/grain. That is, seedoil includes seedoil which is present in the seed/grain or portion thereof, as well as seedoil which has been extracted from the seed/grain. The seedoil is preferably extracted seedoil. Seedoil is typically a liquid at room temperature. Preferably, the total fatty acid (TFA) content in the seedoil predominantly (>50%) comprises fatty acids that are at least 16 carbons in length. More preferably, at least 50% of the total fatty acids in the seedoil are C18 fatty acids

for example, oleic acid. The fatty acids are typically in an esterified form such as for example, TAG, DAG, acyl-CoA or phospholipid. The fatty acids may be free fatty acids and/or in an esterified form. In an embodiment, seedoil in the artificial oil bodies is "substantially purified" or "purified" oil that has been separated from one or more other lipids (with the exception of phospholipids), nucleic acids, polypeptides (with the exception of oleosin), or other contaminating molecules with which it is associated in the seed or in a crude extract. It is preferred that the substantially purified seedoil is at least 60% free, more preferably at least 75% free, and more preferably, at least 90% free from other components with which it is associated in the seed or extract. Seedoil may further comprise non-fatty acid molecules such as, but not limited to, sterols and phenolics. In an embodiment, the seedoil is canola oil (*Brassica napus*, *Brassica rapa ssp.*), mustard oil (*Brassica juncea*), other Brassica oil (e.g., *Brassica napobrassica, Brassica camelina)*, sunflower oil (*Helianthus annus*), linseed oil (*Linum usitatissimum*), soybean oil (*Glycine max*), safflower oil (*Carthamus tinctorius*), corn oil (*Zea mays*), tobacco oil (*Nicotiana tabacum*), peanut oil (*Arachis hypogaea*), palm oil (*Elaeis guineensis)*, cottonseed oil (*Gossypium hirsutum*), coconut oil (*Cocos nucifera*), avocado oil (*Persea americana*), olive oil (*Olea europaea*), cashew oil (*Anacardium occidentale*), macadamia oil (*Macadamia intergrifolia*), almond oil (*Prunus amygdalus*), oat seed oil (*Avena sativa),* rice oil (*Oryza sativa* or *Oryza glaberrima*), or *Arabidopsis* seed oil (*Arabidopsis thaliana*). Seedoil may be extracted from seed/grain by any method known in the art. This typically involves extraction with nonpolar solvents such as diethyl ether, petroleum ether, chloroform/methanol or butanol mixtures, generally associated with first crushing of the seeds. Lipids associated with the starch in the grain may be extracted with water-saturated butanol. The seedoil may be "de-gummed" by methods known in the art to remove polysaccharides or treated in other ways to remove contaminants or improve purity, stability, or colour. The TAGs and other esters in the seedoil may be hydrolysed to release free fatty acids, or the seedoil hydrogenated, treated chemically, or enzymatically as known in the art.

As used herein, “marine oil” is oil obtained from an organism that lives in salt water, particularly marine microalgae, fish or crusteceans. Thus, an oil for use in the invention may be both a marine oil and a fish oil oil derived from a saltwater fish.

The oil comprising polyunsaturated fatty acids with four or more double bonds may be obtained from any source, such as oil extracted from marine micro-organisms, crustaceans, fish or transgenic organisms, such a transgenic plants or yeast, comprising exogenous polynucleotides which enable the organisms to synthesize polyunsaturated

fatty acids with four or more double bonds. Examples of marine micro-organisms from which the PUFAs may be obtained include, but are not limited to, species of *Amphidinium, Amphor, Asterionella, Biddulphia, Ceratium, Chaetoceros, Chlorella, Chroomonas, Cochlodinium, Crisphaera, Crypthecodinium, Cryptomonas, Cyclotella, Cylindrotheca, Duniella, Emiliania, Fragilaria, Glenodinium, Gonyaulax, Gyrodinium, Haematococcus pluvialis, Heteromastix, Isochrysis, Lauderia, Monochrysis, Monodus, Moritella, Mortierella, Nannochloris, Nannochloropis, Navicula, Nitzschia, Odontella, Olisthodiscus, Pavlova, Peridinium, Phaeodactylum, Porphyridium, Prorocentrum, Pseudopedinella, Rhodella, Rhodomas, Schizochytrium, Skelentonema, Stauroneis, Tetraselmis, Thalassiosira, Thrautochytrium,* or *Ulkenia.* Examples of fish oils useful for the invention include, but are not limited to, tuna oil, bonito oil, sea bass oil, halibut oil, spearfish oil, barracuda oil, cod oil, menhaden oil, sardine oil, pilchard oil, anchovy oil, capelin oil, Atlantic cod oil, Atlantic herring oil, Atlantic mackerel oil, Atlantic menhaden oil, salmonids oil, shark oil, squid oil, octopus oil, krill oil, seal oil, whale oil, and the like, including mixtures and combinations thereof. Examples of transgenic organisms producing polyunsaturated fatty acids with four or more double bonds are described in, for example, WO 2005/103253, WO 2007/106905, WO 2010/023202, WO 2010/057246 and WO 2012/000026.

As noted above, techniques that are routinely practiced in the art can be used to extract and process oils produced by cells, plants, seeds, etc. For example, oil extraction may comprise degumming (or acid treatment), neutralization (or alkali treatment), water washing, bleaching, filtering, deodorising, polishing and/or cooling (or winterization). Preferably the purifying comprises acid treatment and/or alkali treatment (degumming and neutralisation). Alternatively, purifying methods may comprise bleaching and/or deodorization. Preferably, however, the purifying will involve bleaching and/or deodorization, and optimally in addition acid and alkali treatment.

Oleosin

Oleosins are hydrophobic proteins present in the membrane of oil bodies in the storage tissues of seeds (see, for example, Huang, 1996; Lin et al., 2005; Capuano et al., 2007; Lui et al., 2009; Shimada and Hara-Nishimura, 2010). They are of low MW (15-26,000), and are abundant in oilseeds. Within each seed species, there are usually two or more oleosins of different MW. Each oleosin molecule contains a relatively hydrophilic N-terminal domain (for example about 48 amino acid residues), a central totally hydrophobic domain (for example of about 70-80 amino acid residues) which is

particularly rich in aliphatic amino acids such as alanine, glycine, leucine, isoleucine and valine, and an amphipathic α-helical domain (for example about of about 33 amino acid residues) at or near the C-terminus. Generally, the central stretch of hydrophobic residues 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 surfactant monolayer and the negatively charged ones exposed to the exterior.

As used herein, the term "oleosin" encompasses caleosins which bind calcium, and steroleosins which bind sterols, as well as polyoleosin (Scott et al., 2010). However, generally a large proportion, if not all, of the oleosins of artificial oil bodies of the invention will not be caleosins and/or steroleosins. Furthermore, the term "oleosin" as used herein refers to either a homogenous population of the same oleosin protein, or more typically a heterogenous, population of different oleosin proteins.

A substantial number of oleosin protein sequences, and nucleotide sequences encoding therefor, are known from a large number of different plant species. Examples include, but are not limited to, oleosins from *Arabidposis*, canola, corn, rice, peanut, castor, soybean, flax, grape, cabbage, cotton, sunflower, sorghum and barley. Examples of some of these oleosins are provided in the Sequence Listing. Thus, in one embodiment, the oleosin comprises a sequence selected from:

a) an amino acid sequence as provided in any one of SEQ ID NOs 1 to 27,

b) an amino acid sequence which is at least 50% identical, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, preferably at least 90%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, identical to any one or more of SEQ ID NOs 1 to 27, and

c) a biologically active fragment of a) or b).

In a further embodiment, the oleosin comprises a sequence selected from:

a) an amino acid sequence as provided in any one of SEQ ID NOs 1 to 18,

b) an amino acid sequence which is at least 50% identical, more preferably at least 60%, more preferably at least 70%, more preferably at least 80%, preferably at least 90%, more preferably at least 95%, more preferably at least 96%, more preferably at least 97%, more preferably at least 98%, more preferably at least 99%, identical to any one or more of SEQ ID NOs 1 to 18, and

c) a biologically active fragment of a) or b).

The % identity of a protein is determined by GAP (Needleman and Wunsch, 1970) analysis (GCG program) with a gap creation penalty=5, and a gap extension

penalty=0.3. The query sequence is at least 50 amino acids in length, and the GAP analysis aligns the two sequences over a region of at least 50 amino acids. More preferably, the query sequence is at least 100 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 100 amino acids. Even more preferably, the query sequence is at least 250 amino acids in length and the GAP analysis aligns the two sequences over a region of at least 250 amino acids. Even more preferably, the GAP analysis aligns the two sequences over their entire length.

As used herein, a "biologically active" fragment is a portion of an oleosin that can be used to prepare an artificial oil body of the invention. Biologically active fragments can be any size as long as they maintain the defined activity.

In a preferred embodiment, the oleosin is obtained from an oilseed such as, but not limited to, canola (*Brassica spp.*), soybean (*Glycine max*), sunflower (*Helianthus annuus*), oil palm (*Elaeis guineeis*), cottonseed (*Gossypium spp.*), groundnut (*Arachis hypogaea*), coconut (*Cocus nucifera*), castor (*Ricinus communis*), safflower (*Carthamus tinctorius*), mustard (*Brassica spp.* and *Sinapis alba*), coriander (*Coriandrum sativum*), squash (*Cucurbita maxima*), linseed/flax (*Linum usitatissimum*), Brazil nut (*Bertholletia excelsa*), jojoba (*Simmondsia chinensis*) or maize (*Zea mays*).

The oleosin may be obtained from a natural, synthetic or recombinant source. Advantageously, the oleosin can be obtained from a natural source such as a plant extract, particularly the meal of an oilseed following oil extraction. Figure 1 shows an example of a procedure used to extract oleosin from plant meal such as canola meal. Approximately 50% of the canola meal protein has been reported to precipitate at pH 6.0-7.0 (Manamperi et al., 2010). Canola meal typically contains up to 40% protein derived mainly from seed cellular protein. Commercial oil extraction processes of mechanical expelling followed by solvent extraction disrupt the canola oil body structure thereby releasing oleosin to the mix of the relatively more abundant cellular proteins. As the isoelectric point of canola oleosin is 6.5, the protein precipitated at the pH range 6.0-7.0 contains oleosin in addition to cellular protein.

The oleosin may also be obtained from a recombinant source, either plants with an exogenous gene(s) encoding an oleosin resulting in enhanced levels of oleosin production, or a recombinant organism comprising an exogenous gene(s) but do not naturally produce an oleosin, for example a recombinant yeast. The production of recombinant organisms producing, and/or with enhanced levels of, oleosin is well within the capability of those skilled in the art (see, for example, Roux et al., 2004; Abenes et al., 1997; Bhatla et al., 2010).

Amino acid sequence mutants of the naturally occurring oleosins can be prepared by introducing appropriate nucleotide changes into a nucleic acid encoding the oleosin, or by *in vitro* synthesis of the desired protein. Such mutants include, for example, deletions, insertions or substitutions of residues within the amino acid sequence. A combination of deletion, insertion and substitution can be made to arrive at the final construct, provided that the final protein product possesses the desired characteristics. The production and identification of functional mutants of naturally occurring oleosins is also well within the capability of those skilled in the art.

Also included within the scope of the invention are proteins which are differentially modified during or after synthesis, e.g., by biotinylation, benzylation, glycosylation, acetylation, phosphorylation, amidation, derivatization by known protecting/blocking groups, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

Olesoins may be prepared using techniques known the art or using a method of the invention. In one embodiment, olesoins are prepared in as a crude extract by

1) adjusting the pH of the extract to a pH of at least about 11.5,

2) separating 1) to produce a solid and liquid phase and selecting the liquid phase,

3) reducing the pH of the liquid phase to precipitate the proteins,

4) separating 3) to produce a solid and liquid phase and selecting the solid phase, and

5) dispersing the solid phase in a carrier.

In an alternate embodiment, filtration methods are used to at least partially purify oleosin from plant material.

Surfactants

Artificial oil bodies of the invention comprise one or more surfactants.

The surfactant may be a non-ionic or anionic surfactant or a combination of two or more thereof. More particularly, the surfactant may be anionic, cationic, zwitterionic or a combination of two or more thereof. For example the surfactant may be selected from the group consisting of, but not limited to, phospholipid, monoglyceride, Perfluorooctanoate (PFOA or PFO), Perfluorooctanesulfonate (PFOS), Sodium dodecyl sulfate (SDS), ammonium lauryl sulfate, and other alkyl sulfate salts, sodium laureth sulphate, also known as sodium lauryl ether sulfate (SLES), Alkyl benzene sulfonate, Soaps, or fatty acid salts, Cetyl trimethylammonium bromide (CTAB), hexadecyl trimethyl ammonium bromide, and other alkyltrimethylammonium

salts, Cetylpyridinium chloride (CPC), Polyethoxylated tallow amine (POEA), Benzalkonium chloride (BAC), Benzethonium chloride (BZT), Dodecyl betaine, Cocamidopropyl betaine, Coco ampho glycinate, Alkyl poly(ethylene oxide), Alkylphenol poly(ethylene oxide), Copolymers of poly(ethylene oxide) and poly(propylene oxide) (commercially called Poloxamers or Poloxamines), Alkyl polyglucosides, including Octyl glucoside, Decyl maltoside, Fatty alcohols, Cetyl alcohol CA), Poly(vinyl alcohol)(PVA), Oleyl alcohol, Cocamide MEA, cocamide DEA, Polysorbates such as Tween 20, Tweeen 40, Tween 80, and Dodecyl dimethylamine oxide.

In one embodiment, the surfactant is a phospholipid. However, when the fatty acid is not a polyunsaturated fatty acid with at least four double bonds a preferred embodiment is that the artificial oil body comprises no phospholipid or phospholipid is present with at least one other surfactant (for example, the at least one other surfactant comprises at least 5%, or at least 25%, or at least 50%, or at least 75%, or at least 90%, of the total surfactant in the artificial oil body).

Phospholipids are a class of lipids and are a major component of all cell membranes as they can form lipid bilayers. Most phospholipids contain a diglyceride, a phosphate group, and a simple organic molecule such as choline. One exception to this rule is sphingomyelin, which is derived from sphingosine instead of glycerol.

Examples of phospholipids useful for the invention include, but are not limited to, phosphatidylethanolamine, phosphatidylcholine, lecithin, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol and mixtures of two or more thereof.

Phospholipids for production of oil bodies of the invention may be from various sources, for example, from seeds, and more typically from the oilseeds. These include lecithin and lyso-lecithin. The lecithin may, for example, be obtained from soy (soy lecithin) or sunflower (sunflower lecithin). Additionally, phospholipids with a modified fatty acid composition can be used. Such phospholipids can be, for example, enzyme modified soy phospholipids. Enzyme modified phospholipids may be prepared, for example, from soy phospholipids (SLP; True Lecithin, Mie, Japan) by treatment with phospholipase $A_2$ (Novo Industry, Bagsvaerd, Denmark). Additionally, phospholipids with a modified fatty acid composition can be obtained from plants or plant seeds that have been genetically modified to produce phospholipids with modified fatty acid compositions. An example of a phospholipid with a modified fatty acid composition is lecithin with a modified fatty acid composition. Other methods that are well known in the art may also be used to modify the fatty acid compositions of phospholipids.

The surfactant layer surrounding the lipid core of the oil body usually is a monolayer.

Any concentration of the surfactant can be used which allows the formation of the artificial oil bodies.

Artificial Oil Bodies

An used herein, the term "artificial oil body" refers to a structure, typically between about 0.1 and about 100 μm in size, comprising oil surrounded by a surfactant layer with oleosins embedded in the surfactant layer. They are referred to as artificial because there is no evidence that the oil bodies of the invention exist in nature. Nonetheless, an "artificial oil body" of the invention can also be referred to as an "oil body".

Typically, an artificial oil body of the invention comprises about 90% to about 98.5% glyceride, about 0.2% to about 5% surfactant, and about 0.5% to about 5% protein. For example, a typical artificial oil body of the invention comprises an oil/ surfactant/oleosin ratio of approximately 97.5/1.0/1.5. As shown herein, reducing the concentration of oleosin results in increased size of the oil body.

In an embodiment, at least 10%, more preferably at least 20%, more preferably at least 30%, of the fatty acids are polyunsaturated fatty acids with four or more double bonds. For example, Hi-DHA tuna oil produced by Numega has about 37% PUFAs, whereas algal oil DHASCO produced by Martek has about 61% PUFAs.

In another embodiment, at least 10%, more preferably at least 20% of the fatty acids are DHA. For example, Hi-DNA tuna oil produced by Numega has about 26% DHA, whereas algal oil DHASCO produced by Martek has about 40% DHA.

The oil bodies can be obtained by preparing a pre-emulsion by mixing oil, surfactant and oleosin with water (for example) using an automatic stirrer operated at 800rpm for 30 minutes. Advantageously, the oleosins can be provided as a protein extract from plants obtained using a method of the invention. In an embodiment, the oleosin is stored as a powder, for example as produced by freeze drying, before being used in the methods of the invention.

Artificial oil bodies of the invention are oxidatively stable. For example, less than 40%, more preferably less than 30%, more preferably less than 20%, and even more preferably less than 15%, of the fatty acids, such as total concentration of EPA and DHA, in the artificial oil bodies are depleted when the oil bodies are stored at 40°C for 15 days. In another example, following storage at 40°C for 15 days, the oil bodies comprise less than 6, more preferably less than 5, and even more preferably less than 4

μg/ml trans,trans-2,4-heptadienal. In yet another example, less than 90 meq/kg oil, more preferably less than 50 meq/kg oil, more preferably less than 25 meq/kg oil, of hydroperoxides are produced after 5 days at 40°C.

Products Comprising Artificial Oil Bodies and Uses Thereof

The oil bodies can be used in a wide variety of products. Examples of such products include, but are limited to, a food or feed product, a drink product, a personal care product, a pharmaceutical product or an industrial product. Processes for the production of these products are well known in the art.

In one preferred embodiment, the subject invention is directed to products which may be ingested by animals and/or humans. Since, these compositions may be ingested they must be of food grade quality. The particular product and the particular form in which the oil bodies are applied, however, is not of critical importance and may be as desired. Examples of a food, feed or drink product, include, but are not limited to, a non-dairy substitutes, a non-dairy cheese, a non-dairy yoghurt, a margarine, a mayonnaise, a vinaigrette, an icing, cream, soup, ice creams (for example as a stabilsation agent), a salad dressing, a mustard, a candy, bechamel sauce, chewing gum, a pudding, a baking product, a condiment, a juice (for example as a naturally clouding agent), baby formula, a flavour carrier, a texturing agent, fish food, petfood and livestock feed.

Examples of a personal care product include, but are not limited to, a soap, a cosmetic product, a skin cream, a facial cream, a tooth paste, a lipstick, a perfume, make-up, a foundation, a blusher, a mascara, an eyeshadow, a sunscreen lotion and a hair care product.

Examples of a pharmaceutical product include, but are not limited to, a therapeutic agent, a diagnostic agent and a delivery agent.

The therapeutic or diagnostic agent can be molecule that one wishes to deliver to a host. In one embodiment, the active ingredient may be a protein or peptide that has therapeutic or diagnostic value. Such peptides include antigens (for vaccine formulations), antibodies or antibody-related molecules, cytokines, blood clotting factors and growth hormones.

Examples of an industrial product include, but are not limited to, a paint, a coating, a lubricant, a film, a gel, a drilling fluid, paper sizing, a latex, a building or road construction material, an ink, a dye, a wax, a polish and an agrochemical formulation.

Artificial oil bodies of the invention can be used to treat or prevent diseases due to the well known benefits of the fatty acids in the oil bodies of the invention. Thus, in a further aspect, the present invention provides a method of treating or preventing a condition which would benefit from fatty acids, in particular a condition which benefits from polyunsaturated fatty acids with four or more double bonds, the method comprising administering to a subject with the condition one or more oil bodies of the invention and/or a composition of the invention. Examples of such conditions include, but are not limited to, cardiac arrhythmia's, angioplasty, inflammation, asthma, psoriasis, osteoporosis, kidney stones, AIDS, multiple sclerosis, rheumatoid arthritis, Crohn's disease, schizophrenia, cancer, foetal alcohol syndrome, attention deficient hyperactivity disorder, cystic fibrosis, phenylketonuria, unipolar depression, aggressive hostility, adrenoleukodystophy, coronary heart disease, hypertension, diabetes, obesity, Alzheimer's disease, chronic obstructive pulmonary disease, ulcerative colitis, restenosis after angioplasty, eczema, high blood pressure, platelet aggregation, gastrointestinal bleeding, endometriosis, premenstrual syndrome, myalgic encephalomyelitis, chronic fatigue after viral infections or an ocular disease.

In an embodiment, the oil bodies or composition is provided by oral administration. Typically, the oil bodies are administered in an amount that is sufficient to deliver at least 10 mg of fatty acid such as omega-3 PUFA per administration event. More preferably, at least 30 mg, even more preferably at least 50 mg and most preferably at 100 mg of of fatty acid such as omega-3 PUFA is delivered per administration event.

It will be understood that the present invention may suitably be employed to treat animals, such as mammals. Most preferably, the present invention is used to treat humans.

In a preferred embodiment, the product is, or comprises, an oil-in-water emulsion. Such emulsions of the invention are physically and oxidatively stable, and do not coalesce on standing.

The oil bodies may be formulated into an emulsion using techniques known in the art. Preferably, at least one additional ingredient is added to the oil body preparation. The additional ingredient may be added as a solution, suspension, a gel or solid and quantities of the additional ingredient will depend on the formulation. The additional ingredient may upon formulation become associated with the oil bodies, remain suspended in solution, or form a suspension in which the oil bodies are dispersed. The ingredient may also penetrate the surfactant layer surrounding the oil body. Ingredients which may penetrate the oil body include oils, waxes and the

colorant Nile Red. In a preferred embodiment, the additional ingredient is a liquid phase. In a further preferred embodiment the liquid phase is water.

Water may be added either directly or through moisture associated with another ingredient. The final amount of water is not critical, as long as upon mixing of the ingredients, a stable emulsion is formed. Generally, the compositions will contain at least 1% of water and up to 99% water. Usually mixing will be required to provide an adequate emulsion and it may be necessary to apply heat or pressure.

In another preferred embodiment, the additional ingredient is an oil or a wax. Oils or waxes may partition within the oil bodies and in this manner lipid soluble ingredients, such as lipid soluble vitamins may be delivered to the oil body. Where oils or waxes comprise the added ingredient, the oil bodies may remain suspended in the lipophilic phase or double emulsions may be formed.

The final compositions may be in solid or in liquid form or of any other desired viscosity. The emulsion may be thickened using gelling agents such as cellulose and derivatives, Carbopol and derivatives, carob, carregeenans and derivatives, xanthane gum, sclerane gum, long chain alkanolamides, and bentone and derivatives, typically present in concentrations less than 2% by weight.

The emulsion may further comprise surfactants to wet, foam, penetrate, emulsify, solubilize and or disperse a selected material. For example anionic surfactants such as sodium coconut monoglyceride sulphonate, cationic surfactants, such as lauryl trimethyl ammonium chloride, cetyl pyridinium chloride and trimethylammonium bromide, nonionic surfactants including pluronics, and polyethylene oxide condensates of alkyl phenols, and zwitterionic surfactants such as derivatives of aliphatic quaternary ammonium, phosmomium and sulphonium compounds may all be added as required.

Chelating agents, capable of binding metal ions, such as tartaric acid, EDTA, citric acid, alkali metal citrates, pyrophosphate salts or anionic polymeric polycarboxylates may be also included in the emulsion formulation as desired.

Generally, the emulsion formulations will be treated such that contamination by bacteria, fungi, mycoplasmas, viruses and the like or undesired chemical reactions, such as oxidative reactions are prevented. In preferred embodiments this is accomplished by the addition of preservatives, for example sodium metabisulfite or other chemical additives or by irradiation, for example by ionizing radiation such as cobalt-60 or cesium-137 irradiation or by ultraviolet irradiation.

In addition, active agents may be added. For example, cosmetic compositions may be formulated as stable suspensions using the present emulsion formulation and

vitamins and moisturizing agents may be included in skin creams. One particularly advantageous way in which an active ingredient may be included in emulsions of the subject invention, is through construction of oleosin gene fusions as detailed in WO 96/21029. These fusion proteins are created by genetically linking the gene encoding oleosin to a gene encoding a peptide or protein of interest. Expression of the fusion gene, in for example an oilseed plant, results in synthesis of a fusion protein which is then used to produce artificial oil bodies of the invention. In principle any desired protein or peptide may be produced using this technology, including polar fish antifreeze peptides or a therapeutic protein may be produced as an oleosin fusion.

An emulsion with film forming properties may also be formulated. Such an emulsion when it is applied to a surface and dried forms a coating. An example of an emulsion where a coated oil body film is applied is in fish food, where oil bodies (for example produced from microalgae oil) may be applied to the fish food to enhance the dietary value. A film forming emulsion is particularly useful in embodiments of the present invention where controlled release of an active ingredient is desirable such as in delivery of pharmaceuticals or volatiles such as fragrances. The release time of the active agent from a film of emulsion, which occurs during drying, depends, among other factors, on the thickness of the film. When a thicker coating is applied a longer drying time will result in a slower release of the active agent. In variant contemplated formulations, release of the agent occurs only when the film is dry.

Other factors, such as the composition of the emulsion and the type and concentration of the active ingredient also determine the characteristics of release. For example, cosolvents, such as ethanol, may be included in the formulation and influence the release time. Release of an active ingredient is also desirable in food applications, where a flavorant entrapped in an emulsion is released during consumption. The release of the flavorant, depending on the exact formulation of the emulsion, may elicit a sudden intense sensation or a more subtle blend of flavours and essences.

The emulsion formulation may also be used in sprays and aerosols. Volatiles, such as alcohol and fragrances may be included in these sprays. Emulsions of this type may also be sprayed onto the surface of dried food preparations such as potato chips and dried soup. The emulsion might include a flavorant and add preservative value or assist in maintaining the appropriate moisture levels of the food.

The stability of the present emulsion formulation may be exploited in formulations of acid emulsions. For example, the emulsion formulation may be used in the preparation of a mayonnaise-like food product, which besides the oil body preparation comprises a vegetable oil, mustard, vinegar and egg yolk, if desired.

Pourable emulsions, such as salad dressings may be prepared by increasing the relative amount of vinegar and/or by the addition of water.

An example of an application where heat may be applied without apparent deleterious effects, is in the preparation of a savory sauce such as a bechamel sauce or in sweet sauces such as chocolate sauces. In these applications, the oil body preparation is employed as a frying substitute. To prepare a bechamel sauce, to 1 part of the heated oil body preparation, 1 part (w/w) of flour is added and stirred until a thick suspension is formed. At moderate heat milk is gradually added until a sauce with a desired viscosity is obtained.

The emulsion formulation may also be used as a butter substitute. In this application, small amounts of water are added to the oil body preparation, for example, less than 10% until a desired viscosity is obtained. Natural butter flavours and thickeners may be added as desired. The butter substitute may be used on sweet corn, bread, in cake mixes or bread making. Salt, which contributes flavour and acts as a preservative may be added typically to a level of about 2.5% (wt/vol). Colour agents, for example, extracts of annatto seed or carotene may be added to deepen the colour as desired. An advantage of this application is that the oil body based butter does not comprise hydrogenated fatty acids, which are used in the formulations of margarines and the like to achieve a desirable consistency, but are also with associated with cardiovascular diseases.

Shortenings may be prepared to various degrees of stiffness, from a foam to a pourable shortening. In this application, air is beaten into the emulsion formulation and the emulsion formulation can be considered to be dispersed into the continuous phase, air. Shortenings may be applied to mixes where creaming and fluffing are desired. These mixes include icings, synthetic creams, ice creams and cake batter.

An imitation fruit juice may be prepared from artificial or natural flavours and nutrients. Such imitation juices do not have the correct appearance and due to transparency appear to be weak or diluted. By adding a small amount, for example 0.1 to 1% (v/v) of the oil body preparation or an emulsion thereof clouding may occur to give the juice a rich appearance. Thus the present oil body preparation may be used as a clouding agent. In another application involving juices, the oil body preparation or an emulsion thereof may be added to juices with settleable solids, such as tomato juice.

Adding a small amount of the oil body preparation, for example 0.1 to 1% (v/v), may decrease the rate of settling of the solids in the juice and assist in maintaining the rich appearance.

Topical applications of the oil body preparation of the present invention are also envisaged. In this embodiment the emulsion is formulated as a dermatologically acceptable emulsion, which may for example be employed to moisturize facial and/or body skin, including nails and lips or may have properties to combat ageing of the skin, acne, pigmentation, hair loss, or promote hair removal or facilitate wound healing and/or restructuring of the skin tissue. The oil body preparation represents preferably 1-99% by weight of the final composition.

The cosmetic compositions of the present invention may comprise additional hydrocarbon compounds such as plant, animal, mineral or synthetic oils or waxes or mixes thereof. They comprise paraffin, petrolatum, perhydrosqualene, arara oil, almond oil, calphyllum oil, avocado oil, sesame oil, castor oil, jojoba oil, olive oil, or cereal germ oil.

Esters may be included such as esters of lanolic acid, oleic acid, lauric acid, stearic acid, myristic acid. It is also possible to include alcohols for example, oleoyl alcohol, linoleyl alcohol or linolenyl alcohol, isostearyl alcohol or octyl dodecanol, alcohol or polyalcohol.

Further hydrocarbons which may be included are octanoates, decanoates, ricinoleates, caprylic/capric triglycerides or C10 to C22 fatty acid triglycerides. Addition of these agents may result in the formation of double emulsions.

Hydrogenated oils, which are solid at 25°C, such as hydrogenated castor oil, palm oil or coconut oil, or hydrogenated tallow; mono- di- tri- or sucroglycerides; lanolins; and fatty acids which are solid at 25°C may also be included in the cosmetic formulations of the present invention. Among the waxes which may be included are animal waxes such as beeswax; plant waxes such as carnauba wax, candelilla wax, ouricurry wax, Japan wax or waxes from cork fibres or sugar cane; mineral waxes, for example paraffin wax, lignite wax, microcrystalline waxes or ozokerites and synthetic waxes.

Pigments may be included and may be white or coloured, inorganic or organic and/or paerlescent. These pigments comprise titanium dioxide, zinc oxide, ziriconium dioxide, black, yellow, red and brown iron oxides, cerium dioxide, chromium oxide, ferric blue, carbon black, barium, strontium, calcium and aluminum lakes and mica coated with titanium oxide or with bismuth oxide.

Active ingredients commonly employed in skin creams, such as vitamins, for example as vitamin A or C and alpha hydroxy acids, such as citric, glycolic, lactic and tartaric, into cosmetic and/or dermatological compositions may be included. For example, US 5,602,183 teaches that vitamin C or ascorbic acid promotes growth of

connective tissue, particularly in the skin strengthens the skin against external aggressions such as from smoke and UV radiation. Moisturizing agents which may be included in skin creams and cosmetics are for example mineral oil and urea. Antioxidants such as the naturally occurring tocopherols and polyphenols, or butylated hydroxytoluene and hydroxyanisole may also be also added. Sunscreens such as octyl methoxycinnamate (Parsol MCX), 3- benzophenone (Uvinul M40) and butylmethoxydibenzoylmethane (Parsol 1789) may be employed to prepare a sun tanning lotion. Pharmaceutically active ingredients which may be used to formulate cosmetic compositions include for example antibiotics, fungicides and anti-inflammatory agents.

The final cosmetic product may be in the form of a free, poured or compacted powder (foundation, blusher or eyeshadow), a relatively greasy product such as lipstick, mascara, or an oil or lotion for the body or face.

The oil body preparation may also be used to serve as an orally acceptable carrier in toothpaste which may further comprise silicas, surfactants, chelating agents, a fluoride, thickeners, sweeteners, flavorants, for example as oil of peppermint, enzymes and biocides.

An example of an industrial product which may be formulated is paint wherein the main resin, such as those based on silicone type compounds, acrylic compounds, polyester, akyd, fluorine, epoxy, polyurethane may be partly or entirely replaced by the oil body preparation of the present invention. Further additives such as pigments, dyes, glass flakes, and aluminum flakes, pigment dispersants, thickeners, levelling agents, hardening catalysts, hardening agents such as dioisocyanates, hardening catalysts, gelling inhibitors, ultraviolet absorbing agents, free radical quenching agents. etc. may be formulated in paint compositions as required.

The oil body preparation may also be to formulate lubricants. For example, the oil body preparation may be used to partially or entirely replace the lubricating oils such as animal oils, vegetable oils, petroleum lubricating oils, synthetic lubricating oils, or the lubricating grease such as lithium grease, urea grease and calcium grease. Other compositions employed in a lubricant formulation comprise antioxidants, detergent dispersants, oilness agents, friction modifiers, viscosity index improvers, pour point depressants, solid lubricant material, rust inhibitors and antifoamers.

Waxes may also be prepared using the oil body preparation of the present invention. These comprise rinse-wax types, such as those providing a stable hydrophobic film-finish onto automobiles and other protective coatings. Other compositions used in the preparation of a wax comprise surfactants, mineral oils, such

as mixed paraffinic and aromatic/naphtenic oils, perfumes, biocides, colouring agents which may be added in compatible amounts as desired.

**EXAMPLES**

**Example 1 - Materials and Methods**

Materials

Canola (*Brassica napus*) seed, oil, and meal were gifts by Cargill Australia. The meal was the residue after canola oil extraction by the usual industrial process of mechanical expelling followed by solvent extraction. On arrival, the seeds were sieved to remove straw and other non-seed material and stored at 4°C until required. The seeds contained 38.0 % oil (information provided by Cargill, Ltd) and 25.2% proteins as determined by LECO® FP-2000 analysis. Canola oil was of refined, bleached and deodorized grade, and had TBHQ (200 ppm) antioxidant added. Winterized tuna oil (HT303-4) was obtained from LYSI HF (Reykjavik, Iceland).

Extraction of oil canola bodies

Natural oil bodies were extracted from canola seeds according to the method described of Tzen (1993) with the exception that the final hexane washing step was omitted. In brief, the seeds were soaked in sodium phosphate buffer (pH 7.5) overnight and homogenized with the grinding medium (10 g dry seeds per 50 ml) for 40 seconds using a kitchen blender (Sunbeam Multiblender, 650W). The grinding medium contained 0.6 M sucrose and 10 mM sodium phosphate buffer (pH 7.5). The homogenate was filtered through three layers of cheesecloth. The filtrate was placed in centrifuge tubes, an equivalent amount of flotation medium (grinding medium containing 0.4 M instead of 0.6 M sucrose) was layered on top, and the tubes were centrifuged at 5,000 *g* for 60 min at 10°C in a swinging-bucket rotor (Beckman J6-HC).

The oil bodies collected at the top were resuspended in twice their volume of detergent washing solution containing 0.1%Tween-20, 0.2 M sucrose, and 5 mM sodium phosphate buffer pH 7.5. The re-suspension was placed at the bottom of centrifuge tubes and 10 mM sodium phosphate buffer pH 7.5 was layered on top in the ratio 1:1, and the tubes were centrifuged. The oil bodies on top were collected and resuspended in twice their volume of ionic elution buffer (grinding medium additionally containing 2 M NaCl). The suspension was placed at the bottom of centrifuge tubes, floating medium (grinding medium containing 2 M NaCl and 0.25 M instead of 0.6 M sucrose) was layered on top (ratio 1:1), and the tubes were

centrifuged. The oil bodies on top were collected and resuspended in their volume of 9 M urea. The re-suspension was vigorously shaken at room temperature for 10 min, then placed at the bottom of centrifuge tubes, 10 mM sodium phosphate buffer pH 7.5 was layered on top (ratio 1:1); and the tubes were centrifuged. The oil bodies on top were collected and resuspended in their volume of grinding medium. The resuspension was placed at the bottom of centrifuge tubes, flotation medium was layered on top (ratio 1:1), and the tubes were centrifuged. The oil bodies on top were collected and resuspended with grinding medium to give a concentration of about 100 mg/ml.

Isolation of oleosin from canola oil bodies

Oleosin was extracted from the purified canola OBs dried to 14% (w/w) moisture content. The oil body preparation was redispersed in a solution of deionised water and ethanol (60% ethanol) and stirred for 1h, and the ethanol was removed using a rotary evaporator (BÜCHI Rotavapor R-124). The emulsion was then centrifuged at 5,000 *g* for 30 min in a swinging-bucket rotor at 20°C (Beckman J6-HC). The top layer containing water and traces of ethanol was removed and the proteins redispersed in deionised water at a concentration of 100 g/L. This aqueous protein dispersion was stored at -18 °C until required.

Protein characterisation

The proteins isolated from canola oil bodies (COBs) were characterised according to their molecular weight by electrophoresis. The electrophoresis was performed using NuPAGE® gel 4-12% BT 1.0 gel (NuPAGE® invitrogen) according to supplier's recommendations. In brief, the concentration of protein samples was adjusted to 1 mg/ml and 25µl placed in eppendorf tubes. Then, 10µl of NuPAGE® LDS sample buffer (4X) and 5µl of NuPAGE® reducing agent (10x) were respectively added and the tubes centrifuged for 3 seconds at 14,000 rpm at room temperature (Eppendorf Centrifuge 5415C) before being heated to 70°C for 10 minutes. Markers (Mark 12™ Unstained Standards) were prepared the same way except that their concentrations were not adjusted. Finally, 10 µl amounts of both markers and samples were added to the gel already placed in an electrophoresis cell (Novex Mini-Cell, Invitrogen) containing NuPAGE® Running Buffer and anti-oxidant. The cell was connected to a generator (BIORAD Power Pac 300) set at 200 Volts and 400 milliampers for 35 min.

Once the electrophoresis was complete, the gel was washed 3 times in deionised water and placed in a plastic container with SimplyBlue™ SafeStain (Invitrogen) for 2

h under slow agitation, 42 rpm (RATEK Platform Mixer model OM6). Afterwards, the gel was washed twice more with deionised water and placed in deionised water for 2 hours in order to remove non-protein bound stain, and photographed with a G:BOX (SYNGENE) and the GeneSnap 7.07 software (SYNGENE).

Extraction of oleosin from canola meal

Oleosin was extracted from canola meal according to a modification of the procedure of Ghodsvali et al. (2005). In brief, canola meal (1.0 kg) was soaked in deionised water (10.0 L) and the pH adjusted to 12.0 with aqueous sodium hydroxide (5%, w/w) and mixed for 10 minutes using an Ultraturax mixer. After standing for 10 minutes, the contents were mixed for a further 10 minutes. The resulting slurry was centrifuged at 4,000 *g* in a swinging-bucket rotor for 30 min at 20°C (Beckman J6-HC). The pH of the supernatant containing crude protein adjusted to pH 6.5 (isoelectric point of oleosin) when protein precipitation occurred. The stirring was continued for a further 30 min before centrifuging at 4,000 *g* when the supernatant and the precipitate were recovered. The precipitate was washed with pH 6.5 water using Ultraturax and centrifuged again for 30 min at 4,000 *g*. The washed precipitate was recovered as a paste and stored in the wet form at -18 °C until required.

Construction of artificial oil bodies

In intial experiements artificial oil body dispersions containing canola oil and tuna oil, respectively, were prepared using oleosin extracts variously obtained from canola meal, both in the presence and absence of phospholipid. First, a pre-emulsion was prepared by mixing oil and water containing the proteins using an automatic stirrer (RZR 2051 Control from Heidolph) operated at 800rpm for 30 minutes. When phospholipids were included, they were dissolved in the oil prior to emulsification. The weight ratios of the ingredients used resembled their ratios in natural canola OBs. Thus, the preemulsions contained 1.5% of proteins, 1% of phospholipids if required, and 10% of oil in order to achieve a particle size between 0.5 and 2μm and oil droplets well covered by proteins and phospholipids. The pre-emulsion was homogenized (RATEK Pilot Lab Homogenizer) three times at 500 bars.

In later experiments artificial oil body dispersions containing canola oil and tuna oil, respectively, were prepared using surfactants and oleosin extracts variously obtained from canola meal. When phospholipids or other surfactants were included, they were mixed with the oil prior to emulsification. First, the pH of the oleosin extract was adjusted to approximately 8.0 by dropwise addition of 40% aqueous sodium

hydroxide while being subject to Ultraturax mixing. Oil (10 g), oleosin extract (1.5 g), surfactant (various) and deionized water (87.5 g) were placed in a glass beaker and warmed in a water bath (60 °C) before being mixed for 2 min using a Silverson mixer under a blanket of nitrogen to minimize oxidation. The mixture was homogenized (EmulsiFlex C5) twice at 1000 bar under nitrogen. The weight ratios of the ingredients used resembled their ratios in natural canola OBs. Thus, the preemulsions contained 1.5% of proteins, 1% of surfactant if required, and 10% of oil.

The oleosin extract from canola meal was "worked up" prior to emulsion preparation as follows. The stored extract was allowed to thaw and mixed using an Ultraturax mixer 40% (w/W) while adding sodium hydroxide dropwise until the pH reached 8.0.

Preparation of control emulsions using Tween40 emulsifier

10% (w/w) o/w emulsions were prepared with canola and tuna oils using Tween40 (6%, w/w) as the emulsifier. Emulsification was performed as above.

Physical characterisation of artificial oil bodies

The physical stability of the AOBs was studied over 12 days at 4°C, ambient temperature (approx. 20°C), 45°C and 60°C respectively, respectively. Particle size of the emulsions was measured with a MASTERSIZER 2000 (Malvern) using a Malvern Small Volume Sample Presentation Unit combined with a Hydro 2000g module. (particle RI : 1,456 / absorption : 0,001 / dispersant RI : 1,330 water). Light microscopy photos were obtained with a light microscope Olympus BH-2 linked to a Color View IIIu camera and processed using AnalySIS getIT 5.0 software. Aggregation of particles was examined by treatment with sodium dodecyl sulfate (SDS). This was performed by mixing three volumes of the emulsion with one volume of a 10% (w/w) solution of SDS for 10 min using a magnetic stirrer at room temperature followed by reanalysis.

Oxidative stability of oil in water emulsions prepared from oil bodies

The oxidative stability of the AOB dispersions were evaluated from the depletion of EPA/DHA, as well as the amount of trans, trans-2,4-heptadienal produced during storage under accelerated oxidation conditions. For the latter purpose samples (2 g) of the emulsions were placed headspace vials (10 ml) and sealed with silicone-lined aluminium caps, and heated at 40 °C or 60 °C inside a dark oven over 15 days.

Samples were withdrawn daily and immediately analyzed for 2,4-heptadienal by solid phase micro extraction and gas chromatography-mass spectrometry as below.

Oxidative volatile compounds generated during the accelerated oxidation of AOB emulsions were determined by headspace solid phase micro extraction (SPME) using a DVB/CAR/PDMS fiber, (50/30 µm, Supelco, Sydney, Australia). The fiber was inserted into the sample headspace and the vials were incubated at 60 °C for 15 min and then withdrawn and transferred to the GC injector (operated in the splitless mode) and held for 7 min to desorb the extracted volatile compounds into the GC column. The entire series of events was performed using a Combi PAL Auto Injector (CTC Analytics, Zwingen, Switzerland). GC-MS was performed using an Agilent Model 6890 GC and Model 5973 MSD (Palo Alto, CA) fitted with a VOC fused silica capillary column (60 mm, 0.32mm i.d., 0.18 mm film thickness, Agilent, Melbourne, VIC, Australia). The GC oven was programmed from 40 °C increasing to 220 °C at the rate of 22 ° C.min -1 and held at that temperature for further 14 minutes. Helium was used as the carrier gas at a constant flow rate of 2.0 mL min-1. The injector was initially operated in the splitless mode and then switched to the split mode (1:20) 2 min after sample injection. The temperature of the injector and the MS detector were both held at 230°C. The MS was operated in scan mode (29-350 amu). Data analyses were performed using Chemstation software and compounds were identified by standards as well as reference to a library of spectra (Wiley 275). Volatile compounds in the emulsions were quantified by using calibration curves established from their corresponding fresh emulsions added with different levels of volatile compound 2,4-heptadienal (trans, trans) standards.

Depletion of ALA (canola) EPA and DHA (tuna) during storage was measured by fatty acid analysis using capillary gas chromatography. For this purpose lipids were extracted from the stored emulsions using iso-propanol/hexane and converted to methyl esters by potassium hydroxide catalysed transesterifcation prior. The esters were analysed on a BPX-70 capillary column and detected by flame ionisation detection.

**Example 2 – Extraction of oleosin from canola meal**

Figure 2 shows electrophoresis results for the protein isolated from canola meal by alkaline extraction (pH 12) followed by precipitation at pH 6.5 and water washing. The molecular weight of oleosin has been reported to in the range 18-20 kDa, and the above isolate contained significant amounts of protein in this molecular range confirming the presence of oleosin. Precipitation of protein at different pH values in the range 3.0 – 12.0, and subsequent electrophoresis showed that the maximum oleosin

recovery was obtained at pH 6.5. Previous studies by other workers have focussed on isolation of oleosin from pre-extracted oil bodies after they have been destabilised by treatment of ethanol or mixtures of chloroform and methanol. To the best of our knowledge, no attempts have been made previously to isolate oleosin from oilseed or meal by aqueous extraction. This could be because oleosins are considered insoluble in water regardless of the pH (Beisson et al., 2001). The apparent water insolubility could be the result of the tendency of oleosin to agglomerate on drying; and once this happens it is hard to dissolve or disperse oleosin in water.

Following the intial experiments, optimisation of extraction of oleosin from canola meal was conducetd. The highest oleosin yield was obtained at pH 12.0 with significantly lower recoveries obtained as the pH was reduced (Figure 3). pH 12.0 also furnished the purest oleosin extracts as shown by SDS-PAGE (Figure 4). Lower pH values tended to extract increasing amounts of two lower molecular weight components of approximately 12 and 5 kDa.

**Example 3 – Particle size of artificial oil bodies**

To eliminate any effects of particle size on the rate of oxidation, the particle size of the two emulsions (Tween40 and oleosin) were matched as far as possible (Figure 5). It was also important to ensure that no separation occurred and the particle size remained relatively constant during storage for the accelerated oxidation study. The droplet size of AOBs made with oleosin as well as that of the Tween40 emulsion remained unchanged during storage over 12 days at 60°C. The uniformity of droplet size during storage at elevated temperatures enabled accelerated oxidation studies based on headspace analysis.

Clearly, the pH 12-soluble extract was primarily composed of cellular protein. The fraction precipitated also would have contained relatively large amounts of non-oleosin protein. Interestingly, however, when AOBs were prepared using either the pH 12-soluble extract or the pH 6.5-precipitated protein re-solubilised at pH 12, there appeared to be a natural selection of oleosin to form the oil body surface (Figure 6). This will have the advantage of not requiring further purification of the precipitated protein to enhance the oleosin content prior to preparation of AOBs.

**Example 4 – Oxidative stability**

The oxidative stabilities of aqueous dispersions of canola and tuna oil AOBs prepared with re-solubilised pH 6.5 precipitated protein both in the absence and presence of PL were compared with a equivalent emulsions prepared with the

corresponding oil and Tween40 emulsifier. For this purpose, the emulsions were stored at an elevated temperature (60°C) and the oxidation levels were measured by three different methods, i.e. primary oxidation products (hydroperoxides), secondary oxidation products (trans,trans-2,4-heptadienal), and depletion of unsaturated fatty acids (linolenic acid for canola and DHA for tuna oil). The hydroperoxide levels of the AOB emulsions were significantly lower than that of the corresponding Tween40 emulsion throughout the storage period tested, showing the greater oxidative stability of the AOB emulsions (data not shown).

The concentration of 2,4-heptadienal in the tuna oil emulsion made with the oleosin extract was significantly lower than that made with Tween40, showing the greater oxidative stability of the oleosin emulsion (Figure 7). This showed that antioxidant effect of canola oleosin is not limited to canola oil but is extended to fish oil such as tuna oil. Additional evidence in support of this was provided by fatty acid data. The EPA and DHA contents of the tuna oil emulsion made with olesoin extracted from canola meal depleted more slowly than those of the Tween40 emulsion (Figure 8), with greater than 90% of DHA remaining after 5 days at 60 °C compared to less than 70% for the Tween40 emulsion.

Figure 9 shows the rate of development of hydroperoxides (as represented by PV) during accelerated oxidation (40 °C, exposed to air) of the oleosin- and Tween 40-stabilised emulsions. When subjected to accelerated oxidation, the PV of the Tween 40-stabilised emulsion rose sharply without an apparent induction period reaching a value of 120 meq/kg oil after 10 days; the PV gradually declined beyond this point which can be explained as been due to the rate of formation of hydroperoxides falling below the rate of their degradation in to secondary oxidation products such as aldehydes and ketones. The PV value of the oleosin-stabilised emulsion did not approach the PV maximum of the Tween 40-stabilised emulsion (120 meq/kg) even after 30 days accelerated at which point the experiment was terminated. The result demonstrates the superior oxidative stability of the oleosin-stabilized emulsion over the Tween 40-stabilized emulsion.

The artificial oil bodies (AOBs) produced from tuna oil and oleosin dispersed easily in water to form tuna oil-in-water emulsions. The emulsions did not separate when stored for 1 week at 4°C irrespective of the secondary surfactant used (phospholipid, monoglyceride or sodium stearoyl lactylate, SSL). In contrast, the emulsions produced from tuna oil and Isolexx (canola protein isolate) with and without phospholipid were unstable and separated within hours of preparation. At 40 °C, the sodium caseinate emulsion was the most stable showing no separation after 1 week.

The oleosin-stabilsed emulsions containing phospholipid or SSL were practically unseparated after 1 week at 40 °C.

**Example 5 – Emulsification and antioxidant efficacy of oleosin relative to other protein ingredients**

Comparisons were made with sodium caseinate, which is widely used as an emulsifier as well as "Isolexx", which is a canola protein isolate produced in Canada and being promoted as a food-grade emulsifier. The following formulations were tested for physical stability and oxidative stability.

1. Tuna oil + oleosin + phospholipid
2. Tuna oil + Isolexx (canola-based protein emulsifier marketed by BioExx, Canada)
3. Tuna oil + Isolexx + phospholipid
4. Tuna oil + sodium caseinate

Accelerated oxidation conditions (storage at 40 °C, exposed to air) were used to compare oxidative stability using trans,trans, 2,4-heptadienal as a marker of oxidative degradation of tuna oil omega-3 fatty acids. Figure 10 shows the development of trans,trans, 2,4-heptadienal within emulsions during storage. All emulsions with the exception of oleosin (plus phospholipid) emulsion reached maximum oxidation within 18 days. The sodium caseinate and Isolexx emulsions reached heptadienal concentration of 3.0 ppm within 9-10 days, whereas the olesosin emulsion did not reach same level of oxidation until after 24 days demonstrating the superior antioxidant properties of oleosin compared with sodium caseinate and Isolexx.

**Example 6 – Role of secondary surfactants on artificial oil body stability**

Based on previous work the presence of phospholipid in addition to oleosin was necessary to produce emulsions that did not coalesce on storage. The present inventors investigated the essentiality of phospholipid for stability of artificial emulsions by preparing emulsions where phospholipid was replaced by Tween40, monoglyceride and SSL as below. Phospholipid, Tween 40, MAG and SSL were used at the same level (1% w/w by weight of oil).

a. Tuna oil + oleosin and phospholipid
b. Tuna oil + oleosin and Tween 40
c. Tuna oil + oleosin and monoglyceride

d. Tuna oil + oleosin and sodium stearoyl lactylate (SSL)

Accelerated oxidation conditions (storage at 40 °C, exposed to air) were used to compare oxidative stability using trans,trans, 2,4-heptadienal as a marker of oxidative degradation of tuna oil omega-3 fatty acids. Figure 11 shows the development of trans,trans, 2,4-heptadienal within emulsions during storage. There was no significant difference in the rate of development of heptadienal between the different emulsions showing that replacement of phospholipid with Tween 40, monoglyceride or SSL did not adversely affect the oxidative stability of oleosin-based artificial oil bodies.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

The present application claims priority from AU 2011900383 filed 7 February 2011, the entire contents of which are incorporated herein by reference.

All publications discussed and/or referenced herein are incorporated herein in their entirety.

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

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**CLAIMS**

1. An artificial oil body comprising oleosin, surfactant, and oil comprising polyunsaturated fatty acids with four or more double bonds.

2. The oil body of claim 1, wherein the surfactant is phospholipid.

3. The oil body of claim 1 or claim 2, wherein at least 5% of the polyunsaturated fatty acids have four or more double bonds.

4. An artificial oil body comprising oleosin, surfactant, and oil comprising fatty acids, wherein at least some of the surfactant is not phospholipid.

5. The oil body according to any one of claims 1 to 4, wherein the oleosin is an oilseed oleosin.

6. The oil body according to any one of claims 1 to 5, wherein the oil is marine oil and/or fish oil.

7. The oil body according to any one of claims 1 to 6, wherein at least 50% of the fatty acids are in the form of glycerides.

8. The oil body according to any one of claims 1 to 7, wherein at least 80% of the dry weight of artificial oil body is oil.

9. The oil body according to any one of claims 1 to 8 which further comprises one or more other molecules.

10. The oil body of claim 9, wherein the other molecule is a bioactive molecule.

11. The oil body according to any one of claims 1 to 10 which has a size of between about 0.1 and about 100 μm.

12. A composition comprising one or more artificial oil bodies according to any one of claims 1 to 11, and a carrier.

13. A method of treating or preventing a condition which would benefit from fatty acids, the method comprising administering to a subject with the condition one or more artificial oil bodies according to any one of claims 1 to 11 and/or the composition of claim 12.

14. Use of one or more artificial oil bodies according to any one of claims 1 to 11 and/or the composition of claim 12 for the manufacture of a medicament for treating or preventing a condition which would benefit from fatty acids.

15. Use of one or more artificial oil bodies according to any one of claims 1 to 11 and/or the composition of claim 12 as a medicament for treating or preventing a condition which would benefit from fatty acids.

16. The method of claim 13, or the use of claim 14 or claim 15, wherein the fatty acids are polyunsaturated fatty acids with four or more double bonds.

17. A product comprising one or more artificial oil bodies according to any one of claims 1 to 11 and/or the composition of claim 12.

18. The product of claim 17 which is a food or feed product, a drink product, a personal care product, a pharmaceutical product or an industrial product.

19. The product of claim 17 or claim 18 which is, or comprises, an oil-in-water emulsion.

20. Use of one or more artificial oil bodies according to any one of claims 1 to 11, and/or the composition of claim 12, for the preparation of a product.

21. A method of preparing a feed, food or drink, the method comprising admixing one or more artificial oil bodies according to any one of claims 1 to 11, and/or the composition of claim 12, with one or more other edible ingredients.

22. A method of producing artificial oil bodies, the method comprising

i) obtaining oleosin, surfactant, and oil comprising polyunsaturated fatty acids with four or more double bonds, and

ii) mixing the oleosin, the surfactant and the oil to produce the artificial oil bodies.

23. A method of producing artificial oil bodies, the method comprising

i) obtaining oleosin, surfactant, and oil comprising fatty acids, wherein at least some of the surfactant is not phospholipid, and

ii) mixing the oleosin, the surfactant and the oil to produce the artificial oil bodies.

24. The method of claim 22 or claim 23 which further comprises,

iii) selecting artificial oil bodies.

25. The method according to any one of claims 22 to 24, wherein step i) comprises

a) obtaining an extract of a plant comprising oleosin, and

b) at least partially purifying protein from the extract, wherein the protein comprises oleosin.

26. The method of claim 25, wherein step b) comprises

1) adjusting the pH of the extract to a pH of at least about 11.5,

2) separating 1) to produce a solid and liquid phase and selecting the liquid phase,

3) reducing the pH of the liquid phase to precipitate the proteins,

4) separating 3) to produce a solid and liquid phase and selecting the solid phase, and

5) dispersing the solid phase in a carrier.

27. A method of partially purifying oleosin from a plant extract, the method comprising

1) adjusting the pH of the extract to a pH of at least about 11.5,

2) separating 1) to produce a solid and liquid phase and selecting the liquid phase,

3) reducing the pH of the liquid phase to precipitate the proteins,

4) separating 3) to produce a solid and liquid phase and selecting the solid phase, and

5) dispersing the solid phase in a carrier.

28. The method of claim 26 or claim 27, wherein step 3) comprises reducing the pH of the liquid phase to less than about 7.

29. The method according to any one of claims 22 to 28 which does not use an organic solvent.

30. The method according to any one of claims 25 to 29, wherein the plant is an oilseed.

31. The method according to any one of claims 25 to 30, wherein the extract is meal produced following the extraction of oil.

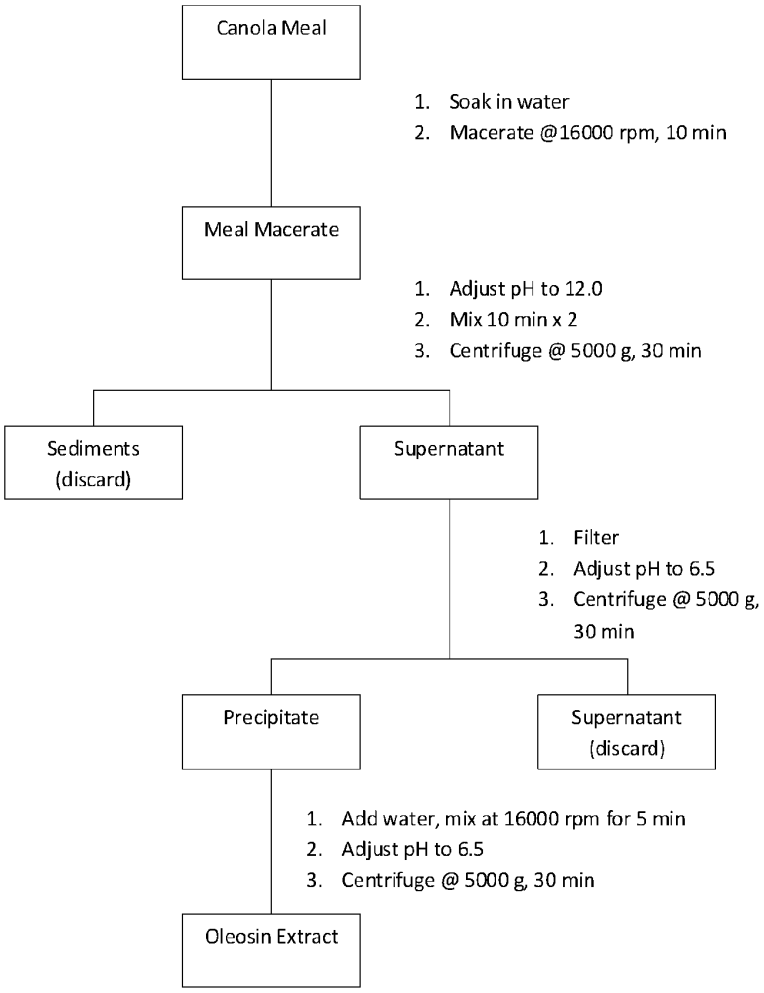
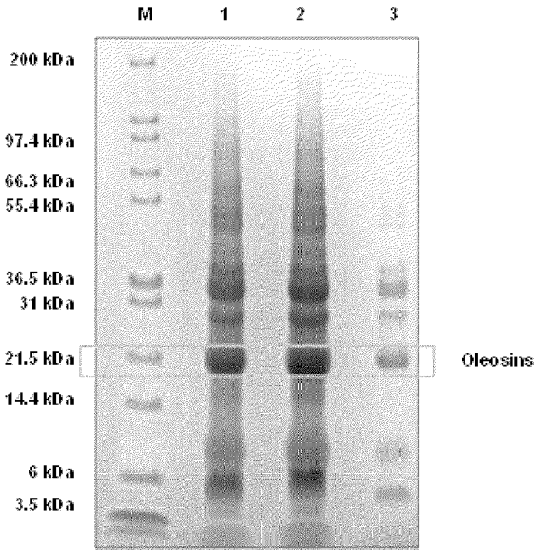

Canola Meal
1. Soak in water
2. Macerate @16000 rpm, 10 min
Meal Macerate
1. Adjust pH to 12.0
2. Mix 10 min x 2
3. Centrifuge @ 5000 g, 30 min
Sediments (discard)
Supernatant
1. Filter
2. Adjust pH to 6.5
3. Centrifuge @ 5000 g, 30 min
Precipitate
Supernatant (discard)
1. Add water, mix at 16000 rpm for 5 min
2. Adjust pH to 6.5
3. Centrifuge @ 5000 g, 30 min
Oleosin Extract

**Figure 1**




| | |
|---|---|
| M | Markers |
| Lane 1 | pH 12 soluble crude extract |
| Lane 2 | pH 12 precipitate before washing |
| Lane 2 | pH 12 precipitate after washing |

**Figure 2**

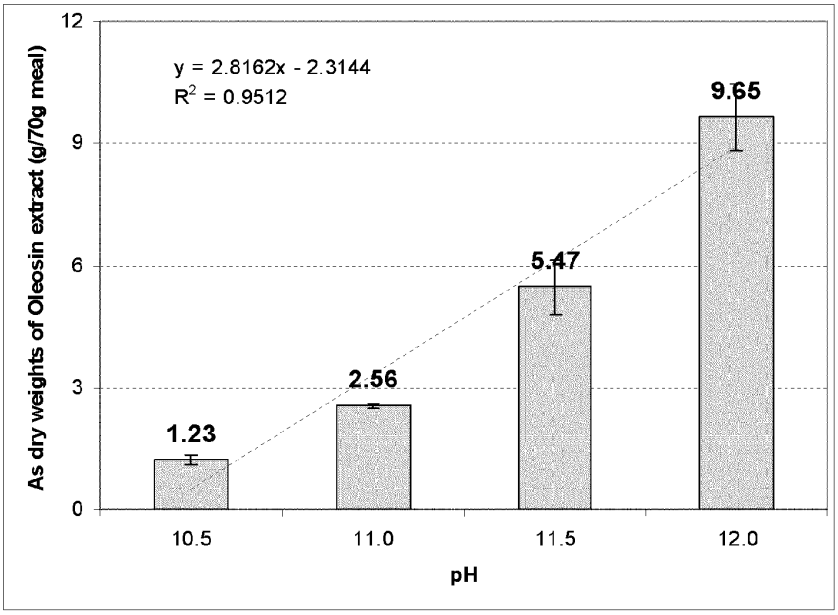

y = 2.8162x - 2.3144
R2 = 0.9512
As dry weights of Oleosin extract (g/70g meal)
12
9
6
3
0
1.23
2.56
5.47
9.65
10.5
11.0
11.5
12.0
pH

**Figure 3**

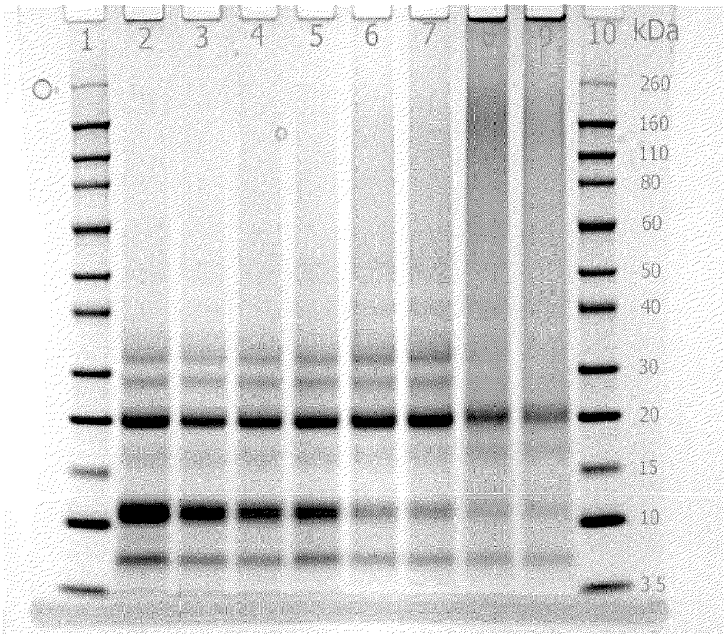



**Figure 4**

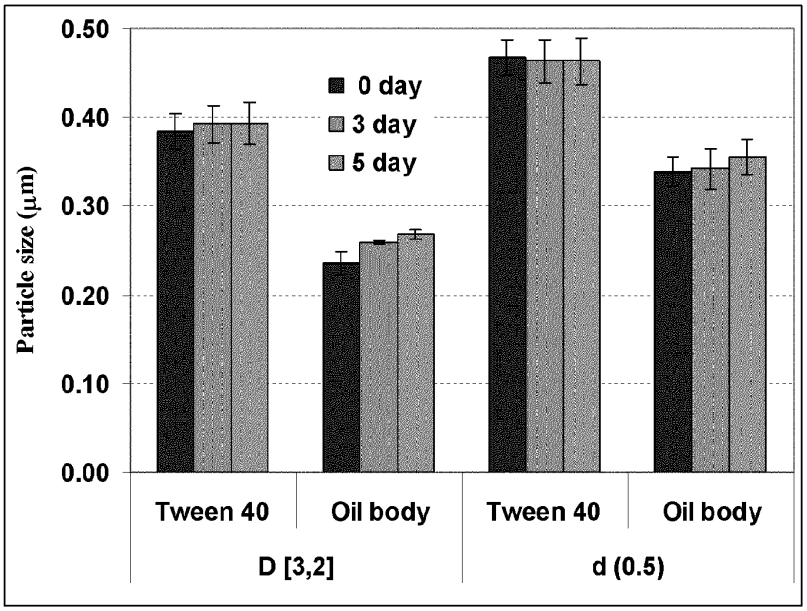



Figure 5A

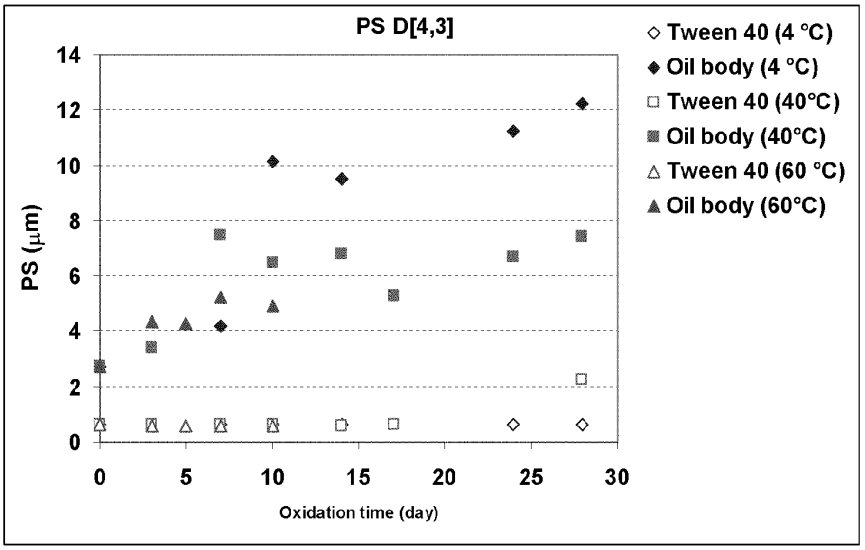



Figure 5B

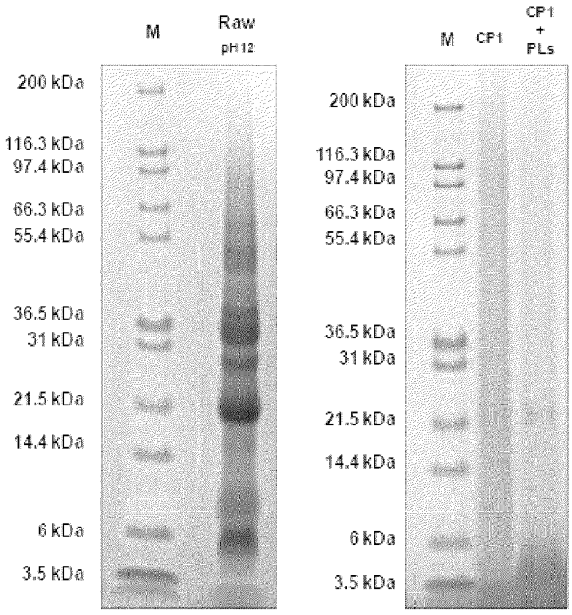

M
Raw
pH12
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31 kDa
21.5 kDa
14.4 kDa
6 kDa
3.5 kDa
M
CP1
CP1 + PLs
200 kDa
116.3 kDa
97.4 kDa
66.3 kDa
55.4 kDa
36.5 kDa
31 kDa
21.5 kDa
14.4 kDa
6 kDa
3.5 kDa

**Figure 6**

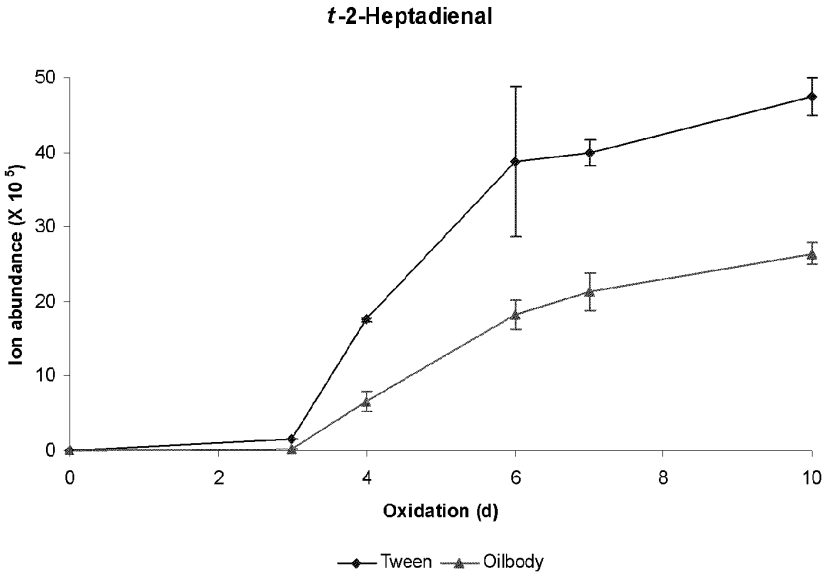



Figure 7A

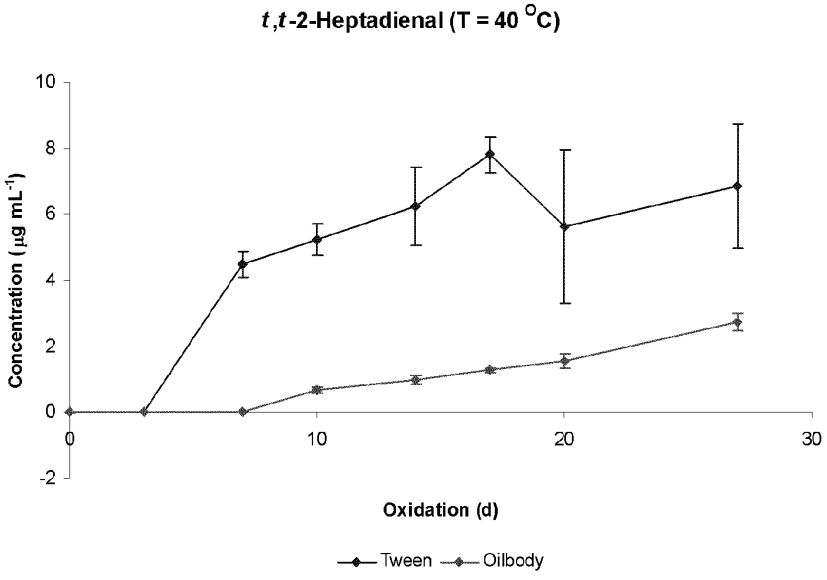



Figure 7B

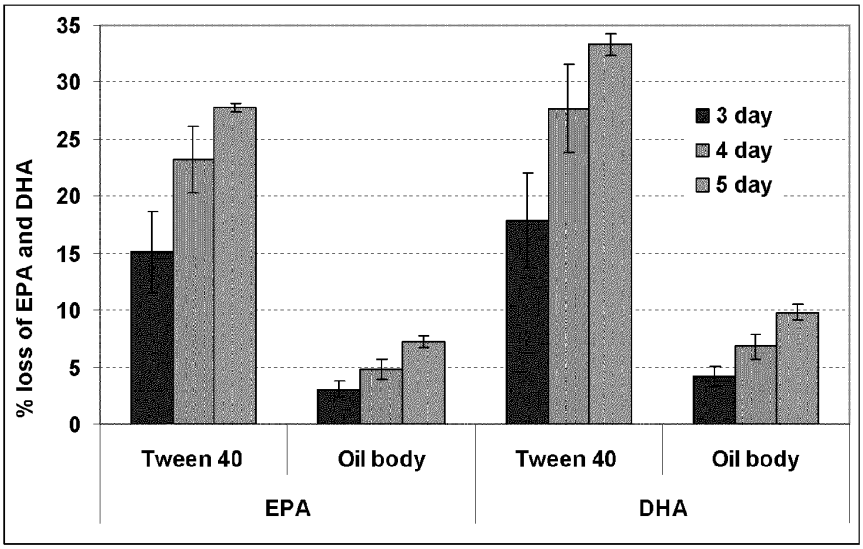



Figure 8A

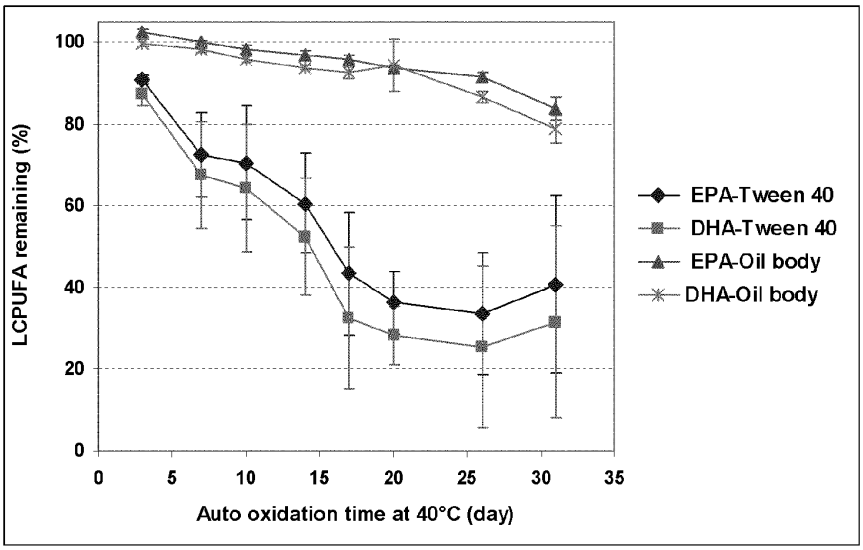



Figure 8B

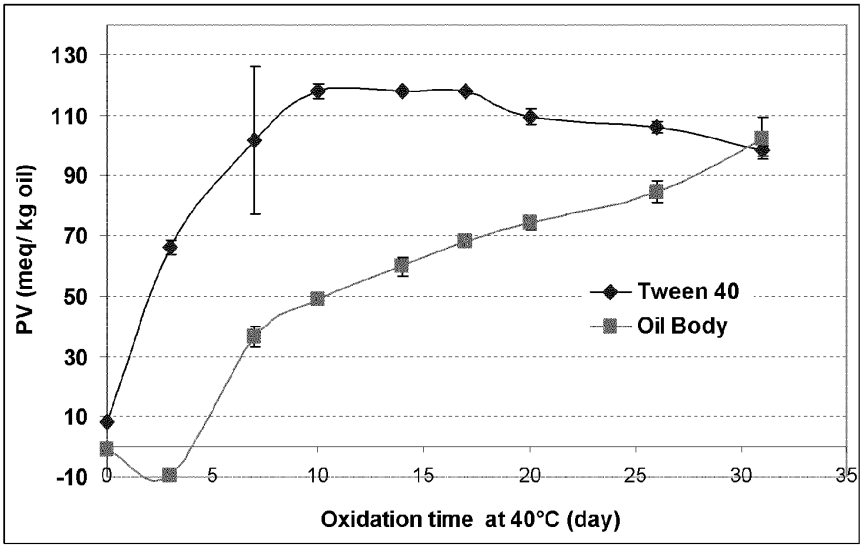

130
110
90
70
50
30
10
-10
PV (meq/ kg oil)
0
5
10
15
20
25
30
35
Oxidation time at 40°C (day)
Tween 40
Oil Body

Figure 9

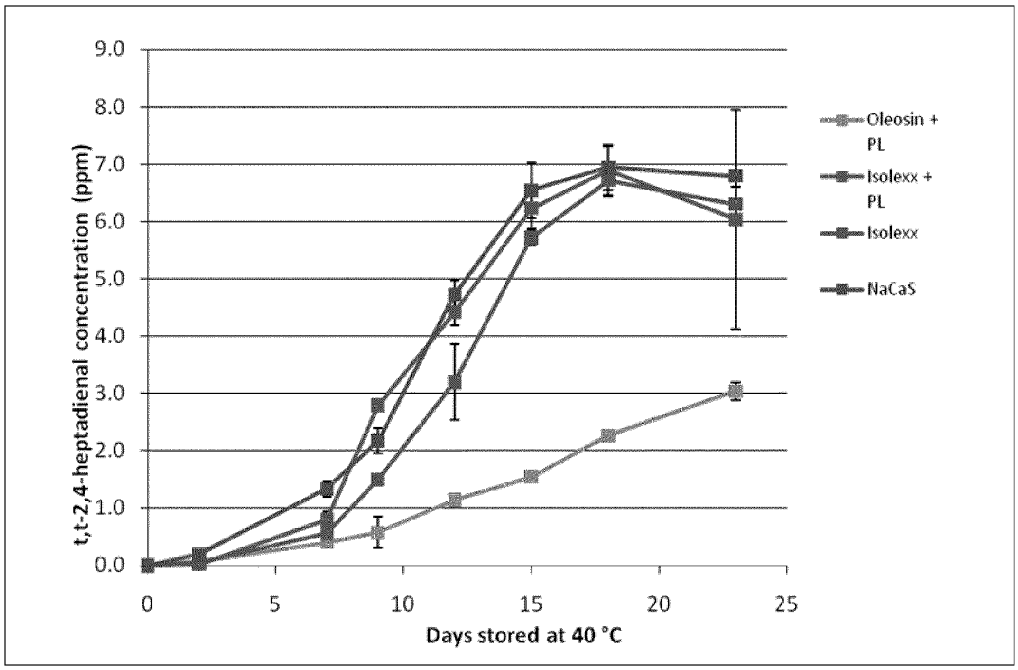

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
t,t-2,4-heptadienal concentration (ppm)
0
5
10
15
20
25
Days stored at 40 °C
Oleosin + PL
Isolexx + PL
Isolexx
NaCaS

**Figure 10**

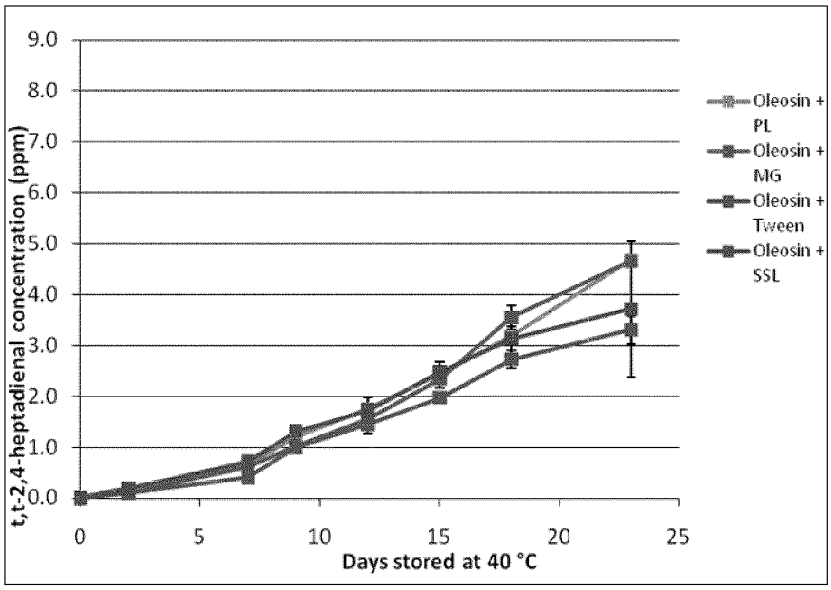

9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
t,t-2,4-heptadienal concentration (ppm)
0
5
10
15
20
25
Days stored at 40 °C
Oleosin + PL
Oleosin + MG
Oleosin + Tween
Oleosin + SSL

**Figure 11**

**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/AU2012/000103

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

***A61K 8/97*** (2006.01) ***A61K 8/98*** (2006.01) ***A61K 47/44*** (2006.01)
***A61K 8/06*** (2006.01) ***A61K 9/107*** (2006.01)
***A61K 8/64*** (2006.01) ***A61K 47/42*** (2006.01)

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)

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)
WPI, EPODOC, MEDLINE, NPL (EPOQUE): oil body, OB, AOB, oleosin, caleosin, steroleosin, polyoleosin, seed_oil, canola oil, mustard oil, brassica oil, sunflower oil, linseed oil, soybean oil, safflower oil, corn oil, tobacco oil, peanut oil, palm oil, cottonseed oil, coconut oil, avocado oil, olive oil, cashew oil, macadamia oil, almond oil, rice oil, arabidopsis oil, surfactant, phospholipid, lecithin, tween, monoglyceride, SSL, sodium stearoyl lactylate, surface active, emulsifier, phosphatidyl choline, SDS, sodium dodecyl sulphate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2009/089115 A1 (HORMEL FOODS CORPORATION) 16 July 2009 (See paragraphs [0006], [0008], [0033], [0035], [0038], [0059], [0079]; Claims 9, 33-36) | 1-25, 30 |
| X | Chen, M.C.M. et al. "Constitution of Stable Artificial Oil Bodies with Triacylglycerol, Phospholipid, and Caleosin" Journal of Agricultural and Food Chemistry (2004) Vol.52 No.12 pages 3982 to 3987 (See Abstract, "Introduction" page 3982, "Materials and Methods" pages 3982 to 3983) | 1-3, 5, 7-22, 24-25, 30 |

[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 application or patent 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 |
|---|---|
| 21 March 2012 | 19/04/2012 |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustralia.gov.au<br>Facsimile No. +61 2 6283 7999 | Authorized officer<br>**MICHAEL GRIEVE**<br>AUSTRALIAN PATENT OFFICE<br>(ISO 9001 Quality Certified Service)<br>Telephone No : +61 2 6283 2267 |

Form PCT/ISA/210 (second sheet) (July 2009)

**INTERNATIONAL SEARCH REPORT**

International application No.
**PCT**/AU2012/000103

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | Chen, M.C.M. et al. "Elevating Bioavailability of Cyclosporine A via Encapsulation in Artificial Oil Bodies Stabilized by Caleosin" Biotechnology Progress (2005) Vol.21 No.4 pages 1297 to 1301 (See Abstract, "Materials and Methods: Production of Recombinant Caleosin in *Escherichia coli*" page 1297, "Materials and Methods: Production of CsA-Loaded AOBs with or without Caleosin" page 1298) | 1-3, 5, 7-10, 12, 16-20, 22, 24, 30 |
| X | Chiang, C.-J. et al. "Selective Delivery of Cargo Entities to Tumor Cells by Nanoscale Artificial Oil Bodies" Journal of Agricultural and Food Chemistry (2010) Vol.58 No.22 pages 11695 to 11702 (See Abstract, "Materials and Methods: Reconstitution of Fluorescent AOBs" page 11696) | 1-3, 5, 7-10, 12, 16-20, 22, 24, 30 |
| X | Tzen, J.T.C. et al. "Surface Structure and Properties of Plant Seed Oil Bodies" The Journal of Cell Biology (1992) Vol.117 pages 327 to 335 (See Abstract, "Materials and Methods: Reconstitution of Oil Bodies from their Constituents" page 328, "Materials and Methods: EM of Isolated Oil Bodies" page 329). | 1-3, 5, 7-8, 11, 22, 24-25, 30 |
| P,X | US 2011/0123448 A1 (CHIANG, C.-J. et al.) 26 May 2011 (See paragraphs [0055], [0058]-[0060], [0067], [0074], [0076], [0094] to [0099], [0133]) | 1-5, 7-12, 15-20, 22-25, 30 |

INTERNATIONAL SEARCH REPORT

International application No.
**PCT**/AU2012/000103

**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 Box 1

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.:

**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.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2012/000103

**Supplemental Box 1**
(To be used when the space in any of Boxes I to IV is not sufficient)

**Continuation of Box No: III**

The present invention is considered to be directed towards two separate inventions:

(a) Claims 1 to 26 (in full), Claims 28 to 31 (in part) are directed to artificial oil bodies (hereinafter referred to as "AOBs") comprising oleosin (which, as presently defined, also encompasses caleosin, steroleosin and polyoleosin), a surfactant, and an oil comprising fatty acids, such as polyunsaturated fatty acids having four or more double bonds. These AOBs may further comprise other molecules such as bioactive molecules.

(b) Claim 27 (in full), Claims 28 to 31 (in part) are directed to a method for the partial purification of oleosin from a plant extract.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.
**PCT/AU2012/000103**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in Search Report | | Patent Family Member | | | | | |
|---|---|---|---|---|---|---|---|
| WO | 2009089115 | US | 2011020519 | US | 2011052680 | US | 2011059164 |
| | | WO | 2009089117 | | | | |
| US | 2011123448 | JP | 2011111456 | TW | 201117837 | | |

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

END OF ANNEX

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 August 2012 (23.08.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/110797 A1

(51) International Patent Classification:
*C11B 1/10* (2006.01) *A61Q 19/00* (2006.01)
*A61K 8/04* (2006.01) *A23L 1/30* (2006.01)

(21) International Application Number:
PCT/GB2012/050323

(22) International Filing Date:
14 February 2012 (14.02.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1102557.4 14 February 2011 (14.02.2011) GB

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published**:

— *with international search report (Art. 21(3))*

(54) **Title**: OIL BODY EXTRACTION AND USES

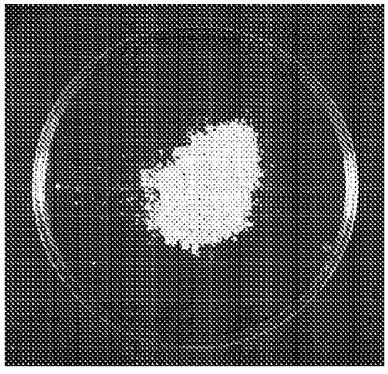


**Figure 2**

(57) **Abstract**: The present invention provides a method of extracting naturally occurring oil bodies comprising obtaining material containing naturally occurring oil bodies,recovering the oil bodies in a wet preparation and drying the oil bodies;and dried oil bodies obtained by the method and uses thereof.

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# OIL BODY EXTRACTION AND USES

The present invention relates to a method for extracting oil bodies, to dried extracted oil bodies and to the use of dried oil bodies

Oil bodies are subcellular droplets of oil (1-3 μm in diameter), covered with an oleosin-protein-rich half unit membrane. The oleosin proteins, in addition to a hydrophobic domain that associates with the entrapped oil, have hydrophilic N-terminal and C-terminal regions. These regions are enriched in basic amino acids that appear to associate with acidic phospholipids in the half unit membrane, thus forming a protective coat over much of the oil body surface. Tocopherol molecules (and other bioactive micronutrients) are also intrinsically associated with oil bodies. It is likely that these molecules are positioned at the interface between the oil body and the cytosol of the oilseed cell.

The combination of a robust layer of proteins (e.g. oleosins) and the presence of tocopherols is likely to render the oil bodies stable to oxidation *in-vivo*. Oilseeds are resistant to desiccation; oil bodies remain intact and resistant to lipid oxidation in this dry environment. Maturing oilseed cells can accumulate sugars that appear to assist in preserving biomolecules during this drying or vitrification process. However, when removed from the seeds the oil bodies become less physically stable and vulnerable to spoilage by microorganisms. It is an aim of the present invention to provide more stable oil bodies.

An example of oils found in oil bodies which are of great interest because of their medical/dietary benefits are the omega-3 fatty acids. Two major challenges face manufacturers when incorporating omega-3 fatty acids into food. One is the dwindling supply of fish oil (the most common source of such acids) with its associated impact on cost, the other is the tendency for highly unsaturated omega-3 fatty acids in food products to oxidise, a chemical reaction that leads to the generation of off flavours, and ultimately to product rejection. For these reasons functional foods containing omega-3 fatty acids often only contain very low concentrations of the active fatty acid; this undermines the potential benefit of omega-3-enriched functional foods to the health of the consumer. There is clearly a need and a market for chemically stable

omega-3 rich oils from sustainable sources. It is an aim of the present invention to provide a potential solution to these problems.

The seeds from the plant *Echium plantageneum* (Echium) contain oil enriched with an omega-3 fatty acid called stearidonic acid (SDA). Recent research has strongly indicated that in terms of human and fish health, SDA is better than other plant-derived omega-3 fatty acids (i.e. α-linolenic acid), and is almost as potent as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in its physiological benefits. Echium oil is naturally encapsulated in mature seeds within droplets called oil bodies (see Figure 1). In this structure within seeds the oil can be protected against deterioration for many years.

This specific example alone could have an impact in two major growing markets – fortification of human food, and aquaculture feed. The global value of the omega-3 oils supplying these markets is currently worth an estimated £600M. A dried, powder product, as provided by the invention, would be considered safe to eat if the source material is eaten routinely.

It is an aim of the present invention to provide a novel application for the use of oil bodies/oleosomes derived from plant material, in particular the recovery of oil bodies from seeds containing omega-3 fatty acids (e.g. from soya, linseed and echium), and the processing of such oil bodies to yield a material of low water activity. The low water activity of the product and surface chemistry of the oil bodies protect the oil against oxidation and the material against microbial spoilage. This is a bio-innovative solution to the challenge of protecting highly unsaturated edible oils against oxidation: natural emulsions enriched in omega-3 fatty acids from a renewable source are used. The invention described herein also offers functionality that could be harnessed in other products such as cosmetics and pharmaceuticals. The concept described could equally be applied to oil bodies/oleosomes from any source, and to any lipid-rich organelle, cell or microorganism.

According to a first aspect the present invention provides a method of extracting naturally occurring oil bodies comprising:

(i) obtaining material containing naturally occurring oil bodies;

(ii) recovering the oil bodies in a wet preparation;

(iii) drying the oil bodies.

Preferably the dried oil bodies have a water activity (Aw) of less than 0.3.

The method of the invention has the advantage that the dried oil bodies are easier to transport than the wet preparation, thus reducing costs. The dry powder is also easier for an end user to handle, thus reducing logistics and therefore costs. The dry oil bodies also display improved storage properties, for example, they may demonstrate microbial stability for several months or indeed several years, preferably at least two years. The dry oil bodies may also display improved stability with respect to oxidation when compared to the wet preparation.

The material containing the oil bodies may be selected from one or more of seeds, pollen, flowers, roots and stems of flowering plants, the spores and vegetative organs of non-flowering plants, algae, microalgae, animal cells, fungi and protists such as *Euglena*. Preferably in this invention the oil bodies are extracted from seeds or algae, more preferably from seeds.

The seeds may be seeds or kernels from one or more of the following plants, sunflower, soybean, oil palm, safflower, almond, macadamia, cotton seed, ground nut, coconut, oil seed rape, echium, borage, linseed/flax/hemp, evening primrose, rice, wheat, oat, maize and barley. Preferably the material containing the oil bodies is echium seeds.

The material containing the oil bodies used in the method of the invention could all be from the same source or it could be from different sources. For example, more than one type of seed could be used.

The dried oil bodies could all be derived from the same source material or from a mixture of sources, such as sunflower seeds and echium seeds.

Oil bodies are organelles sometimes also referred to as oil droplets, lipid droplets, olesomes or spherosomes.

Preferably the oil bodies contain triacylglycerol molecules enriched in unsaturated fatty acids. Other lipid rich organelles, cells or microorganisms could also be dried to produce stable powders enriched in functional lipids.

In one embodiment the aim of the invention is to extract oil bodies containing omega-3 or other essential fatty acids. The intention being to isolate the oil bodies for administration to humans or animals.

By keeping the oils in the oil bodies they can be added to food stuffs or pharmaceuticals without imparting a flavour. It is well known in the food industry that the application of polyunsaturated fatty acids can give rise to serious off-flavour problems. These off-flavour problems are associated with oxidation of the fatty acids, leading to the formation of volatile potent flavour molecules, such as unsaturated aldehydes. Flavour attributes associated with such oxidation include fish, cardboard, paint, rancid and metallic.

For example, omega-3 has an unpleasant taste if added as the raw oil, but if it is retained in an oil body this taste is hidden and the health benefits of ingesting the oil can be achieved without any taste problems.

Preferably the dried oil bodies are in a powder form. The powder form is preferably free flowing,

The dried oil powders made by the method of the invention may be rehydrated for use or may be used in the dried/powder form.

The oil bodies may be recovered from the material containing them into a wet preparation by grinding the material in a water based medium in which the pH, viscosity and ionic strength can be controlled, filtering out the larger material, and then centrifuging the filtrate. The oil bodies will float on the surface of the filtrate forming a thick, cream-like pad (crude oil bodies) that can be easily removed.

The removed oil bodies (crude oil bodies) may be washed several times by dispersing them is a washing medium (in which the pH, viscosity and ionic strength can be

controlled, which may or may not contain a chaotropic agent such as salt), and re-centrifuged and recovered, to clean the oil bodies and/or to remove contaminants.

The recovered oil body material (thick cream) may be a concentrated oil-in-water emulsion with a solids content of between about 35% and about 75%, mostly made up of triacylglycerol. This cream may be dispersed to form a more dilute emulsion if required.

The recovered oil bodies may be dried by any suitable means. Suitable means include spray drying, drum drying, freeze drying and vacuum drying. Preferably the oil bodies are dried by spray drying.

Preferably in the dried oil bodies the oil bodies occupy the core of the particles that form the powder, this is in contrast to processed oil droplets that are microencapsulated where the oil is often found in the shell layer of hollow structures. Preferably spray drying oil bodies, compared with encapsulation of oil using surfactants/carriers, results in a novel distribution of oil that may increase its protection against oxidation; preferably the oil in the oil body is not only covered in its natural protective layer of proteins and phospholipids, it is removed from the surface of the particle. Preferably a carrier used in the spray drying process forms the surface of the particle. This spatial arrangement prevents inadvertent oil release on the surface of the particle through prolonged handling, and may further reduce the exposure of the oil to oxygen.

When drying the oil bodies in the wet preparation or emulsion a carrier may be used. The carrier may be a protein or a sugar. The carrier may be, for example, maltodextrin, any other dextrin, whey protein, casein protein, cellulose, a modified starch or trehalose.

Preferably the dried oil bodies can be stored without phase separation, oxidation and/or microbial spoilage for at least 6 months, preferably at least a year, preferably at least 18 months, preferably at least 2 years. The oil bodies may be stored at 4°C, 5°C or room temperature.

Preferably the dried oil bodies can be stored at room temperature for at least 6 months

Preferably the dried oil bodies produced by the method of the invention are able to be rehydrated to produce a stable suspension of oil bodies. Preferably the dried oil bodies are physically intact when resuspended or rehydrated.

According to a further aspect, the invention provides a dried oil body obtained or obtainable by the method of the invention.

According to another aspect, the invention provides a composition comprising dried naturally occurring oil bodies.

The oil bodies may be prepared according to the first aspect of the invention.

The oil bodies may be derived from any of the aforementioned sources.

According to a yet further aspect, the invention provides the use of dried naturally occurring oil bodies according to the invention in the manufacture of another product, such as a personal care product, a food product or a pharmaceutical product.

According to another aspect, the invention provides a pharmaceutical composition comprising dried oil bodies and a pharmaceutically acceptable excipient. The pharmaceutical product may be a powder, a tablet, a capsule or any other dry formulation. Alternatively, the dried oil bodies may be added dried or rehydrated to a liquid or gel or other non-dry pharmaceutical composition.

According to yet another aspect, the invention provides a food stuff or ingredient comprising dried oil bodies. Preferably the foodstuff is a dried foodstuff or ingredient, such as a cereal or a dehydrated food, or a mix of dried ingredients that include dried oil bodies that could provide their own nutritional value (for example for a baby milk formulation) and/or be loaded with primary ingredients such as natural antioxidants, vitamins, flavourings and/or colourants. The dried oil bodies may also be added dried or rehydrated to any other food or animal feed products, for example sauces, spreads (for example peanut butter, margarines etc), salad dressings, dips, humous, cereals, heath bars, crisps, snack products, confectionery products (for example caramels, ganaches etc), baked products (for example breads, doughs,

muffins, pastries, pizza bases etc) dairy products (for example yoghurts, milk, ice creams etc) health drinks (for example smoothies, fruit juices, drinkable yoghurt etc), canned food (for example baked beans, soups etc), fish food etc.

According to another aspect, the invention provides a personal care product comprising dried oil bodies or rehydrated dried oil bodies.

Personal care products may include body butters, shampoos, body lotions, body creams, sun creams etc.

According to a further aspect the invention provides the use of a dried oil body, or a rehydrated dried oil body, in the manufacture of one or more of a foodstuff, a pharmaceutical or a personal care product.

The skilled man will appreciate that the preferred features of any aspect of the invention, or recited in any claim, can be applied to all aspects of the invention.

Embodiments of the invention will now be described, by way of example only, with reference to the following figures.

**Figure 1** – is a transmission electron micrograph of a mature Echium seed. The scale bar = 20µm. The light-grey circles represent spherical oil bodies

**Figure 2** – is a photograph of spray dried echium seed oil bodies, comprising 10% maltodextrin and 7.5% wet/wt. crude oil bodies (COB) in the spray drying feed liquid.

**Figure 3** – is a scanning electron microscopy image of the spray dried oil bodies of Figure 2.

**Figure 4** – shows scanning electron microscopy images of the internal structure of the spray dried oil bodies of Figure 2.

**Figures 5a and 5b** – are scanning electron microscopy images of the internal structure of microencapsulated oil material formed by spray drying. HV-

Hollow void. Figure 5a is spray-dried soya oil encapsulated with sodium caseinate and corn syrup (DE 28), reproduced from Hogan et al. (2001) International Dairy Journal 11(3): 137-144. Figure 5b is spray-dried ethyl caprylate encapsulated with whey protein and corn syrup (DE 24), reproduced from Sheu and Rosenberg (1995) Journal Of Food Science 60(1): 98-103.

**Figure 6** – shows the lipid hydroperoxide concentration in samples during storage trial at 40°C.

**Figure 7** – shows the hexanal concentration in samples during storage trial at 40°C.

**Figures 8a and 8b** – are light microscopy images of oil bodies. Figure 8a shows rehydrated spray dried oil bodies, and Figure 8b shows an oil body parent emulsion. The scale bars represent 10 μm.

**Figure 9** – shows confocal microscopy images of rehydrated oil bodies. Red indicates protein, yellow indicates lipid. The scale bar represents 10 μm.

**Isolation of oil bodies in a wet preparation**

The biochemistry of oil bodies has been studied since the early 1970's and therefore the methods used to recover them into wet preparations are well known. In principle the seed is ground in a water based medium in which the pH, viscosity and ionic strength is controlled. This crude preparation can be cleaned by resuspension in water or chaotropic agents such as salt or urea, followed by further centrifugation. This assists in removing proteins that are not intrinsic to the oil bodies. In the present invention crude or clean oil bodies can be used.

**Resuspending isolated oil bodies to form an emulsion**

The buoyant oil body pad can be resuspended as part of a washing regime (see above) or as a means to generate a final emulsion. The resuspension of oil bodies can be achieved through a range of devices such as a high pressure homogenizer, Potter Elvenheim homogenizer or a Silverson mixer. The oil content of such oil-in-water emulsions can be varied over a wide range by simply changing the ratio of oil body pad to water or resuspension medium. The pH of the continuous aqueous phase can be

set over a wide range since the oil bodies manifest a pH reversible aggregation at pH 5-7, but they are immune from coalescence under these general conditions. A range of preservatives can be included in the emulsions to prevent microbial spoilage of oil body preparation at high water activities, or the emulsion can be pasteurised.

**Drying oil Bodies and their performance**

Drying oil bodies by any means has never before been reported or exploited. The data presented herein demonstrates the dried material to be resistant to oxidation and to microbial spoilage over several months, and even years. The data also demonstrates that a stable oil body emulsion can be re-formed by simply re-hydrating the powder. This rehydrated oil body dispersion has more-or-less the same physical and chemical properties as the original oil body emulsion.

**RESULTS**

Crude echium oil bodies were encapsulated with maltodextrin (DE 15) through spray drying. The spray dried powder was optimised by determining the optimum inlet temperature and flow rate of the spray dryer and maltodextrin concentration in the liquid feed. These conditions were determined by assessing the lipid and moisture content, size and initial hexanal production. The optimum liquid feed contained 7.5% wet/weight crude oil body and 10% maltodextrin and was spray dried at an inlet temperature of 180°C with a liquid feed flow rate of 320 mL $h^{-1}$ to produce a free flowing powder with 20% lipid (Figure 2). These conditions were used to produce powder for further analysis. Higher total solids concentrations in the feed-liquid could be used to increase the rate of dry powder production; this would necessitate further optimisation of the spray dryer operating parameters.

SEM imaging was used to determine the surface properties of the spray dried oil bodies (see Figure 3). These images show spray dried particles with a spherical shape with a combination of smooth and crinkled surfaces with no cracks apparent. A coating surface free of cracks is important as this can act as a barrier to oxygen which in turn may prevent oxidation of oils. The morphology of the spray dried particle surface is directly affected by the specification of maltodextrin used. It has been shown that the molecular weight of maltodextrin plays a major part in the surface structure of spray dried particles, as the molecular weight decreases (DE increases) the smaller oligosaccharides form a less porous more uniform coating (Sankarikutty et

al. 1988 Journal of Food Science and Technology 25(6): 352-356; Rosenberg et al. 1995 Food Microstructure 7(1): 15-23). Sheu and Rosenberg ((1995) Journal Of Food Science 60(1): 98-103) emulsified ethyl caprylate with whey protein and encapsulated these emulsions with maltodextrins at a range of DE. It was found that a DE of 15 or above was sufficient to produce a surface free of cracks. The images presented here support these findings as the encapsulated spray dried oil body material encapsulated by maltodextrin with a DE of 15 showed no apparent cracks on the surface of the particle.

The internal structure of the spray dried material was observed by fracturing the particles, and viewing under SEM (see Figure 4). These images show a maltodextrin coat with a hollow centre where the oil body is hypothesised to be present.

The cross-sectional image of the spray dried oil body powder is quite different to the cross-sectional image of a powder formed by the microencapsulation of refined or crude oils through spray-drying with a carrier. In these latter materials small oil droplets are embedded into the carrier that forms a shell around a hollow void (see Figures 5a & b) (Buma 1971 Netherlands Milk and Dairy Journal 25(3): 159-72; Sheu and Rosenberg 1995 Journal Of Food Science 60(1): 98-103; Hogan et al. 2001 International Dairy Journal 11(3): 137-144; Soottitantawat et al. 2003 Journal Of Food Science 68(7): 2256-2262). This central void is produced by the "ballooning" of the drying droplet which occurs when steam is formed in the interior of the drying droplet causing the particle to puff and drastic increase in size compared to the parent emulsion (Rulkins and Thijssen 1972 International Journal of Food Science and Technology 7(1): 95-105; Finney et al. 2002 Journal Of Food Science 67(3): 1108-1114). The SEM images of the oil body spray dried powder does not show oil droplets embedded in the wall of the particles but does show a hollow void where the oil body is situated. This is due to particle size of the powder not showing drastic increases in size compared to the parent emulsion that is commonly associated with ballooning (increases from approximately 3.2 μm to 6.1 μm).

Powder samples were stored at 40°C for a period of 3 weeks and markers for oxidation measured. The high temperature used during spray drying may have had a negative effect on the oxidative stability of the highly polyunsaturated lipids found in echium oil. Hydroperoxide concentrations and volatile secondary oxidation products were

determined to assess the oxidative stability of encapsulated spray dried echium oil body powders. In addition to spray dried powder, fresh echium oil body emulsions (10% lipid weight) and bulk echium oil were also stored and assessed so comparisons could be drawn.

Hydroperoxide formation did not increase in the encapsulated spray dried oil body samples over storage (Figure 6). In comparison, hydroperoxide formation in bulk echium oil increased rapidly over the first 7 days then plateaued. Formation of hydroperoxides in oil body emulsions followed a similar trend to spray dried echium oil body powder for the first 7 days then subsequently increased with storage. The formation of secondary oxidation volatiles in spray dried oil body powders was also low, reflecting the low hydroperoxide formation. There was no 2,4 heptadienal detected in the headspace volatiles and only small amounts of hexanal (Figure 7). Headspace hexanal concentrations in bulk oil increased rapidly over storage and reflects the initial rapid hydroperoxide formation. Hexanal formation in oil bodies showed a small increase in the latter stages of storage which may have been caused by the accumulation of hydroperoxides also in the later stages of storage. The oxidation data shows encapsulated spray dried echium oil bodies are extremely oxidatively stable over long term storage which suggests the elevated temperature used in spray drying does not accelerate oxidation of the dried product. This stability was associated with the maltodextrin coat formed around the core preventing oxygen from entering, and the surface chemistry of oil bodies which slow down oxidation reactions. The low water activity of the powder had a major impact of the microbiological stability of the powder as it was low enough to prevent growth of microorganisms.

To assess if whole oil bodies had been spray dried with their structure intact the resultant powder was rehydrated in water (10% lipid weight) and compared to crude oil bodies in emulsion using light microscopy. The micrographs show rehydrated spray dried oil bodies are spherical single entities present in an aqueous phase and have similar size and morphology to crude oil bodies in suspension there is also no apparent free oil present in the rehydrated solution (see Figure 8a & b). The microscope images suggest that whole oil bodies are encapsulated by maltodextrin during spray drying and that the powdered material can be rehydrated to produce whole oil bodies.

To determine if free oil was present in the rehydrated oil bodies Nile blue was applied to samples which allows lipids and proteins to fluoresce under confocal microscopy. Sequential imaging of fluorescently stained rehydrated oil bodies was performed so structural information could be determined (Figure 9). The images show that lipid (yellow) is surrounded by a layer of protein (red). These images suggest that that no free lipid is present in the suspension as all lipid is surrounded by a layer of protein. This protein would be anticipated to be oleosins and possibly other surface proteins such as caleosin and steroleosin which suggest that intact oil bodies are present in the suspension and thus were spray dried as whole entities.

The spray dried 'encapsulated' oil bodies produced according to the invention were more stable than oil bodies in an emulsion, both oxidatively and microbially while still having the ability to be rehydrated to form an emulsion of the same oil droplet size and behaviour as that formed when isolate from the seed in a wet preparation. These results prove the commercial applications of dried oil bodies, as a shelf stable product enriched in omega-3 oil.

**Materials and Methods**

**Materials**

Seeds from *E. plantagineum* were obtained from Technology Crops International, Essex, UK. All chemicals were analytical grade or higher, and sourced from Fisher UK (Loughborough, UK) unless otherwise stated.

**Echium Oil Body Extraction**

Echium oil bodies were recovered as described previously (Tzen et al. 1997 Journal of Biochemistry 121(4): 762-768) but modified. Echium oil bodies were extracted by adding 100g of Echium seed and 500ml $dH_2O$ into a blender (Krups, UK) at maximum speed for 2min. The solution was filtered under vacuum through 3 layers of cheese cloth. The solid residue was discarded and the filtrate isolated and centrifuged for 20 min at 10400 g, 5 °C (Beckman Coulter, Buckinghamshire, UK). The oil body pad were removed from the surface and placed into a clean bottle; these oil bodies produced were classed as the crude oil bodies (COB) and stored until use at 4 °C.

Water-washed oil bodies (WWOB) were obtained by re-suspending the COB pad in deionised water at a ratio of 1:4 (oil body:water); this solution was vortexed and centrifuged for 20 min at 2600 g, 5 °C. After removing the oil body pad the process was repeated twice more and stored until use at 4°C. Urea-washed oil bodies (UWOB) were obtained by first re-suspending the crude oil body pad in a 9 M urea solution at a ratio of 1:4 (oil body:urea solution). The dispersion was then vortexed and centrifuged for 20 min at 2600 g, 5 °C. The pad was removed and the oil bodies were washed three times using deionised water as described above for the water-washed step and the oil body pad stored until use at 4°C.

**Drying Oil Bodies**

**Emulsion Formation for Drying**

Emulsions prepared for drying were a blend COB and maltodextrin (dextrose equivalent 15) (Brenntag, Leeds) prepared in $dH_2O$. The emulsions were homogenised using a shear mixer (Silverson L 5 M, Bukinghamshire, UK) for 5min at 7500rpm.

**Spray Drying**

Spray-drying was performed using a Buchi B-191 laboratory spray dryer (Flawid. Switzerland). Various temperatures, flow rates and emulsion compositions were used (Table 1.1). Consistent operating parameters were as follows; aspirator = 100%, 650ml.min$^{-1}$ of filtered air and filter pressure = < 50mBar.

| Inlet (°C) | COB / % | Maltodextrin / % | Flow / % | Outlet (≈°C) |
|---|---|---|---|---|
| 160 | 7.5 | 10 | 10 | 97 |
| | | | 20 | 85 |
| | | | 30 | 70 |
| 180 | 7.5 | 10 | 10 | 125 |
| | | | 20 | 95 |
| | | | 30 | 70 |
| 180 | 7.5 | 7.5 | 20 | 95 |
| | | 5 | | |
| | | 2.5 | | |

Table 1.1 Varied drying conditions

**Moisture**

The moisture content of the powders were determined gravimetrically by vacuum oven-drying at 40 °C for 48 h

**Water Activity (aw)**

Water activity of spray dried powders was measured using AquaLab Model Series 3 TE (AquaLab, USA.).

**Relative hexanal concentrations of spray dried powder**

Relative hexanal concentrations was measured by APcI-MS for analysis of static headspace intensity are described previously (Linforth 1998; Linforth 1999 - Linforth, R. S. T. G., Taylor, Andrew John (GB) (1998). Apparatus and methods for the analysis of trace constituents in gases, Univ, Nottingham (GB); Linforth, R. S. T. K., GB), Taylor, Andrew John (Kegworth, GB) (1999). Apparatus and methods for the analysis of trace constituents in gases. United States, Micromass UK Limited (Manchester, GB2)) but modified, in brief, samples (1g) were placed in a capped glass bottle (volume = 20mL) with a plugged hole in the lid, after equilibrium (2hr) the plug was removed and the interface probe for the APcI-MS was passed though the hole. The interface sampled the headspace and measured the relative concentration of hexanal present in the headspace.

**Scanning electron microscopy (SEM)**

A JSM-6490LV model (JEOL Co., Ltd., Tokyo, Japan) scanning electron microscope was used to investigate the microstructural properties of the spray-dried products. The powders were placed on the SEM stubs using a 2-sided adhesive tape (Nisshin EM Co. Ltd., Tokyo, Japan). In order to examine the inner structure, the powders (attached to the stub) were fractured by attaching a 2nd piece of adhesive tape on top of the microcapsules and then quickly ripping it off (Moreau *et al.* 1993 Food Structure 12(4): 457-468). The specimens were subsequently coated with gold using a SC7620 sputter coater (Quorum Technologies Ltd, Sussex, UK). The coated samples were then analyzed using the SEM operating at 15 kV.

**Confocal Laser Microscopy**

Images were collected using a Nikon Eclipse Ti inverted Confocal microscope, supplier: Nikon UK Ltd., Kingston upon Thames. The equipment comprises

lasers: Argon Ion 488 nm, Green Helium- Neon 543 nm, Blue diode 405 nm and is fitted with a C1 detector unit (3 PMT), a C1 transmitter detector unit (transmitted light), and the data collected and analysed with EZ-C1 control software. The samples were stained prior to imaging with Nile blue (excitation 561 nm and emission 567-650 nm).

**Spray Dried Powder Oxidation**

Powder samples (5 g) were place into 40ml containers and stored at 40 °C in an incubator (Sanyo, Loughborough, UK)) with restricted light. Three containers were used for spray dried samples and samples were removed at each time point. Hydroperoxide and volatile detection were performed as previously below using equal amounts of spray dried powder instead of emulsion sample.

**Hydroperoxide Detection Assay**

Hydroperoxides were detected according to the method by Shantha and Decker ((1994) Journal of Aoac International 77(2): 421-424)and adapted by Nuchi et al ((2001) Journal of Agricultural and Food Chemistry 49(10): 4912-4916). Isooctane/2-propanol (3:1 v/v) (1.5 ml) was added to Emulsion solution (200 µl). The solution was vortexed for 10 s every 2 min for 10 min, followed by centrifuging at 2000 g for 2 min. The organic phase (200 µl) was then removed and added to methanol/1-butanol (2:1 v/v) (2.8 ml); this was followed by the addition of ammonium thiocyanate (3.94 M) (15 µl) and iron (II) solution (0.072 M) (15 µl ) (formed by mixing equal volume of 0.132 M $BaCl_2$ (in 0.4 M HCl) and 0.144 M $FeSO_4.7H_2O$). After 20 min, the solution absorbance was measured at 510 nm against a blank which contained everything but the sample emulsion solution. The concentration of hydroperoxide was calculated from a standard curve produced using cumene hydroperoxide. The weight of lipid was determined gravimetrically by taking a further 200 µL of the above organic phase, and evaporating the solvent on a hot plate (200 °C).

**Volatile Detection**

Volatiles from the process of secondary oxidation were measured by solid-phase microextraction and detected using gas chromatography mass spectrometry (SPME GC-MS). Emulsion solution (1 ml) was placed in a 20 ml vial together with 10 µl of 1, 2 dichlorobenzene (internal standard at 100 ppm) and sealed with a magnet cap lined with a silicone/PTFE seal (Chromacol, Hertfordshire, UK). SPME GC-MS was

performed using a CTS Analytics PAL system autosampler and a DSQ and TRACE GC Ultra (Thermo Electron, Loughborough, UK). Volatiles were extracted onto a SPME fibre assembly (50/30 µm DVB/Carboxen/PDMS StableFlex, Sigma Ltd., Gillingham, United Kingdom). The sample was pre-incubated (5 min at 60 °C) prior to extraction (20 min at 60 °C), desorption was achieved in 5 min (250 °C). Compounds were separated using a ZB-5 Phenomenex gas chromatography column (Macclesfield, UK) with 30 ml $min^{-1}$ helium. Oven temperatures were controlled at 40 °C (5 min) then ramped (3 °C $min^{-1}$) to 140 °C, ramped (15 °C $min^{-1}$) to 210 °C and held for 1 min. Volatiles were quantified with authentic standards.

## CLAIMS

1. A method of extracting naturally occurring oil bodies comprising:
   (i) obtaining material containing naturally occurring oil bodies;
   (ii) recovering the oil bodies in a wet preparation;
   (iii) drying the oil bodies.

2. The method of claim 1 wherein the dried oil bodies have a water activity of less than 0.3.

3. The method of claim 1 or 2 wherein the material containing the naturally occurring oil bodies is selected from one or more of seeds, pollen, flowers, roots and stems of flowering plants, the spores and vegetative organs of non-flowering plants, algae, microalgae, animal cells, fungi and protists such as Euglena.

4. The method of claim 3 wherein the seeds are seeds or kernels from one or more of the following plants, sunflower, soybean, oil palm, safflower, almond, macadamia, cotton seed, ground nut, coconut, oil seed rape, echium, borage, linseed/flax/hemp, evening primrose, rice, wheat, oat, maize and barley.

5. The method of any preceding claim wherein the oil bodies contain triacylglycerol molecules enriched in unsaturated fatty acids

6. The method of any preceding claim wherein the oil bodies contain omega-3 or other essential fatty acids

7. The method of any preceding claim wherein the oil bodies are spray dried.

8. The method of any preceding claim wherein before drying the oil bodies a carrier is added to the wet preparation.

9. The method of claim 8 wherein the carrier is a protein or a sugar.

10. The method of claim 9 wherein the carrier is maltodextrin.

11. The method of any preceding claim wherein the dried oil bodies are in a powder form.

12. The method of claim 11 wherein the dried oil bodies occupy the core of the particles which form the powder.

13. Dried oil bodies obtained or obtainable by the method of any preceding claim.

14. Dried oil bodies of claim 13 which can be stored at room temperature for at least 6 months without phase separation, oxidation and/or microbial spoilage.

15. Dried oil bodies of claim 13 or 14 which can be rehydrated to produce a stable oil body suspension.

16. A composition comprising dried naturally occurring oil bodies.

17. The composition of claim 16 comprising dried oil bodies as defined in any of claims 13 to 15.

18. The composition according to claim 16 or 17 wherein the dried oil bodies are in a dried powder form.

19. The composition according to claim 16 or 17 wherein the oil bodies have been rehydrated.

20. Use of dried naturally occurring oil bodies according to any of claims 13 to 15 in the manufacture of another product, such as a personal care product, a food product or a pharmaceutical product.

21. A pharmaceutical composition comprising dried oil bodies and a pharmaceutically acceptable excipient.

22. The composition of claim 21 comprising dried oil bodies according to any of claims 13 to 15.

23. A food stuff or ingredient comprising dried oil bodies or rehydrated oil bodies.

24. A personal care product comprising dried oil bodies or rehydrated dried oil bodies.

25. The food stuff or ingredient of claim 23 or the personal care product of claim 24 comprising oil bodies according to any of claims 13 to 15.

26. Use of a dried oil body, or a rehydrated dried oil body, in the manufacture of one or more of a foodstuff, a pharmaceutical or a personal care product.

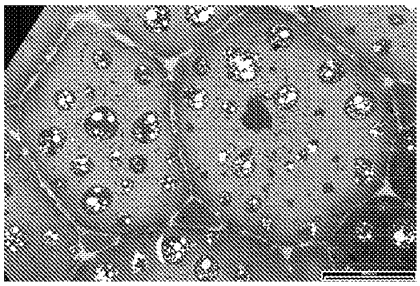


Figure 1

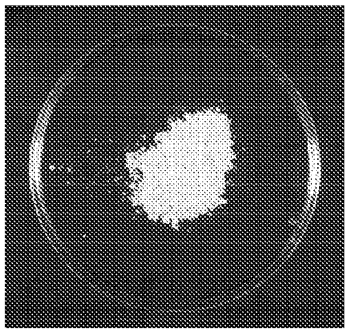


Figure 2

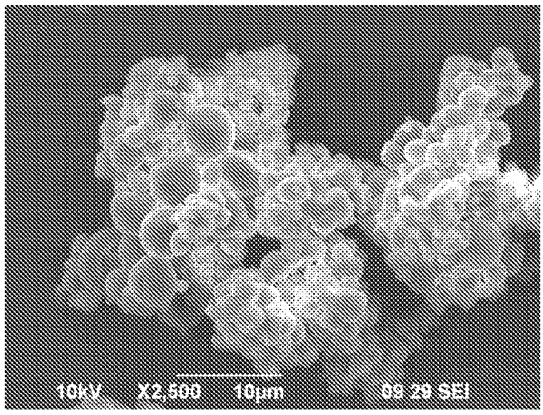



Figure 3

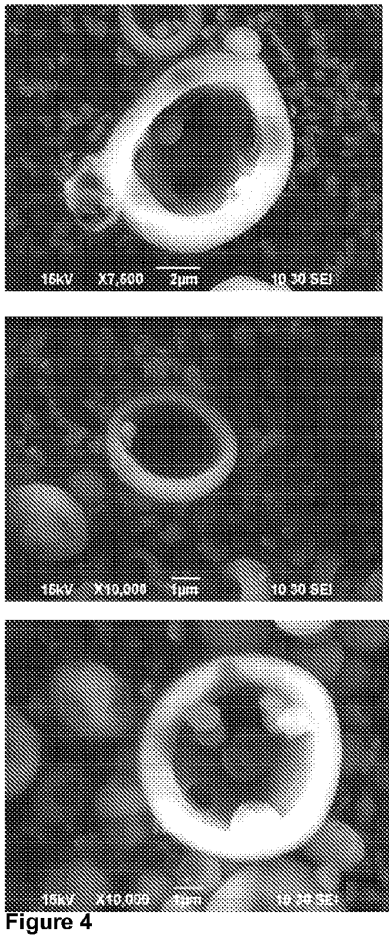



Figure 4

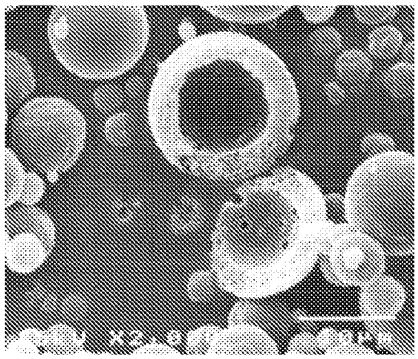


Figure 5a

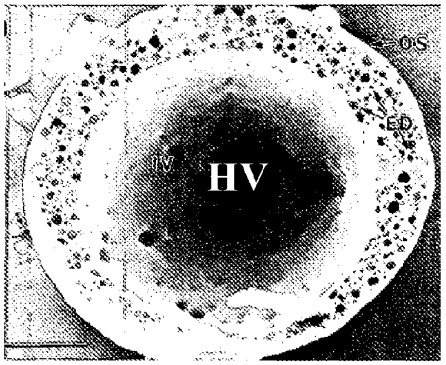



Figure 5b

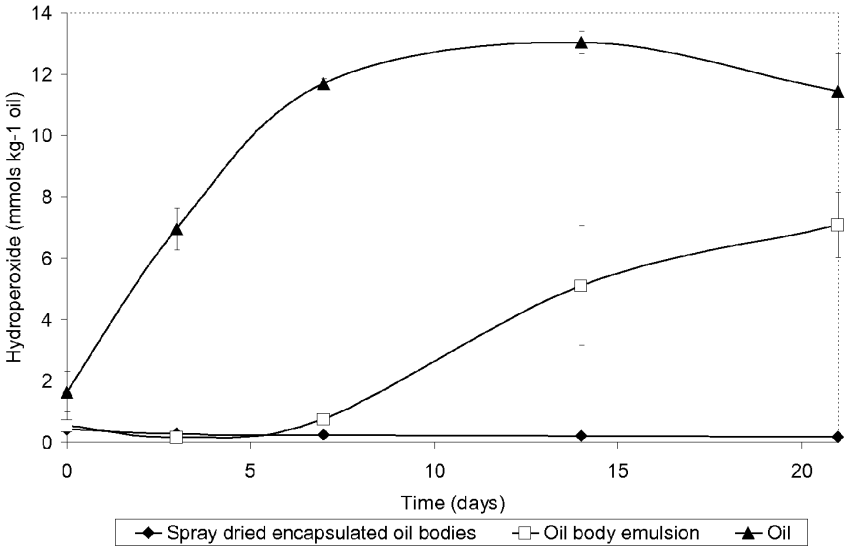



**Figure 6**

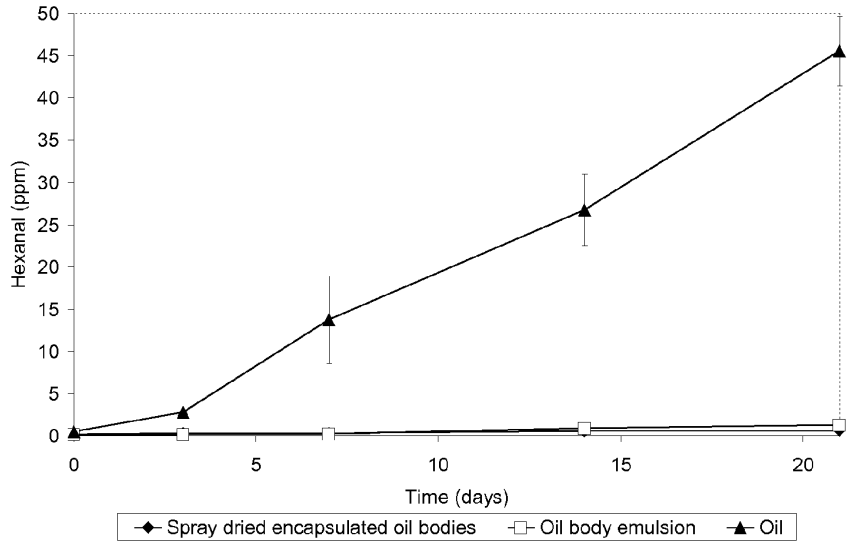



**Figure 7**

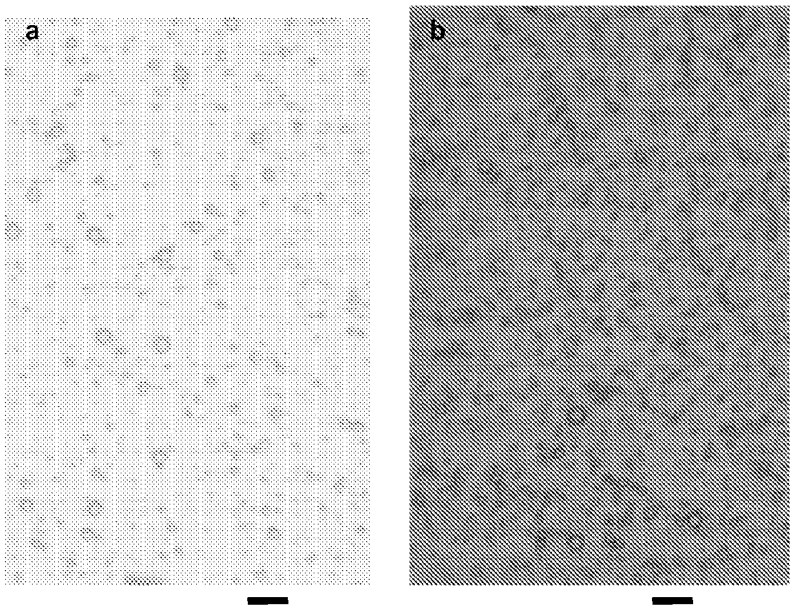



Figure 8

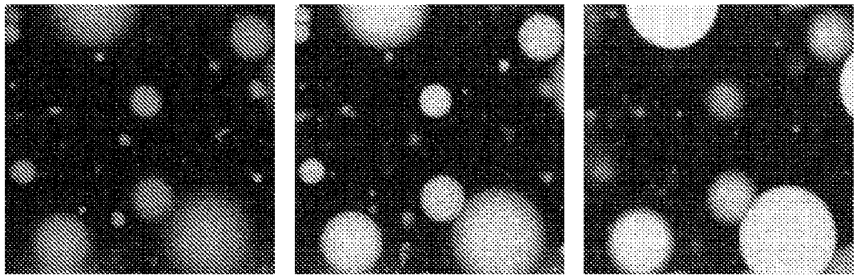


Figure 9

# INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/050323

A. CLASSIFICATION OF SUBJECT MATTER
INV. C11B1/10 A61K8/04 A61Q19/00 A23L1/30
ADD.

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)
C11B A61K A61Q 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 practicable, search terms used)
EPO-Internal, BIOSIS, COMPENDEX, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 2009/089115 A1 (HORMEL FOOD CORP [US]; HENDRICKSON WILLIAM A [US]; FINNEY JOHN M [US];) 16 July 2009 (2009-07-16) | 1,3-6, 13-17, 20-26 |
| Y | claims 1,16-18; example 1 the whole document | 7-10 |
| X | GRAY, D.A., ET AL.: "Oxidative stability of Echium plantagineum seed oil bodies", EUROPEAN JOURNAL OF LIPID SCIENCE AND TECHNOLOGY., vol. 112, 2010, pages 741-749, XP002676409, DEWILEY VCH VERLAG, WEINHEIM. ISSN: 1438-7697 | 1,3-6, 13-17, 20-26 |
| Y | page 742, column 2 | 7-10 |

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

[X] See patent family annex.

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"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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"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 |
|---|---|
| 23 May 2012 | 05/06/2012 |

| Name and mailing address of the ISA/ | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040,<br>Fax: (+31-70) 340-3016 | Rooney, Kevin |

Form PCT/ISA/210 (second sheet) (April 2005)

## INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2012/050323

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | BEISSON, F. ET AL.: "oil-bodies as substrates for lipolytic enzymes", BIOCHIMICA ET BIOPHYSICA ACTA., vol. 1531, 2001, pages 47-58, XP002676410, ELSEVIER ISSN: 0006-3002 abstract page 48, column 2 ----- | 1-6, 11-26 |

Form PCT/ISA/210 (continuation of second sheet) (April 2005)

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2012/050323

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 2009089115 A1 | 16-07-2009 | US 2011052680 A1 | 03-03-2011 |
| | | US 2011059164 A1 | 10-03-2011 |
| | | WO 2009089115 A1 | 16-07-2009 |
| | | WO 2009089117 A1 | 16-07-2009 |

Form PCT/ISA/210 (patent family annex) (April 2005)

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 September 2012 (07.09.2012)

WIPO | PCT

(10) International Publication Number
WO 2012/116703 A1

(51) International Patent Classification:
*A23J 1/00* (2006.01) *A23L 1/211* (2006.01)
*A23J 1/14* (2006.01) *A23L 1/216* (2006.01)
*A23L 1/20* (2006.01)

(21) International Application Number:
PCT/DK2012/050067

(22) International Filing Date:
1 March 2012 (01.03.2012)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PA 2011 70106 1 March 2011 (01.03.2011) DK

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(74) Agents: **HEEBØLL-NIELSEN, Anders** et al.; Awapatent A/S, Rigensgade 11, DK-1316 København K (DK).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published**:

— *with international search report (Art. 21(3))*

(54) **Title**: A PROCESS FOR THE MANUFACTURE OF A PRODUCT FROM A PLANT MATERIAL

(57) **Abstract**: The present invention relates to a process for the manufacture of a product from a plant material comprising the steps of providing a disrupted plant material comprising <10 %(w/w) starch and <10 %(w/w) oil/lipids; adjusting the pH of the disrupted plant material to a value of pH 3.5 or below to provide an acidic suspension; heating the acidic suspension to a temperature in the range of about 50°C to about 80°C; isolating the product from the heated, acidic suspension. The product may be a protein product or a non-protein product. In particular, the process of the invention provides a protein product with reduced contents of non-protein components of negative nutritional value.

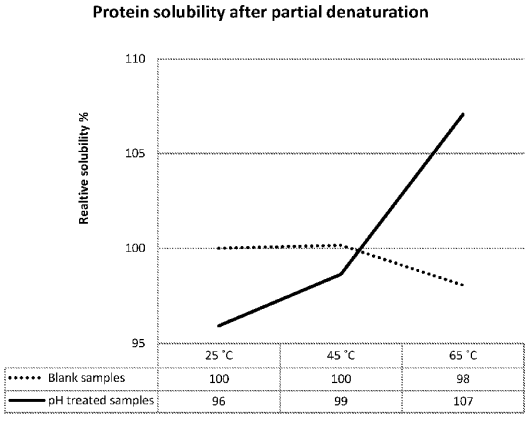



**Fig. 1**

WO 2012/116703 A1

# A process for the manufacture of a product from a plant material

## Field of the invention

This invention relates to processes for the manufacture of a product from a plant material. The product may be a protein product or a non-protein product. The processes of the invention provide plant products of use in the animal feeds, non-food products or for human consumption. Non-food products include adhesives, paint, etc. In particular, the protein products have reduced contents of non-protein components of negative nutritional value.

## Prior art

Processes for the production of plant products, e.g. protein isolates or concentrates or the like, are well known in the prior art. Such plant products are commonly used for animal feeds although they may also be used for human consumption or other purposes. In addition to nutritionally valuable components many plants also comprise components of negative nutritional value. Components of plant material may have negative nutritional value because they decrease the digestibility of an animal feed, or these components may generally inhibit the digestive system, or specific functions of the metabolism system in humans and animals. Some components may even possess an inherent toxicity.

Many plants contain too high levels of hydrolase or protease inhibitors, the removal of which is considered beneficial for e.g. a protein product to be used in an animal feed. Protease inhibitors, e.g. trypsin inhibitors, are found in most of legumes but they are also present in potatoes. Protease inhibitors are commonly small sized hydrophilic proteins of high solubility that may be glycosylated. They may therefore be difficult to precipitate, but they can normally be partly heat inactivated. Protease inhibitors tend to follow soluble protein in the processing of plant material.

A significant proportion of a plant material will be made up of variously sized polymers of sugars. Thus, for example a plant material may comprise oligo- and polysaccharides which may be found as soluble or insoluble fibres. One example of a soluble fibre is the raffinose family oligosaccharides. The

raffinose family oligosaccharides includes the compounds raffinose, stachyose, verbascose, and ajugose. They are found in all legumes and to a minor extent in potatoes. They are neutral compounds, which may be separated from a solution using membrane filtration, such as ultra- or nanofiltration.

Saponins are neutral or anionic compounds that tend to follow the proteins during purification processes. The solubility of saponins is relatively high, although they may precipitate along with the proteins. It is of interest to be able to separately isolate the saponins when processing plant material so that their presence in final products can be strongly reduced or avoided if desired. The effects of saponins vary from causing unacceptable, e.g. bitter, taste to being toxic.

Phytate is a common component of plant material having negative nutritional value, since it may *inter alia* reduce the bioavailability of various minerals. The negative nutritional value of phytate is especially pronounced for non-ruminant animals. Phytate is highly negatively charged and may to a certain extent be extracted from the protein matrices of plants using a mechanical treatment of the plant cell walls in combination with low pH, which makes the cell wall swell. In general, the solubility of phytate is high at low pH, e.g. below 5.

Alkaloids and heteroaromatics are low molecular weight nitrogen containing compounds found too high concentrations in many plants, and these may be toxic. For example, lupine seeds represent one type of plant material of legume origin comprising toxic alkaloids. Alkaloids are neutral compounds and they may be removed from a plant material using ultra- or nanofiltration or by adsorption to a hydrophobic material such as hydrophobic column materials. They can also be removed by use of activated carbon.

Glycoalkaloids are glycosylated alkaloids compounds present in plants, such as potatoes. Glycoalkaloids may have a bitter taste or even be toxic. Therefore the control of their presence in a plant product is desirable.

Phenolics such as polyphenolics, lignin or tannins, the group of flavonoids, isofavonoids, o-diphenolics, are compounds present in plant material and may be causing quality problems such as reduced protein bioavailability, off colour or taste in processed plant material. Current processes within the field typically only focus on individual contaminants or undesirable compo-

nents. Thus, current processes commonly fail to account for other components than the component of interest and there is a need to provide better processes considering more than a single contaminant component.

Moreover, current protein products from plants exhibit a number of drawbacks. One major drawback is that the recovery of protein from the plant raw materials is commonly carried out on an industrial scale by heat coagulation. Due to the presently applied heat coagulation processes the protein becomes denatured and as a consequence it loses functional properties, i.e. emulsifying capacity, foaming capacity, thermogelling capacity, water binding capacity, etc. Even the most essential requirement for its application in the food industry, i.e. solubility in water, can barely be met by heat denatured proteins.

WO2008/049385 describes a process for producing coagulated leguminous protein fractions. These are produced from fruit juice of the legumes, which is generated by squeezing relevant plant sections, especially fruits, or by liquid/liquid extraction of milled plant parts. The method of WO2008/049385 comprises from one process step to increase precipitation conditions, which may comprise heat coagulation of the proteins, i.e. at a temperature between 50 and 85°C, in the fruit juice, or precipitation of the proteins from the fruit juice at acidic pH, i.e. 2 to 7, with 5-7 being preferred. In particular, a target protein fraction is isolated by adjusting to a pH value which is suitable for precipitation together with a thermal precipitation of the supernatant of the previous step; a pH value is selected around the isoelectric point of the protein, in combination with a temperature increase above room temperature. WO2008/049385 lists negative substances, the removal of which is considered relevant. These comprise trypsin inhibitors, phytic acid, lectins and other enzyme inhibitors, digestion inhibitors, tannins/tannic acid, protease inhibitors, polyphenols and special sugars such as legume sugars. However, data provided in WO2008/049385 only concerns the protein profile of the product, and little support is provided relating to the negative nutritional components.

US2008/226810 discloses a pea protein composition with a high protein content with a characteristic molecular weight distribution profile and a characteristic soluble protein content. The composition may be produced in a process comprising preparing a flour by grinding dry peas, suspending the

pea flour in water, fractionating the suspension to isolate a protein-rich fraction, isolating a protein component of this fraction by a thermal flocculation technique at the isoelectric point of the proteins, i.e. about 4.5, and at a temperature ranging from 40 to 70°C.

The pea protein compositions of US2008/226810 are characterised only with respect to protein components, and other negatively nutritional components are not considered.

WO2008/144939 describes a process of aqueous protein extraction from *Brassicaceae* oilseed meal. The process may include an aqueous extraction of a *Brassicaceae* oilseed meal at a pH of from 2.5 to 5.0 at a temperature in the range of 40 to 60°C. As for US2008/226810, the products of WO2008/144939 are concerned with the protein profile and non-protein components are not considered.

US 4,697,004 describes a soy protein isolate with a reduced aluminium content and which is free of phytic acid and phytate complexes. This isolate is prepared from a soy protein raw material, such as defatted soy flour or defatted soy flakes in a process involving forming an aqueous solution of soy protein at an alkaline pH from the soy protein containing raw material. For example, the pH may be of 8 to 10 and the temperature above 65°C. Thereafter, the temperature is reduced rapidly to 25 to 65°C, and the slurry maintained at this temperature to continue extraction of the soy protein from the raw material. The insoluble fraction with phytates and carbohydrates is separated from the solubilised protein fraction by filtration or centrifugation. These temperature ranges are considered optimal values for dissociation of the soluble soy protein from the phytic acid complex and for maintaining the phytates and phytic acid derivatives substantially insoluble. According to US 4,697,004 low pH will results in the formation of a bond between the phytate and the protein.

EP 1528068 relates to a method for producing an isolated-soy protein comprising the step of acid-washing defatted soybeans with an aqueous medium in a region of pH 3.0 to 5.0 to extract and remove whey components. The washing temperature may be from 10 to 60°C which is considered a range of temperature at which protein is not denatured; the most preferred temperature range is 40 to 50°C. In an exemplary embodiment of EP 1528068 low-denatured defatted soybean flake is resuspended in water

at 45°C followed by adjustment of the pH to 4.2 with hydrochloric acid.

EP 1323353 provides a method for producing a fractionated soybean protein. The method comprises warming a solution containing a soybean protein to a temperature of 30 to 75°C under a weakly acidic pH of 3.8 to 6.8. The method may further comprise decomposing phytate by a phytase during the manufacturing process. The preferred pH of 4.2 to 6.2 encompasses the isoelectric point of the protein, e.g. 4.5 to 5.3, and the method of EP 1323353 is thus an isoelectric precipitation.

There is therefore an interest in providing improved processes capable of producing plant products of increased nutritional value. The challenge of obtaining such plant products has not been fully addressed. In particular, there is a need for a process capable of an efficient and simple method of fractionating a plant material to separate nutritionally valuable components from components of negative nutritional value. It is especially of interest to provide processes capable of accounting for, and reducing the content of, more than a single contaminant component, and likewise there is a need for a process capable of handling both protein and non-protein components.

The present invention addresses these points.

## Disclosure of the invention

The present invention relates to a process for the manufacture of a product from a plant material comprising the steps of:

process for the manufacture of a product from a plant material comprising the steps of:

- providing a disrupted plant material comprising <10 %(w/w) starch and <10 %(w/w) oil/lipids;
- adjusting the pH of the disrupted plant material to a value of pH 3.5 or below to provide an acidic suspension;
- heating the acidic suspension to a temperature in the range of about 50°C to about 80°C;
- isolating the product from the heated, acidic suspension.

The present inventors have found that when a plant material containing less than 10% starch and less than 10% lipids is subjected to a treatment comprising lowering of the pH to below pH 3.5, such as the range of about 1.0 to about 3.5, e.g. to about 2.0, while increasing the temperature by

heating the plant material to be in the range of about 50°C to about 80°C, preferably about 60°C to about 80°C, the solubility of protein in the plant material increases compared to it when the plant material is extracted at increased temperatures without lowering the pH or when the plant material is extracted at a lowered pH without increasing the temperature. Thus, for example the present inventors have surprisingly found that when a plant material is extracted at 65°C after adjusting the pH to 2.0 an amount of protein is extracted corresponding to 107% of the amount of protein extracted at pH 7.0 at ambient temperature. In comparison, extraction from the pH-adjusted material, e.g. a pH of 2.0, at ambient temperature yields less protein, 96%, and extraction at increased temperature, 65°C, from plant material at pH 7.0 also yields less protein, 98%, compared to the reference extraction at ambient temperature and pH 7.0.

The results indicate that both heat and low pH change the protein structure in a way that results in a decrease in the protein solubility. Surprisingly, however, combining the effects of both heat and low pH result in changed protein structures resulting in increased protein solubility. Without being bound by theory it is believed that some large proteins, e.g. above 100 kDa molecular weight, may dissociate into smaller subunits, of e.g. about 10 to about 30 kDa in size. Such subunits are expected to be of higher solubility and this effect may therefore help explain the advantageous effect.

Furthermore, the combination of low pH and high temperature has also been found to have a beneficial effect on the interaction between non-protein components, e.g. phytate, phenolics, saponins etc., and protein components in the material. In particular, it has been found that the interactions between phytate and protein are hindered allowing easier separation of the two components. Likewise, the interactions between proteins and other non-protein components, e.g. alkaloids, glycoalkaloids, saponins, phenolics and soluble fibres, etc., have also been found to be modified by the combined effect of low pH and high temperature, so that protein may easily be separated from these. Furthermore, hindering the interactions between phytate and protein allows that phytate is recovered from the plant material, and the degradation of the phytate, e.g. by present phytase enzymes, is thus prevented. The method advantageously provides a product from the plant material which is lowered in phytate content, and in particular the addition of a phytase en-

zyme is less needed in the process to provide a product which is low in phytate content. In a specific embodiment no phytase enzyme is added in the process. Likewise, since the interactions between phytate and protein are avoided it is also possible to recover phytate from the plant material, and phytate can be recovered without requiring addition of a phytase enzyme inhibitor in the process. In a specific embodiment of the process no phytase inhibitor is added.

High temperature and low pH may further, reversibly or irreversibly, inhibit various enzymes of a plant material. Some enzymes, such as polyphenol oxidases, participate in reactions causing undesirable enzymatic discolouration or browning and their inhibition may advantageously prevent browning of plant products.

The method used to adjust the pH is not limited to any particular principle. Any acid may be used. For example the acid may be an inorganic acid, such as sulphuric acid, hydrochloric acid or phosphoric acid, or the acid may be organic, such as citric acid or acetic acid. It is also possible to combine different inorganic acids or organic acids or to use combinations thereof, for example sulphuric acid and citric acid may be used in combination. The acid may be added at a final, desired concentration or the acid may be added in a concentrated form to an aqueous suspension of the plant material. For example, 1 M sulphuric acid may be added to an aqueous suspension of a plant material in a volume sufficient to adjust the pH as desired, or the dry material may be suspended in e.g. 20 to 25 mM citric acid supplemented with sulphuric acid, e.g. at about 50 mM concentration. The acid may also be added in the form of a buffer, or the conditions may be buffered once the acid has been added in order to maintain the pH at the desired value. It is furthermore also preferred that the acid used for adjusting the pH is compatible with applications for foods and beverages for human consumption or with applications for animal feed.

When the acid is also capable of serving as a chelating agent, e.g. citric acid, oxalic acid, lactic acid, malic acid, maleonic acid, tartaric acid, succinic acid, the combination of low pH and the chelating effect may further serve to prevent discolouration of the plant material caused by enzymes, such as polyphenol oxidases, or discolouration occurring non-enzymatically.

Addition of acid may also be accompanied with addition of a further

volume of another liquid, e.g. water, or the acid may be added in the desired final concentration. When the plant material for processing exists as a dry material, e.g. in the form of a powder, flakes, granules, etc., the material may be suspended in water prior to addition of acid. The plant material is typically suspended at about 50 g/L to about 500 g/L. Some plant materials, e.g. fruit juices or the like, may already be in an appropriate liquid form and addition of acid, e.g. in a concentrated form, such as 1 M sulphuric acid, may be sufficient to adjust the pH as desired.

The process of the invention may be used with any plant material. However, it is especially advantageous with plant materials derived from legumes. Some plants, e.g. some legumes like soy and lupine, contain large amounts of lipids, typically up to about 20% of the dry weight, and the lipid content of the plant material must then be reduced prior to processing according to the invention. The plant material to be processed according to the present invention must be reduced to comprise no more than 10 % (w/w) lipids or oil. Processes for removing lipids from, or "defatting", legumes and other plants with high lipid contents are well known in the art.

Likewise, the content of starch in the plant material to be processed must be below 10 % (w/w). Starch is a mixture of amylose and amylopectin; these are both complex carbohydrate polymers of glucose. Starch is often found in the fruit, seeds, rhizomes or tubers of plants. Starch is a fibre which is insoluble in cold water but which swells upon increase of the temperature, so that its presence above 10 % (w/w) will be detrimental to the processing. Plants with high contents of starch must therefore have the starch content reduced. Removal of starch is well known in the art.

Some plants may have inherent contents of lipids and starch already below the required limit and may thus not need any processing prior to subjecting to the method of the invention. In general, removal of the shell from e.g. leguminous plants such as soy, lupin, horse bean and pea may increase the protein yield and the purity.

It is preferred that the plant material has been disrupted prior to treating with the combination of low pH and high temperature. Alternatively, a step to disrupt the plant material may also be comprised in the method of the invention, in particular when the plant material inherently has less than 10 % (w/w) starch and lipids. Disruption of the plant material will increase

the specific surface area available for extraction of components of the plant material, so that extraction will proceed more efficiently and faster. Likewise, the contents, such as protein and non-protein components, of a disrupted plant material are heated faster than plant materials with lower specific surface areas.

The process of the invention may have an impact on several components in a plant material and their subsequent separation from each other. In particular, the process of the invention advantageously allows that protein and non-protein components may be more easily separated from each other after the initial treatment at high temperature and low pH. For example, the initial processing minimises interactions between protein and non-protein components, such as phytate, saponins, alkaloids, glycoalkaloids, and soluble fibres etc. The product manufactured in the process may therefore be a protein product or a non-protein product. Protein products comprise protein isolates, protein concentrates, purified protein components and so forth. A protein product may have any desired purity and the water content may also be controlled as desired. Protein products prepared according to the process advantageously have lowered contents of non-protein components. Thus for example, the relative amount of a non-protein component to protein will be lower than in the starting plant material. The product produced in the process may also be a non-protein product. For example, the product may be a fibre product, e.g. a product of soluble or insoluble fibres, e.g. dietary fibres, or the product may be phenolics, phytate, alkaloids, heteroaromatics or glycoalkaloids etc.

The treatment of a plant material with low pH and high temperature according to the invention may advantageously be included as an initial process step in various processes for the production of plant materials. In another embodiment the process further comprises

- subjecting the heated, acidic suspension to a solid-liquid separation to provide an acid precipitated fraction and an acid soluble liquid fraction.

- isolating the product from the acid precipitated fraction or from the acid soluble liquid fraction.

The acid precipitated fraction will comprise the majority of the insoluble fibres from the plant material. Depending on the conditions and unit opera-

tion employed to separate the acid precipitated fraction and the acid soluble liquid fraction a product enriched in insoluble fibres, e.g. an insoluble fibre product, may be obtained. In particular, the insoluble fibres will not be solubilised under the acidic conditions in the initial processing steps, and the insoluble fibres tend to follow the precipitated fractions. The insoluble fibres may be removed, selectively, using solid-liquid separation unit operations for removing coarse materials, such as sieving or filtering through coarse filters or using centrifugation with low centrifugal force or by a combination thereof, e.g. using centrifugal sieving or using a peeler centrifuge. In one embodiment the heated, acidic suspension is subjected to sieving in order to primarily separate the insoluble fibres from the remaining components thus providing a product enriched in insoluble fibres. In a specific embodiment several solid-liquid separation steps are employed in series before other unit operations are used to separate components from each other. For example, after adjusting the pH of the plant material and heating it, the heated, acidic suspension may be subjected to a sieving operation to remove coarse material, such as insoluble fibres, followed by a filtration or centrifugation to remove finer material, such as protein precipitates. This allows provision of a fraction with enriched insoluble fibres and a fraction with enriched protein and reduced insoluble fibres and other non-protein components from the respective solid-liquid operations.

The heated, acidic suspension may also be subjected to homogenisation, e.g. wet milling or the like. Homogenisation may be performed before or after or between any solid-liquid separation operation(s). Some soluble carbohydrate fibres, e.g. pectin, tend to swell upon heating and acidification, and therefore a homogenisation may advantageously increase the extraction of protein and non-protein components and thus provide an increased yield. Moreover, homogenisation may advantageously decrease the viscosity of the suspension. Likewise, a homogenisation operation may be included after any step in the processes. For example, when a precipitate is re-suspended or resolubilised it may appropriately also be homogenised to improve resuspension or resolubilisation.

In addition to insoluble fibres, the acid precipitated fraction will typically comprise protein and a reduced relative amount of non-protein components over the starting material. In one embodiment the insoluble fibres have been

removed prior to subsequent processing of the acid precipitated fraction. This acid precipitated fraction may be a product in itself, e.g. a "protein isolate" or the acid precipitated fraction may also be treated further, and in another embodiment the process further comprises

- suspending the acid precipitated fraction in a liquid and adjusting the pH to a value above pH 4.9, such as from about 7.0 to about 12.0 to provide an alkaline suspension;

- subjecting the alkaline suspension to a solid-liquid separation to provide an alkaline precipitated fraction and an alkaline soluble liquid fraction;

- isolating the product from the alkaline precipitated fraction or from the alkaline soluble liquid fraction.

A product may be isolated from the alkaline precipitated fraction, and as for the acid precipitated fraction, the alkaline precipitated fraction will typically comprise fibrous material with a reduced relative amount of other non-protein components and protein over the starting material. This product may also be referred to as a "fibre isolate". Any base may be used to increase the pH, but the base is preferably compatible with those used in food products.

Either of the liquid phases, e.g. the heated, acidic suspension, the acid soluble liquid fraction, or the alkaline soluble liquid fraction may be processed further. Thus, in one embodiment the process further comprises

- adjusting the pH of the heated, acidic suspension or the acid soluble liquid fraction or the alkaline soluble liquid fraction to a value from about 4.0 to about 6.0 to provide a neutral suspension.

The heated, acidic suspension or the acid soluble liquid fraction or the alkaline soluble liquid fraction may be concentrated prior to pH adjustment by any given concentration method as e.g. evaporation or filtration.

Likewise, the step of adjusting the pH to a value of about 4.0 to about 6.0 may also be applied to the alkaline suspension. The solid fractions of the previous steps, i.e. the acid precipitated fraction or the alkaline precipitated fraction may also be subjected to this step. In all cases the step will provide a neutral suspension. In a preferred embodiment the pH is adjusted to a value close to the average isoelectric points of the proteins in the plant material; for legumes, such as soy, peas, beans or the like, the pH is adjusted

to about 4.7. Other preferred ranges are from about 4.5 to about 5.0. Acids and bases for adjusting the pH are as described above.

Subsequent to adjusting the pH a product may be isolated from the neutral suspension. In one embodiment the neutral suspension may be subjected to a solid-liquid separation to provide a neutral precipitate and a neutral supernatant, and the product may be isolated from either of these. The neutral precipitate may also be referred to in general terms as a protein isolate; this protein isolate will, as for the protein isolates prepared from the acid precipitated fraction or the alkaline precipitated fraction comprise protein and a reduced relative amount of non-protein components over the starting material.

For the protein isolates prepared according to the above processes in the various precipitation operations, the content of non-protein components, such as phytate, phenolics, saponins, alkaloids, glycoalkaloids, will advantageously be reduced over the starting material. Such non-protein components tend to stay in solution, or possibly suspension, in the liquid fractions. Therefore, their content will be reduced in the precipitated fractions. In general the proteins precipitated in each step will mainly be proteins of low solubility at the pH employed in the previous step, e.g. the acid precipitated fraction will comprise proteins of low solubility at low pH and the alkaline precipitated fraction will comprise proteins of low solubility at high pH; in general, however, most of the protein can be precipitated from the neutral suspension, i.e. in the neutral precipitate, e.g. at a pH close to the isolectric points of the bulk protein of the plant material, if a step to adjust the pH to a value from about 4.0 to about 6.0 is included in the process. The protein contents of the precipitates may be at least 70 % (w/w), such as at least 80 % (w/w) or at least 90 % (w/w). In a preferred embodiment where the insoluble fibres have been separated a protein isolate of at least 90 % (w/w) is provided. Other major components may be insoluble fibres and salts. All of the protein isolates prepared according to the invention have reduced contents of non-protein components, such as phytate, phenolics, saponins, alkaloids, heteroaromatics, glycoalkaloids and oligosaccharides. They may therefore be used to prepare animal feed or food of increased value.

The embodiments disclosed above may comprise solid-liquid separation unit operations. In general according to the invention a solid-liquid separa-

tion step may be included at any stage where a liquid contains a solid material. For example, the starting material comprises solids, and following the adjustments of pH values solid materials may be formed in suspension. In general, any solid-liquid separation may be employed. It is preferred that the solid-liquid separation operation is chosen to selectively remove a component of interest. For example, insoluble fibres may be removed using sieving whereas protein precipitates generally require stronger separation power. Following sedimentation of protein from the plant material, the protein component may be isolated using any solid-liquid separation principle. Specific embodiments further employ addition of flocculating agents and/or filter aids prior to or during the solid-liquid separation. Exemplary filter aids comprise starch, e.g. potato starch, plant fibres or plant protein, or a combination thereof.

The protein isolates may be further processed following the solid liquid operations. In one embodiment, the separated protein is subsequently dried.

In other embodiments of the present invention any liquid phase, i.e. suspensions or supernatants from solid liquid operations, may be subjected to further operations to obtain products with particularly desired characteristics, such as purity or further reduction of non-protein components. Isolation of protein may also take place using ultrafiltration, e.g. via 100 kDa cut-off membranes to isolate high molecular weight proteins (optionally together with high-molecular weight sugars, primarily pectins), followed by separation via finer membranes (e.g. 75, 50, 25, 10 and/or 3 kDa) to isolate protein from low molecular weight compounds, such as oligosaccharides, phytate, saponin etc.

Thus, according to an embodiment the process of the invention further comprises

- optionally adjusting the pH of the alkaline or heated, acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension or the neutral supernatant to a value from about 3.0 to about 4.0;

- contacting the alkaline or heated acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension or the neutral supernatant with a cation-exchange chromatography resin to separate a cationic binding fraction from a cationic non-binding fraction.

The pH of the material to be contacted with the cation-exchange resin

may be adjusted to adjust the cationic binding properties of components in the material. Thus, by adjusting the pH to a value from about 3.0 to about 4.0, e.g. to about 3.5, it can be ensured that a large proportion of proteins present will bind as cations to the negative charges of the cation-exchange resin. The material bound to the cation-exchange resin is referred to as the "the cationic binding fraction" regardless of the condition under which the suspension or liquid fraction is contacted with the cation-exchange resin. However, if purification of a specific component from the material is desired, the material may be contacted with the cation-exchange resin at a higher pH. Phytate and saponins will generally be negatively charged and will not bind to the cation-exchange resin, i.e. they will be present in the cationic non-binding fraction. Likewise, soluble fibres will generally not be charged, and thus they are also cationic non-binding.

Any cation-exchange resin is appropriate for the present invention, and in this context a cation-exchange membrane is also considered relevant for the invention.

Following contacting of the cation-exchange resin with the plant material, the cation-exchange resin may be contacted with an eluent solution to elute the product from the cationic binding fraction from the cation-exchange chromatography resin. Elution conditions from the cation-exchange resin may be any appropriate condition, and such appropriate conditions are well known to the skilled person. In one embodiment, the eluent solution has a pH higher, e.g. about 8.0, than the pH of the liquid fraction or suspension contacted with the cation-exchange chromatography resin. This will bring cationic bound components to an uncharged or anionic stage, so that they will be repelled by the negative charges on, and thereby eluted from, the cation-exchange chromatography resin. In other embodiments, the eluent solution has an increased salt, e.g. NaCl, concentration. The eluate may be subjected to further processing steps, e.g. steps to reduce the volume, desalting, concentrating steps, drying operations and so forth, as desired. The eluate will generally comprise at least 90 protein %(w/w) protein, such as at least 95 protein %(w/w) protein or even about 99 protein %(w/w) protein with the balance generally being represented by ash; this product may be referred to as a "protein isolate" or a "protein concentrate". The protein isolate will generally be significantly reduced in non-protein components, such as

phytate, phenolics, saponins, alkaloids, heteroaromatics, glycoalkaloids, and soluble fibres; in one embodiment the product is reduced in phytate below the detection level of the measurement method used.

The cationic non-binding fraction, or run through, from the step of contacting the suspension or liquid fraction with the cation-exchange chromatography resin will generally account for the non-protein components, such as phytate, phenolics, saponins, alkaloids, heteroaromatics, glycoalkaloids, and soluble fibres. However, this run through may also be subjected to further processing steps, and another embodiment also comprises the isolation of the product from the cationic non-binding fraction. These further processing steps may be ultrafiltration or nanofiltation - e.g. at a cut-off of 100 kDa to 10 kDa or even 1 kDa to separate high-molecular weight pectins from the pool of low molecular weight compounds. In its simplest form this embodiment involves concentration and drying of the cationic non-binding fraction. However, the process may further comprise

- optionally adjusting the pH of the cationic non-binding fraction to a value from about 7.0 to about 9.0;

- contacting the cationic non-binding fraction with an anion-exchange chromatography resin to separate an anionic binding fraction from an anionic non-binding fraction.

The cationic non-binding fraction will comprise a significant amount of the phytate and saponins and also the soluble fibres. Phytate and saponins will likely be negatively charged and thereby found in the anionic binding fraction. Various non-protein components, such as soluble fibres and, depending on matrix, pH, and emulsifying properties, also some alkaloids and glycoalkaloids may be found in the anionic non-binding fraction. The anionic binding fraction may be eluted using any appropriate eluent solution, and such are well known within the art. These may comprise elution at low pH or high salt concentration. However, as the anionic binding fraction is generally not proteinaceous the eluent solution may also advantageously comprise organic solvents. As for the cation-exchange resin, "anion-exchange resin" is not considered limiting and anion-exchange membranes are also considered relevant.

Certain embodiments of the invention provide integrated processes for processing a plant material employing the above processes in an integrated

set-up. An integrated process may comprise any combination of the above processes, but need not comprise all the above processes; the exact processes for a given set-up will depend on the starting material and the components of interest contained in the starting material. In the integrated process set-up, each of the above processes will typically yield a fraction comprising a component of interest for that specific process and another fraction, which may be used as a starting material in another process described above.

The invention also relates to the products obtainable in the disclosed embodiments of the process of the invention. Thus, in one aspect the invention relates to a protein isolate or protein concentrate with a reduced relative content of non-protein components. In particular, the invention relates to a protein isolate or protein concentrate with a reduced relative content of phytate. In another aspect, the invention relates to a protein isolate with a reduced relative content of saponins. A further aspect relates to a product of a non-protein component with a reduced relative content of protein, e.g. a phytate product with a reduced relative content of protein, a saponin product with a reduced relative content of protein, an alkaloid or a glycoalkaloid product with a reduced relative content of protein. Likewise, a product of soluble fibres and carbohydrates like raffinose family oligosaccharides with a reduced relative content of protein and/or other non-protein components is also considered within the scope of the invention.

## Brief description of the figures

In the following the invention will be explained in greater detail with the aid of examples of embodiments and with reference to the schematic drawings, in which

Fig. 1 shows the effect of high temperature and low pH on extraction of protein;

Fig. 2 shows a process diagram of an embodiment of the invention;

Fig. 3 shows a process diagram of an embodiment of the invention.

Fig. 4 shows a process diagram of an embodiment of the invention.

## Detailed description of the invention

The present invention relates to a process for the manufacture of a

product from a plant material comprising the steps of:

process for the manufacture of a product from a plant material comprising the steps of:

- providing a disrupted plant material comprising <10 %(w/w) starch and <10 %(w/w) oil/lipids;

- adjusting the pH of the disrupted plant material to a value of pH 3.5 or below to provide an acidic suspension;

- heating the acidic suspension to a temperature in the range of about 50°C to about 80°C;

- isolating the product from the heated, acidic suspension.

In the context of the present invention, the term "plant material" refers to any plant material, and it may refer to whole plants, certain parts of plants, such as fruits, leaves, stems, roots, tubers, nuts, berries etc., or any mixtures of these. In general, the plant material will be subjected to some degree of disruptive processing prior to subjecting to the methods of the inventions. This disruptive processing (or "disruption" or derived forms of this term) may be any processing intended to reduce the size of parts or particles of the plant material, and typical disruptive processing involves cutting, pressing, chopping, milling, grinding, crushing, grating, shredding etc. In particular the disruption aims to degrade or disrupt the cell walls of the plant material to make the contents of the cells accessible. The terms plant material and disrupted plant material may also refer to any liquid produced in the disruptive processing, and the term "plant material" may thus refer to any whole plant, any part of a plant, a solid or liquid material obtained during disruptive processing or a mixture of these.

The content of starch in the plant material to be processed must be below 10 %(w/w). It is preferred that the starch content is lower than this, e.g. below 8 %(w/w), below 6 %(w/w) or below 5 %(w/w). Likewise the content of lipids and/or oils must be below 10 % (w/w). As for the starch content it is preferred that the oil/lipid content is even lower, than this, e.g. below 8 %(w/w), below 6 %(w/w) or below 5 %(w/w).

The present invention is not limited to a specific plant or group of plants, and material from any plant is relevant to the invention. However, legumes are a preferred source of plant material. Legumes, i.e. plants of the family *Fabaceae* (or *Leguminosae*), commonly have dry fruits in pods; the

fruits have high contents of proteins and lipids. Common legumes include alfalfa, clover, peas, beans, lentils, lupins, mesquite, carob, soy, and peanuts. More specifically legumes include dry beans (*Phaseolus spp*. including several species now in Vigna), such as kidney bean, haricot bean, pinto bean, navy bean (*Phaseolus vulgaris*), lima bean, butter bean (*Phaseolus lunatus*), azuki bean, adzuki bean (*Vigna angularis*), mung bean, golden gram, green gram (*Vigna radiata*), black gram, urad (*Vigna mungo*), scarlet runner bean (*Phaseolus coccineus*), ricebean (*Vigna umbellata*), moth bean (*Vigna aconti-folia*), tepary bean (*Phaseolus acutifolius*); dry broad beans (*Vicia faba*), such as horse bean (*Vicia faba equina*), broad bean (*Vicia faba*), field bean (*Vicia faba*); dry peas (*Pisum spp.*), such as garden pea (*Pisum sativum var. sativum*), protein pea (*Pisum sativum var. arvense*); chickpea (*Cicer arietinum*), dry cowpea (*Vigna unguiculata* ), pigeon pea (*Cajanus cajan*), lentil (*Lens culinaris*), peanut (*Arachis hypogaea*), lupins (*Lupinus spp.*), soy (*Glycine max*).

Some legumes of particular interest comprise soy, lupine, faba beans (or broad bean) and peas.

Lupine has the same general composition as soy bean, but the saponin content is very low. Before processing the hull fraction can be removed. This increases the protein concentration of the protein concentrates. Lupines contain oil in concentrations from a few percent up to around 15 % (W/W) depending on the specific species. Some of the species can be processed directly whereas the high oil containing species may need to be defatted before processing. Lupines also contain toxic alkaloids that may be removed. Sweet lupine species has reduced content of these alkaloids and are preferred over bitter species. The alkaloid compounds will generally be present in the eluate after cation exchange chromatography where they can be eluted in a specific fraction by a skilled person or in the soluble carbohydrate fraction after cation- and anion exchange chromatography. They can be removed from this fraction by ultra- or nanofiltration or by adsorption to a hydrophobic material such as hydrophobic column materials. They can also be removed by use of activated carbon.

Faba bean or broad bean contains starch instead of lipids/oil. This must be reduced to 10 % (w/w) or less prior to heating, and removal of the hull fraction is also advantageous. This can e.g. be performed by wind sieving af-

ter milling, or the starch (and hull fraction) can be removed by wet fractionation before heating e.g. by centric sieves or decanters. Faba beans or broad beans may also contain too high concentrations of heteroaromatics acting as toxic compounds such as vicine and convicine. In addition some faba bean phenolics/tannins can create nutritional problems when present in food and feed. Species with low concentration of these compounds have been developed and are preferred for the process of the present invention.

Pea contains both starch and high concentrations of saponins. Starch and hulls can be removed as described for the faba beans and lupine seeds, whereas the saponins are (partly) removed by the process in general and are isolated in the anionic binding fraction in the elution from the anion-exchange resin.

Some legumes contain large proteins, e.g. soy contains glycinin and conglycinin of about 400 kDa molecular weight, that are known to create immunological response in calves. The present inventors have now found that the combined effect the acidic pH and high temperature may dissociate these into their constituent subunits and thereby reduce their antigenecity. Thus, the invention provides a further beneficial effect in an animal feed for calves prepared using a protein product of the invention.

Without being bound by theory it is believed that a combined effect of the increased temperature and the decreased pH may be that large proteins, e.g. proteins of molecular weight above 100 kDa, such as above 200 or 300 kDa, e.g. proteins of about 400 kDa molecular weight, are dissociated to units of about 10 to about 30 kDa in size. This in turn may explain the increase in solubility of protein.

In certain embodiments the plant material is derived from potatoes, and the process is also adaptable for potato protein purification, also in relation to starch production. Homogenisation and acid treatment are durable on the whole potatoes, and starch can be removed by a combination of centrifugal sieves and decanters. The patatin protein fraction is to a minor extent precipitating in the acidic pH range, but due to partial glycosylation the precipitate is relatively difficult to isolate in the native form. Traditionally isolation of the patatin protein fraction is performed at very low pH by addition of high concentration of sulphuric acid followed by high temperature treatment (>80°C). This creates a denatured protein with low solubility and loss

of the water binding capacity, foaming capability and other characteristics. The process of the present invention reduces the denaturing effects on the patatin proteins and preserves to a greater extent the physico-chemical properties of the patatin proteins.

In potatoes glycoalkaloids are present, and these toxic compounds will generally be extracted from the potato matrix along with the proteins. The glycoalkaloids may bind cationic to the cation exchange resin together with the proteins that are not precipitated. Separation of the glycoalkaloids from the cationic bound protein fraction can be performed by use of a controlled pH elution of the cation exchanger.

Potatoes also contain phenolic compounds that may create colouring of the protein and fibre compounds. This is primary chlorogenic acid and caffeic acid. These compounds bind anionic to the anion exchange resin if used and they can be isolated by controlled pH elution from this resin.

Plant material is a complex mixture of soluble and insoluble material comprising proteins and non-protein components, such as carbohydrates, e.g. starch, pectin, cellulose and hemicellulose, minerals and other organic components, such phytate, glycoalkaloides, alkaloids, flavour components, simple organic acids, etc., and monomeric and polymeric reactive phenols.

Phytate is the hexaorthomonophosphate ester of myo-inositol and it may occur as a calcium magnesium salt, phytin. In the context of the present invention the words "phytate" and "phytic acid" may be used interchangeably. In particular, the exact form of phytate will depend on the surrounding pH so that phytate may be changed to phytic acid and phytic acid to phytate simply by adjusting the pH. Phytate has a strong binding affinity to important minerals such as calcium, magnesium, iron, and zinc. When a mineral binds to phytate, it becomes insoluble and will be nonabsorbable in the intestines, and it may thus reduce the bioavailability of various minerals, such as zinc, magnesium, calcium, iron, etc. Removal of phytate and its derivatives and alternative forms is desirable because phytate phosphorous is not readily available to humans or animals and it interferes with the absorption of cations, such as zinc, magnesium, calcium, iron.

The content of phytate in plant materials may be up to about 5% of the dry weight or higher. In legumes the content of phytate is typically from about 0.5% to about 2.5% of the dry weight.

Other components of typical plant materials are soluble and insoluble fibres. Insoluble fibres are often relatively easily collected by e.g. gravity/sedimentation and are subsequently used as either fertiliser/soil conditioner or as animal feed. The majority of these fibres are derived from cell wall material. However, these insoluble fibres could potentially become a valuable product for use in the food industry, provided that simple methods were provided enabling extraction of such fibres preferably without components of negative nutritional value, such as alkaloids, glycoalkaloids, phytate, phenolics, inhibitors of digestive enzymes. Such fibre products could potentially have widespread applications within the food industry as an inexpensive additive with the ability to improve the nutritional and/or digestive qualities of food products and/or to function as a bulking agent and/or as a replacement for other constituents such as e.g. sugar, sugar alcohols, and/or fat.

Soluble fibres may be desirable to remove from a protein product, e.g. a protein isolate or concentrate, to be used to prepare an animal feed. However, soluble fibres may themselves constitute a potentially commercially interesting component from plant materials. Soluble fibres can be used as e.g. a sugar replacement agent. Soluble fibres of current commercial interest comprise e.g. fructans, inulin, oligofructose, polydextrose, indigestible dextrins, etc. Due to the solubility in water, these products find widespread use in the food industry, in particular as a low calorie sugar substitute in combination with e.g. high intensity sweeteners. Soluble fibres can also be used to improve the nutritional qualities of various food products.

The methods of the present invention comprise a step to adjust the pH of the plant material to a value of from about 1.0 to about 3.5. In the context of the present invention this range is generally referred to as "low pH" or "acidic pH". Other pH ranges relevant to the process of the present invention are values such as from about 1.5 to about 3.0 or about 1.5 to about 2.5, e.g. about 1.5 to 2.0 or about 2.0 to about 2.5. The pH adjustment may be performed prior to or after heating the plant material, or the two steps may be performed simultaneously. Likewise, the pH adjustment may be performed simultaneously with or after the disruption of the plant material, if this step is also performed. The pH may be adjusted by adding any acid as is appropriate depending on the plant material and the component of interest

in the process. It is also possible to adjust the pH using a buffer. The acid should preferably be compatible with food applications, and it may be inorganic or organic. Preferred inorganic acids are sulphuric acid, hydrochloric acid or phosphoric acid.

In some embodiments the acid also has a chelating effect and it may in combination with the low pH prevent discolouration of the plant material caused by contact with oxygen, such as enzymatic oxidation caused by polyphenol oxidase or non-enzymatic discolouration. Combinations of pH-values and concentrations of chelating acids for preventing discolouration are found in WO 2010/006621, which is hereby incorporated by reference. In particular, relevant pH-ranges are found on page 13 of WO 2010/006621 A1 and relevant concentrations of the chelating agents on page 12 of WO 2010/006621 A1. The contents of these pages are hereby included by reference.

The pH-range of about 1.0 to about 3.5 may also be referred to in the context of the present invention as "acidic". In parallel with this definition, the pH-range from about 4 to about 8 is referred to as "neutral", and the pH-range above 8, e.g. from 8-14 is referred to as "alkaline" or "basic". Likewise, these terms may be used to describe specific fractions occurring in the different process steps of the invention.

When a base is required it should preferably be compatible with food applications. Relevant bases are hydroxides of alkali metals, e.g. NaOH, KOH, ammonia, or $Ca(OH)_2$. Basic conditions may also be provided using a buffer.

Any method of heating the plant material is appropriate in the process of the present invention as long as the temperature of the plant material can reach a value in the range of about 50°C to about 80°C. In the context of the invention, this range is generally referred to as "high" temperature. In specific embodiments of the invention the plant material is heated to a temperature at or above about 55 °C, about 60°C, about 65°C, or about 70°C or about 75°C. In a preferred embodiment, the plant material is in a dry form without any added liquid, and the acid is heated before mixing with the plant material so that the plant material is brought to the temperature of the heated acid. This will ensure that the plant material is heated fast within the time to mix the plant material with the liquid, such as within 5 minutes or

less, e.g. within 3 minutes or within 1 minute. In order to ensure that the plant material is heated efficiently the volume of liquid added will be such that the plant material constitutes no more than half the total mass, e.g. the plant material is suspended at about 50 g/L to about 500 g/L, such as about 100 g/L to about 300 g/L, e.g. about 100 g/L or about 200 g/L. Alternatively, the plant material may be suspended in water at the desired temperature and the pH may be adjusted by adding acid to the desired pH. It is furthermore also possible to suspend the plant material in liquid, i.e. water or acid, and subsequently increase the temperature using external heating means. Regardless of how the heating is initially provided the process of the invention may involve further auxiliary heating means to maintain the temperature as desired once the plant material has been mixed with a heated liquid or once it has been heated externally.

The duration of the acid treatment is not limiting to the invention. Once the pH of the plant material has been adjusted to the desired value it can be retained at this value until a subsequent procedural step requires a change in the pH or until solid and liquid material are separated in a solid-liquid separation. However, it is also possible to adjust the pH, e.g. to increase it, before lowering the temperature.

The duration of the period the plant material is maintained at the increased temperature should be sufficient to ensure that the effect of the combined low pH and high temperature sets in. This will generally occur after about 1 minute. However, in some embodiments the high temperature is maintained for about 5 minutes or for about 10 minutes. In other embodiments the time it takes to handle the liquids and bring them to the next procedural step is considered sufficient. In yet other embodiments, the plant material is subjected to homogenisation after heating the plant material, preferably after adding a liquid at high temperature to the plant material. For example, the plant material may be homogenised for about 1 second, about 1 minute, e.g. about 2 minutes, about 5 minutes or for about 10 minutes before isolating a product from the heated, acidic suspension.

Some embodiments of the invention comprise solid-liquid separation steps to separate solids from liquids in a liquid suspension. In general, any solid-liquid separation unit operation may be employed, although it is preferred that the solid-liquid separation operation is chosen based on the na-

ture of, especially, the solids to be removed. For example, coarse material, such as insoluble fibres, may be separated from a suspension using a sieve or screen with a mesh size of e.g. about 500 µm, about 250 µm, about 125 µm, about 100 µm or about 80 µm. This will allow selective separation of insoluble fibres while retaining most protein precipitates in suspension.

Sedimented protein components may be isolated by filtration. Filtration may be performed through any commercially available filter material, and it may be performed in any type of filtration operation, such as the types known as vacuum filtration, pressure filtration, cross-flow filtration, basket centrifugation, deep bed filtration, etc. The protein component may also be isolated using centrifugation, e.g. in a solid bowl centrifuge, tubular bowl centrifuge, decanter centrifuge, disk-stack centrifuge etc.

Certain embodiments of the present invention involve cation- and anion-exchange resins. Any unit operation for contacting a liquid material with a chromatography resin is appropriate for these aspects of the invention. For example, the plant material may be contacted with the cation-exchange chromatography resin in a stirred tank reactor, or in a packed or expanded bed column. Depending on the nature of the unit operation used in the contacting step the plant material may be clarified, such as by screening, filtration or centrifugation, prior to applying the plant material to the cation- or anion-exchange unit operation or to other unit operations. The plant material may further be concentrated prior to applying the plant material to the cation- or anion-exchange unit operation or to other unit operations. In either aspect, it may be relevant to adjust the pH of the plant material prior to contacting with the cation-exchange chromatography resin. For example, the pH may be further adjusted to a value from about 2.0 to about 3.5. Such adjustment may be provided by the addition of an acid or base, e.g. $H_2SO_4$ or NaOH, respectively, as appropriate, or by addition of a buffer. Ion-exchange membranes are also relevant for the present invention.

The ion-exchange resins may be contacted with an eluent solution to eluate ionic bound components. For the cation-exchange resin, the eluent solution may have a higher pH, a higher ionic strength or a combination of a higher pH and a higher ionic strength than material contacted with the resin. It is preferred to use an eluent solution with a higher pH than the pH of the suspension or liquid fraction contacted with the chromatography resin. The

eluent solution for the anion-exchange chromatography resin follows the same principles as relevant for anion-exchange. Thus, it may contain a lower pH than the material contacted with the resin, or the salt concentration may be increased. It is also possible for the eluent solution to elute anionic bound components to contain an organic solvent. For example the eluent solution may be 0.5 M formic acid in 50% ethanol.

However, in certain embodiments the cationic or anionic bound components are further fractionated by contacting the cation- or anion-exchange chromatography resins, respectively, with bound material with gradually changing elution conditions, for example for the cationic bound material by first contacting the resin with a first eluent solution of a higher pH than the material contacted with the resin, then with another eluent solution of a higher pH than the first eluent solution. The cation-exchange chromatography resin may then be contacted with yet another eluent solution. The same considerations apply for the anion-exchange chromatography resin. The changes from binding material, or an optional washing solution, to the series of eluent solutions may take place in a step-wise fashion, or the conditions may be changed gradually in a so-called gradient-type elution.

Protein isolates or concentrates produced in a process according to the invention may also be subjected to further steps to remove traces of non-protein components or to concentrate the product. For example, the process may also further comprise a concentrating step capable of removing water from a liquid suspension; a preferred concentrating step is ultrafiltration. Suitable ultrafiltration membranes are those with cut-off values of 100 kDa, 20 kDa, 15 kDa, 10 kDa, 8 kDa, 5 kDa or even finer membranes depending on the specific application. Other methods such as e.g. diafiltration or nanofiltration may also be employed.

Liquid may also be removed from a liquid suspension using other appropriate methods, such as a solid-liquid separation method, e.g. centrifugation or filtration, or the liquid may be removed from a suspension, e.g. by evaporation, vacuum evaporation, freeze drying, spin flash drying, spray drying, floating bed drying or the like. It is also possible to combine several of these principles to obtain a product in a dry form.

The invention will now be explained in the following non-limiting examples. As will be evident to the skilled person variations are possible without

deviating from the invention.

## Examples

### 1. Effect of temperature and pH on protein solubility

2.5 gram white flake soybean meal was mixed with 25.00 mL 20 mM phosphate buffer at pH 7.0. Extraction was performed by shaking for 10 min. at room temperature followed by centrifugation for 15 min at 2350 x *g*.

750 µL supernatant were transferred to Eppendorf tubes and the pH was reduced to 2.0 by addition of 37.5 µL 1 M $H_2SO_4$ to the sample tubes. Reference samples were added 37.5 µL milli-Q water. After mixing the samples were placed on a heating block for 10 min. before pH adjustment to pH 7.0 by addition of 75 µL 1 M NaOH. Reference samples were added 70 µL milli-Q water. The samples were centrifuged and protein content in the supernatants was measured by UV spectrophotometry at 280 nm.

Protein solubility (estimated as concentration of soluble protein) as a function of pH adjustment and heat treatment (25°C, 45°C and 65°C) were calculated relative to the protein solubility of the reference sample (25°C without pH adjustments). The results are shown in Fig. 1.

Heat treatment without pH adjustment has no effect on protein solubility up to 45°C. At higher temperatures the protein solubility decreases slightly and a heat treatment at 65°C results in a 2% reduction of the protein concentration.

At room temperature low pH treatment results in decreased in protein solubility at pH 7.0 of 4% compared to the blank sample. Increasing the temperature during low pH treatment results in an increased protein solubility at pH 7.0, and heat treatments at temperatures above approximately 50°C results in higher protein solubility than the reference samples. Heat treatment at 65°C during low pH treatment thus result in a 7% increased protein solubility compared to the reference sample (25°C) and an approximately 10% increase in protein solubility compared to the 65°C heat treated reference sample.

The results indicate that both heat and low pH are capable of changing the protein structure in a way that results in a decrease in the protein solubility, but combining the denaturing effects of both heat and low pH results in a different kind of protein solubilisation resulting in increased protein solu-

bility.

**2. Production of protein concentrates in laboratory scale**

Production of proteins, insoluble dietary fibre fraction, soluble dietary fibre fraction and soy anions by the full process in laboratory scale

Fig. 2 illustrates a laboratory scale embodiment of the invention. The major unit operations are shown in Fig. 2, however, certain details as evident from the text below are not included in the figure. The various unit operations employed in the example may be varied and the specific unit operations easily replaced with other unit operations known within the art. Such variations will be within the understanding of the skilled person.

50.0 g gram starting material (HP-soy: defatted and toasted soy or WF-soy: defatted soy) were pre-heated to 70°C followed by addition of 250 mL hot (70°C) acid solution A (20 mM citric acid + 50 mM sulphuric acid). The sample was homogenised by ultra turrax for 1 min. at 6500 rpm followed by centrifugation at 1700 x *g* for 2 min. The supernatant was decanted and the pellet was submitted to further two extractions using similar conditions as above but only 200 mL hot acid solution A. The three acidic supernatants were pooled and stored at room temperature.

The pellet was added 200 mL hot (70°C) 50 mM NaOH solution and homogenised by ultra turrax (6500 rpm for 1 min.). The pH was adjusted to 9.0 ± 0.2 with 1 M HCl and the sample was centrifuged (1700 x *g* for 2 min.). The alkaline supernatant was decanted and the pellet was submitted to further two extractions using similar conditions as above using 200 mL hot (70°C) water. The three alkaline and water supernatants were pooled and stored at room temperature.

The pellet was added 200 mL acidic solution A and pH was adjusted to 4.7 ± 0.2 using 1 M NaOH. The suspension was centrifuged (1700 x *g* for 2 min). The neutral supernatant was stored at room temperature for further purification and the pellet (Protein 1A) was frozen (-20°C) and lyofilised.

The acid soluble proteins were precipitated from the acidic supernatants by adjusting the pH to 4.7 ± 0.2 with 1 M NaOH and the solution was centrifuged (1700 x *g*; 2 min). The pellet (acid soluble and neutral precipitated protein; Protein 2) was frozen and lyophilised. The supernatant was pooled with the neutral supernatant.

The alkaline soluble proteins were precipitated from the alkaline supernatants by adjusting the pH to 4.7 ± 0.2 with 1 M sulphuric acid and the solution was centrifuged (1700 x *g*; 2 min). The pellet (alkaline soluble and neutral precipitated protein; Protein 1B) was frozen and lyophilised. The supernatant was pooled with the neutral supernatant.

The pH of the neutral supernatant was adjusted to 3.5 ± 0.2 and submitted to cation-exchange chromatography using a 100 mL column (Amersham SP sepharose FF (Fastflow) on $H^+$ form). Unbound material was eluted by use of 20 mM acetate pH 3.5. The run through fraction was collected and stored for further purification. Elution of bound compounds was performed by use of 150 mL linear gradient (from 20 mM acetate pH 3.5 to 20 mM phosphate pH 8.2). Eluted proteins (neutral non-precipitated protein; Protein 3) were collected and pooled before freezing and lyophilisation.

The pH of the run through fraction after cation exchange chromatography was adjusted to 8.0 ± 0.2 and submitted to anion exchange chromatography using a 100 mL column (Amersham Q sepharose FF on acetate form). Unbound material was eluted by use of 10 mM phosphate pH 8.0. The run through fraction was collected, frozen and lyophilised (neutral non-protein constituents (Soluble carbohydrates). Elution of bound compounds was performed by use of 150 mL 0.5 M formic acid in 50 % ethanol. The eluted fraction was dried by evaporation.

0.5 – 1 gram sample (Protein 1A, Protein 1B and Protein 2) was heated at 550°C for 4 hours. Ash content was measured gravimetrically. The ash was quantitatively transferred into 50 mL flasks by use of 5 mL milli-Q water and 5.00 mL concentrated hydrochloric acid. The samples were heated in boiling water for 10 min prior to filtering (Frisenette AGF 165 – 50 mm) and quantitatively transferred into 100 mL measuring flasks by use of 5 x 1 mL milli-Q water. The flasks were added 10.0 mL 2 M KOH prior to filling with milli-Q water.

100 µL of each sample was transferred to a 50 mL measuring flask and 20 mL milli-Q water was added along with 5.00 mL phosphate reagents consisting of 1.7 g ascorbic acid in 300 mL ammonium molybdate solution (12.8 gram ammonium molybdate, 0.3 gram potassium timoyltartrate, 158 mL concentrated sulphuric acid and water op to 2000 mL). The flask was filled with milli-Q water and mixed. The absorbance was measured spectropho-

tometrically after 15 min. at 890 nm. Phytate content was calculated from the total content of phosphate (P) by use of a phosphate standard.

Table 1 Gravimetric yield (% of start mass) as well as product composition (% of dry matter).

| | Gravimetric yield % | Protein % | IDF % | SDF % | Sucrose % | Ash % |
|---|---|---|---|---|---|---|
| HP products | | | | | | |
| Protein 1A | 54 | 57 | 39 | 0 | 0 | 4 |
| Protein 1B | 10 | 91 | 2 | 0 | 0 | 7 |
| Protein 2 | 7 | 73 | 21 | 0 | 0 | 6 |
| Protein 3 | 5 | 96 | 0 | 0 | 0 | 4 |
| Soluble carbohydrates | 13 | 0 | 0 | 54 | 44 | 2 |
| WF products | | | | | | |
| Protein 1A | 16 | 47 | 45 | 0 | 0 | 8 |
| Protein 1B | 30 | 81 | 13 | 0 | 0 | 6 |
| Protein 2 | 20 | 83 | 13 | 0 | 0 | 5 |
| Protein 3 | 7 | 96 | 0 | 0 | 0 | 4 |
| Soluble carbohydrates | 14 | 0 | 0 | 50 | 48 | 2 |

*Protein determination based on %N*6,25; IDF = insoluble dietary fibres; SDF = soluble dietary fibres*

Table 2 Concentration of phytate in protein concentrates. Figures are calculated as the highest possible concentration based on total phosphate in the samples. Results are given in % relative to the concentration found in WF starting material. The recovery (%) is relative to the content found in WF starting material.

| | Phytate concentration[1] % | Recovery[2] % |
|---|---|---|
| HP products | | |
| Protein 1A | 22 | 12 |
| Protein 1B | 35 | 3 |
| Protein 2 | 65 | 5 |
| Protein 3 | 0 | 0 |
| Soluble carbohydrates | 0 | 0 |
| WF products | | |
| Protein 1A | 17 | 3 |
| Protein 1B | 65 | 19 |
| Protein 2 | 28 | 6 |
| Protein 3 | 0 | 0 |
| Soluble carbohydrates | 0 | 0 |

*1) Estimate based on total P in sample relative to P in WF starting material*

*2) Recovery relative to content in WF starting material*

The mass balances for the processing of WF-soy and HP soy are provided in Tables 3 and 4

Table 3 Mass balance for lab-scale processing of HP-soy

| Fraction | Gravimetric yield | Protein | IDF | SDF | Sucrose | Phytate recovery |
|---|---|---|---|---|---|---|
| Start | 100 | | | | | |
| Protein 1A | 54 | 30.8 | 21.1 | 0 | 0 | 12 |
| Protein 1B | 10 | 9.1 | 0.2 | 0 | 0 | 3 |
| Protein 2 | 7 | 5.1 | 1.5 | 0 | 0 | 5 |
| Protein 3 | 5 | 4.8 | 0 | 0 | 0 | 0 |
| Soluble carbohydrates | 13 | 0 | 0 | 7.0 | 5.7 | 0 |
| Subtotal | | 49.8 | 22.8 | 7.0 | 5.7 | 20 |
| Total | 89 | 85.3 | | | | |

Table 4 Mass balance for lab-scale processing of WF-soy

| Fraction | Gravimetric yield | Protein | IDF | SDF | Sucrose | Phytate recovery |
|---|---|---|---|---|---|---|
| Start | 100 | | | | | |
| Protein 1A | 16 | 7.5 | 7.2 | 0 | 0 | 3 |
| Protein 1B | 30 | 24.3 | 3.9 | 0 | 0 | 19 |
| Protein 2 | 20 | 16.6 | 2.6 | 0 | 0 | 6 |
| Protein 3 | 7 | 6.7 | 0 | 0 | 0 | 0 |
| Soluble carbohydrates | 14 | 0 | 0 | 7 | 6.7 | 0 |
| Subtotal | | 55.1 | 13.7 | 7 | 6.7 | 28 |
| Total | 87 | 82.5 | | | | |

As seen from the mass balances in Tables 3 and 4 all protein fractions were reduced in phytate content over the starting material. In particular only about 20 to about 30 percent of the phytate was accounted for in the protein fractions. The majority of the phytate was present in the eluate from the anion-exchange step. This fraction would also contain the saponins and anions, such as citrate and sulphate used in the process.

**3. Production of protein concentrates in pilot scale**

3.1. Production of soy protein concentrates and isolates from white flake soy in pilot scale

Fig. 3 illustrates a pilot scale embodiment of the invention. The major unit operations are shown in Fig. 3, however, certain details as evident from the text below are not included in the figure. The various unit operations employed in the example may be varied and the specific unit operations easily replaced with other unit operations known within the art. Such variations will be within the understanding of the skilled person.

15.0 kg defatted soybean flakes (white flakes; WF-soy) was added 75 L hot (60°C) 25 mM citric acid and pH was adjusted to 2.0 ± 0.2 with sulphuric acid. The suspension was homogenised by a fryma mill (0.25 mm). The homogenate was drained in a centrifugal sieve (ø 25 cm; pore 125 µm). The undissolved material was washed by re-suspending the filter cake with 2 times 50 L hot (60°C) water followed by homogenisation and draining as described above. The filter cake was finally re-suspended in water and pH was

adjusted to 4.7 ± 0.2 with sodium hydroxide prior to draining in the centrifugal sieve and drying by use of spin flash dryer (Protein 1).

The filtrates from the centrifugal sieve were pooled and pH was adjusted to 4.7 ± 0.2 with sodium hydroxide. The solution was stored at 4°C overnight. The clear upper fraction was decanted and the precipitate was purified by two times centrifugation at 1700 x *g* for 3 min. with intermediate re-suspension in water (1:1 V/V). The decanted clear soluble phase and the supernatants were pooled. The pellet was thereafter dried by use of spin flash dryer (Protein 2).

The soluble phase was submitted to cation exchange chromatography using SP-Sepharose BB (Big Beads) column material (GE Healthcare, Denmark). Unbound material was flushed out of the column using water (Run through) while the bound material was eluted by use of 10 mM sodium hydroxide. The protein elution profile was monitored by UV detection at 280 nm. Eluted proteins were pooled and pH adjusted to 5.0 ± 0.2 prior to spray drying (Protein 3).

Table 5 Gravimetric yield (% of start mass) as well as product composition (% of dry matter).

| | Gravimetric yield % | Protein % | IDF % | SDF % | Sucrose % | Ash % |
|---|---|---|---|---|---|---|
| Protein 1 | 40 | 71 | 23 | 0 | 0 | 6 |
| Protein 2 | 30 | 95 | 3 | 0 | 0 | 2 |
| Protein 3 | 4 | 99 | 0 | 0 | 0 | 1 |
| Run through | 27 | 0 | 0 | 50 | 48 | 2 |

*Protein determination based on %N*6,25; IDF = insoluble dietary fibres; SDF = soluble dietary fibres*

3.2. Production of soy protein concentrates and isolates from HP-soy in pilot scale

Fig. 4 illustrates a pilot scale embodiment of the invention. The major unit operations are shown in Fig. 4, however, certain details as evident from the text below are not included in the figure. The various unit operations employed in the example may be varied and the specific unit operations easily replaced with other unit operations known within the art. Such variations

will be within the understanding of the skilled person.

15.0 kg de-fatted and toasted soybean flakes (HP-soy) was added 75 L hot (60°C) 25 mM citric acid and pH was adjusted to 1.7 ± 0.2 with sulphuric acid. The suspension was homogenised by a fryma mill (0.25 mm). The homogenate was drained in a centrifugal sieve (ø 25 cm; pore 125 µm). The un-dissolved material was washed by re-suspending the cake with 2 times 50 L hot (60°C) water followed by homogenisation and draining as described above. The filter cake was finally re-suspended in water and pH was adjusted to 4.7 ± 0.2 with sodium hydroxide prior to draining in the centrifugal sieve (Protein 1).

The filtrates from the centrifugal sieve were pooled and pH was adjusted to 4.7 ± 0.2 with sodium hydroxide. The solution was stored at 4°C overnight. The clear upper fraction was decanted and the precipitate was purified by two times frame filtering (BECO –KD7 Depth filter sheet) with intermediate re-suspension in water (1:1 V/V). The decanted clear soluble phase and the filtrate were pooled. The filter cake (Protein 2) was pooled with Protein 1 and dried by use of spin flash dryer (Protein 1+2).

The soluble phase was submitted to cation-exchange chromatography using SP-Sepharose BB column material (GE Healthcare, Denmark). Unbound material was flushed out of the column using water (Run through) while the bound material was eluted by use of 10 mM sodium hydroxide. The protein elution profile was monitored by UV detection at 280 nm. Eluted proteins were pooled and pH adjusted to 5.0 ± 0.2 prior to spray drying (Protein 3).

Table 6 Gravimetric yield (% of start mass) as well as product composition (% of dry matter).

| | Gravimetric yield % | Protein % | IDF % | SDF % | Sucrose % | Ash % |
|---|---|---|---|---|---|---|
| Protein 1 + 2 | 59 | 67 | 27 | 0 | 0 | 6 |
| Protein 3 | 13 | 99 | 0 | 0 | 0 | 1 |
| Run through | 28 | 0 | 0 | 49 | 49 | 2 |

*Protein determination based on %N*6,25; IDF = insoluble dietary fibres; SDF = soluble dietary fibres*

3.3. Recovery of trypsin inhibitors in protein concentrates after pilot plant protein isolation

0.5 g Protein (WF protein 1, WF protein 2 (section 3.1) and HP protein 1+2 (section 3.1)) is extracted in 2 x 5 mL 100 mM acetate buffer pH 4.9 for 2 x 1 min by use of ultra turrax. The extracts are centrifuged (two 3000 x *g* for 2 min.) and the clear supernatants of each protein concentrate is pooled and stored overnight at 4°C. The solutions are re-centrifuged to remove precipitations and the clear supernatants are decanted. The content of trypsin inhibitors are measured by a spectrophotometric assay using porcine trypsin and Nα-benzoyl-L-arginine-4-nitroanilid (L-BAPA) as substrate measuring the colour of the hydrolysate at 410 nm. Trypsin activity is defined as the amount of enzyme needed to hydrolyse 1 µmol L-BAPA in 1 min (25°C; pH 8.2). Trypsin inhibitor unit (TIU) is defined as the amount of inhibitor protein needed to inhibit 1 trypsin unit.

The extract was further analysed for the content of saponins by use of TLC chromatography using silica gel sheets.

Table 7 Content of trypsin inhibitors in protein concentrates. Trypsin inhibitor units (TIU) is defined in the text.

| Protein sample | TIU/g |
|---|---|
| HP products | |
| Start | 0.2 |
| Protein 1+2 | 0.1 |
| WF products | |
| Start | 12.4 |
| Protein 1 | 0.4 |
| Protein 2 | 0.6 |

# PATENT CLAIMS

1. A process for the manufacture of a product from a plant material comprising the steps of:

- providing a disrupted plant material comprising <10 %(w/w) starch and <10 %(w/w) oil/lipids;

- adjusting the pH of the disrupted plant material to a value of pH 3.5 or below to provide an acidic suspension;

- heating the acidic suspension to a temperature in the range of about 50°C to about 80°C;

- isolating the product from the heated, acidic suspension.

2. The process for the manufacture of a product according to claim 1, wherein the pH is in the range of about 1.0 to about 3.5.

3. The process for the manufacture of a product according to claim 1 or 2, wherein the pH is about 2.0.

4. The process for the manufacture of a product according to any one of claims 1 to 3, wherein the temperature is in the range of about 60°C to about 80°C.

5. The process for the manufacture of a product according to any one of claims 1 to 4, wherein the temperature is about 65°C.

6. The process for the manufacture of a product according to any one of claims 1 to 5, wherein the pH is about 2.0 and the temperature is about 65°C.

7. The process for the manufacture of a product according to any one of claims 1 to 6, wherein the pH is adjusted with an inorganic acid or an organic acid or a combination of inorganic acids, organic acids or inorganic acids and organic acids.

8. The process for the manufacture of a product according to any one of claims 1 to 7, wherein the pH is adjusted with an acid capable of serving as a chelating agent.

9. The process for the manufacture of a product according to any one of claims 1 to 8, wherein the acid comprises and acid selected from the group consisting of citric acid, oxalic acid, lactic acid, malic acid, maleonic acid, tartaric acid, succinic acid or a combination thereof.

10. The process for the manufacture of a product according to any one of claims 1 to 9, wherein the acidic suspension is buffered.

11. The process for the manufacture of a product according to any one of claims 1 to 10, wherein the pH adjustment is performed prior to, simultaneously with or after heating the plant material.

12. The process for the manufacture of a product according to any one of claims 1 to 11, wherein the plant material is derived from a legume.

13. The process for the manufacture of a product according to claim 12, wherein the legume is selected from the group consisting of soy, lupine, faba beans, broad bean and peas.

14. The process for the manufacture of a product according to any one of claims 1 to 11, wherein the plant material is derived from potatoes.

15. The process for the manufacture of a product according to any one of claims 1 to 14, wherein the plant material is in dry form and is suspended in liquid at about 50 g/L to about 500 g/L.

16. The process for the manufacture of a product according to any one of claims 1 to 15, wherein the duration of the period the plant material is maintained at the increased temperature is from about 1 minute to about 10 minutes.

17, The process for the manufacture of a product according to any one of claims 1 to 16, wherein no phytase enzyme or phytase enzyme inhibitor is added.

18. A process for the manufacture of a product according to any one of claims 1 to 17, comprising the steps of:

- subjecting the heated, acidic suspension to a solid-liquid separation to provide an acid precipitated fraction and an acid soluble liquid fraction.

- isolating the product from the acid precipitated fraction or from the acid soluble liquid fraction.

19. A process for the manufacture of a product according to claim 18, comprising the steps of:

- suspending the acid precipitated fraction in a liquid and adjusting the pH to a value above pH 4.9, such as from about 7.0 to about 12.0 to provide an alkaline suspension;

- subjecting the alkaline suspension to a solid-liquid separation to provide an alkaline precipitated fraction and an alkaline soluble liquid fraction;

- isolating the product from the alkaline precipitated fraction or from the alkaline soluble liquid fraction.

20. A process for the manufacture of a product according to any one of claims 1 to 19, comprising the steps of:

- adjusting the pH of the heated, acidic suspension or the acid soluble liquid fraction or the alkaline soluble liquid fraction to a value from about 4.0 to about 6.0 to provide a neutral suspension;

- isolating the product from the neutral suspension.

21. A process for the manufacture of a product according to claim 20, comprising the step of:

- subjecting the neutral suspension to a solid-liquid separation to provide a neutral precipitate and a neutral supernatant;

- isolating the product from the neutral precipitate or from the neutral supernatant.

22. The process for the manufacture of a product according to any one of claims 1 to 21, wherein the heated, acidic suspension, the acid precipitated fraction, the acid soluble liquid fraction, the alkaline precipitated fraction, the alkaline soluble liquid fraction, the neutral suspension, the neutral precipitate, and/or the neutral supernatant is subjected to homogenisation.

23. The process for the manufacture of a product according to any one of claims 1 to 22, wherein the alkaline or heated, acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension and/or the neutral supernatant is clarified.

24. A process for the manufacture of a product according to any one of claims 1 to 23, comprising the steps of:

- optionally adjusting the pH of the alkaline or heated, acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension or the neutral supernatant to a value from about 3.0 to about 4.0;

- contacting the alkaline or heated acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension or the neutral supernatant with a cation-exchange chromatography resin to separate a cationic binding fraction from a cationic non-binding fraction;

- contacting the cation-exchange chromatography resin with an eluent solution to elute the product from the cationic binding fraction from the

cation-exchange chromatography resin.

25. A process for the manufacture of a product according to claim 24, wherein the eluent solution has a higher pH and/or a higher ionic strength than the pH and the ionic strength, respectively, of the liquid fraction or suspension contacted with the cation-exchange chromatography resin.

26. A process for the manufacture of a product according to any one of claims 1 to 23, comprising the steps of:

- optionally adjusting the pH of the alkaline or heated, acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension or the neutral supernatant to a value from about 3.0 to about 4.0;

- contacting the alkaline or heated acidic suspension, or the acid or the alkaline soluble liquid fraction or the neutral suspension or the neutral supernatant with a cation-exchange chromatography resin to separate a cationic binding fraction from a cationic non-binding fraction;

- isolating the product from the cationic non-binding fraction.

27. A process for the manufacture of a product according to claim 26, comprising the steps of:

- optionally adjusting the pH of the cationic non-binding fraction to a value from about 7.0 to about 9.0;

- contacting the cationic non-binding fraction with an anion-exchange chromatography resin to separate an anionic binding fraction from an anionic non-binding fraction;

- contacting the anion-exchange chromatography resin with an eluent solution to elute the product from the anionic binding fraction from the anion-exchange chromatography resin.

28. A process for the manufacture of a product according to claim 27, wherein the eluent solution has a lower pH and/or a higher ionic strength than the pH and the ionic strength, respectively, of the cationic non-binding fraction contacted with the anion-exchange chromatography resin and/or the eluent solution comprises an organic solvent.

29. A process for the manufacture of a product according to claim 26, comprising the steps of:

- optionally adjusting the pH of the cationic non-binding fraction to a value from about 7.0 to about 9.0;

- contacting the cationic non-binding fraction with an anion-exchange chromatography resin to separate an anionic binding fraction from an anionic non-binding fraction;

- isolating the component from the anionic non-binding fraction.

30. The process for the manufacture of a product according to any one of claims 24 to 29, wherein the plant material is contacted with the cation-exchange or the anion-exchange chromatography resin in a stirred tank reactor, or in a packed or expanded bed column, or wherein the cation-exchange or the anion-exchange chromatography resin is a membrane.

31. The process for the manufacture of a product according to any one of claims 17 to 30, wherein the solid-liquid separation is selected from filtration, such as vacuum filtration, pressure filtration, cross-flow filtration, basket centrifugation, deep bed filtration, or centrifugation, e.g. in a solid bowl centrifuge, tubular bowl centrifuge, decanter centrifuge, disk-stack centrifuge or a combination of filtration and centrifugation, e.g. a centrifugal sieve or a peeler centrifuge.

32. The process for the manufacture of a product according to any one of claims 17 to 31 further comprising the step of adding flocculating agents and/or filter aids prior to or during the solid-liquid separation.

33. The process for the manufacture of a product according to any one of claims 1 to 32, wherein a liquid fraction or suspension is further subjected to a concentrating step.

34. The process for the manufacture of a product according to claim 33, wherein the concentrating step is selected from ultrafiltration, diafiltration, nanofiltration, evaporation, vacuum evaporation, freeze drying, spin flash drying, spray drying, floating bed drying or a combination thereof.

35. A process for the manufacture of a product according to any one of claims 17 to 34, wherein the product isolated from the acid precipitated fraction is an insoluble fibre product.

36. A process for the manufacture of a product according to any one of claims 17 to 34, wherein the product isolated from the acid precipitated fraction is a protein isolate.

37. A process for the manufacture of a product according to any one of claims 18 to 34, wherein the product isolated from the alkaline precipitated fraction is a fibre isolate.

38. A process for the manufacture of a product according to any one of claims 21 to 34, wherein the product isolated from the neutral precipitate is a protein isolate.

39. A process for the manufacture of a product according to any one of claims 24 to 34, wherein the product isolated from the cationic binding fraction is a protein concentrate or a protein isolate or a glycoalkaloid product or an alkaloid product.

40. A process for the manufacture of a product according to any one of claims 27 to 34, wherein the product isolated from the anionic binding fraction is a phytate product or a saponin product or a phenolic product.

41. A process for the manufacture of a product according to any one of claims 26 to 34, wherein the product isolated from the cationic or the anionic non-binding fraction is a soluble fibre or a carbohydrate product.

42. A process for the manufacture of a product according to any one of claims 26 to 34, wherein the product isolated from the cationic or the anionic non-binding fraction is an alkaloid or a glycoalkaloid product.

43. An integrated process for the manufacture of a product from a plant material comprising the process according to claim 19 combined with one or more of the processes according to 20 to 34.

44. A product obtainable in a process according to any one of claims 1 to 34 or 43, wherein the product is a protein isolate, a protein concentrate with a reduced relative content of phytate and/or saponins, a phytate product, a saponin product, a phenolics product, an alkaloid or a glycoalkaloid product with a reduced relative content of protein, or a fibre product or a carbohydrate product with a reduced relative content of protein.

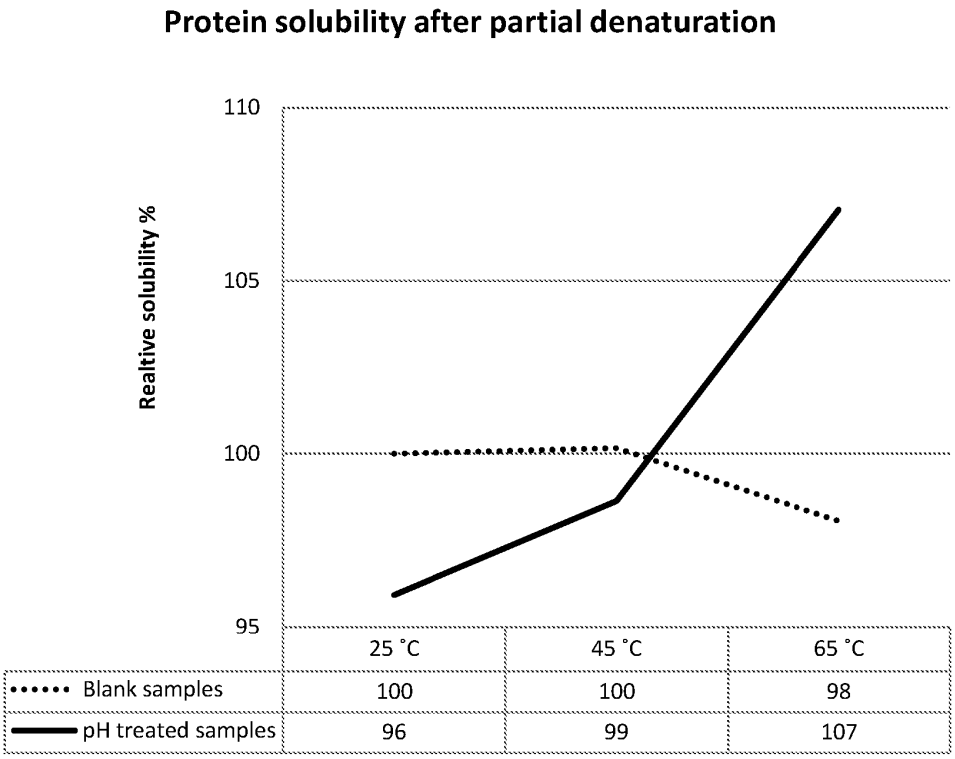

Protein solubility after partial denaturation
Realtive solubility %
110
105
100
95
25 ˚C
45 ˚C
65 ˚C
Blank samples
100
100
98
pH treated samples
96
99
107

**Fig. 1**