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PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
351	PHE0006171_16491.pep	Glyoxalase	70	214	136.8	5.90E-38
353	PHE0006906_16796.pep	Proteasome	10	206	145.9	1.00E-40
354	PHE0006649_16180.pep	AP2	46	110	149.5	8.60E-42
355	PHE0006809_16390.pep	Prismane	1	548	886.8	9.80E-264
356	PHE0006918_16808.pep	SWIM	321	353	42.8	1.10E-09
357	PHE0007813_18219.pep	MtN3_slv	9	98	79.8	8.50E-21
357	PHE0007813_18219.pep	MtN3_slv	132	218	125.3	1.80E-34
359	PHE0006825_16405.pep	Cytochrom_C552	27	476	1333.6	0
360	PHE0006964_16864.pep	tRNA_anti	43	122	55.2	2.10E-13
360	PHE0006964_16864.pep	tRNA-synt_2	140	557	208.2	1.90E-59
361	PHE0008105_18407.pep	Histone	152	225	69.9	8.40E-18
361	PHE0008105_18407.pep	CBFD_NFYB_HMF	158	222	77.9	3.10E-20
362	PHE0006630_16159.pep	Ldh_1_N	90	239	202	1.30E-57
362	PHE0006630_16159.pep	Ldh_1_C	241	413	258.1	1.80E-74
363	PHE0006966_16868.pep	DNA_photolyase	12	183	298.7	1.10E-86
363	PHE0006966_16868.pep	FAD_binding_7	214	492	536.3	3.30E-158
365	PHE0006608_16123.pep	GSHPx	42	150	237.8	2.30E-68
366	PHE0010462_21459.pep	NUDIX	17	159	111.7	2.20E-30
366	PHE0010462_21459.pep	NUDIX	190	322	100.5	4.90E-27
367	PHE0009211_21774.pep	Auxin_inducible	1	106	149.5	8.80E-42
368	PHE0006256_8775.pep	RPE65	105	591	864.5	5.10E-257
369	PHE0006989_16918.pep	Pkinase	88	396	224.8	1.90E-64
370	PHE0007649_18166.pep	Ank	108	140	50.4	6.20E-12
370	PHE0007649_18166.pep	Pkinase	196	455	115.1	2.00E-31
370	PHE0007649_18166.pep	Pkinase_Tyr	196	455	98.9	1.50E-26
371	PHE0006708_16264.pep	DEAD	57	223	202.7	8.40E-58
371	PHE0006708_16264.pep	Helicase_C	291	367	127.8	3.10E-35
372	PHE0001117_19094.pep	Homeobox	79	133	68.2	2.70E-17
372	PHE0001117_19094.pep	HALZ	134	178	66.7	7.20E-17
373	PHE0006266_8814.pep	Fe_bilin_red	16	244	209.5	7.50E-60
374	PHE0006795_16378.pep	Na_H_antipport_1	4	380	875.9	1.90E-260
375	PHE0006956_16853.pep	Molybdop_Fe4S4	2	56	92	1.90E-24
375	PHE0006956_16853.pep	Molybdopterin	59	482	377.8	1.60E-110
375	PHE0006956_16853.pep	Molydop_binding	578	689	159.8	7.10E-45
375	PHE0006956_16853.pep	Fer2_BFD	825	877	62	2.00E-15
377	PHE0006824_16404.pep	Globin	14	154	93.4	7.00E-25
378	PHE0006812_16393.pep	Bac_globin	3	118	188.4	1.70E-53
379	PHE0006815_16396.pep	Bac_globin	26	146	191.4	2.10E-54
380	PHE0006806_16387.pep	Bac_globin	26	146	196.8	5.10E-56
381	PHE0006624_16153.pep	NAD_binding_2	3	178	282.5	7.90E-82
381	PHE0006624_16153.pep	6PGD	182	473	713.8	1.20E-211
382	PHE0005006_15823.pep	Chloroa_b-bind	63	242	241.4	1.90E-69
384	PHE0006683_16229.pep	Response_reg	10	151	73.8	5.40E-19
385	PHE0006884_16703.pep	Glyco_transf_5	83	344	462.7	4.80E-136
385	PHE0006884_16703.pep	Glycos_transf_1	384	567	40.2	7.10E-09
386	PHE0006691_16237.pep	NAD_Gly3P_dh_N	6	165	285	1.40E-82

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386	PHE0006691_16237.pep	ApbA	8	169	6.6	0.00011
386	PHE0006691_16237.pep	NAD_Gly3P_dh_C	183	327	289.1	8.20E-84
387	PHE0006790_16372.pep	Rieske	220	317	96.7	7.10E-26
387	PHE0006790_16372.pep	PaO	408	507	180.7	3.60E-51
388	PHE0009143_19932.pep	Histone_HNS	21	129	170.1	5.70E-48
389	PHE0008406_18830.pep	AA_permease	57	511	619.8	2.40E-183
390	PHE0008279_18708.pep	Mit_rib_S27	14	93	135.3	1.70E-37
391	PHE0009142_19931.pep	Histone_HNS	21	130	175.1	1.70E-49
393	PHE0007639_17991.pep	ELFV_dehydrog_N	84	214	298.9	9.20E-87
393	PHE0007639_17991.pep	ELFV_dehydrog	229	474	469.7	3.60E-138
394	PHE0007646_18170.pep	F-box	36	83	51.8	2.20E-12
394	PHE0007646_18170.pep	Kelch_1	131	185	25.1	0.00026
394	PHE0007646_18170.pep	Kelch_1	187	233	44.2	4.40E-10
394	PHE0007646_18170.pep	Kelch_2	187	233	26.1	0.00012
395	PHE0007765_18182.pep	GST_N	5	79	69.6	1.00E-17
395	PHE0007765_18182.pep	GST_C	101	204	28.8	1.90E-05
396	PHE0008167_18465.pep	Pkinase	8	262	210	5.60E-60
396	PHE0008167_18465.pep	Pkinase_Tyr	8	262	247.9	2.10E-71
398	PHE0008183_18479.pep	LRRNT_2	23	66	49.3	1.30E-11
398	PHE0008183_18479.pep	LRR_1	71	93	12.1	1.6
398	PHE0008183_18479.pep	LRR_1	95	117	10.3	3.3
398	PHE0008183_18479.pep	LRR_1	119	142	13	1
398	PHE0008183_18479.pep	LRR_1	144	166	19.5	0.012
398	PHE0008183_18479.pep	LRR_1	168	190	10.6	3
398	PHE0008183_18479.pep	LRR_1	192	214	8.8	6.4
398	PHE0008183_18479.pep	LRR_1	289	311	17.3	0.055
398	PHE0008183_18479.pep	LRR_1	313	335	10.6	2.9
398	PHE0008183_18479.pep	LRR_1	337	359	10.8	2.8
398	PHE0008183_18479.pep	LRR_1	361	384	12	1.6
398	PHE0008183_18479.pep	LRR_1	409	431	10.4	3.2
398	PHE0008183_18479.pep	LRR_1	457	479	11.9	1.7
398	PHE0008183_18479.pep	LRR_1	481	503	10.2	3.4
398	PHE0008183_18479.pep	LRR_1	505	527	10.4	3.3
398	PHE0008183_18479.pep	LRR_1	529	551	10.9	2.6
398	PHE0008183_18479.pep	LRR_1	553	575	9.3	5.2
398	PHE0008183_18479.pep	LRR_1	577	598	11	2.4
398	PHE0008183_18479.pep	Pkinase	695	966	133.8	4.80E-37
398	PHE0008183_18479.pep	Pkinase_Tyr	695	966	134.8	2.30E-37
399	PHE0007585_17875.pep	LEA_3	2	98	138.9	1.30E-38
400	PHE0006804_16385.pep	zf-CCCH	13	39	35.4	1.90E-07
400	PHE0006804_16385.pep	zf-CCCH	149	174	24.3	0.00045
401	PHE0006703_15751.pep	PFK	72	347	650.2	1.70E-192
402	PHE0007410_17653.pep	Acyltransferase	27	249	29.2	1.00E-05
403	PHE0006854_16456.pep	YABBY	40	278	249.1	9.30E-72
404	PHE0007721_21293.pep	KNOX1	102	146	90.4	5.50E-24
404	PHE0007721_21293.pep	KNOX2	153	204	101.2	3.10E-27

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404	PHE0007721_21293.pep	ELK	242	263	37	6.50E-08
405	PHE0006563_15990.pep	GATase_2	2	161	103.9	4.70E-28
405	PHE0006563_15990.pep	Asn_synthase	209	450	328.7	9.80E-96
406	PHE0006619_16147.pep	AA_kinase	15	261	192.3	1.20E-54
407	PHE0006813_16394.pep	Bac_globin	7	124	123	8.50E-34
408	PHE0006688_16234.pep	Ribosomal_S2	13	229	448.6	8.00E-132
409	PHE0006635_16164.pep	MGS	28	121	99.4	1.10E-26
411	PHE0008416_18838.pep	PTR2	115	504	350.4	3.00E-102
413	PHE0008599_19166.pep	Isoamylase_N	24	115	93.4	6.60E-25
413	PHE0008599_19166.pep	Alpha-amylase	164	578	75.8	1.40E-19
414	PHE0008375_18732.pep	Pribosyltran	93	237	141.8	1.80E-39
416	PHE0008394_18763.pep	Gln-synt_N	68	148	147.3	4.20E-41
416	PHE0008394_18763.pep	Gln-synt_C	154	406	287.7	2.20E-83
417	PHE0008395_18764.pep	Gln-synt_N	68	148	147.3	4.20E-41
417	PHE0008395_18764.pep	Gln-synt_C	154	406	285	1.50E-82
418	PHE0008444_18846.pep	Gln-synt_N	17	97	150.4	4.70E-42
418	PHE0008444_18846.pep	Gln-synt_C	103	355	283.9	3.00E-82
419	PHE0006819_16400.pep	Lactamase_B	47	236	77.1	5.60E-20
419	PHE0006819_16400.pep	Flavodoxin_1	267	397	8.1	0.0041
419	PHE0006819_16400.pep	Flavin_Reduct	424	573	254.7	1.90E-73
420	PHE0007417_17663.pep	EIN3	60	467	777.5	7.80E-231
421	PHE0008443_18845.pep	GATase_2	2	161	98.7	1.70E-26
421	PHE0008443_18845.pep	Asn_synthase	209	450	331	2.00E-96
422	PHE0006818_16399.pep	Lactamase_B	34	227	105.3	1.80E-28
422	PHE0006818_16399.pep	Flavodoxin_1	256	389	86.4	8.80E-23
422	PHE0006818_16399.pep	Rubredoxin	423	471	89.8	8.10E-24
423	PHE0006579_16031.pep	Thi4	63	306	552.2	5.40E-163
423	PHE0006579_16031.pep	DAO	91	319	-9	1.90E-05
424	PHE0006808_16389.pep	CCT	354	392	75.3	1.90E-19
425	PHE0008160_18460.pep	TPP_enzyme_N	45	221	286.5	5.20E-83
425	PHE0008160_18460.pep	TPP_enzyme_M	243	376	82.1	1.70E-21
425	PHE0008160_18460.pep	TPP_enzyme_C	431	578	34.8	2.80E-09
426	PHE0007571_17834.pep	GSH_synth_ATP	29	499	836.5	1.30E-248
426	PHE0007571_17834.pep	GSH_synthase	229	331	200	5.50E-57
427	PHE0007586_17877.pep	Chloroa_b-bind	64	261	255.3	1.20E-73
429	PHE0009729_21717.pep	Homeobox	67	123	68.9	1.60E-17
429	PHE0009729_21717.pep	HALZ	124	168	53.4	7.60E-13
430	PHE0006424_15520.pep	PsbW_2	1	133	382.1	8.60E-112
431	PHE0006392_15480.pep	PsbP	106	322	252.9	6.50E-73
432	PHE0006589_16093.pep	Thioredoxin	64	167	130.3	5.20E-36
433	PHE0009258_21487.pep	Na_H_Exchanger	22	441	181.7	1.70E-51
434	PHE0006680_16226.pep	2OG-FeII_Oxy	187	287	158	2.40E-44
435	PHE0006481_16072.pep	adh_short	33	181	28.8	7.00E-08
436	PHE0006269_8820.pep	TPR_1	74	107	21.3	0.0035
436	PHE0006269_8820.pep	TPR_2	74	107	24.7	0.00034
436	PHE0006269_8820.pep	TPR_1	108	141	37.4	4.80E-08

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436	PHE0006269_8820.pep	TPR_2	108	141	35.3	2.10E-07
436	PHE0006269_8820.pep	TPR_1	142	175	15	0.086
436	PHE0006269_8820.pep	TPR_2	142	175	23.8	0.00063
436	PHE0006269_8820.pep	TPR_1	179	212	7.9	0.61
436	PHE0006269_8820.pep	TPR_4	247	272	23.6	0.00069
436	PHE0006269_8820.pep	TPR_1	281	314	16.2	0.063
436	PHE0006269_8820.pep	TPR_2	281	314	26	0.00014
436	PHE0006269_8820.pep	TPR_1	349	382	13.6	0.13
437	PHE0006783_16365.pep	Aa_trans	32	469	574.9	7.90E-170
438	PHE0003151_18392.pep	AP2	318	391	149.5	8.70E-42
438	PHE0003151_18392.pep	AP2	420	485	125.8	1.20E-34
439	PHE0008272_18723.pep	HSF_DNA-bind	18	188	213.9	3.50E-61
440	PHE0008277_18706.pep	SNF5	172	400	471.8	8.60E-139
441	PHE0008109_18411.pep	Sad1_UNC	203	330	195.6	1.10E-55
442	PHE0008280_18826.pep	Alpha-amylase	14	452	198.9	1.20E-56
443	PHE0002424_15825.pep	adh_short	43	215	24.9	1.30E-07
443	PHE0002424_15825.pep	KR	43	200	-69.1	0.0024
444	PHE0006914_16804.pep	AAA	204	391	298.6	1.10E-86
445	PHE0006642_16172.pep	p450	42	496	213.5	4.80E-61
447	PHE0009926_21079.pep	DSPc	24	152	137.5	3.60E-38
448	PHE0006206_19159.pep	Sina	106	305	426.4	4.00E-125
449	PHE0008274_18705.pep	BURP	56	280	380	3.60E-111
450	PHE0009510_20526.pep	Spermine_synth	38	283	500.4	2.10E-147
451	PHE0009937_21097.pep	Spermine_synth	38	283	500.4	2.10E-147
452	PHE0009927_21080.pep	DSPc	24	162	146.6	6.60E-41
454	PHE0006445_15956.pep	Allene_ox_cyc	74	253	401.6	1.20E-117
455	PHE0006987_16893.pep	efhand	86	114	25	0.00026
455	PHE0006987_16893.pep	efhand	167	195	27.6	4.30E-05
456	PHE0004230_15865.pep	E2F_TDP	146	211	133.8	4.60E-37
457	PHE0006814_16395.pep	Bac_globin	26	144	206	8.80E-59
458	PHE0002773_15881.pep	NTP_transferase	88	366	415.4	8.30E-122
459	PHE0006060_15842.pep	ubiquitin	5	73	138	2.50E-38
459	PHE0006060_15842.pep	ubiquitin	81	149	138	2.50E-38
459	PHE0006060_15842.pep	ubiquitin	157	225	138	2.50E-38
459	PHE0006060_15842.pep	ubiquitin	233	301	138	2.50E-38
460	PHE0006850_16451.pep	YABBY	6	166	342.9	5.20E-100
461	PHE0008043_18199.pep	Homeobox	25	85	64.6	3.20E-16
461	PHE0008043_18199.pep	START	164	374	198.3	1.80E-56
461	PHE0008043_18199.pep	MEKHLA	693	840	296.7	4.40E-86
462	PHE0000125_18852.pep	Response_reg	28	153	25.4	3.80E-05
462	PHE0000125_18852.pep	CCT	457	495	70.6	5.10E-18
463	PHE0008269_18720.pep	Pkinase	23	279	234	3.30E-67
464	PHE0008264_18712.pep	zf-UBR	40	109	73.4	7.10E-19
465	PHE0002782_20059.pep	PGI	2	457	172.8	8.80E-49
466	PHE0009438_20404.pep	GATA	191	226	72.7	1.10E-18
467	PHE0009429_20392.pep	ParBc	70	151	47.5	4.50E-11

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468	PHE0009440_20406.pep	ParBc	11	126	55.3	2.00E-13
469	PHE0008543_18945.pep	TP_methylase	222	435	272.6	7.70E-79
470	PHE0006960_16857.pep	Oxidored_molyb	116	301	289.2	7.70E-84
470	PHE0006960_16857.pep	Mo-co_dimer	326	459	300.9	2.30E-87
470	PHE0006960_16857.pep	Cyt-b5	515	588	93.3	7.20E-25
470	PHE0006960_16857.pep	FAD_binding_6	633	740	212.6	9.20E-61
470	PHE0006960_16857.pep	NAD_binding_1	759	872	169.6	8.00E-48
471	PHE0009427_20389.pep	IGPD	103	247	372.6	6.20E-109
472	PHE0006437_15906.pep	Pkinase	39	307	15.8	1.70E-09
473	PHE0007409_17652.pep	DAGAT	64	340	278.2	1.60E-80
474	PHE0009426_20387.pep	PHP	2	281	104.4	3.30E-28
475	PHE0009430_20393.pep	ParBc	44	128	45.7	1.60E-10
476	PHE0007003_16906.pep	PTPA	96	396	524	1.60E-154
477	PHE0008378_18741.pep	bZIP_1	223	280	76.5	8.20E-20
477	PHE0008378_18741.pep	bZIP_2	224	277	47.5	4.40E-11
478	PHE0008273_18725.pep	zf-CCCH	80	106	39.3	1.30E-08
479	PHE0008547_18953.pep	Fer2	20	95	95	2.20E-25
480	PHE0006922_16812.pep	Hexokinase_1	44	251	260.7	2.90E-75
480	PHE0006922_16812.pep	Hexokinase_2	258	501	261.2	2.10E-75
481	PHE0006920_16810.pep	AAA	267	458	270	4.60E-78
481	PHE0006920_16810.pep	Vps4_C	464	514	4.5	0.00031
482	PHE0008396_18765.pep	Gln-synt_N	68	148	147.3	4.20E-41
482	PHE0008396_18765.pep	Gln-synt_C	154	406	285.4	1.10E-82
483	PHE0006957_16854.pep	Gln-synt_N	68	148	147.3	4.20E-41
483	PHE0006957_16854.pep	Gln-synt_C	154	406	285.4	1.10E-82
484	PHE0006404_15936.pep	RRM_1	8	77	81.4	2.80E-21
484	PHE0006404_15936.pep	RRM_1	112	182	81.8	2.10E-21
485	PHE0006820_16401.pep	Lactamase_B	47	236	82.8	1.10E-21
485	PHE0006820_16401.pep	Flavin_Reduct	423	572	222.8	7.90E-64
486	PHE0006690_16236.pep	MFS_1	57	421	44.3	4.30E-10
487	PHE0006395_15932.pep	LRR_1	185	206	11.9	1.7
487	PHE0006395_15932.pep	LRR_1	208	229	17.1	0.065
487	PHE0006395_15932.pep	LRR_1	231	252	13.5	0.77
487	PHE0006395_15932.pep	LRR_1	254	272	12.6	1.2
487	PHE0006395_15932.pep	LRR_1	278	299	8.4	7.8
487	PHE0006395_15932.pep	LRR_1	349	370	20.8	0.0049
487	PHE0006395_15932.pep	LRR_1	372	394	7.8	9.8
488	PHE0006759_16384.pep	SHMT	55	453	1032.6	0
489	PHE0006576_16028.pep	Myb_DNA-binding	11	57	59.3	1.30E-14
489	PHE0006576_16028.pep	Myb_DNA-binding	63	108	48.9	1.70E-11
490	PHE0006979_16885.pep	Tryp_alpha_amyl	33	111	55.3	2.00E-13
491	PHE0009786_21754.pep	HLH	33	80	30	8.20E-06
492	PHE0006572_16007.pep	Pkinase	11	269	317	3.30E-92
494	PHE0006588_16092.pep	Thioredoxin	56	161	128.8	1.50E-35
495	PHE0006985_16891.pep	DUF1716	13	118	159.3	1.00E-44
496	PHE0006875_16688.pep	PSI_PSAK	44	138	191.6	1.90E-54

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497	PHE0006626_16155.pep	Glyco_hydro_17	30	335	587.5	1.20E-173
498	PHE0006689_16235.pep	Aa_trans	165	548	224.1	3.20E-64
499	PHE0006799_16382.pep	B12-binding	10	135	104.3	3.70E-28
499	PHE0006799_16382.pep	Radical_SAM	202	368	91.2	3.10E-24
500	PHE0006959_16856.pep	Oxidored_molyb	143	328	286.7	4.40E-83
500	PHE0006959_16856.pep	Mo-co_dimer	353	486	297.6	2.40E-86
500	PHE0006959_16856.pep	Cyt-b5	543	616	90.4	5.60E-24
500	PHE0006959_16856.pep	FAD_binding_6	660	767	231.6	1.70E-66
500	PHE0006959_16856.pep	NAD_binding_1	786	899	186.4	7.00E-53
501	PHE0011615_23861.pep	Pkinase	267	523	297.5	2.50E-86
501	PHE0011615_23861.pep	Pkinase_Tyr	267	523	127.5	3.70E-35
502	PHE0006925_16814.pep	Tim17	4	133	198.1	2.10E-56
503	PHE0006713_16274.pep	APC8	1	161	401.5	1.20E-117
503	PHE0006713_16274.pep	TPR_1	339	372	34.7	3.20E-07
503	PHE0006713_16274.pep	TPR_2	339	372	23.7	0.00066
503	PHE0006713_16274.pep	TPR_2	373	406	23.7	0.00068
503	PHE0006713_16274.pep	TPR_1	373	406	34.1	4.80E-07
503	PHE0006713_16274.pep	TPR_2	407	440	22.1	0.002
503	PHE0006713_16274.pep	TPR_1	407	440	24.3	0.00043
504	PHE0008447_18848.pep	Lir1	1	126	178.6	1.60E-50
505	PHE0006674_16221.pep	PTR2	96	505	222.7	8.20E-64
506	PHE0010613_22398.pep	DAGK_cat	43	184	106.4	8.50E-29
507	PHE0002720_22558.pep	DnaJ	90	151	137.6	3.40E-38
507	PHE0002720_22558.pep	DnaJ_CXXCXGXG	218	301	100.5	5.20E-27
507	PHE0002720_22558.pep	DnaJ_C	314	436	210.6	3.60E-60
508	PHE0011760_24005.pep	Pkinase	400	656	297.5	2.50E-86
508	PHE0011760_24005.pep	Pkinase_Tyr	400	656	127.5	3.70E-35
509	PHE0010612_22397.pep	G-patch	15	59	62	1.90E-15
511	PHE0007411_17654.pep	Acyltransferase	136	283	86.4	8.50E-23
512	PHE0010617_22402.pep	Isochorismatase	27	210	-41	0.0027
513	PHE0010610_22395.pep	zf-C3HC4	103	144	36.3	1.10E-07
514	PHE0006883_16702.pep	Glyco_transf_5	78	339	461.4	1.10E-135
514	PHE0006883_16702.pep	Glycos_transf_1	379	562	40.5	5.80E-09
515	PHE0010636_22408.pep	UAA	96	387	-125.2	0.00037
515	PHE0010636_22408.pep	DUF6	105	230	32.1	2.00E-06
515	PHE0010636_22408.pep	TPT	239	384	188.8	1.30E-53
516	PHE0011082_23038.pep	adh_short	19	187	111.5	2.50E-30
516	PHE0011082_23038.pep	KR	19	202	-67.5	0.0019
517	PHE0009475_20462.pep	2OG-FeII_Oxy	223	322	166.9	5.20E-47
518	PHE0007415_17661.pep	EIN3	60	467	781.6	4.80E-232
519	PHE0006614_16129.pep	Cellulase	19	358	-25.9	1.00E-05
520	PHE0010611_22396.pep	FMO-like	25	424	-364.7	5.40E-07
521	PHE0011719_23946.pep	Acyltransferase	69	222	30	8.30E-06
522	PHE0006861_16463.pep	NPH3	166	409	387.6	1.80E-113
523	PHE0006568_16005.pep	DUF26	80	134	56.9	6.70E-14
523	PHE0006568_16005.pep	DUF26	199	253	62.1	1.80E-15

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PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
523	PHE0006568_16005.pep	Pkinase_Tyr	346	615	114	4.20E-31
523	PHE0006568_16005.pep	Pkinase	346	615	163.2	6.70E-46
524	PHE0010652_22429.pep	FtsJ	21	211	282.4	8.80E-82
525	PHE0008158_18448.pep	PLATZ	22	144	232.3	1.00E-66
526	PHE0003316_20755.pep	Glyco_hydro_2_N	9	180	238.7	1.30E-68
526	PHE0003316_20755.pep	Glyco_hydro_2	182	272	129.9	7.00E-36
526	PHE0003316_20755.pep	Glyco_hydro_2_C	274	593	612.1	5.00E-181
527	PHE0009478_20470.pep	2OG-FeII_Oxy	229	328	171.2	2.50E-48
528	PHE0010395_21760.pep	DUF716	109	266	180.5	4.10E-51
529	PHE0010391_21753.pep	DUF640	28	160	296.6	4.70E-86
530	PHE0010396_21761.pep	DUF716	109	265	175.2	1.60E-49
531	PHE0009511_22422.pep	SAM_decarbox	5	337	710.3	1.30E-210
532	PHE0010614_22399.pep	SOUL	28	211	330.7	2.50E-96
534	PHE0011454_23667.pep	Histone	56	126	103.3	7.20E-28
535	PHE0011443_24001.pep	Histone	61	131	103.3	7.20E-28
536	PHE0011452_23665.pep	Histone	58	132	142.7	9.80E-40
537	PHE0010394_21758.pep	SAP18	29	150	260.5	3.30E-75
540	PHE0010100_21467.pep	Pyr_redox_2	5	285	134.4	3.10E-37
540	PHE0010100_21467.pep	Pyr_redox	147	240	91	3.50E-24
540	PHE0010100_21467.pep	Fer2_BFD	420	472	81.5	2.60E-21
540	PHE0010100_21467.pep	NIR_SIR_ferr	554	621	89.9	7.80E-24
540	PHE0010100_21467.pep	NIR_SIR	629	775	176.5	6.70E-50
541	PHE0011503_23734.pep	GAF	158	307	91.9	1.90E-24
541	PHE0011503_23734.pep	HisKA	343	408	87.5	4.10E-23
541	PHE0011503_23734.pep	HATPase_c	455	586	123.9	4.50E-34
542	PHE0010099_21319.pep	Molybdop_Fe4S4	1	55	91.2	3.30E-24
542	PHE0010099_21319.pep	Molybdopterin	58	475	479.9	3.00E-141
542	PHE0010099_21319.pep	Molydop_binding	570	681	140	6.60E-39
542	PHE0010099_21319.pep	Fer2_BFD	811	863	74.8	2.70E-19
543	PHE0011269_23400.pep	Homeobox	21	78	70.4	5.80E-18
543	PHE0011269_23400.pep	START	215	437	167.3	3.90E-47
546	PHE0011613_23858.pep	AP2	62	136	129.6	8.70E-36
546	PHE0011613_23858.pep	AP2	165	230	109.9	7.50E-30
547	PHE0001582_22064.pep	GAF	195	344	57.2	5.30E-14
547	PHE0001582_22064.pep	HisKA	380	445	22.2	0.00068
547	PHE0001582_22064.pep	Response_reg	645	761	69.4	1.20E-17
548	PHE0007444_22314.pep	Spermine_synth	90	349	414.5	1.50E-121
549	PHE0010543_22298.pep	PBP	19	165	216.7	5.30E-62
550	PHE0010543_22323.pep	PBP	19	157	186	9.30E-53
551	PHE0011081_23033.pep	Sugar_tr	33	465	-7.2	1.20E-06
552	PHE0011075_23026.pep	P-II	73	175	181.5	2.10E-51
553	PHE0010100_21462.pep	Pyr_redox_2	5	285	134.4	3.10E-37
553	PHE0010100_21462.pep	Pyr_redox	147	240	91	3.50E-24
553	PHE0010100_21462.pep	Fer2_BFD	420	472	81.5	2.60E-21
553	PHE0010100_21462.pep	NIR_SIR_ferr	554	621	89.9	7.80E-24
553	PHE0010100_21462.pep	NIR_SIR	629	775	176.5	6.70E-50

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PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
554	PHE0009640_21775.pep	Auxin_inducible	1	102	151.5	2.10E-42
555	PHE0010092_21307.pep	S1	13	88	51.4	3.00E-12
555	PHE0010092_21307.pep	EIF_2_alpha	125	256	217.3	3.50E-62
556	PHE0004611_24123.pep	PTR2	13	406	283.1	5.40E-82
557	PHE0010093_21308.pep	S1	13	88	50	8.10E-12
557	PHE0010093_21308.pep	EIF_2_alpha	125	256	217.3	3.50E-62
558	PHE0008605_23084.pep	Histone	58	128	105.5	1.60E-28
559	PHE0009939_21100.pep	GDC-P	81	508	1024.4	0
560	PHE0009941_21105.pep	mTERF	2	262	117.5	3.70E-32
561	PHE0009951_21135.pep	Ribosomal_L10e	1	176	487.9	1.20E-143
562	PHE0009943_21127.pep	Ribosomal_L10e	1	176	460.5	2.20E-135
563	PHE0009948_21132.pep	Ribosomal_L10e	1	176	476.4	3.40E-140
564	PHE0011084_23040.pep	Peptidase_S10	54	482	561	1.20E-165
565	PHE0008233_23042.pep	Phi_1	40	314	616.9	1.80E-182
566	PHE0010854_22730.pep	RGS	294	412	23.2	2.80E-06
567	PHE0003797_23051.pep	zf-Dof	34	96	127	5.20E-35
568	PHE0010194_21769.pep	Glyco_hydro_9	52	509	1007.2	5.70E-300
570	PHE0011665_23899.pep	Chloroa_b-bind	63	260	255.3	1.20E-73
571	PHE0012178_24443.pep	Whirly	53	191	263.2	5.30E-76
573	PHE0011064_22994.pep	TPR_1	73	106	33.7	6.50E-07
573	PHE0011064_22994.pep	TPR_2	73	106	33.4	7.80E-07
577	PHE0011446_23659.pep	Histone	58	132	142.9	8.50E-40
578	PHE0001424_22077.pep	Agglutinin	55	193	46.9	6.30E-12
579	PHE0011445_23658.pep	C2	6	87	74.9	2.50E-19
580	PHE0010090_21297.pep	LRRNT_2	33	73	34.2	4.60E-07
580	PHE0010090_21297.pep	LRR_1	78	101	11.5	2
580	PHE0010090_21297.pep	LRR_1	103	127	11.2	2.3
580	PHE0010090_21297.pep	LRR_1	129	151	21.9	0.0024
580	PHE0010090_21297.pep	LRR_1	177	199	16	0.14
580	PHE0010090_21297.pep	LRR_1	226	248	10.3	3.3
580	PHE0010090_21297.pep	LRR_1	274	296	18.9	0.018
580	PHE0010090_21297.pep	LRR_1	298	320	12.1	1.6
580	PHE0010090_21297.pep	LRR_1	322	341	11.2	2.3
580	PHE0010090_21297.pep	LRR_1	395	417	9.8	4.1
580	PHE0010090_21297.pep	LRR_1	444	466	8.2	8.3
580	PHE0010090_21297.pep	LRR_1	468	490	12.8	1.1
580	PHE0010090_21297.pep	LRR_1	492	514	7.7	10
580	PHE0010090_21297.pep	LRR_1	516	538	15.7	0.17
580	PHE0010090_21297.pep	LRR_1	540	562	7.8	10
580	PHE0010090_21297.pep	LRR_1	564	585	8.5	7.4
580	PHE0010090_21297.pep	Pkinase	678	952	107.9	3.00E-29
580	PHE0010090_21297.pep	Pkinase_Tyr	678	952	110.4	5.10E-30
581	PHE0012180_24445.pep	B3	516	619	90.3	6.00E-24
582	PHE0011083_23039.pep	adh_short	44	200	46.7	7.60E-11
583	PHE0011085_23041.pep	Peptidase_S10	31	459	551.6	8.20E-163
584	PHE0012175_24440.pep	CXC	457	498	80.2	6.60E-21

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PEP SEQ ID NO	Gene ID	Pfam domain name	Begin	Stop	Score	E-value
584	PHE0012175_24440.pep	CXC	543	584	83.5	6.40E-22
585	PHE0012177_24442.pep	Whirly	91	229	325.2	1.10E-94
587	PHE0010194_21426.pep	Glyco_hydro_9	52	509	1013.8	5.70E-302
588	PHE0011666_23900.pep	Spermine_synth	46	291	503.1	3.20E-148
589	PHE0011448_24160.pep	Histone	25	90	35.4	1.90E-07
590	PHE0008324_18636.pep	DUF1005	256	460	524.8	9.30E-155
591	PHE0006971_16875.pep	Myb_DNA-binding	14	61	44.2	4.40E-10
591	PHE0006971_16875.pep	Myb_DNA-binding	67	112	51.3	3.20E-12
592	PHE0009939_21147.pep	GDC-P	81	508	1024.4	0
593	PHE0009953_21137.pep	Na_sulph_symp	110	578	831.2	5.40E-247
595	PHE0008557_18970.pep	NAD_binding_1	234	350	138.6	1.60E-38
596	PHE0006443_15955.pep	YDG_SRA	360	519	403.5	3.00E-118
596	PHE0006443_15955.pep	Pre-SET	543	639	156.7	6.00E-44
596	PHE0006443_15955.pep	SET	641	771	181.7	1.80E-51

Table 11. pfam module annotation

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
299	PHE0007721_18998.pep	KNOX1::KNOX2::ELK	102-146::153-204::242-263
300	PHE0007756_18009.pep	RPE65	105-591
302	PHE0004988_15925.pep	F-box::LRR_1::LRR_2	4-52::157-179::290-314
303	PHE0007140_21771.pep	MtN3_slv::MtN3_slv	9-98::134-221
304	PHE0006823_16403.pep	Globin	10-149
305	PHE0007440_22555.pep	Ras	14-175
306	PHE0000206_22432.pep	Pkinase::efhand::efhand::efhand	79-337::384-412::456-484::490-518
307	PHE0000598_16824.pep	HLH	385-434
308	PHE0007590_17883.pep	S1	2-70
309	PHE0007588_17881.pep	S1	2-86
310	PHE0007592_17885.pep	S1	1-75
311	PHE0007587_17880.pep	S1	3-75
312	PHE0007624_17923.pep	eIF-1a	5-70
313	PHE0007591_17884.pep	S1	2-68
314	PHE0007623_17922.pep	CN_hydrolase	11-184
315	PHE0007583_17871.pep	MtN3_slv::MtN3_slv	9-98::132-218
316	PHE0006618_16146.pep	S10_pectin	3-98
317	PHE0006791_16374.pep	GUN4	84-227
318	PHE0007620_17918.pep	peroxidase	89-321
319	PHE0006659_16195.pep	MFS_1	10-391
321	PHE0006274_15867.pep	E2F_TDP	130-195

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
322	PHE0006637_16166.pep	PP2C	7-305
323	PHE0006926_16815.pep	VPS28	18-209
324	PHE0006821_16402.pep	PALP	49-358
325	PHE0007687_18057.pep	p450	35-462
326	PHE0007642_17999.pep	PAS_3::PAS_3::Pkinase	211-303::489-581::663-952
327	PHE0007677_18052.pep	FAD_binding_3	5-372
328	PHE0007644_18001.pep	LRRNT_2::LRR_1::LR R_1::LRR_1::LRR_1::L RR_1::Pkinase	23-64::97-119::121-143::145- 167::169-192::194-216::414- 716
329	PHE0007640_17992.pep	CorA	66-448
330	PHE0007678_18053.pep	4HBT	66-151
331	PHE0007649_18006.pep	Ank::Pkinase	108-140::196-455
332	PHE0006726_16298.pep	WD40::WD40	45-83::135-173
333	PHE0006218_8776.pep	Pyr_redox_2::Thioredoxi n	85-370::423-528
335	PHE0002554_17876.pep	Chloroa_b-bind	63-261
336	PHE0006807_16388.pep	CCT	352-390
337	PHE0003359_8487.pep	p450	51-489
338	PHE0006542_15765.pep	PMSR	92-245
339	PHE0007622_17921.pep	Spermine_synth	34-279
340	PHE0007630_17956.pep	2OG-FeII_Oxy	190-291
341	PHE0007593_17886.pep	Spermine_synth	28-273
342	PHE0006475_15588.pep	PsbQ	3-162
343	PHE0007619_17915.pep	MBD	11-84
344	PHE0006846_16447.pep	Sterol_desat	1-186
345	PHE0007584_17874.pep	MBD	4-77
346	PHE0003695_17913.pep	LRRNT_2::LRR_1::LR R_1::LRR_1::LRR_1::L RR_1::LRR_1::LRR_1:: LRR_1::LRR_1::LRR_1 ::LRR_1::LRR_1::LRR_ 1::LRR_1::LRR_1::LRR _1::LRR_1::LRR_1::LR R_1::Pkinase	23-64::93-115::117-139::141- 163::165-186::189-211::213- 235::237-259::260-281::284- 306::308-330::332-354::356- 378::380-402::404-426::428- 450::452-474::476-498::500- 521::523-542::648-917
347	PHE0003695_17879.pep	LRRNT_2::LRR_1::LR R_1::LRR_1::LRR_1::L RR_1::LRR_1::LRR_1:: LRR_1::LRR_1::LRR_1 ::LRR_1::LRR_1::LRR_ 1::LRR_1::LRR_1::LRR _1::LRR_1::LRR_1::LR R_1::Pkinase	23-64::93-115::117-139::141- 163::165-186::189-211::213- 235::237-259::260-281::284- 306::308-330::332-354::356- 378::380-402::404-426::428- 450::452-474::476-498::500- 521::523-542::648-917
349	PHE0007645_18002.pep	AP2	115-180
350	PHE0006784_16366.pep	PALP	12-301

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
351	PHE0006171_16491.pep	Glyoxalase	70-214
353	PHE0006906_16796.pep	Proteasome	10-206
354	PHE0006649_16180.pep	AP2	46-110
355	PHE0006809_16390.pep	Prismane	1-548
356	PHE0006918_16808.pep	SWIM	321-353
357	PHE0007813_18219.pep	MtN3_slv::MtN3_slv	9-98::132-218
359	PHE0006825_16405.pep	Cytochrom_C552	27-476
360	PHE0006964_16864.pep	tRNA_anti::tRNA-synt_2	43-122::140-557
361	PHE0008105_18407.pep	CBFD_NFYB_HMF	158-222
362	PHE0006630_16159.pep	Ldh_1_N::Ldh_1_C	90-239::241-413
363	PHE0006966_16868.pep	DNA_photolyase::FAD_binding_7	12-183::214-492
365	PHE0006608_16123.pep	GSHPx	42-150
366	PHE0010462_21459.pep	NUDIX::NUDIX	17-159::190-322
367	PHE0009211_21774.pep	Auxin_inducible	1-106
368	PHE0006256_8775.pep	RPE65	105-591
369	PHE0006989_16918.pep	Pkinase	88-396
370	PHE0007649_18166.pep	Ank::Pkinase	108-140::196-455
371	PHE0006708_16264.pep	DEAD::Helicase_C	57-223::291-367
372	PHE0001117_19094.pep	Homeobox::HALZ	79-133::134-178
373	PHE0006266_8814.pep	Fe_bilin_red	16-244
374	PHE0006795_16378.pep	Na_H_antiport_1	4-380
375	PHE0006956_16853.pep	Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD	2-56::59-482::578-689::825-877
377	PHE0006824_16404.pep	Globin	14-154
378	PHE0006812_16393.pep	Bac_globin	3-118
379	PHE0006815_16396.pep	Bac_globin	26-146
380	PHE0006806_16387.pep	Bac_globin	26-146
381	PHE0006624_16153.pep	NAD_binding_2::6PGD	3-178::182-473
382	PHE0005006_15823.pep	Chloroa_b-bind	63-242
384	PHE0006683_16229.pep	Response_reg	10-151
385	PHE0006884_16703.pep	Glyco_transf_5::Glycos_transf_1	83-344::384-567
386	PHE0006691_16237.pep	NAD_Gly3P_dh_N::NAD_Gly3P_dh_C	6-165::183-327
387	PHE0006790_16372.pep	Rieske::PaO	220-317::408-507
388	PHE0009143_19932.pep	Histone_HNS	21-129
389	PHE0008406_18830.pep	AA_permease	57-511
390	PHE0008279_18708.pep	Mit_rib_S27	14-93
391	PHE0009142_19931.pep	Histone_HNS	21-130

PEP SEQ ID NO	Gene ID	Pfam domain module	Position
393	PHE0007639_17991.pep	ELFV_dehydrog_N::ELFV_dehydrog	84-214::229-474
394	PHE0007646_18170.pep	F-box::Kelch_1::Kelch_1	36-83::131-185::187-233
395	PHE0007765_18182.pep	GST_N::GST_C	5-79::101-204
396	PHE0008167_18465.pep	Pkinase_Tyr	8-262
398	PHE0008183_18479.pep	LRRNT_2::LRR_1::LR_R_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr	23-66::71-93::95-117::119-142::144-166::168-190::192-214::289-311::313-335::337-359::361-384::409-431::457-479::481-503::505-527::529-551::553-575::577-598::695-966
399	PHE0007585_17875.pep	LEA_3	35827
400	PHE0006804_16385.pep	zf-CCCH::zf-CCCH	13-39::149-174
401	PHE0006703_15751.pep	PFK	72-347
402	PHE0007410_17653.pep	Acyltransferase	27-249
403	PHE0006854_16456.pep	YABBY	40-278
404	PHE0007721_21293.pep	KNOX1::KNOX2::ELK	102-146::153-204::242-263
405	PHE0006563_15990.pep	GATase_2::Asn_synthas e	2-161::209-450
406	PHE0006619_16147.pep	AA_kinase	15-261
407	PHE0006813_16394.pep	Bac_globin	7-124
408	PHE0006688_16234.pep	Ribosomal_S2	13-229
409	PHE0006635_16164.pep	MGS	28-121
411	PHE0008416_18838.pep	PTR2	115-504
413	PHE0008599_19166.pep	Isoamylase_N::Alpha-amylase	24-115::164-578
414	PHE0008375_18732.pep	Pribosyltran	93-237
416	PHE0008394_18763.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
417	PHE0008395_18764.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
418	PHE0008444_18846.pep	Gln-synt_N::Gln-synt_C	17-97::103-355
419	PHE0006819_16400.pep	Lactamase_B::Flavodoxi n_1::Flavin_Reduct	47-236::267-397::424-573
420	PHE0007417_17663.pep	EIN3	60-467
421	PHE0008443_18845.pep	GATase_2::Asn_synthas e	2-161::209-450
422	PHE0006818_16399.pep	Lactamase_B::Flavodoxi n_1::Rubredoxin	34-227::256-389::423-471
423	PHE0006579_16031.pep	Thi4	63-306
424	PHE0006808_16389.pep	CCT	354-392
425	PHE0008160_18460.pep	TPP_enzyme_N::TPP_e nzyme_M::TPP_enzyme _C	45-221::243-376::431-578
426	PHE0007571_17834.pep	GSH_synth_ATP	29-499

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
427	PHE0007586_17877.pep	Chloroa_b-bind	64-261
429	PHE0009729_21717.pep	Homeobox::HALZ	67-123::124-168
430	PHE0006424_15520.pep	PsbW_2	1-133
431	PHE0006392_15480.pep	PsbP	106-322
432	PHE0006589_16093.pep	Thioredoxin	64-167
433	PHE0009258_21487.pep	Na_H_Exchanger	22-441
434	PHE0006680_16226.pep	2OG-FeII_Oxy	187-287
435	PHE0006481_16072.pep	adh_short	33-181
436	PHE0006269_8820.pep	TPR_2::TPR_1::TPR_2:: TPR_1::TPR_4::TPR_2:: TPR_1	74-107::108-141::142- 175::179-212::247-272::281- 314::349-382
437	PHE0006783_16365.pep	Aa_trans	32-469
438	PHE0003151_18392.pep	AP2::AP2	318-391::420-485
439	PHE0008272_18723.pep	HSF_DNA-bind	18-188
440	PHE0008277_18706.pep	SNF5	172-400
441	PHE0008109_18411.pep	Sad1_UNC	203-330
442	PHE0008280_18826.pep	Alpha-amylase	14-452
443	PHE0002424_15825.pep	adh_short	43-215
444	PHE0006914_16804.pep	AAA	204-391
445	PHE0006642_16172.pep	p450	42-496
447	PHE0009926_21079.pep	DSPc	24-152
448	PHE0006206_19159.pep	Sina	106-305
449	PHE0008274_18705.pep	BURP	56-280
450	PHE0009510_20526.pep	Spermine_synth	38-283
451	PHE0009937_21097.pep	Spermine_synth	38-283
452	PHE0009927_21080.pep	DSPc	24-162
454	PHE0006445_15956.pep	Allene_ox_cyc	74-253
455	PHE0006987_16893.pep	efhand::efhand	86-114::167-195
456	PHE0004230_15865.pep	E2F_TDP	146-211
457	PHE0006814_16395.pep	Bac_globin	26-144
458	PHE0002773_15881.pep	NTP_transferase	88-366
459	PHE0006060_15842.pep	ubiquitin::ubiquitin::ubiq uitin::ubiquitin	5-73::81-149::157-225::233- 301
460	PHE0006850_16451.pep	YABBY	6-166
461	PHE0008043_18199.pep	Homeobox::START::M EKHLA	25-85::164-374::693-840
462	PHE0000125_18852.pep	Response_reg::CCT	28-153::457-495
463	PHE0008269_18720.pep	Pkinase	23-279
464	PHE0008264_18712.pep	zf-UBR	40-109
465	PHE0002782_20059.pep	PGI	2-457
466	PHE0009438_20404.pep	GATA	191-226

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
467	PHE0009429_20392.pep	ParBc	70-151
468	PHE0009440_20406.pep	ParBc	11-126
469	PHE0008543_18945.pep	TP_methylase	222-435
470	PHE0006960_16857.pep	Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1	116-301::326-459::515-588::633-740::759-872
471	PHE0009427_20389.pep	IGPD	103-247
472	PHE0006437_15906.pep	Pkinase	39-307
473	PHE0007409_17652.pep	DAGAT	64-340
474	PHE0009426_20387.pep	PHP	2-281
475	PHE0009430_20393.pep	ParBc	44-128
476	PHE0007003_16906.pep	PTPA	96-396
477	PHE0008378_18741.pep	bZIP_1	223-280
478	PHE0008273_18725.pep	zf-CCCH	80-106
479	PHE0008547_18953.pep	Fer2	20-95
480	PHE0006922_16812.pep	Hexokinase_1::Hexokinase_2	44-251::258-501
481	PHE0006920_16810.pep	AAA::Vps4_C	267-458::464-514
482	PHE0008396_18765.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
483	PHE0006957_16854.pep	Gln-synt_N::Gln-synt_C	68-148::154-406
484	PHE0006404_15936.pep	RRM_1::RRM_1	8-77::112-182
485	PHE0006820_16401.pep	Lactamase_B::Flavin_Re duct	47-236::423-572
486	PHE0006690_16236.pep	MFS_1	57-421
487	PHE0006395_15932.pep	LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1	185-206::208-229::231-252::254-272::278-299::349-370::372-394
488	PHE0006759_16384.pep	SHMT	55-453
489	PHE0006576_16028.pep	Myb_DNA-binding::Myb_DNA-binding	11-57::63-108
490	PHE0006979_16885.pep	Tryp_alpha_amyl	33-111
491	PHE0009786_21754.pep	HLH	33-80
492	PHE0006572_16007.pep	Pkinase	11-269
494	PHE0006588_16092.pep	Thioredoxin	56-161
495	PHE0006985_16891.pep	DUF1716	13-118
496	PHE0006875_16688.pep	PSI_PSAK	44-138
497	PHE0006626_16155.pep	Glyco_hydro_17	30-335
498	PHE0006689_16235.pep	Aa_trans	165-548
499	PHE0006799_16382.pep	B12-binding::Radical_SAM	10-135::202-368

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
500	PHE0006959_16856.pep	Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1	143-328::353-486::543-616::660-767::786-899
501	PHE0011615_23861.pep	Pkinase	267-523
502	PHE0006925_16814.pep	Tim17	4-133
503	PHE0006713_16274.pep	APC8::TPR_1::TPR_1::TPR_1	1-161::339-372::373-406::407-440
504	PHE0008447_18848.pep	Lir1	1-126
505	PHE0006674_16221.pep	PTR2	96-505
506	PHE0010613_22398.pep	DAGK_cat	43-184
507	PHE0002720_22558.pep	DnaJ::DnaJ_CXXCXGXG::DnaJ_C	90-151::218-301::314-436
508	PHE0011760_24005.pep	Pkinase	400-656
509	PHE0010612_22397.pep	G-patch	15-59
511	PHE0007411_17654.pep	Acyltransferase	136-283
512	PHE0010617_22402.pep	Isochorismatase	27-210
513	PHE0010610_22395.pep	zf-C3HC4	103-144
514	PHE0006883_16702.pep	Glyco_transf_5::Glycos_transf_1	78-339::379-562
515	PHE0010636_22408.pep	DUF6::TPT	105-230::239-384
516	PHE0011082_23038.pep	adh_short	19-187
517	PHE0009475_20462.pep	2OG-FeII_Oxy	223-322
518	PHE0007415_17661.pep	EIN3	60-467
519	PHE0006614_16129.pep	Cellulase	19-358
520	PHE0010611_22396.pep	FMO-like	25-424
521	PHE0011719_23946.pep	Acyltransferase	69-222
522	PHE0006861_16463.pep	NPH3	166-409
523	PHE0006568_16005.pep	DUF26::DUF26::Pkinase	80-134::199-253::346-615
524	PHE0010652_22429.pep	FtsJ	21-211
525	PHE0008158_18448.pep	PLATZ	22-144
526	PHE0003316_20755.pep	Glyco_hydro_2_N::Glyco_hydro_2::Glyco_hydro_2_C	9-180::182-272::274-593
527	PHE0009478_20470.pep	2OG-FeII_Oxy	229-328
528	PHE0010395_21760.pep	DUF716	109-266
529	PHE0010391_21753.pep	DUF640	28-160
530	PHE0010396_21761.pep	DUF716	109-265
531	PHE0009511_22422.pep	SAM_decarbox	5-337
532	PHE0010614_22399.pep	SOUL	28-211
534	PHE0011454_23667.pep	Histone	56-126
535	PHE0011443_24001.pep	Histone	61-131

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
536	PHE0011452_23665.pep	Histone	58-132
537	PHE0010394_21758.pep	SAP18	29-150
540	PHE0010100_21467.pep	Pyr_redox_2::Fer2_BFD ::NIR_SIR_ferr::NIR_SIR	5-285::420-472::554-621::629-775
541	PHE0011503_23734.pep	GAF::HisKA::HATPase_c	158-307::343-408::455-586
542	PHE0010099_21319.pep	Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD	1-55::58-475::570-681::811-863
543	PHE0011269_23400.pep	Homeobox::START	21-78::215-437
546	PHE0011613_23858.pep	AP2::AP2	62-136::165-230
547	PHE0001582_22064.pep	GAF::HisKA::Response_reg	195-344::380-445::645-761
548	PHE0007444_22314.pep	Spermine_synth	90-349
549	PHE0010543_22298.pep	PBP	19-165
550	PHE0010543_22323.pep	PBP	19-157
551	PHE0011081_23033.pep	Sugar_tr	33-465
552	PHE0011075_23026.pep	P-II	73-175
553	PHE0010100_21462.pep	Pyr_redox_2::Fer2_BFD ::NIR_SIR_ferr::NIR_SIR	5-285::420-472::554-621::629-775
554	PHE0009640_21775.pep	Auxin_inducible	1-102
555	PHE0010092_21307.pep	S1::EIF_2_alpha	13-88::125-256
556	PHE0004611_24123.pep	PTR2	13-406
557	PHE0010093_21308.pep	S1::EIF_2_alpha	13-88::125-256
558	PHE0008605_23084.pep	Histone	58-128
559	PHE0009939_21100.pep	GDC-P	81-508
560	PHE0009941_21105.pep	mTERF	2-262
561	PHE0009951_21135.pep	Ribosomal_L10e	1-176
562	PHE0009943_21127.pep	Ribosomal_L10e	1-176
563	PHE0009948_21132.pep	Ribosomal_L10e	1-176
564	PHE0011084_23040.pep	Peptidase_S10	54-482
565	PHE0008233_23042.pep	Phi_1	40-314
566	PHE0010854_22730.pep	RGS	294-412
567	PHE0003797_23051.pep	zf-Dof	34-96
568	PHE0010194_21769.pep	Glyco_hydro_9	52-509
570	PHE0011665_23899.pep	Chloroa_b-bind	63-260
571	PHE0012178_24443.pep	Whirly	53-191
573	PHE0011064_22994.pep	TPR_1	73-106
577	PHE0011446_23659.pep	Histone	58-132
578	PHE0001424_22077.pep	Agglutinin	55-193

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PEP SEQ ID NO	Gene ID	Pfam domain module	Position
579	PHE0011445_23658.pep	C2	6-87
580	PHE0010090_21297.pep	LRRNT_2::LRR_1::LR R_1::LRR_1::LRR_1::L RR_1::LRR_1::LRR_1:: LRR_1::LRR_1::LRR_1 ::LRR_1::LRR_1::LRR_ 1::LRR_1::LRR_1::Pkin ase_Tyr	33-73::78-101::103-127::129- 151::177-199::226-248::274- 296::298-320::322-341::395- 417::444-466::468-490::492- 514::516-538::540-562::564- 585::678-952
581	PHE0012180_24445.pep	B3	516-619
582	PHE0011083_23039.pep	adh_short	44-200
583	PHE0011085_23041.pep	Peptidase_S10	31-459
584	PHE0012175_24440.pep	CXC::CXC	457-498::543-584
585	PHE0012177_24442.pep	Whirly	91-229
587	PHE0010194_21426.pep	Glyco_hydro_9	52-509
588	PHE0011666_23900.pep	Spermine_synth	46-291
589	PHE0011448_24160.pep	Histone	25-90
590	PHE0008324_18636.pep	DUF1005	256-460
591	PHE0006971_16875.pep	Myb_DNA- binding::Myb_DNA- binding	14-61::67-112
592	PHE0009939_21147.pep	GDC-P	81-508
593	PHE0009953_21137.pep	Na_sulph_symp	110-578
595	PHE0008557_18970.pep	NAD_binding_1	234-350
596	PHE0006443_15955.pep	YDG_SRA::Pre- SET::SET	360-519::543-639::641-771

Table 12. Description of pfam domains

Pfam domain name	Accession number	Gathering cutoff	Domain description
2OG-FeII_Oxy	PF03171.10	11.5	2OG-Fe(II) oxygenase superfamily
4HBT	PF03061.12	20.6	Thioesterase superfamily
6PGD	PF00393.9	-232.3	6-phosphogluconate dehydrogenase, C-terminal domain
AAA	PF00004.19	12.3	ATPase family associated with various cellular activities (AAA)
AA_kinase	PF00696.18	-40	Amino acid kinase family
AA_permease	PF00324.11	-120.8	Amino acid permease
AP2	PF00847.10	0	AP2 domain
APC8	PF04049.4	-19.8	Anaphase promoting complex subunit 8 / Cdc23
Aa_trans	PF01490.8	-128.4	Transmembrane amino acid transporter protein
Acyltransferase	PF01553.11	-0.4	Acyltransferase
Agglutinin	PF07468.2	25	Agglutinin

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Pfam domain name	Accession number	Gathering cutoff	Domain description
Allene_ox_cyc	PF06351.2	25	Allene oxide cyclase
Alpha-amylase	PF00128.14	-92.6	Alpha amylase, catalytic domain
Ank	PF00023.20	0	Ankyrin repeat
ApbA	PF02558.6	-16.2	Ketopantoate reductase PanE/ApbA
Asn_synthase	PF00733.11	-52.8	Asparagine synthase
Auxin_inducible	PF02519.5	-15	Auxin responsive protein
B12-binding	PF02310.9	19.6	B12 binding domain
B3	PF02362.12	26.5	B3 DNA binding domain
BURP	PF03181.6	-52	BURP domain
Bac_globin	PF01152.11	-12.7	Bacterial-like globin
C2	PF00168.20	3.7	C2 domain
CBFD_NFYB_HMF	PF00808.13	18.4	Histone-like transcription factor (CBF/NF-Y) and archaeal histone
CCT	PF06203.4	25	CCT motif
CN_hydrolase	PF00795.12	-13.9	Carbon-nitrogen hydrolase
CXC	PF03638.5	25	Tesmin/TSO1-like CXC domain
Cellulase	PF00150.8	-49.3	Cellulase (glycosyl hydrolase family 5)
Chloroa_b-bind	PF00504.12	-31.9	Chlorophyll A-B binding protein
CorA	PF01544.9	-61.3	CorA-like Mg ²⁺ transporter protein
Cyt-b5	PF00173.18	4	Cytochrome b5-like Heme/Steroid binding domain
Cytochrom_C552	PF02335.6	-166	Cytochrome c552
DAGAT	PF03982.3	-151.7	Diacylglycerol acyltransferase
DAGK_cat	PF00781.14	-5.7	Diacylglycerol kinase catalytic domain (presumed)
DAO	PF01266.14	-35	FAD dependent oxidoreductase
DEAD	PF00270.19	7.2	DEAD/DEAH box helicase
DNA_photolyase	PF00875.8	26.1	DNA photolyase
DSPc	PF00782.11	-21.8	Dual specificity phosphatase, catalytic domain
DUF1005	PF06219.3	25	Protein of unknown function (DUF1005)
DUF1716	PF08216.2	25	Eukaryotic domain of unknown function (DUF1716)
DUF26	PF01657.8	0	Domain of unknown function DUF26
DUF6	PF00892.11	20.8	Integral membrane protein DUF6
DUF640	PF04852.3	2.4	Protein of unknown function (DUF640)
DUF716	PF04819.3	-18.4	Family of unknown function (DUF716)
DnaJ	PF00226.21	-8	DnaJ domain
DnaJ_C	PF01556.9	-24	DnaJ C terminal region
DnaJ_CXXCXGXG	PF00684.9	1	DnaJ central domain (4 repeats)
E2F_TDP	PF02319.11	17	E2F/DP family winged-helix DNA-binding domain
EIF_2_alpha	PF07541.2	-4.2	Eukaryotic translation initiation factor 2 alpha subunit
EIN3	PF04873.4	-137.6	Ethylene insensitive 3
ELFV_dehydrog	PF00208.11	-27	Glutamate/Leucine/Phenylalanine/Valine dehydrogenase

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Pfam domain name	Accession number	Gathering cutoff	Domain description
ELFV_dehydrog_N	PF02812.8	31.8	Glu/Leu/Phe/Val dehydrogenase, dimerisation domain
ELK	PF03789.4	25	ELK domain
F-box	PF00646.23	13.9	F-box domain
FAD_binding_3	PF01494.9	-136.6	FAD binding domain
FAD_binding_6	PF00970.14	-11.4	Oxidoreductase FAD-binding domain
FAD_binding_7	PF03441.4	25	FAD binding domain of DNA photolyase
FMO-like	PF00743.9	-381.6	Flavin-binding monooxygenase-like
Fe_bilin_red	PF05996.3	25	Ferredoxin-dependent bilin reductase
Fer2	PF00111.17	7	2Fe-2S iron-sulfur cluster binding domain
Fer2_BFD	PF04324.5	25	BFD-like [2Fe-2S] binding domain
Flavin_Reduct	PF01613.8	-18	Flavin reductase like domain
Flavodoxin_1	PF00258.15	6.3	Flavodoxin
FtsJ	PF01728.9	-50.2	FtsJ-like methyltransferase
G-patch	PF01585.13	18.3	G-patch domain
GAF	PF01590.16	23	GAF domain
GATA	PF00320.17	28.5	GATA zinc finger
GATase_2	PF00310.11	-95.1	Glutamine amidotransferases class-II
GDC-P	PF02347.6	-306.2	Glycine cleavage system P-protein
GSHPx	PF00255.10	-16	Glutathione peroxidase
GSH_synth_ATP	PF03917.8	-129.9	Eukaryotic glutathione synthase, ATP binding domain
GSH_synthase	PF03199.6	25	Eukaryotic glutathione synthase
GST_C	PF00043.15	22.3	Glutathione S-transferase, C-terminal domain
GST_N	PF02798.10	14.6	Glutathione S-transferase, N-terminal domain
GUN4	PF05419.3	25	GUN4-like
Gln-synt_C	PF00120.14	-124	Glutamine synthetase, catalytic domain
Gln-synt_N	PF03951.9	9	Glutamine synthetase, beta-Grasp domain
Globin	PF00042.12	-8.8	Globin
Glutaredoxin	PF00462.14	17.2	Glutaredoxin
Glyco_hydro_17	PF00332.9	-152.3	Glycosyl hydrolases family 17
Glyco_hydro_2	PF00703.11	-19.3	Glycosyl hydrolases family 2, immunoglobulin-like beta-sandwich domain
Glyco_hydro_2_C	PF02836.7	-127.7	Glycosyl hydrolases family 2, TIM barrel domain
Glyco_hydro_2_N	PF02837.8	-46.9	Glycosyl hydrolases family 2, sugar binding domain
Glyco_hydro_9	PF00759.9	-246.7	Glycosyl hydrolase family 9
Glyco_transf_5	PF08323.2	-114.9	Starch synthase catalytic domain
Glycos_transf_1	PF00534.10	-7.3	Glycosyl transferases group 1
Glyoxalase	PF00903.15	12.1	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily
HALZ	PF02183.8	17	Homeobox associated leucine zipper

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Pfam domain name	Accession number	Gathering cutoff	Domain description
HATPase_c	PF02518.16	22.4	Histidine kinase-, DNA gyrase B-, and HSP90-like ATPase
HLH	PF00010.16	8.3	Helix-loop-helix DNA-binding domain
HSF_DNA-bind	PF00447.8	-70	HSF-type DNA-binding
Helicase_C	PF00271.21	2.1	Helicase conserved C-terminal domain
Hexokinase_1	PF00349.11	-110.3	Hexokinase
Hexokinase_2	PF03727.6	-131.3	Hexokinase
HisKA	PF00512.15	10.3	His Kinase A (phosphoacceptor) domain
Histone	PF00125.14	17.4	Core histone H2A/H2B/H3/H4
Histone_HNS	PF00816.11	25	H-NS histone family
Homeobox	PF00046.19	-4.1	Homeobox domain
IGPD	PF00475.9	25	Imidazoleglycerol-phosphate dehydratase
Isoamylase_N	PF02922.8	-6.5	Isoamylase N-terminal domain
Isochorismatase	PF00857.11	-46	Isochorismatase family
KNOX1	PF03790.4	25	KNOX1 domain
KNOX2	PF03791.4	25	KNOX2 domain
KR	PF08659.1	-74.3	KR domain
Kelch_1	PF01344.15	7.8	Kelch motif
Kelch_2	PF07646.5	14	Kelch motif
LEA_3	PF03242.4	25	Late embryogenesis abundant protein
LRRNT_2	PF08263.3	18.6	Leucine rich repeat N-terminal domain
LRR_1	PF00560.23	7.7	Leucine Rich Repeat
LRR_2	PF07723.3	6	Leucine Rich Repeat
Lactamase_B	PF00753.17	24.6	Metallo-beta-lactamase superfamily
Ldh_1_C	PF02866.8	-13	lactate/malate dehydrogenase, alpha/beta C-terminal domain
Ldh_1_N	PF00056.13	-31.3	lactate/malate dehydrogenase, NAD binding domain
Lir1	PF07207.2	25	Light regulated protein Lir1
MBD	PF01429.10	12.9	Methyl-CpG binding domain
MEKHLA	PF08670.1	-59.7	MEKHLA domain
MFS_1	PF07690.6	23.5	Major Facilitator Superfamily
MGS	PF02142.12	3	MGS-like domain
Miro	PF08477.3	10.8	Miro-like protein
Mit_rib_S27	PF08293.2	25	Mitochondrial ribosomal subunit S27
Mo-co_dimer	PF03404.6	25	Mo-co oxidoreductase dimerisation domain
Molybdop_Fe4S4	PF04879.6	13.6	Molybdopterin oxidoreductase Fe4S4 domain
Molybdopterin	PF00384.12	-50	Molybdopterin oxidoreductase
Molydop_binding	PF01568.11	1.1	Molybdopterin dinucleotide binding domain
MtN3_slv	PF03083.6	9.7	MtN3/saliva family
Myb_DNA-binding	PF00249.21	14	Myb-like DNA-binding domain
NAD_Gly3P_dh_C	PF07479.4	-50.8	NAD-dependent glycerol-3-phosphate dehydrogenase C-terminus
NAD_Gly3P_dh_N	PF01210.13	-44	NAD-dependent glycerol-3-phosphate

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Pfam domain name	Accession number	Gathering cutoff	Domain description
			dehydrogenase N-terminus
NAD_binding_1	PF00175.11	-3.9	Oxidoreductase NAD-binding domain
NAD_binding_2	PF03446.5	-63.5	NAD binding domain of 6-phosphogluconate dehydrogenase
NIR_SIR	PF01077.12	-19.6	Nitrite and sulphite reductase 4Fe-4S domain
NIR_SIR_ferr	PF03460.7	2.4	Nitrite/Sulfite reductase ferredoxin-like half domain
NPH3	PF03000.5	25	NPH3 family
NTP_transferase	PF00483.13	-90.5	Nucleotidyl transferase
NUDIX	PF00293.18	0.2	NUDIX domain
Na_H_Exchanger	PF00999.11	-67.9	Sodium/hydrogen exchanger family
Na_H_antiport_1	PF06965.2	-270.8	Na ⁺ /H ⁺ antiporter 1
Na_sulph_symp	PF00939.9	-259	Sodium:sulfate symporter transmembrane region
Oxidored_molyb	PF00174.9	-39.6	Oxidoreductase molybdopterin binding domain
P-II	PF00543.12	-29	Nitrogen regulatory protein P-II
PALP	PF00291.15	-70	Pyridoxal-phosphate dependent enzyme
PAS	PF00989.14	0	PAS fold
PAS_3	PF08447.1	13.4	PAS fold
PBP	PF01161.10	-20.6	Phosphatidylethanolamine-binding protein
PFK	PF00365.10	-132	Phosphofructokinase
PGI	PF00342.9	-168.9	Phosphoglucose isomerase
PHP	PF02811.9	13.8	PHP domain
PLATZ	PF04640.4	20	PLATZ transcription factor
PMSR	PF01625.11	-62	Peptide methionine sulfoxide reductase
PP2C	PF00481.12	-44	Protein phosphatase 2C
PSI_PSAK	PF01241.9	25	Photosystem I psaG / psaK
PTPA	PF03095.5	-106	Phosphotyrosyl phosphate activator (PTPA) protein
PTR2	PF00854.12	-50	POT family
PaO	PF08417.2	25	Pheophorbide a oxygenase
ParBc	PF02195.8	25	ParB-like nuclease domain
Peptidase_S10	PF00450.12	-198	Serine carboxypeptidase
Phi_1	PF04674.3	25	Phosphate-induced protein 1 conserved region
Pkinase	PF00069.15	-70.3	Protein kinase domain
Pkinase_Tyr	PF07714.7	65	Protein tyrosine kinase
Pre-SET	PF05033.6	3.9	Pre-SET motif
Pribosyltran	PF00156.17	2	Phosphoribosyl transferase domain
Prismane	PF03063.10	-169.3	Prismane/CO dehydrogenase family
Proteasome	PF00227.16	-36.7	Proteasome A-type and B-type
PsbP	PF01789.7	25	PsbP
PsbQ	PF05757.2	25	Oxygen evolving enhancer protein 3 (PsbQ)

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Pfam domain name	Accession number	Gathering cutoff	Domain description
PsbW_2	PF07123.2	-25.8	Photosystem II reaction centre W protein (PsbW)
Pyr_redox	PF00070.17	5	Pyridine nucleotide-disulphide oxidoreductase
Pyr_redox_2	PF07992.4	-20	Pyridine nucleotide-disulphide oxidoreductase
RGS	PF00615.9	10	Regulator of G protein signaling domain
RPE65	PF03055.6	-156.5	Retinal pigment epithelial membrane protein
RRM_1	PF00076.12	17.7	RNA recognition motif. (a.k.a. RRM, RBD, or RNP domain)
Radical_SAM	PF04055.11	8.5	Radical SAM superfamily
Ras	PF00071.12	-69.9	Ras family
Response_reg	PF00072.14	4	Response regulator receiver domain
Ribosomal_L10e	PF00826.8	25	Ribosomal L10
Ribosomal_S2	PF00318.10	-22	Ribosomal protein S2
Rieske	PF00355.16	-7	Rieske [2Fe-2S] domain
Rubredoxin	PF00301.10	12.1	Rubredoxin
S1	PF00575.13	16.8	S1 RNA binding domain
S10_plectin	PF03501.5	25	Plectin/S10 domain
SAM_decarbox	PF01536.7	-154	Adenosylmethionine decarboxylase
SAP18	PF06487.3	-49.7	Sin3 associated polypeptide p18 (SAP18)
SET	PF00856.18	23.5	SET domain
SHMT	PF00464.10	-238.5	Serine hydroxymethyltransferase
SNF5	PF04855.3	25	SNF5 / SMARCB1 / INI1
SOUL	PF04832.3	25	SOUL heme-binding protein
START	PF01852.10	-20.7	START domain
SWIM	PF04434.8	11	SWIM zinc finger
Sad1_UNC	PF07738.3	-20.4	Sad1 / UNC-like C-terminal
Sina	PF03145.7	-48.4	Seven in absentia protein family
Spermine_synth	PF01564.7	-93.8	Spermine/spermidine synthase
Sterol_desat	PF01598.8	-13	Sterol desaturase
Sugar_tr	PF00083.14	-85	Sugar (and other) transporter
TPP_enzyme_C	PF02775.11	19.7	Thiamine pyrophosphate enzyme, C-terminal TPP binding domain
TPP_enzyme_M	PF00205.12	-8.1	Thiamine pyrophosphate enzyme, central domain
TPP_enzyme_N	PF02776.8	-70	Thiamine pyrophosphate enzyme, N-terminal TPP binding domain
TPR_1	PF00515.18	7.7	Tetratricopeptide repeat
TPR_2	PF07719.7	20.1	Tetratricopeptide repeat
TPR_4	PF07721.5	15	Tetratricopeptide repeat
TPT	PF03151.7	-15.3	Triose-phosphate Transporter family
TP_methylase	PF00590.10	-38	Tetrapyrrole (Corrin/Porphyrin) Methylases
Thi4	PF01946.7	-65.1	Thi4 family
Thioredoxin	PF00085.10	-25.7	Thioredoxin

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Pfam domain name	Accession number	Gathering cutoff	Domain description
Tim17	PF02466.9	2.7	Tim17/Tim22/Tim23 family
Tryp_alpha_amyl	PF00234.12	-4	Protease inhibitor/seed storage/LTP family
UAA	PF08449.2	-146.2	UAA transporter family
VPS28	PF03997.3	25	VPS28 protein
Vps4_C	PF09336.1	-4.6	Vps4 C terminal oligomerisation domain
WD40	PF00400.22	21.5	WD domain, G-beta repeat
Whirly	PF08536.2	25	Whirly transcription factor
YABBY	PF04690.4	25	YABBY protein
YDG_SRA	PF02182.8	25	YDG/SRA domain
adh_short	PF00106.15	-40.2	short chain dehydrogenase
bZIP_1	PF00170.11	24.5	bZIP transcription factor
bZIP_2	PF07716.5	15	Basic region leucine zipper
eIF-1a	PF01176.9	20	Translation initiation factor 1A / IF-1
efhand	PF00036.22	21.7	EF hand
mTERF	PF02536.5	-60	mTERF
p450	PF00067.12	-105	Cytochrome P450
peroxidase	PF00141.13	-10	Peroxidase
tRNA-synt_2	PF00152.11	-168.2	tRNA synthetases class II (D, K and N)
tRNA_anti	PF01336.15	8	OB-fold nucleic acid binding domain
ubiquitin	PF00240.13	19.4	Ubiquitin family
zf-C3HC4	PF00097.15	16	Zinc finger, C3HC4 type (RING finger)
zf-CCCH	PF00642.15	0	Zinc finger C-x8-C-x5-C-x3-H type (and similar)
zf-Dof	PF02701.6	25	Dof domain, zinc finger
zf-UBR	PF02207.10	25	Putative zinc finger in N-recognin (UBR box)

Example 9. Selection of transgenic plants with enhanced agronomic trait(s)

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This example illustrates the preparation and identification by selection of transgenic seeds and plants derived from transgenic plant cells of this invention where the plants and seed are identified by screening for a transgenic plant having an enhanced agronomic trait imparted by expression of a protein selected from the group including the homologous proteins identified
5 in Example 6. Transgenic plant cells of corn, soybean, cotton, canola, alfalfa, wheat and rice are transformed with recombinant DNA for expressing each of the homologs identified in Example 6. Plants are regenerated from the transformed plant cells and used to produce progeny plants and seed that are screened for enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and
10 enhanced seed oil. Plants are identified exhibiting enhanced traits imparted by expression of the homologous proteins.

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- _1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr, Gln-synt_N::Gln-synt_C, SAP18, TPP_enzyme_N::TPP_enzyme_M::TPP_enzyme_C, zf-C3HC4, eIF-1a, RPE65, PBP, Pkinase, AA_permease, F-box::LRR_1::LRR_2, zf-CCCH::zf-CCCH, Lactamase_B::Flavodoxin_1::Flavin_Reduct, Bac_globin, DSPc, adh_short, Tim17,
- 5 Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1, ubiquitin::ubiquitin::ubiquitin::ubiquitin, MBD, CXC::CXC, HSF_DNA-bind, Spermine_synth, AP2, Peptidase_S10, PALP, EIN3, Gln-synt_N::Gln-synt_C, 2OG-FeII_Oxy, Glyco_hydro_9, GDC-P, B3, PTPA, Acyltransferase, Isochorismatase, FMO-like, Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD, Lir1, Prismane, Fer2,
- 10 DEAD::Helicase_C, Molybdop_Fe4S4::Molybdopterin::Molydop_binding::Fer2_BFD, KNOX1::KNOX2::ELK, Glyoxalase, Sad1_UNC, Bac_globin, VPS28, PP2C, Pkinase::efhand::efhand::efhand, LEA_3, Peptidase_S10, Pkinase, CBFD_NFYB_HMF, Gln-synt_N::Gln-synt_C, Pyr_redox_2::Fer2_BFD::NIR_SIR_ferr::NIR_SIR, NUDIX::NUDIX, FAD_binding_3, GST_N::GST_C, SAM_decarbox, Acyltransferase, NTP_transferase, G-patch,
- 15 2OG-FeII_Oxy, Gln-synt_N::Gln-synt_C, AAA::Vps4_C, Histone, Pkinase, TPR_1, F-box::Kelch_1::Kelch_1, Spermine_synth, Bac_globin, Bac_globin, zf-UBR, Homeobox::HALZ, Whirly, NAD_binding_1, PTR2, EIN3, 4HBT, adh_short, 2OG-FeII_Oxy, P-II, Myb_DNA-binding::Myb_DNA-binding, DAGK_cat, AP2, MFS_1, Chloroa_b-bind, DUF716, zf-Dof, CCT, Homeobox::HALZ, Histone, 2OG-FeII_Oxy, Globin,
- 20 Pyr_redox_2::Fer2_BFD::NIR_SIR_ferr::NIR_SIR, Whirly, PsbP, bZIP_1, LRRNT_2::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::LRR_1::Pkinase_Tyr, Phi_1, BURP, Sterol_desat, DSPc, SNF5, Acyltransferase, GATase_2::Asn_synthase, adh_short, Homeobox::START, Pkinase, ParBc, SOUL, DNA_photolyase::FAD_binding_7, Pkinase_Tyr,
- 25 NAD_Gly3P_dh_N::NAD_Gly3P_dh_C, Na_H_Exchanger, peroxidase, Oxidored_molyb::Mo-co_dimer::Cyt-b5::FAD_binding_6::NAD_binding_1, Hexokinase_1::Hexokinase_2, DUF716, S10_plectin, Thi4, p450, CCT, adh_short, PSI_PSAK, DUF640, Thioredoxin, Globin, Ank::Pkinase, DAGAT, RPE65, Ank::Pkinase, GSHPx, Gln-synt_N::Gln-synt_C, MtN3_slv::MtN3_slv, Allene_ox_cyc, IGPD, MBD, CorA, Response_reg, Histone, AAA,
- 30 Ribosomal_L10e, Pkinase, DUF26::DUF26::Pkinase, p450, mTERF, AA_kinase, PBP, GUN4, Lactamase_B::Flavodoxin_1::Rubredoxin, C2, RRM_1::RRM_1, Histone, Alpha-amylase,

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tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil.

3. A transgenic plant cell nucleus comprising a recombinant DNA construct of claim 2.
- 5 4. A transgenic plant cell having a plant cell nucleus of claim 3.
5. The transgenic plant cell of claim 4 wherein said transgenic plant cell is homozygous for said recombinant DNA construct.
- 10 6. The transgenic plant cell of claim 4 further comprising DNA expressing a protein that provides tolerance from exposure to an herbicide applied at levels that are lethal to a wild type of said plant cell.
- 15 7. The transgenic plant cell of claim 5 wherein said herbicide is a glyphosate, dicamba, or glufosinate compound.
8. A transgenic plant comprising a plurality of plant cells of claim 4.
- 20 9. The transgenic plant of claim 8 wherein said transgenic plant is homozygous for said recombinant DNA construct.
10. A transgenic seed comprising a recombinant DNA construct of claim 2.
- 25 11. The transgenic seed of claim 10 from a corn, soybean, cotton, canola, alfalfa, wheat or rice plant.
12. A transgenic pollen grain comprising a recombinant DNA construct of claim 2.
- 30 13. A method for manufacturing transgenic seeds that can be used to produce a crop of transgenic plants with an enhanced trait resulting from expression of a DNA segment in a plant

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cell nucleus comprising a recombinant DNA construct of claim 2, wherein said method comprises:

- (a) screening a population of plants for said enhanced trait and said recombinant DNA construct, wherein individual plants in said population can exhibit said trait at a level less than,
5 essentially the same as or greater than the level that said trait is exhibited in control plants which do not contain said recombinant DNA construct, wherein said enhanced trait is selected from the group of enhanced traits consisting of enhanced water use efficiency, enhanced cold tolerance, enhanced heat tolerance, enhanced high salinity tolerance, enhanced shade tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil;
10 (b) selecting from said population one or more plants that exhibit said trait at a level greater than the level that said trait is exhibited in control plants; and
(c) collecting seeds from selected plant selected from step b.

14. The method of claim 13, wherein said method further comprises:

- 15 (a) verifying that said recombinant DNA construct is stably integrated in said selected plants; and
(b) analyzing tissue of said selected plant to determine the expression of a protein having the function of a protein having an amino acid sequence selected from the group consisting of one of SEQ ID NO: 299 through SEQ ID NO: 30468.

20

15. The method of claim 14 wherein said seed is corn, soybean, cotton, alfalfa, canola wheat or rice seed.

16. A method of producing hybrid corn seed comprising:

- 25 (a) acquiring hybrid corn seed from a herbicide tolerant corn plant which also has a stably-integrated, recombinant DNA construct of claim 2;
(b) producing corn plants from said hybrid corn seed, wherein a fraction of the plants produced from said hybrid corn seed is homozygous for said recombinant DNA construct, a fraction of the plants produced from said hybrid corn seed is hemizygous for said recombinant
30 DNA construct, and a fraction of the plants produced from said hybrid corn seed has none of said recombinant DNA construct;

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- (c) selecting corn plants which are homozygous or hemizygous for said recombinant DNA construct by treating with an herbicide;
- (d) collecting seeds from herbicide-treated-surviving corn plants and planting said seed to produce further progeny corn plants;
- 5 (e) repeating steps (c) and (d) at least once to produce an inbred corn line; and
- (f) crossing said inbred corn line with a second corn line to produce hybrid corn seed.

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Fig. 1

SEQ ID NO:	homolog	SEQ ID NOS
4210	-----	-----
4213	-----	-----
5	23796	-----MASNIMQRCNVFMSESVKLHAAMLALQFGYAGFHIVS
	5323	-----MSP-----VPERTKLHIAMAVFQTGYAGNHVIM
	9197	-----MVHGRLCNRDGWILTAMVVTEFSNMGVNTLV
	22010	-----MVHGRLCNRDGWILTAMVVTEFSNMGVNTLV
	2187	-----
10	26922	-----MAGAVSLWRREAVFLTAMLAGETSIVGLSTLF
	12208	-----MAGAVSLWRREAVFLTAMLAGETSIVGLSTLF
	27336	-----MAGAVSLWRREAVFLTAMLAGETSIVGLSTLF
	19871	-----MPIMAGTASPWRREAVFLTAMLVVETSIVGISTLF
	11517	-----
15	8202	-----MPIMAGTASPWRREAVFLTAMLVVETSIVGISTLF
	6010	-----
	16802	-----MARTVSLWRREAVFLTAMLATETGVVISTLF
	301	-----
	2040	-----MTRKYFQREVLPVTALVIMECANVGLNTLF
20	6920	-----
	5087	-----MKKEMI-----EEVVIVGGLVMVQFVYAGNSLLM
	5965	MEGLLSYRSNVLRNIMREREKDRKKMLISEIFSENEDEEVVIGGLVMVQFVYAGNSLLM
	3372	-----MAAVAMADGGGKKPYVVAIAQINTGTFFVS
	consensus	-----XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
25	30526	-----
	-----	-----
	RAALNMGVSKVFPVYRNILALMLIGPCAYFLEKKERPALTLISFLIQFFLLALCGITGQS	
30	RFALNLGVSKLIFPLYRNIIALSVLAPSAFFLEKKERPRMTSLLIQFFLLGLVGITLNG	
	KAATSKGLSPFVVLVYSYTFGSLLLLPLTFFSFRSLPPLTFSILCNMGILGLIASAFQ	
	KAATSKGLSPFVVLVYSYTFGSLLLLPLTFFSFR-----SAFQ	
	-----MYV	
	KVATSKGLNIYPFLSYSYLLASLLLLPSLFFTN-----SASILSKIGLLGFLGSMYV	
35	KVATSKGLNIYPFLSYSYLLASLLLLPSLFFTNRSRSLPPLSASILSKIGLLGFLGSMYV	
	KVATSKGLNIYPFLSYSYLLASLLLLPSLFFTNRSRSLPPLSASILSKIGLLGFLGSMYV	
	KFATSKGLNIYPFLGYSYLLASLLLLPSLFFTNRSSSLPPLSVSILSKIGLLGFLGSMYV	
	-----MYV	
	KFATSKGLNIYPFLGYSYLLASLLLLPSLFFTNRSSSLPPLSVSILSKIGLLGFLGSMYV	
40	-----MYV	
	KVATSKGLNLYAFLGYSYLLASLLLLPSLFFTDNRSLPPLSILSKIGLLGFLGSMYV	
	KAATLQGMGFHVIVYSYGLAALLLPSLFFSYRSRTLPPMNFSSILYKVVLGLIGCCSN	

45	SYLMSLGLGPFTIVIFSTFATFLILSPFAILFERKQWPKELSARLIGKLVLISFAGVTLF	
	SYLMSLGLGPFTIVIFSTFATFLILSPFAILFERKQWPDELSPRLIGKLVLISFAGVTLF	
	KAAFDSGMYTYIFVFYRLAAATALLVPAILINCACRRSQSTSTPAQAMSCRLFLKFLC	
	XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX	
50	-----	

	RIL-----SLR-IVLHIPTFA	
	GFY-----IFG-LDNTSPTFA	
55	ILG-----YNG-IKYSSPTLS	
	ILG-----YNG-IKYSSPTLS	

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ITG-----GIG-IEYSNPTLA
ITG-----GIG-IEYSNPTLA
ITG-----GIG-IEYSNPTLA
5 ITG-----GIG-IEYSNPTLA
ITG-----YIG-IEYSSPTLA
ITG-----YIG-IEYSSPTLA
ITG-----YIG-IEYSSPTLA
ITG-----YIG-IEYSSPTLA
ITG-----YIG-IEYSSPTLA
10 ITG-----YIG-IEYSSPTLA
-----
IMG-----YTG-INYSSPTLA
-----
QSL-----FLEGIRLTSPAMA
15 QTL-----FLEGIRLTSPAMA
AFLGSAGLTLQALTKFVKKSCQEVGGFTSLLSSLLQGKAHLHPQVYHASLKQTSATVA
xxx-----xxx-ixxxxptxa

20 -----
-----
SAIQNSVPAITFIMAAALRLEKVHISRRDGLAKIIGTVACVSGATIITLYKGPPITHIWR
SATENAVPAVSFLMAALLGIEKVELKRRDGVAKVVGTFVSVAGSLAITLYKGP---TIYQ
SAMSNNVPAFTFILAVVFRMENISLGKKSSVAKVLGTILSIIGALVVTLYHGP-----
25 SAMSNNVPAFTFILAVVFRMENISLGKKSSVAKVLGTILSIIGALVVTLYHGP-----
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFASS
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFASS
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFASS
SAIGNIVPALTFILAVIFRMEKVSFKERSSVAKVMGTILSLIGAFVVIFYHGPRVFASS
30 SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFASS
SAINNITPALTFILAIIFRMEKVSFKERSSLAKLMGTILSLIGALVVIFYHGPRVFASS
SAISNITPALTFILAIIFRMEKVSFKERSSVAKVMGTILSLIGALVVIFYHGPRVFASS
35 -----MEKASFKEKSSVAKVMGTIVSLVGALVVIFYHGPRVFASS
SAISNLTPAFTFLAILFRMESVSFKRTSSVAKMLGTIVSIGGAFIVTLYNGPVPVIMSP
-----MERIYWRHFSSQAKAIGTIVSMAGAFVVILYKGPPILKIHS
TAMPNLAPGLIFFIAWIVRLEKMNMKCVYSKLKILGTLLCVFGALTMSLMHSASIIQDEK
TAMPNLAPGLIFFIAWMVRLEKMDMKCVYSKLKILGTLLCVFGALTMSLMHSASIIQDEK
40 SAATNSMPVVTFLALVLRMETIKLRRRSGLGKLAGVALCLAGVLVIAFYVGPSIRPLAH
saxnxxpxxxxfxaxxxrmexxxxxxxxxsaxxgtxsxxgaxxxxxyxgpxxxxxxx

-----FLPDSKHVTASEDDNWILGCLLLLASSVFWSCWMIMQVPISSSCPDHVLST
45 -----FLPDSKHVTASEDDNWILGCLLLLASSVFWSCWMIMQVPISSSCPDHVLST
PNLEVTASYFKAFQGNLDAKSENWTLGCIYLLGNCLAWSGWIVLQAPVLKRYPARLSVT
PSLRSM---OPTNAGEAEEQKNWTLGCVCLMGHCLCWSSWIVLQSPLLKRYPARYSFV
-----MLMSSHSDWIIGGGLLALQYILVSVSYLVMAHTMGRYPSAVVVT
-----MLMSSHSDWIIGGGLLALQYILVSVSYLVMAHTMGRYPSAVVVT
50 P-----PVLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQTHIMREYPEAFTVS
P-----PVLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQTHIMREYPEAFTVS
P-----PVLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQTHIMREYPEAFTVS
P-----PVLNFRQLSPPLSSSKSDWLIGGAILTIQGIFVSVSFILQAHIMREYPEAFTVS
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Transgenic Plants With Enhanced Agronomic Traits
Figure 1 of 4

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 GPLFCAMFKPIGIIITVFLGSIFLGDSELYLGSVLGGILISLGFYVVMWGKA-----
 55 GPVVMVSMFSPFATVISVGFSLVTLGESICLGSVGGMALMFGLYLVWAKG-----
 GPVVMVSMFSPATVISVGLSVVTLGEPVRIGSVGGMALMFGLYLVWAKG-----

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Transgenic Plants With Enhanced Agronomic Traits
Figure 1 of 4

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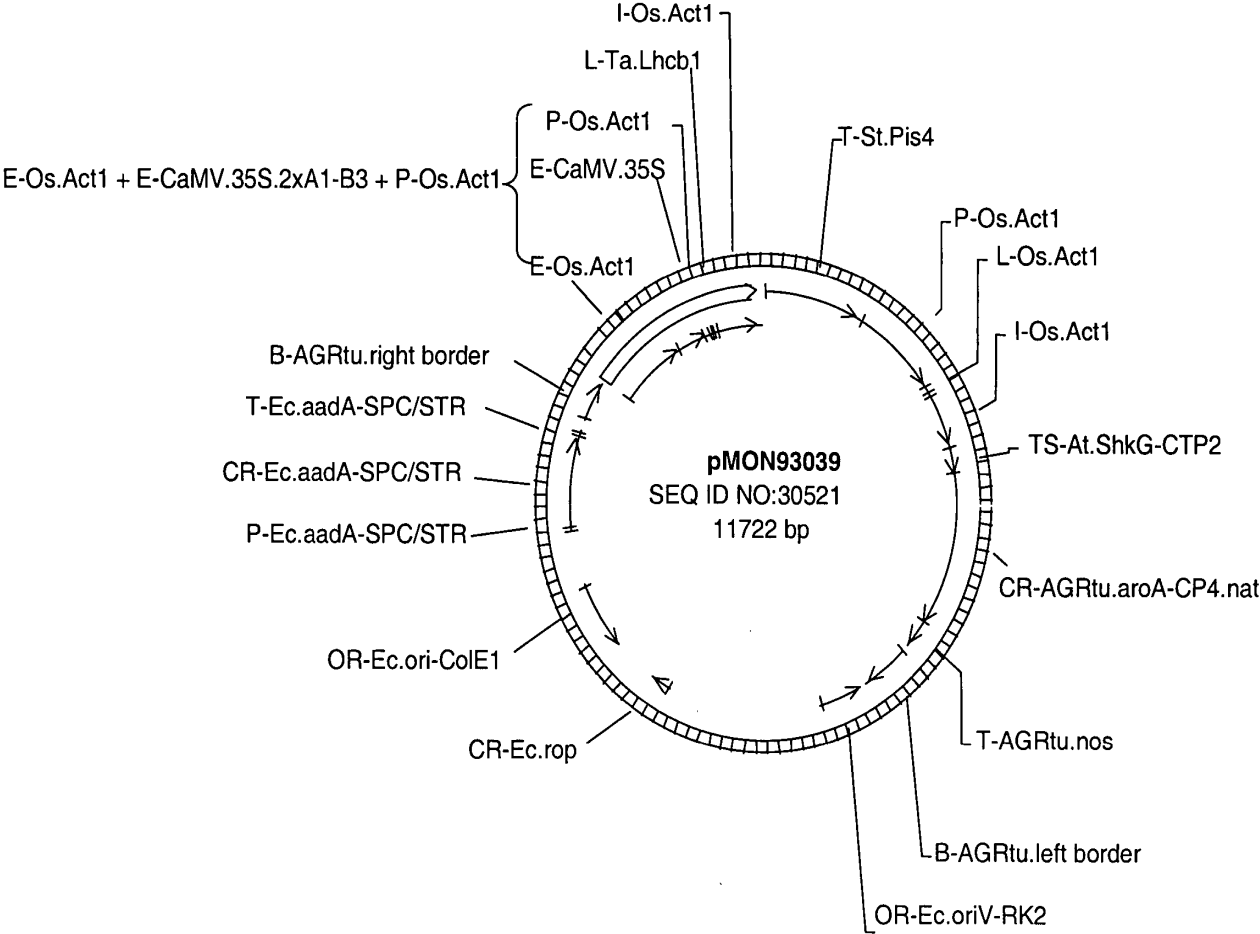
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15  ----NEEKDKLL---SFSGKEKTPLLLSGKNEQV-----
   -----
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20  ----NEEKDQLL---LVSGKERTPLLLNG-----
   ----KEGKTQFL---SLS--EETPLLDENIDDRI-----
   NKANNEDATNEADHDSPLASQKAPLLESYKNDQV-----
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30  -----*
   -----*
   DEQPQ*
   -----*
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35  -----*
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40  -----*
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45  -----*
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50  -----*
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Transgenic Plants With Enhanced Agronomic Traits
Figure 2 of 4

Fig. 2 Plasmid map of pMON93039



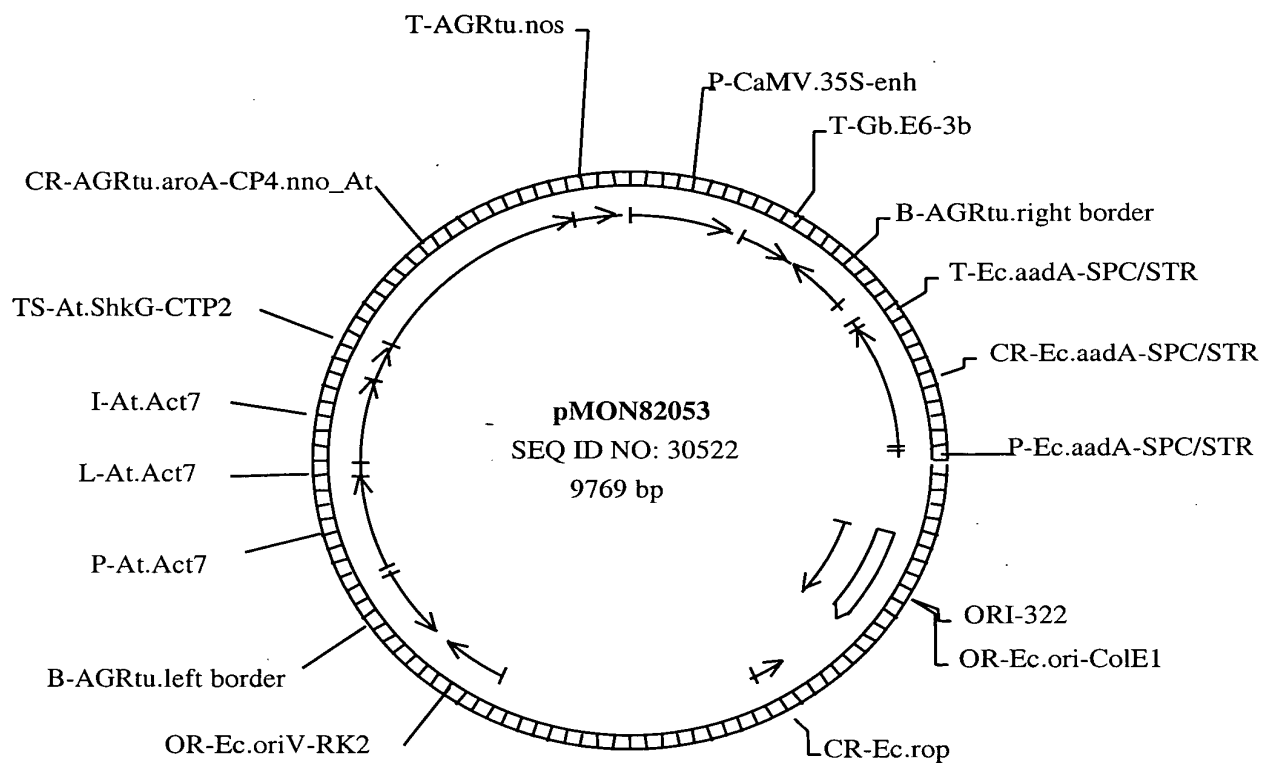
5/7

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Transgenic Plants With Enhanced Agronomic Traits
Figure 3 of 4

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Fig. 3 Plasmid map of pMON82053

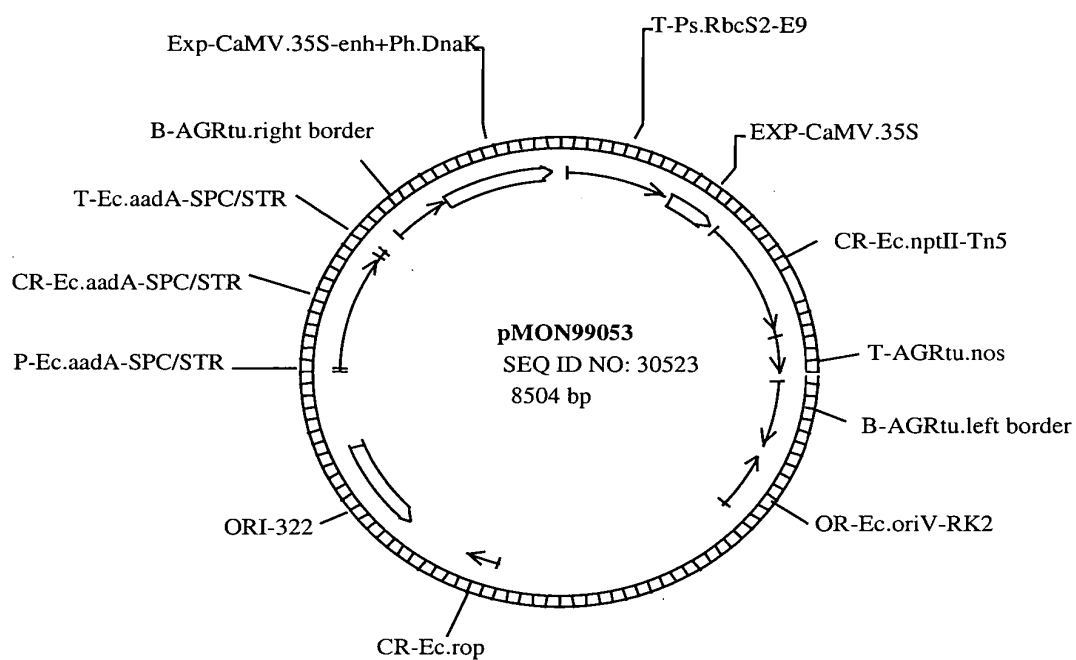


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Figure 4 of 4

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Fig. 4 Plasmid map of pMON99053



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- (71) **Applicant (for all designated States except US):** **MON-SANTO TECHNOLOGY, LLC** [US/US]; 800 North Lindbergh Boulevard, St. Louis, MO 63167 (US).
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[Continued on next page]

(54) **Title:** TRANSGENIC PLANTS WITH ENHANCED AGRONOMIC TRAITS

(57) **Abstract:** This invention provides recombinant DNA constructs, transgenic plant nuclei and cells with such recombinant DNA construct for expression of proteins that are useful for imparting enhanced agronomic trait(s) to transgenic crop plants. This invention also provides transgenic plants and progeny seed comprising the transgenic plant cells where the plants are selected for having an enhanced trait selected from the group of traits consisting of enhanced water use efficiency, enhanced cold tolerance, increased yield, enhanced nitrogen use efficiency, enhanced seed protein and enhanced seed oil. Also disclosed are methods for manufacturing transgenic seed and plants with enhanced traits.

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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, 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.

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Published:

- *with international search report*
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- *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

(88) **Date of publication of the international search report:**
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INTERNATIONAL SEARCH REPORT

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PCT/US2008/008557**A. CLASSIFICATION OF SUBJECT MATTER***A01H 1/00(2006.01)i, C12N 15/82(2006.01)i, C12N 5/04(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)

IPC A01H 1/00, A01H 4/00, A01H 5/00

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

Korean Utility models and applications for Utility models since 1975

Japanese Utility models and applications for Utility models since 1975

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

eKIPASS(KIPO internal) "recombinant DNA", "SEQ ID NO:309", "enhanced trait",

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KENNETH J. McDOWALL et al. 'The <i>ams-1</i> and <i>mre-3071</i> Temperature-Sensitive Mutations in the <i>ams</i> Gene Are in Close Proximity to Each Other and Cause Substitutions within a Domain That Resembles a Product of the <i>Escherichia coli</i> <i>mre</i> Locus.' Journal of Bacteriology. July 1993, pages 4245-4249. See the Abstract and figures 1-3	1
A	JP 15-310071 A(KUMHO PETROCHEM CO LTD) 5 NOV.2003 See the Claims 1-5 and figure 1	1
A	SWAINE L. CHEN et al. 'Identification of genes subject to positive selection in uropathogenic strains of <i>Escherichia coli</i> : A comparative genomics approach.' PNAS. April 2006, Vol. 103, no.15, pages 5977-5982. See the Abstract and tables 1-2, figures 1-3, column 10	1

☐ 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 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 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

06 FEBRUARY 2009 (06.02.2009)

Date of mailing of the international search report

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Name and mailing address of the ISA/KR

Korean Intellectual Property Office
Government Complex-Daejeon, 139 Seonsa-ro, Seo-
gu, Daejeon 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

CHO, Hyun Kyung

Telephone No. 82-42-481-5629



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2008/008557

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.: 2-16
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:

: Claim 2 is too broad to make meaningful search regarding to the expression of constitutional elements (b) and (c). Since claims 3-16 are dependent on claim 2, they are broad too.

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:

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

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

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

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

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

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C07K 5/083 (2006.01) *G01N 33/53* (2006.01)
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C12N 15/63 (2006.01) *C07K 1/22* (2006.01)

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kind of national protection available): AE, AG, AL, AM,
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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,
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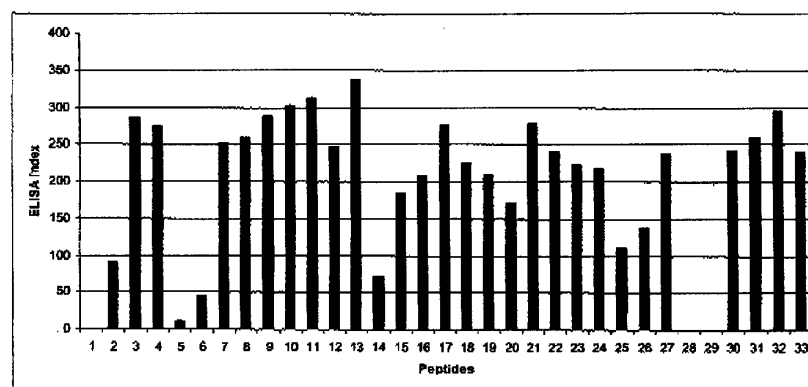


FIGURE 2

(57) Abstract: The present invention relates to anchor peptides for linking a ligand molecule to a solid support. The anchor peptide includes an amino acid motif having at least one neutral polar residue and at least one basic polar residue. In one aspect of the invention the anchor peptide is ATKASK (SEQ ID NO: 6). The anchor peptide of the present invention may be used in methods for immobilizing a ligand molecule to a solid support. Ligand molecules include Protein A.

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NOVEL AMINO ACID LINKER SEQUENCES FOR LIGAND IMMOBILIZATION

Field of the Invention

[0001] This invention relates to novel amino acid sequences that can function as linkers or anchors to facilitate immobilization of a protein ligand or another macromolecule onto a solid support.

Background of the Invention

[0002] Protein A is a membrane protein encoded by a bacterium (*Staphylococcus aureus*). Protein A consists of five homologous domains arranged as E-D-A-B-C. Protein A is unique in its ability to bind to most antibodies (including IgG, IgM, IgE, IgA) from various species including humans. Thus, Protein A has been widely used as a protein ligand immobilized on a solid support (eg., resin) to form an affinity column for antibody purification for research and therapeutic applications.

[0003] To fully exploit the antibody-binding property of Protein A for both pharmaceutical and biotechnological applications, methods to improve the immobilization efficiency of Protein A on a solid resin have been under investigation. Immobilization of a protein onto a solid support may be mediated through the ϵ -amine of the Lys side-chain. Although convenient, this method is non-specific, inefficient and may lead to loss of protein function. US Patent Nos. 5,084,559 and 5,260,373 describe methods of immobilizing Protein A in which a single cysteine residue, or a single arginine residue, is added to the N-terminus of Protein A, or a domain, or multiple domains thereof, to facilitate their immobilization.

[0004] Despite the foregoing, there remains a need to develop improved methods for protein immobilization and enhanced ligand stability.

[0005] This invention focused on identifying short amino acid sequence motifs that may function as site-specific linker or anchor for a protein or another biomolecule. By flanking a Lys residue with polar, neutral amino acids such as Ser and Thr, we have identified a series of novel short peptide sequences that exhibit superior efficiency in coupling the peptide to a solid support.

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[0006] These motifs can function as linkers to couple a protein, DNA molecules such as aptamers, or another biomolecule to the surface of a solid support in a site-specific and oriented manner. Immobilized ligands may be used in affinity purification of proteins and other biomolecules for research or therapeutic uses. Depending on the type of molecules immobilized and surface employed, they can also be developed into research or diagnostic tools.

Summary of the Invention

[0007] In one embodiment the present invention provides for an anchor peptide for linking a ligand molecule to a solid support, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.

[0008] In another embodiment the present invention provides for a fused product comprising a ligand molecule and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.

[0009] In another embodiment the present invention provides for a solid support having an immobilized fused product, the fused product comprising a ligand molecule and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.

[0010] In another embodiment the present invention provides for a method of immobilizing a ligand molecule to a solid support comprising: (a) forming a fused product comprising a ligand molecule and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue; and (b) contacting the fused product with a solid support under conditions to immobilize the fused product to the solid support.

[0011] In another embodiment the present invention provides for a method for purifying a molecule of interest in a sample suspected to include the molecule of interest, characterized in that the method comprises: (a) contacting the sample with a fused product immobilized to a solid support, the fused product comprising a ligand molecule

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that binds to the molecule of interest and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue, said contacting being under conditions wherein a complex is formed between the molecule of interest and the ligand molecule if said molecule of interest is present in the sample; and (b) recovering the molecule of interest from the complex thereby purifying the molecule of interest from the sample.

[0012] In another embodiment the present invention provides for a method for detecting a molecule of interest in a sample suspected to include the molecule of interest, characterized in that the method comprises: (a) contacting the sample to a fused product immobilized to a solid support, the fused product comprising a ligand molecule that binds to the molecule of interest and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue, said contacting being under conditions wherein a complex is formed between the molecule of interest and the ligand molecule if said molecule of interest is present in the sample; (b) recovering the molecule of interest from the complex; and (c) detecting the recovered molecule of interest.

[0013] In another embodiment the present invention provides for a vector comprising at least one copy of a gene of a ligand molecule and at least one gene capable of coding for an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.

[0014] In one embodiment of the present invention the at least one neutral polar residue is serine (S) or threonine (T), and wherein said at least one basic polar residue is lysine (K).

[0015] In one embodiment of the present invention the amino acid motif comprises the formula XX, wherein one X is a K and the other X is S or T.

[0016] In one embodiment of the present invention the anchor peptide further comprises at least one neutral non-polar residue.

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[0017] In one embodiment of the present invention the at least one neutral polar residue is S or T, the at least one basic polar residue is K and the at least one neutral non-polar residue is alanine (A).

[0018] In one embodiment of the present invention the amino acid motif comprises at least 2 amino acid residues.

[0019] In one embodiment of the present invention the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 3), wherein each X is K, S or T and wherein U is any amino acid.

[0020] In one embodiment of the present invention the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 4), wherein each X is A, K, S or T and wherein U is any amino acid.

[0021] In one embodiment of the present invention the amino acid motif comprises the formula XX-GA-XX (SEQ ID NO: 5), wherein each X is K, S or T.

[0022] In one embodiment of the present invention the amino acid motif comprises the sequence ATK (SEQ ID NO: 1) or ASK (SEQ ID NO: 2).

[0023] In one embodiment of the present invention the amino acid motif comprises the sequence ATKASK (SEQ ID NO: 6).

[0024] In one embodiment of the present invention the amino acid motif comprises the sequence ATKGATK (SEQ ID NO: 7).

[0025] In one embodiment of the present invention the ligand molecule includes an immunoglobulin-binding protein, an enzyme, a target protein binding partner or a nucleic acid molecule.

[0026] In one embodiment of the present invention the ligand molecule is protein A or a domain of protein A.

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[0027] In one embodiment of the present invention the anchor peptide is labelled with a detectable label.

[0028] In one embodiment of the present invention said anchor peptide is labelled with a cytotoxic molecule, a fluorescent molecule or radioactive molecule.

[0029] These and other aspects of the invention will become apparent from the detailed description by reference to the following Figures.

Brief Description of the Figures

[0030] Figure 1 is a table of a peptide library constructed by chemically synthesizing short peptides containing 14-15 amino acid residues with different motifs in accordance to Example 1.

[0031] Figure 2 shows the efficiency of immobilization of different peptides (the sequences of which are provided in Table 1) onto an epoxy-activated 96-well plate as evaluated by an ELISA assay.

[0032] Figure 3 A illustrates the anchor peptide ATKASK (SEQ ID NO: 6) motif linked to a single Z domain. Figure 3 B illustrates eight fusion products (SEQ ID NOS: 11 - 18) according to one embodiment of the present invention. The fusion products labelled 901 and 700 contain the ATKASK (SEQ ID NO: 6) motif.

[0033] Figure 4 illustrates a dot staining analysis of the immobilization efficiency of six of the eight fusion products illustrated in Figure 3 as well as a control bovine serum albumin (BSA) sample on an epoxy resin. On strip A, 901 and 902 fused products were dotted; on strip B, 903 and 904 fused products were dotted; on strip C, 123 fused product was dotted; on strip C, 201 fused product was dotted; and on strip E, control BSA was dotted. Ori, original sample without dilution.

[0034] Figure 5 is a graph showing a BCA protein analysis of the fused products referred to in Figure 4 using a protein assay kit (Pierce Co.). The protein concentrations in those samples were measured. The immobilization efficiency of a protein ligand was

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illustrated as percentages of a protein ligand concentration before and after immobilization process.

[0035] Figure 6 panels A to D show the IgG binding capacities of eight affinity columns composed of the protein A ligand (Z) fused to various N-linkers illustrated in Fig 3. A. fusion products 901 and 902, B. fusion products 903 and 904, C. fusion products 285 and 700, and D. fusion products 123 and 201.

Detailed Description of the Invention

Definitions

[0037] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Also, unless indicated otherwise, except within the claims, the use of "or" includes "and" and vice versa. Non-limiting terms are not to be construed as limiting unless expressly stated or the context clearly indicates otherwise (for example "including", "having" and "comprising" typically indicate "including without limitation"). Singular forms including in the claims such as "a", "an" and "the" include the plural reference unless expressly stated otherwise.

[0038] The term "functionally equivalent derivative" is used herein to refer to derivatized versions which retain the function of anchor to immobilize the selected protein without substantial loss of protein function.

[0039] The term "ligand" as used herein refers to a molecule or group of molecules capable of binding to one or more specific sites of a receptor, another molecule or target analyte. There can exist more than one ligand for a given target. The ligands may differ from one another in their binding affinities for the target molecule.

Overview of the Invention

[0041] The present invention relates in general to an anchor peptide. The anchor peptide may be used for linking a ligand molecule to a solid support. The anchor peptide

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may comprise an amino acid motif having at least one neutral polar residue and at least one basic polar residue. In one embodiment of the present invention the anchor peptide may further comprise at least one neutral non-polar residue. The neutral polar residue may be a threonine (T) or a serine (S) residue, the basic polar residue may be a lysine (K) residue, and the neutral non-polar residue may be an alanine (A) residue.

[0042] In another aspect, the present invention provides for an anchor peptide for linking a ligand molecule to a solid support, said anchor peptide comprising an amino acid motif having at least one neutral polar residue, at least one basic polar residue and at least one neutral non-polar residue.

[0043] The present method is not particularly restricted with respect to the ligand molecule that may be immobilized, and is therefore useful for immobilizing any protein of interest. Of particular interest is use of the method to immobilize immunoglobulin-binding proteins, including but not limited to, Protein A, Protein G, Protein A/G and Protein L. Other ligand molecules of interest may include nucleic acid ligands, including aptamers.

[0044] *The Anchor Peptide*

[0045] The anchor peptide may be suitable to immobilize the selected ligand molecule onto a solid support while substantially retaining the function of the selected ligand molecule, i.e. retaining at least about 50% of ligand molecule function, such as immunoglobulin binding, in its immobilized state. In one embodiment of the present invention, the anchor or linker may comprise a motif having at least 2 amino acid residues. The amino acid motif of the anchor may comprise equally three groups of amino acids- neutral non-polar, neutral polar, basic polar residues. An anchor sequence may also contain only the neutral-polar and basic residues in different combinations and arrangements.

[0046] In one embodiment of the present invention the anchor peptide may comprise an amino acid sequence ATK (SEQ ID NO. 1) or ASK (SEQ ID NO. 2).

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[0047] In one embodiment the present invention provides for a novel peptide including an amino acid formula XX-UU-XX (SEQ ID NO. 3), wherein each X may be K, S or T and U may be any amino acid. In one embodiment of the present invention the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 4), wherein each X is A, K, S or T and wherein U is any amino acid. In another embodiment the formula may be XX-GA-XX (SEQ ID NO. 5) wherein each X may be K, S or T.

[0048] In one embodiment of the invention, the anchor peptide may comprise an amino acid sequence ATKASK (SEQ ID NO. 6), or a functionally equivalent derivative thereof including at least 50% sequence homology.

[0049] In one embodiment the present invention provides for a novel peptide including the amino acid sequence SEQ ID NO. 6.

[0050] In one embodiment of the invention, the anchor peptide may comprise an amino acid sequence ATKGATK (SEQ ID NO. 7), or a functionally equivalent derivative thereof including at least 50% sequence homology.

[0051] In one embodiment the present invention provides for a novel peptide including the amino acid sequence SEQ ID NO. 7.

[0052] The anchor peptides of the present invention may also be provided as a fused product comprising the anchor peptide and a ligand molecule. The anchor peptide may be an N- or C-terminal extension of a ligand protein.

[0053] The anchor peptide or the fused product comprising the anchor peptide and a ligand protein may be synthesized by any known method in the art of peptide synthesis including solid phase synthesis (Merrifield (1964) J. Am. Chem. Assoc. 65:2149; J. Amer. Chem. Soc. 85:2149 (1963); and Int. J. Peptide Protein Res. 35:161-214 (1990)) or synthesis in homogenous solution (Methods of Organic Chemistry, E. Wansch (Ed.) Vol. 15, pts. I and II, Thieme, Stuttgart (1987) to generate synthetic peptides.

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[0054] The anchor peptide or the fused product comprising the anchor peptide linked to a ligand protein may be made by recombinant methods known in the art. In this regard, the anchor peptide may generally be bound or fused to a terminus of the selected ligand protein such that the function of the protein may substantially be retained. Thus, depending on the ligand protein, the anchor may be bound to the N-terminus or the C-terminus of the selected ligand protein. In certain aspects, the anchor peptide may be bound to both termini, or may additionally be bound to an amino acid residue which may not be a terminal residue. In another aspect, the anchor peptide may be bound to a solid support prior to the coupling of the protein ligand by chemical or recombinant means or through the use of an enzyme.

[0055] The anchor peptides of the invention may be labelled with a label to facilitate their detection in an assay as is understood by one of skill in the art. Such labels may include but are not limited to radioactive label, fluorescent label or cytotoxic label. The anchor peptides of the invention may be provided with a carrier such as for example coupled to bovine serum albumin (BSA) or keyhole limpet haemocyanin. The anchor peptides may be preceded by a Biotin-GGYG (SEQ ID NO: 55) N-terminal sequence that may facilitate peptide concentration determination by OD280 (of Tyr or Y) measurement. The N-terminal biotin moiety may provide a ligand for ELISA assay (see Figure 2) using avidin-HRP.

[0056] Once the ligand molecule is bound or fused to the anchor peptide of the present invention, the fused product may be immobilized onto a solid support. Suitable solid supports include epoxy-based supports, e.g. beads or resins, or solid supports having the surface chemistry of an epoxy group, or materials that are functionalized on the surface to allow coupling of a protein via the anchor sequence.

[0057] To immobilize the fused product to the solid support, the solid support may be treated in an aqueous solution. The aqueous solution may include a binding buffer, such as 0.1 M sodium phosphate/0.5 M Na₂SO₄, pH, e.g. in the range of about 6.5 to about 7.2. In one aspect the pH is 6.8. Suitable salts for use in this regard may include salts that may be compatible with the solid support and fused product, non-toxic

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with regard to the intended use of the immobilized protein and otherwise suitable for use. The solid support may be mixed with the fused product, for example at room temperature, for a sufficient period of time for immobilization to occur.

[0058] In embodiment, the present invention relates to a matrix for affinity separation. One end of the anchor peptides of the present invention may be bound to the matrix, and the other end of the anchor peptide may be bound to the ligand molecule. The matrix of the present invention, when compared to a matrix having the ligand directly bound to the matrix, may exhibit an increased immobilization capacity during two or more separations.

[0059] Examples of the material for the solid support is based on polymers having hydrophilic surface, such as polymers having hydroxyl group (-OH), carboxyl group (-COOH), aminocarbonyl group (-CONH₂, possibly in N-substituted forms), amino group (-NH₂, possibly in substituted form), oligo or polyethyleneoxy group on their external surface and if present, also on internal surface. In one embodiment, the polymer may be a synthetic polymer (e.g., polymethacrylate, polyacrylamide, or styrene-divinylbenzene copolymer). Such a synthetic polymer may be easily produced by a known method (see J. MATER. CHEM 1991, 1(3), 371-374, for example). Alternatively, a commercially available product such as TOYOPEARL™ may also be used. In another embodiment, the polymer is a polysaccharide (e.g., dextran, starch, cellulose, pullulan, or agarose). Such a polysaccharide may be easily produced by a known method (see Japanese Patent No. 4081143, for example). Alternatively, a commercially available product such as Sepharose (manufactured by GE Healthcare Biosciences) may also be used. In still another embodiment, an inorganic support (e.g., silica or zirconium oxide) may be used.

[0060] The solid support may be in the shape of particles. The particles may be porous or nonporous. The solid support in the shape of particles may be used as a packed bed, or may be used in a suspended form. The suspended form may be an expanded bed or a pure suspension, in which the particles can move freely. When using a packed bed, or an expanded bed, separation process used in known affinity chromatographic methods is used. When using a pure suspension, a batch method is used.

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[0061] The solid support in the shape of particles according to this embodiment may preferably have a particle size (volume average particle size) of about 20 to about 200 micrometers, and more preferably about 30 to about 100 micrometers. If the solid support has a particle size of less than about 20 micrometers, the pressure of column packed with this support may increase to an impractical level at a high flow rate. If the solid support has a particle size of more than about 200 micrometers, the binding capacity may deteriorate. Note that the term "particle size" used herein refers to a volume average particle size determined using a laser diffraction/scattering particle size distribution analyzer.

[0062] The solid support according to this embodiment may preferably be porous, and having a specific surface area larger than about 50-1000 m²/g, and more preferably about 80-400 m²/g. If the affinity chromatography packing material has a specific surface area of less than about 50 m²/g, the binding capacity may deteriorate. If the affinity chromatography packing material has a specific surface area of more than about 1000 m²/g, the packing material may be destroyed at a high flow rate due to a decrease in strength, so that the column pressure may increase. Note that the term "specific surface area" used herein refers to a value of the surface area of pores (pore size: 10 to 5000 nm) determined using a mercury porosimeter by the dry weight of the particles.

[0063] The solid support according to this embodiment may preferably have a volume average pore size of about 50 to about 400 nm, and more preferably about 100 to about 300 nm. If the affinity solid support has a volume average pore size of less than about 50 nm, the binding capacity may significantly deteriorate at a high flow rate. If the affinity chromatography packing material has a volume average pore size of more than about 400 nm, the binding capacity may deteriorate irrespective of the flow rate. Note that the term "volume average pore size" used herein refers to the volume average pore size of pores (pore size: 10 to 5000 nm) determined using a mercury porosimeter.

[0064] Specific examples of porous particles used as the solid support according to this embodiment may include porous organic polymer particles including a copolymer of 20 to 50 wt% of a crosslinkable vinyl monomer, 3 to 80 wt% of an epoxy group-

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containing vinyl monomer, and 20 to 80 wt% of a diol group-containing vinyl monomer, and having a particle size of 20 to 80 micrometers, a specific surface area of 50 to 150 m²/g, and a volume average pore size of 100 to 400 nm.

[0065] In yet another form embodiment, the solid support may be in another form such as a surface, a monolith, a chip, capillaries, or a filter.

[0066] The ligand molecule may be attached to the support via conventional coupling techniques utilizing, e.g. amino and/or carboxy groups present in the ligand. Bisepoxides, epichlorohydrin, CNBr, N-hydroxysuccinimide (NHS) etc. are well-known coupling reagents. Between the support and the ligand, a molecule known as a spacer may be introduced, which will improve the availability of the ligand and facilitate the chemical coupling of the ligand to the support. Alternatively, the ligand may be attached to the support by non-covalent bonding, such as physical adsorption or biospecific adsorption.

[0067] In one embodiment of the present invention, the molecule ligands may be peptide ligands that may be used in the purification of immunoglobulins of interest from a composition comprising at least one immunoglobulin of interest and at least one other substance from which the immunoglobulin is to be separated.

[0068] The fused product comprising the anchor peptides of the present invention bound to peptide ligands, may be immobilized on to a solid support or may be used in solution. The solid support may then be contacted with a composition containing an immunoglobulin, the immunoglobulin then binding to the peptide ligand.

[0069] When using the fused products immobilized on a solid support, the composition containing the immunoglobulin may be contacted to the fused products-solid support either before or after the solid support material is packed into a column. For example, the fused products-solid support material may be incubated with the composition containing the immunoglobulin and then this mixture may be packed into a column for further purification steps. Alternatively, the fused product-solid support may be first packed into the column followed by the addition of the composition containing

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the immunoglobulin of interest. The fused products-solid support material may first be packed in the column followed by the introduction of the composition containing the immunoglobulin of interest.

[0070] The recovery of the immunoglobulin from the fused products-solid support may involve any technique conventionally used in affinity chromatography to remove compounds from solid supports, including changes in pH or ionic strength. In one preferred embodiment of this invention, the immunoglobulin is eluted using a buffer at or below pH 4.

[0071] In an additional embodiment of the invention, the fused products may be used in assays for the detection of molecules of interest such as antibodies in a solution or composition suspected of containing the same. The fused products may be coupled to a detectable group or label. The contacting and separating steps may be carried out in solution or with immobilized fused products. Methods of detecting labelled fused products include those for the detection of labelled immunoglobulin. When carried out in solution, fused products bound immunoglobulin may be separated and/or detected from the solution using a variety of techniques including, but not limited to, fluorescence-activated cell sorting, membrane filtration and mass spectrometry.

[0072] One embodiment of this invention includes methods of using a fused product of the present invention in methods for determining the presence or concentration of a molecule of interest in a sample. The ligand component of the fused product being a ligand of the molecule of interest. The method may include:

[0073] (a) contacting said sample to the fused product under conditions wherein a molecule of interest/ligand complex is formed if said molecule of interest is present in the sample; and

[0074] (b) detecting of the molecule of interest by detecting the molecule of interest/ligand complex, thereby determining the presence of the molecule of interest in the sample. Step (b) may also include measuring the amount of the molecule of interest in the sample by quantitatively detecting the complex.

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[0075] One embodiment of this invention includes methods of using a fused product of the present invention in an affinity column for the determination of presence and/or concentration of molecules of interest in a sample suspected of containing the same. The methods may include the following steps:

[0076] (a) immobilizing a fused product of the present invention to an affinity column, the ligand component of the fused product being a ligand of the molecule of interest;

[0077] (b) running the sample through the affinity column under conditions whereby the molecule of interest is captured by the fused product if said molecule of interest is present in the sample, thereby ;

[0078] (c) recovering the molecule of interest from the column with a recovering agent; and

[0079] (d) measuring the quantity of molecule of interest captured by the column by methods such as direct fluorescence measurement, high performance liquid chromatography and mass spectrometry of the molecule of interest. Alternatively, prior to the recovering step, the molecule of interest/ligand complex formation within the column may be detected by the use of fluorescence, or fluorescence in combination with quenchers, or fluorescence polarization, and electro-affinity analysis.

[0080] Embodiments of the invention are described in the following specific example which is not to be construed as limiting.

EXAMPLES

Example 1 – Chemical synthesis of a peptide library

[0081] A peptide library was constructed by chemically synthesizing short peptides containing 14-15 amino acid residues with different motifs (Figure 1). These peptides were synthesized by the Fmoc chemistry based solid-phase peptide synthesis and labelled at the N-terminus with biotin. After synthesis, the peptides were dissolved in

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water and the identities (eg., molecular masses) were confirmed by MALDI-TOF mass analysis.

Peptides 1 to 24 of Figure 1 are designed in such a way that a lysine (K) is next to an Ser (S) or Thr (T) residue or flanked by different combinations of serine (S), threonine (T) and other types of amino acids. The motif length may be six as in XX-UU-XX (SEQ ID NO: 3) or XX-GA-XX (SEQ ID NO: 5) (peptides 9 to 16, wherein at least one K is in the X positions). The GA dipeptide may serve as a spacer between the XX motif, but GA can be replaced by other amino acid types as in the motif XX-UU-XX (SEQ ID NO: 3), where U represents any amino acid. The motif can be as short as containing two amino acid as in the motif XX, wherein one X is K, and the other X is S or T. The peptide core sequence may be preceded by a Biotin-GGYG (SEQ ID NO: 55) N-terminal sequence that may facilitate peptide concentration determination by OD280 (of Tyr or Y) measurement. The N-terminal biotin moiety may provide a ligand for ELISA assay (see Figure 2) using avidin-HRP.

Example 2 – Identification of peptide motifs that allow efficient ligand immobilization onto a solid support

[0082] The synthetic peptides are each dissolved in ddH₂O and the pH value of the solution adjusted to pH 7-8 using a 0.1N NaOH solution. The resulting peptide solutions are added, respectively, to different wells of a 96-well plate containing epoxy groups on the surface and incubated at 4 °C overnight. The plate is washed in PBS and then blocked with 5% skim milk at room temperature for 1 hour. After blocking, a avidin-HRP solution (1:40,000 dilution) was added to the plate and incubated at room temperature for 1 hour. The plate is washed again three times with PBS and incubated with tetramethylbenzidine (TMB), a colorless substrate. After 20 minutes, the reaction is stopped by adding 2M H₂SO₄. The OD at 405 nm was recorded for the plate using a Multi-label reader (Perkin-Elmmer). As shown in Figure 2, a higher OD₄₀₅ value corresponds to a greater amount of a peptide immobilized in a well. As illustrated in Figure 2, a number of peptides showed efficient ligand immobilization to the solid support. Peptides that exhibited the greatest immobilization efficiency contained the ATK (SEQ ID NO: 1) and ASK (SEQ ID NO: 2) motifs (highlighted in Figure 1).

Example 3 – Protein ligand immobilization on the epoxy resin

[0083] Using a molecular cloning method, a single Z domain was fused with various N-linker sequences as illustrated in Fig. 3. These fused protein ligands were expressed in a bacterial expression system. The target proteins were purified with the Ni-NTA affinity column (GE Healthcare) to highly homology. Protein ligand's purity was examined by SDS-PAGE analysis.

[0084] The purified protein ligands were prepared in the immobilization buffer (0.1 M sodium phosphate/0.5 M Na₂SO₄, pH 6.8). Five milligram of each protein ligand solution was mixed with 1 millilitre of the epoxy-activated resin (JSR). The protein ligand/resin mixture was incubated at about room temperature overnight with a rotating mixer. Immobilization of the fused product in each case may be monitored by the dot staining method (FIG. 4) and by the BCA protein measuring kit (FIG. 5).

[0085] The results illustrated in FIGS. 4 and 5 confirmed that the Z protein ligand fused with a N-linker containing either "ATKASK" (SEQ ID NO: 6) (ligand 901) motif or "ATKGATK" (SEQ ID NO: 7) (ligand 902) motif was successfully immobilized on the beads, while the Z ligand fused with other N-linker sequences without the motifs "ATKASK" (SEQ ID NO: 6) or "ATKGATK" (SEQ ID NO: 7) were less efficient to be immobilized onto the beads under the same conditions.

Example 4 – IgG binding capacity of Immobilized Protein A containing an ATKASK (SEQ ID NO: 6) or ATKGATK (SEQ ID NO: 7) anchor

[0086] Eight affinity columns of 1 ml bed volume, packed with the epoxy beads (JSR) immobilized with eight protein ligands with various N-linkers, were analyzed to determine their human IgG binding capacity (Fig. 5). The IgG solution at 1 mg/ml of PBS was added to each affinity column. The flow through samples were collected. OD280 values were monitored between the loading IgG solution and the flow through samples. The differences of the OD280 values between the initial IgG solution and the flow through samples are the indication of the affinity column's IgG binding capacity. The higher an affinity column's IgG binding capacity, the lower the percentage of the

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initial IgG OD280 reading and the flow through sample OD280 reading. Results are depicted in Figure 6. Figure 6 A and 6 C show that in both 901 and 902 columns as well as 700 columns, the percentage of eluted IgG (unbound IgG) was approximately at 25-30 % at the 20 mg IgG loading, while the rest of the columns showed the percentage above 50%. These three columns contain the protein ligands fused with the N-linker containing the motif of "ATKASK" (SEQ ID NO: 6) (901 and 700) or the motif of "ATKGATK" (SEQ ID NO: 7) (902). These data indicate that the N-linker containing the motif of "ATKASK" (SEQ ID NO: 6) would greatly facilitate a protein ligand to be immobilized on an epoxy bead and thus enhance the IgG binding capacity.

Methods and Materials for Examples 3 and 4

[0087] **(1) Vector constructs for Protein A expression** – The Z domain of protein A fused to a different anchor peptide was cloned into the pETM11 vector (European Molecular Biology Laboratory) or pET302/N-His (Invitrogen). The original Protein A gene was amplified by PCR method using a TAP-Tag plasmid (EMBL) containing Protein A gene as a template. The Z domain was created by the PCR assisted QuickChange Site-Directed Mutagenesis method (Stratagene Cloning System).

[0088] All protein A ligands with various N-linkers listed in FIG. 3B (201, 123, 901, 902, 903, 904, 285, 700) were manufactured in the E. coli expression system, BL21(DE3) pLysS competent cells, as described in the following paragraphs.

[0089] The eight expression vectors listed in Fig. 3B were constructed as follows:

[0090] 201 expression vector was constructed by PCR the single Z domain with the restriction enzyme sites: EcoRI/BamHI and cloned the single Z domain into the expression vector of pET302/N-His (Invitrogen).

[0091] 123 expression vector was constructed by PCR the single Z domain with the restriction enzyme sites: NcoI/BamHI and cloned the single Z domain into the expression vector of pETM11 (EMBL).

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[0092] 901, 902, 903, and 904 expression vectors were constructed by deleting V and introducing ATKASK (SEQ ID NO: 6) (901), ATKGATK (SEQ ID NO: 7) (902), AHKGAHK (SEQ ID NO: 56) (903) and GHTKGHTK (SEQ ID NO: 57)(904) into the 123 by using the QuickChange Site-Directed Mutagenesis method (Stratagene Cloning system).

[0093] 285 expression vector was constructed by deleting MSDYDIPTT (SEQ ID NO: 58) in 123 by using the QuickChange Site-Directed Mutagenesis method (Stratagene Cloning system).

[0094] 700 expression vector was constructed by deleting MV and adding ATKASK (SEQ ID NO: 6) in 285 by using the QuickChange Site-Directed Mutagenesis method (Stratagene Cloning system).

[0095] **(2) Recombinant protein expression and purification** - BL21(DE3) pLysS competent cells allow high-efficiency protein expression of any gene that is under the control of a T7 promoter and has a ribosome binding site. BL21(DE3)pLysS is lysogenic for λ -DE3, which contains the T7 bacteriophage gene I, encoding T7 RNA polymerase under the control of the lac UV5 promoter. BL21(DE3)pLysS also contains a plasmid, pLysS, which carries the gene encoding T7 lysozyme. T7 lysozyme lowers the background expression level of target genes under the control of the T7 promoter but does not interfere with the level of expression achieved following induction by IPTG. Target DNA plasmids were used to transfect BL21 competent cells. 0.5 μ l of plasmid DNA containing about 0.5 ug of DNA was used for transfection. The BL21 competent cells were stored at - 80 degree Celsius. An aliquot of BL21 cells (20 μ l) was thawed and put on ice. 0.5 μ l of plasmid DNA was mixed with the competent cells and put on ice for 60 minutes. After incubation on ice, these cells containing plasmid DNA were "heat shocked" in 45 degree C water bath for 45 seconds and immediately put on ice for another 2 minutes. Then, 30 μ l of LB medium was added to the bacterial cells and shaken at 37 degree C for 1 hour. These cells were then spread on agar /LB plates containing kanamycine (final concentration: 100 μ g/ml). These plates were incubated at 37 degree C overnight.

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[0096] For expression of recombinant Protein A domains, the DNA plasmids were transformed into *E. coli* BL21. Overnight cultures were inoculated into fresh medium and grown to an OD_{600nm} of approximately 0.5. Isopropyl beta-D-1-thiogalactopyranoside was added to a concentration of 1 mM, and the culture was grown for a further 15 hours at 18 degree C. Cells were harvested by centrifugation at 7,000 rpm for 10 min in a Sorvall GS-3 rotor.

[0097] The pellet was resuspended in phosphate-buffered saline (PBS) containing protease inhibitor (Roche Applied Science), lysozyme (200 µg/ml), and DNase I (3 µg/ml). Cells were lysed by repeated freeze/thaw cycles. Cell debris was removed by centrifugation.

[0098] Recombinant proteins expressed in pETM11 vector contained an amino-terminal His-Tag. His-Tag fusion proteins were purified using the Ni-NTA columns (Qiagen) and dialyzed against PBS.

[0099] **(3) Protein Analysis by Polyacrylamide gel electrophoresis (SDS-PAGE)** - Electrophoresis was performed on polyacrylamide gels in the presence of sodium dodecyl sulphate (SDS). Samples (10 µl) were mixed with 10 µl of sample buffer (62.5mM Tris-HCl, pH 6.8, 2% SDS, 5% mercaptoethanol, 10% glycerol and 0.0025% brophenol blue), heated at 90 C for 2 min, cooled and loaded on gels. Electrophoresis was performed at 75 mA in an apparatus purchased from Bio-Rad. Gels were stained in a solution of 0.5 mg/ml Coomassie blue in 5:5:1 methanol/water/acidic acid and de-stained in water heated in a microwave.

[00100] **(4) Peptide synthesis** -Fmoc solid-phase peptide synthesis method was used to synthesize all peptides. Protected amino acid derivatives were purchased from AnaSpec (US); N,N-diisopropylethylamine (DIEA), HBTU, HOBT, piperidine were purchased from EMD (Canada); and Tentel gel resin was purchased from Intavis (Canada). Other reagents and solvents were purchased from Sigma (Canada). MALDI-TOF-mass spectra were recorded on a Micromass® bench-top MALDI-TOF mass analysis with reflection ion mode.

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[00101] **(5). Synthesis of porous beads** - 8.2 g of glycidyl methacrylate (manufactured by Mitsubishi Rayon Co., Ltd.), 65.9 g of trimethylolpropane trimethacrylate (manufactured by Sartomer), and 90.6 g of glycerol monomethacrylate (manufactured by NOF Corporation) were dissolved in 245.8 g of 2-octanone (manufactured by Toyo Gosei Co., Ltd.) and 62 g of acetophenone (manufactured by Wako Pure Chemical Industries, Ltd.), and 2 g of 2,2'-azoisobutyronitrile (manufactured by Wako Pure Chemical Industries, Ltd.) was added to the solution to prepare an organic monomer solution.

[00102] 8.5 g of polyvinyl alcohol ("PVA-217" manufactured by Kuraray Co., Ltd.), 0.43 g of sodium dodecyl sulfate ("Emal 10G" manufactured by Kao Corporation), and 21.3 g of sodium sulfate (manufactured by Wako Pure Chemical Industries, Ltd.) were added to 4240 g of distilled water. The mixture was stirred overnight to prepare an aqueous solution.

[00103] A separable flask (7 l) was charged with the resulting aqueous solution. The separable flask was equipped with a thermometer, a stirring blade, and a cooling tube, and placed on a hot water bath. The aqueous solution was stirred at 600 rpm in a nitrogen atmosphere. When the temperature of the aqueous solution had reached 85°C, the organic monomer solution was added to the aqueous solution using a dropping funnel. The mixture was stirred for 5 hours.

[00104] After cooling the reaction solution, the reaction solution was transferred to a polypropylene bottle (5 l). The reaction solution was allowed to stand until the beads floated. Unnecessary water was then sucked out from the bottom of the bottle. Acetone was then added to the reaction solution to precipitate the beads. After allowing the reaction solution to stand for 3 minutes, acetone was removed by decantation. After repeating this operation twice, water was added to precipitate the beads. After allowing the mixture to stand for 3 minutes, the mixture was subjected to decantation. After replacing the dispersion medium of the bead dispersion with acetone, the mixture was air-dried overnight. The mixture was then dried using a vacuum dryer to obtain 90 g of

porous beads (hereinafter referred to as "PB"). The average particle size of the PB was 43 micrometers, and the specific surface area of the PB was 83 m²/g.

[00105] **(6) Immobilization of protein ligands on the epoxy beads-** Epoxy-activated beads obtained as described in the previous section, were used for immobilization. The epoxy beads were washed with the immobilization buffer before the immobilization. Five milligram of protein ligand fused to an anchor peptide was added to 1 ml of the epoxy resin. The protein/resin mixture was incubated at room temperature overnight with a rotating shaker. After overnight immobilization, the protein ligand fused to an anchor peptide (such as 901, 902, etc) resin was blocked with 1M thioglyceol in 0.1 M Na₂SO₄, pH 8.3 for 4 hours at room temperature. After blocking, the resin was washed with 1 x PBS (+ 0.5% Tween 20). After washing, a protein A affinity resin was ready for further use.

[00106] **(7) Dot analysis** – Referring to Figure 4, products 901 (Figure 4 A), 902 (Figure 4 A), 903 (Figure 4 B), 904 (Figure 4 B), 123 (Figure 4 C) and 201 (Figure 4 D) before immobilization (top row) and after immobilization (product left in solution, bottom row) on the epoxy beads were diluted 1:1 in PBS respectively and dotted on a nitrocellulose filter paper. The filter strips were stained by Coomassie blue. In a control experiment, BSA (bovine serum albumin, Pierce Co.) was immobilized on the same epoxy beads and samples were taken before and after the immobilization reaction and dotted on a nitrocellulose filter paper, as illustrated in Figure 4 E (Top row: before immobilization; bottom row: after immobilization).

(8) BCA protein analysis – Referring to Figure 5 a BCA protein analysis of the fused products referred to in Figure 4 was performed using a protein assay kit (Pierce). Briefly, BCA Protein Assay combines the well-known reduction of Cu²⁺ to Cu¹⁺ by protein in an alkaline medium with the highly sensitive and selective colorimetric detection of the cuprous cation (Cu¹⁺) by bicinchoninic acid. The first step is the chelation of copper with protein in an alkaline environment to form a light blue complex. In this reaction, known as the biuret reaction, peptides containing three or more amino acid residues form a colored chelate complex with cupric ions in an alkaline environment containing sodium

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potassium tartrate. In the second step of the color development reaction, bicinchoninic acid (BCA) reacts with the reduced (cuprous) cation that was formed in step one. The intense purple-colored reaction product results from the chelation of two molecules of BCA with one cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations.

[00107] For the BCA assay, 200 μ l of the reaction solution was added into each well on a 96-well plate. Then, 25 μ l of BSA standard samples or unknown protein samples was added into each well. The 96-well plate containing samples was incubated at 37 °C, for 30 minutes. The purple color was measured at OD595nm using the microplate reader (Perkin-Elmer). A BSA standard curve was constructed by using protein concentration as X axis and OD595 as Y axis. Unknown protein samples' concentrations were deduced based on the BSA standard curve.

[00108] The protein concentrations in those samples were measured. The immobilization efficiency of a protein ligand was illustrated as percentages of a protein ligand concentration before and after immobilization process.

[00109] **(9) IgG binding capacity of a Protein A affinity column**-Protein A affinity columns were evaluated for their IgG binding capacities. Human IgG solution at 1 mg/ml of PBS was loaded on 1 ml of bed volume Protein A affinity resin. The flow-through samples were collected. OD280 value was measured in both loading IgG solution and flow-through samples to calculate the IgG content in samples. The percentage of IgG content in the loading solution and in the flow-through samples indicated a Protein-A affinity column's IgG binding capacity (illustrated at Y axis of Figure 6).

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CLAIMS

We Claim:

1. An anchor peptide for linking a ligand molecule to a solid support, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.
2. The anchor peptide of claim 1, characterized in that said at least one neutral polar residue is serine (S) or threonine (T), and wherein said at least one basic polar residue is lysine (K).
3. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the formula XX, wherein one X is a K and the other X is S or T.
4. The anchor peptide of claim 1, characterized in that said anchor peptide further comprises at least one neutral non-polar residue.
5. The anchor peptide of claim 4, characterized in that said at least one neutral polar residue is S or T, said at least one basic polar residue is K and said at least one neutral non-polar residue is alanine (A).
6. The anchor peptide of claim 1, characterized in that the amino acid motif comprises at least 2 amino acid residues.
7. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 3), wherein each X is K, S or T and wherein U is any amino acid.
8. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 4), wherein each X is A, K, S or T and wherein U is any amino acid.
9. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the formula XX-GA-XX (SEQ ID NO: 5), wherein each X is K, S or T.

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10. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the sequence ATK (SEQ ID NO: 1) or ASK (SEQ ID NO: 2).
11. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the sequence ATKASK (SEQ ID NO: 6).
12. The anchor peptide of claim 1, characterized in that the amino acid motif comprises the sequence ATKGATK (SEQ ID NO: 7).
13. The anchor peptide of claim 1, characterized in that the ligand molecule includes an immunoglobulin-binding protein, an enzyme, a target protein binding partner or a nucleic acid molecule.
14. The anchor peptide of claim 1, characterized in that the ligand molecule is protein A or a domain of protein A.
15. The anchor peptide of claim 1, characterized in that the anchor peptide is labelled with a detectable label.
16. The anchor peptide of claim 1, characterized in that said anchor peptide is labelled with a cytotoxic molecule, a fluorescent molecule or radioactive molecule.
17. A fused product comprising a ligand molecule and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.
18. The fused product of claim 17, characterized in that said at least one neutral polar residue is serine (S) or threonine (T), and wherein said at least one basic polar residue is lysine (K).
19. The fused product of claim 17, characterized in that the amino acid motif comprises the formula XX, wherein one X is a K and the other X is S or T.
20. The fused product of claim 17, characterized in that said anchor peptide further comprises at least one neutral non-polar residue.

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21. The fused product of claim 20, characterized in that said at least one neutral polar residue is S or T, said at least one basic polar residue is K and said at least one neutral non-polar residue is alanine (A).
22. The fused product of claim 17, characterized in that the amino acid motif comprises at least 2 amino acid residues.
23. The fused product of claim 17, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 3), wherein each X is K, S or T and wherein U is any amino acid.
24. The fused product of claim 17, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 4), wherein each X is A, K, S or T and wherein U is any amino acid.
25. The fused product of claim 17, characterized in that the amino acid motif comprises the formula XX-GA-XX (SEQ ID NO: 5), wherein each X is K, S or T.
26. The fused product of claim 17, characterized in that the amino acid motif comprises the sequence ATK (SEQ ID NO: 1) or ASK (SEQ ID NO: 2).
27. The fused product of claim 17, characterized in that the amino acid motif comprises the sequence ATKASK (SEQ ID NO: 6).
28. The fused product of claim 17, characterized in that the amino acid motif comprises the sequence ATKGATK (SEQ ID NO: 7).
29. The fused product of claim 17, characterized in that the ligand molecule includes an immunoglobulin-binding protein, an enzyme, a target protein binding partner or a nucleic acid molecule.
30. The fused product of claim 17, characterized in that the ligand molecule is protein A or a domain of protein A.
31. The fused product of claim 17, characterized in that the fused product is labelled with a detectable label.

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32. The fused product of claim 17, characterized in that the fused product is labelled with a cytotoxic molecule, a fluorescent molecule or radioactive molecule.
33. The fused product of claim 17, characterized in that the ligand molecule is a protein and said anchor peptide is bound to the N-terminus or the C-terminus of the ligand protein.
34. A solid support having an immobilized fused product, the fused product comprising a ligand molecule and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.
35. The solid support of claim 34, characterized in that said at least one neutral polar residue is serine (S) or threonine (T), and wherein said at least one basic polar residue is lysine (K).
36. The solid support of claim 34, characterized in that the amino acid motif comprises the formula XX, wherein one X is a K and the other X is S or T.
37. The solid support of claim 34, characterized in that said anchor peptide further comprises at least one neutral non-polar residue.
38. The solid support of claim 37, characterized in that said at least one neutral polar residue is S or T, said at least one basic polar residue is K and said at least one neutral non-polar residue is alanine (A).
39. The solid support of claim 34, characterized in that the amino acid motif comprises at least 2 amino acid residues.
40. The solid support of claim 34, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 3), wherein each X is K, S or T and wherein U is any amino acid.
41. The solid support of claim 34, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 4), wherein each X is A, K, S or T and wherein U is any amino acid.

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42. The solid support of claim 34, characterized in that the amino acid motif comprises the formula XX-GA-XX (SEQ ID NO: 5), wherein each X is K, S or T.
43. The solid support of claim 34, characterized in that the amino acid motif comprises the sequence ATK (SEQ ID NO: 1) or ASK (SEQ ID NO: 2).
44. The solid support of claim 34, characterized in that the amino acid motif comprises the sequence ATKASK (SEQ ID NO: 6).
45. The solid support of claim 34, characterized in that the amino acid motif comprises the sequence ATKGATK (SEQ ID NO: 7).
46. The solid support of claim 34, characterized in that the ligand molecule includes an immunoglobulin-binding protein, an enzyme, a target protein binding partner or a nucleic acid molecule.
47. The solid support of claim 34, characterized in that the ligand molecule is protein A or a domain of protein A.
48. The solid support of claim 34, characterized in that the fused product is labelled with a detectable label.
49. The solid support of claim 34, characterized in that fused product is labelled with a cytotoxic molecule, a fluorescent molecule or radioactive molecule.
50. The solid support of claim 34, characterized in that the ligand molecule is a protein and said anchor peptide is fused to the N-terminus or the C-terminus of the ligand protein.
51. The solid support of claim 34, characterized in that the solid support includes porous or nonporous particles.
52. The solid support of claim 51, characterized in that the particles are packed in a column or suspended in a solution.
53. The solid support of claim 34, characterized in that the solid support is in the form of a surface, a monolith, a chip, a capillary or a filter.
54. A method of immobilizing a ligand molecule to a solid support comprising:

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- a. forming a fused product comprising a ligand molecule and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue; and
 - b. contacting the fused product with a solid support under conditions to immobilize the fused product to the solid support.
55. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that said at least one neutral polar residue is serine (S) or threonine (T), and wherein said at least one basic polar residue is lysine (K).
56. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that said anchor peptide further comprises at least one neutral non-polar residue.
57. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that said at least one neutral polar residue is S or T, said at least one basic polar residue is K and said at least one neutral non-polar residue is alanine (A).
58. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises at least 2 amino acid residues.
59. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 3), wherein each X is K, S or T and wherein U is any amino acid.
60. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises the formula XX-UU-XX (SEQ ID NO: 4), wherein each X is A, K, S or T and wherein U is any amino acid.
61. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises the formula XX-GA-XX (SEQ ID NO: 5), wherein each X is K, S or T.

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62. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises the sequence ATK (SEQ ID NO: 1) or ASK (SEQ ID NO: 2).
63. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises the sequence ATKASK (SEQ ID NO: 6).
64. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the amino acid motif comprises the sequence ATKGATK (SEQ ID NO: 7).
65. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the ligand molecule includes an immunoglobulin-binding protein, an enzyme, a target protein binding partner or a nucleic acid molecule.
66. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the ligand molecule is protein A or a domain of protein A.
67. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the fused product is labelled with a detectable label.
68. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that fused product is labelled with a cytotoxic molecule, a fluorescent molecule or radioactive molecule.
69. The method of immobilizing a ligand molecule to a solid support of claim 54, characterized in that the ligand molecule is a protein and said anchor peptide is bound to the N-terminus or the C-terminus of the ligand protein.
70. A method for purifying a molecule of interest in a sample suspected to include the molecule of interest, characterized in that the method comprises:
 - (a) contacting the sample with a fused product immobilized to a solid support, the fused product comprising a ligand molecule that binds to the molecule of interest and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue, said contacting being under

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conditions wherein a complex is formed between the molecule of interest and the ligand molecule if said molecule of interest is present in the sample; and

(b) recovering the molecule of interest from the complex thereby purifying the molecule of interest from the sample.

71. A method for detecting a molecule of interest in a sample suspected to include the molecule of interest, characterized in that the method comprises:

(a) contacting the sample to a fused product immobilized to a solid support, the fused product comprising a ligand molecule that binds to the molecule of interest and an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue, said contacting being under conditions wherein a complex is formed between the molecule of interest and the ligand molecule if said molecule of interest is present in the sample;

(b) recovering the molecule of interest from the complex; and

(c) detecting the recovered molecule of interest.

72. The method for detecting a molecule of interest of claim 71, characterized in that said method further comprises (d) measuring the quantity of recovered molecule of interest.

73. The method for detecting a molecule of interest of claim 71, characterized in that prior to the recovering step, the method further comprises detecting the complex.

74. The methods according to any one of claims 69 and 70, characterized in that said at least one neutral polar residue is serine (S) or threonine (T), and wherein said at least one basic polar residue is lysine (K).

75. The methods according to any one of claims 70 and 71, characterized in that said molecule of interest is IgG and said ligand molecule is protein A or a domain of protein A.

76. A vector comprising at least one copy of a gene of a ligand molecule and at least one gene capable of coding for an anchor peptide, said anchor peptide comprising an amino acid motif having at least one neutral polar residue and at least one basic polar residue.

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I.D.	Structure	Sequence
1	X-A-X-G-A-X-G-X	biotin-G-G-Y-G-K-A-K-G-A-K-G-K-A-G
2		biotin-G-G-Y-G-K-A-S-G-A-K-G-S-A-G
3		biotin-G-G-Y-G-S-A-S-G-A-K-G-K-A-G
4		biotin-G-G-Y-G-K-A-S-G-A-K-G-S-A-G
5		biotin-G-G-Y-G-S-A-S-G-A-S-G-S-A-G
6		biotin-G-G-Y-G-T-A-T-G-A-T-G-T-A-G
7		biotin-G-G-Y-G-K-A-T-G-A-K-G-T-A-G
8		biotin-G-G-Y-G-H-A-K-G-A-H-G-K-A-G
9	X-X-G-A-X-X	biotin-G-G-Y-G-A-K-K-G-A-K-K-G-A-G
10		biotin-G-G-Y-G-A-K-S-G-A-K-S-G-A-G
11		biotin-G-G-Y-G-A-S-K-G-A-S-K-G-A-G
12		biotin-G-G-Y-G-A-K-T-G-A-K-T-G-A-G
13		biotin-G-G-Y-G-A-T-K-G-A-T-K-G-A-G
14		biotin-G-G-Y-G-A-S-T-G-A-S-T-G-A-G
15		biotin-G-G-Y-G-A-H-K-G-A-H-K-G-A-G
16		biotin-G-G-Y-G-A-H-H-G-A-K-K-G-A-G
17	X-X-X-G-X-X-X	biotin-G-G-Y-G-A-K-K-K-G-K-K-K-G-A
18		biotin-G-G-Y-G-A-K-S-K-G-K-S-K-G-A
19		biotin-G-G-Y-G-A-K-T-K-G-K-T-K-G-A
20		biotin-G-G-Y-G-A-K-H-K-G-K-H-K-G-A
21		biotin-G-G-Y-G-A-K-K-S-G-K-K-S-G-A
22		biotin-G-G-Y-G-A-K-K-T-G-K-K-T-G-A
23		biotin-G-G-Y-G-A-S-K-K-G-S-K-K-G-A
24		biotin-G-G-Y-G-A-T-K-K-G-T-K-K-G-A
25	Random	biotin-G-K-D-Y-D-I-P-K-K-E-N-L-Y-F-Q
26		biotin-G-H-H-Y-D-I-P-K-K-E-N-L-Y-F-Q
27		biotin-G-S-K-Y-K-I-P-T-T-K-K-L-Y-F-Q
28		biotin-G-S-S-Y-S-A-P-T-T-S-N-L-Y-F-Q
29		biotin-G-S-D-Y-D-A-K-T-T-E-N-L-Y-F-Q
30		biotin-G-S-D-Y-D-A-P-T-T-E-K-K-Y-F-Q
31		biotin-G-S-D-Y-D-A-P-T-T-E-K-K-Y-K-K
32		biotin-G-S-D-Y-D-A-P-T-T-E-K-K-T-K-K
33		biotin-G-S-D-Y-D-A-P-T-T-E-K-K-H-K-K

FIGURE 1

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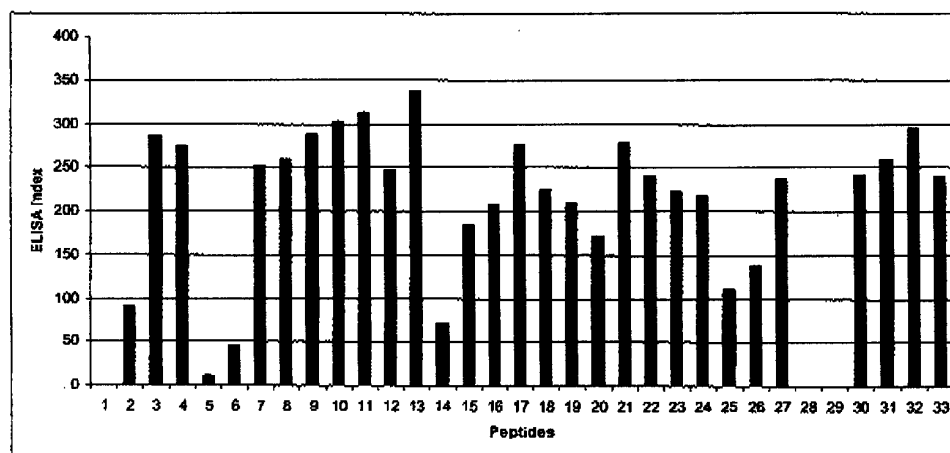


FIGURE 2

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A

ATKASK- Z domain

B

201	MHHHHHHVNSLE-----	VDNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQGSK
123	MKHHHHHHHPMSDYDIPTTENLYFQGAMV-----	ADNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQGSK
901	MKHHHHHHHPMSDYDIPTTENLYFQGAM-ATK-ASK	VDNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQGSK
902	MKHHHHHHHPMSDYDIPTTENLYFQGAM-ATKGATK	VDNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQGSK
903	MKHHHHHHHPMSDYDIPTTENLYFQGAM-AHKGAAK	VDNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQGSK
904	MKHHHHHHHPMSDYDIPTTENLYFQSGAMGHTKGHTK	VDNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQGSK
285	MKHHHHHHH-----ENLYFQGAMV-----	VDNKFNKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQAPK
700	MKHHHHHHH-----ENLYFQG--ATK-ASK	VDHKFDKEQQNAFYIILHLFNLNNEQORNAFIQSLKDDPSQSANLLAEAKKLNDAAQAPK

FIGURE 3

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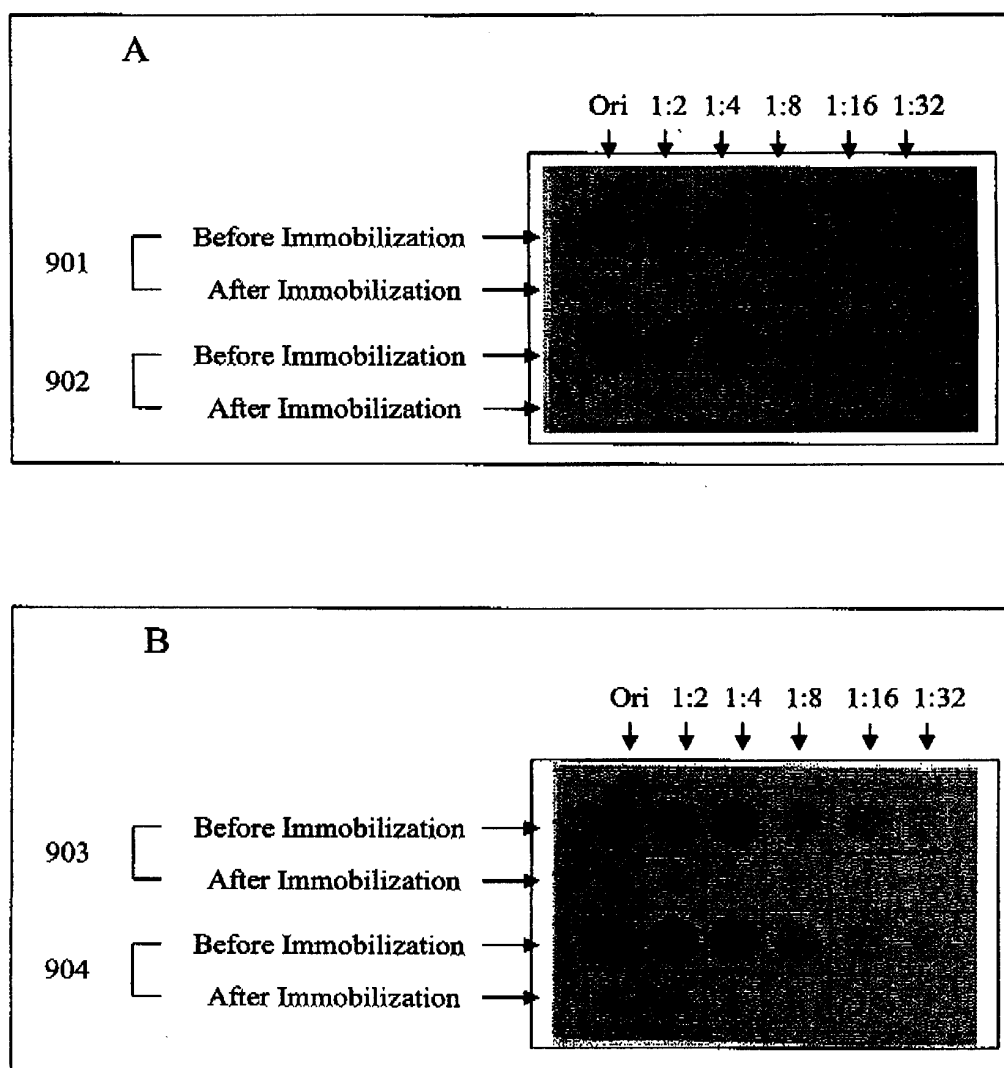


FIGURE 4

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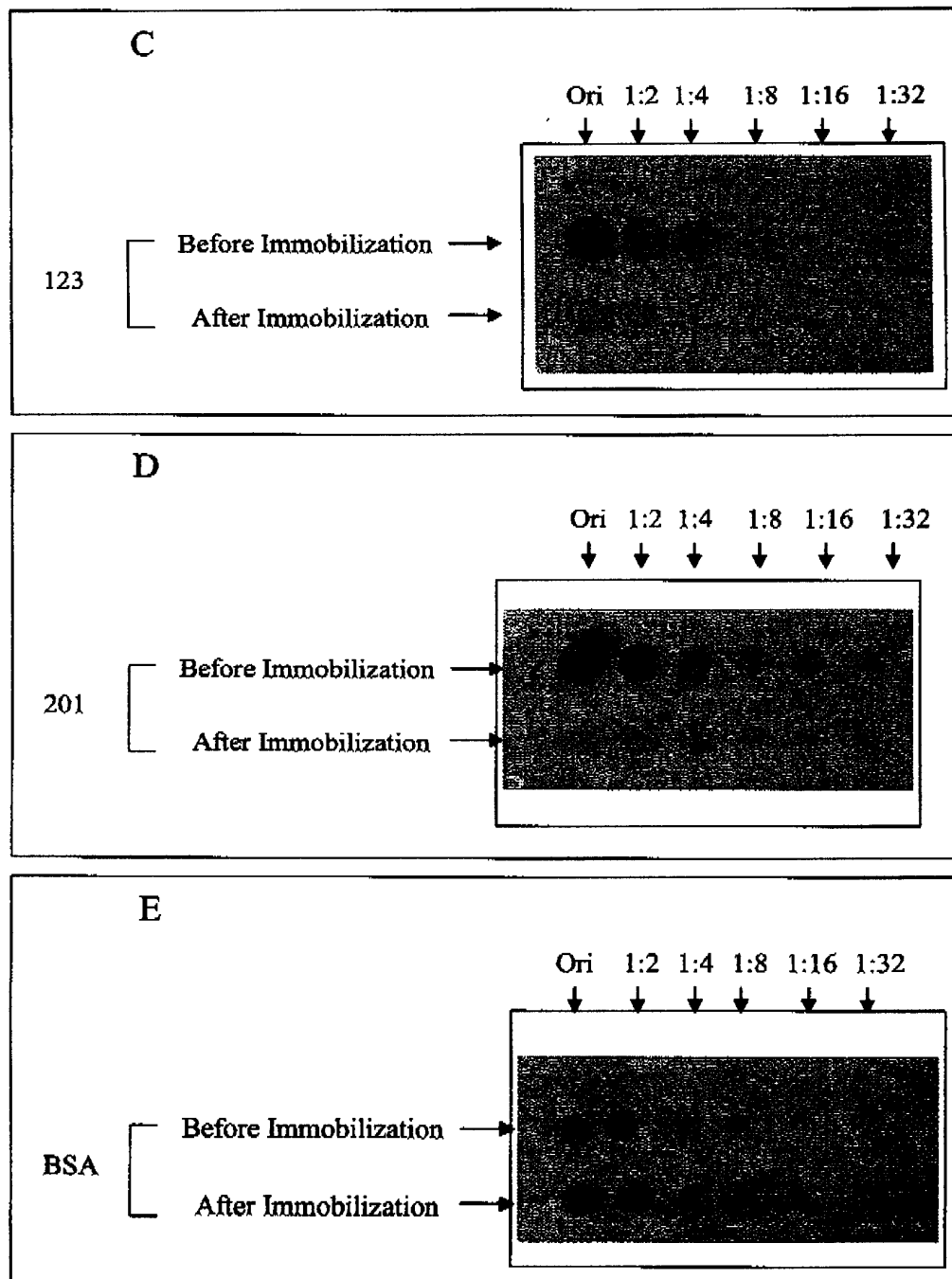


FIGURE 4 (Continued)

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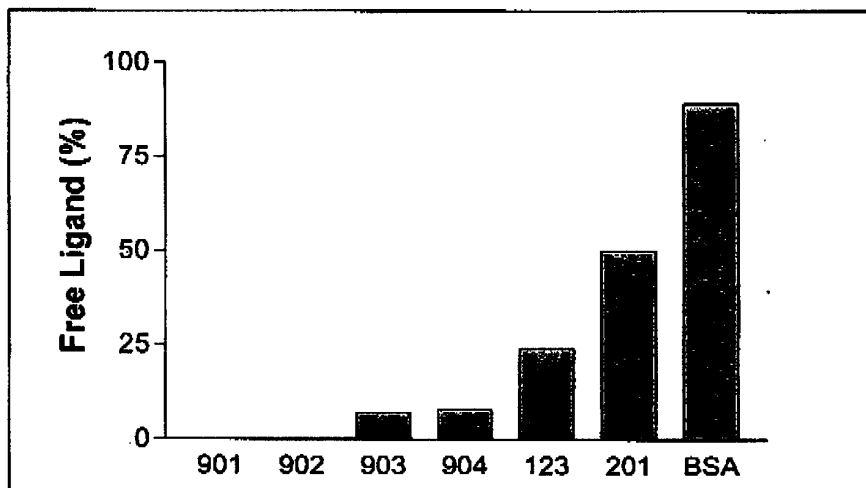


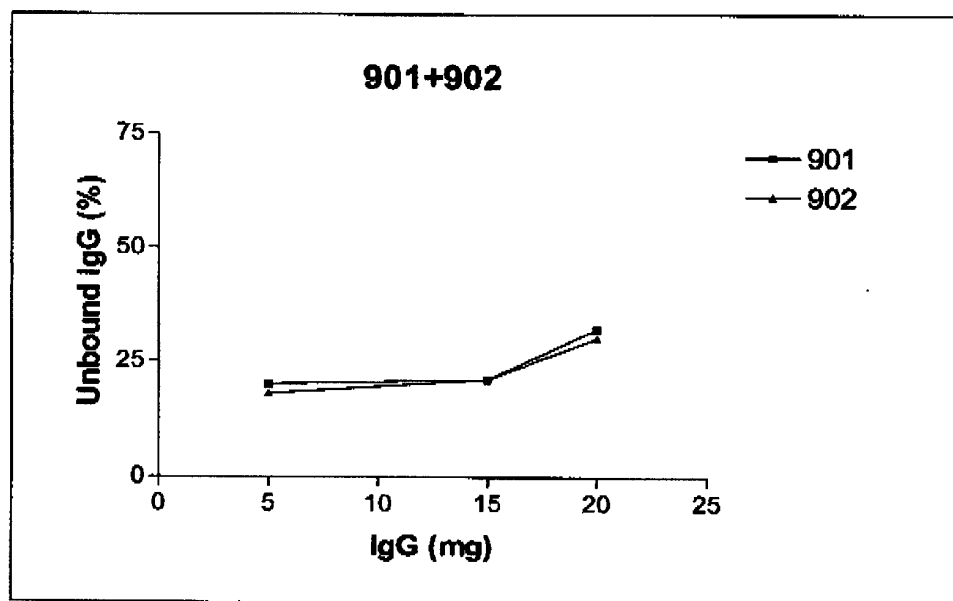
FIGURE 5

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A.



B.

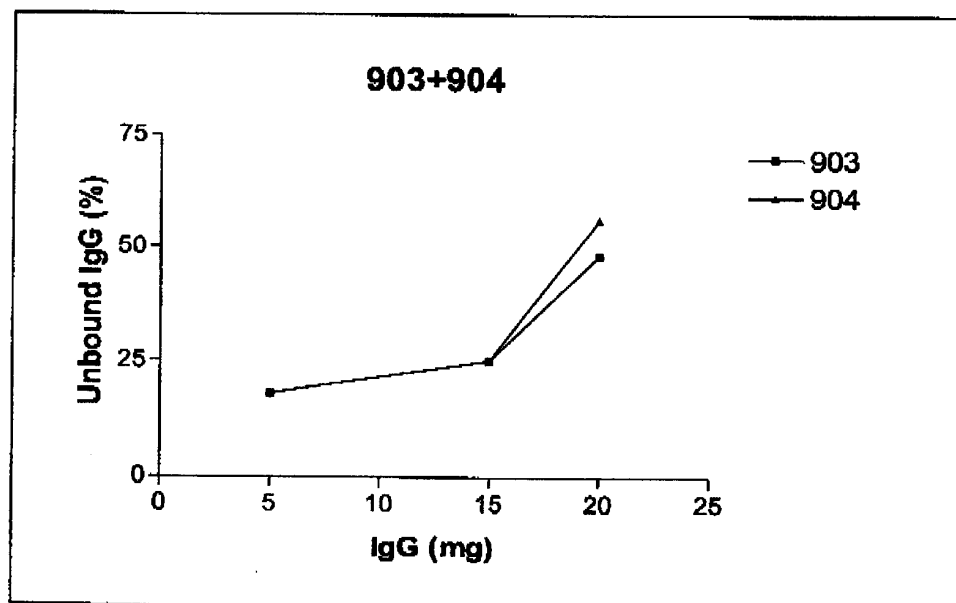


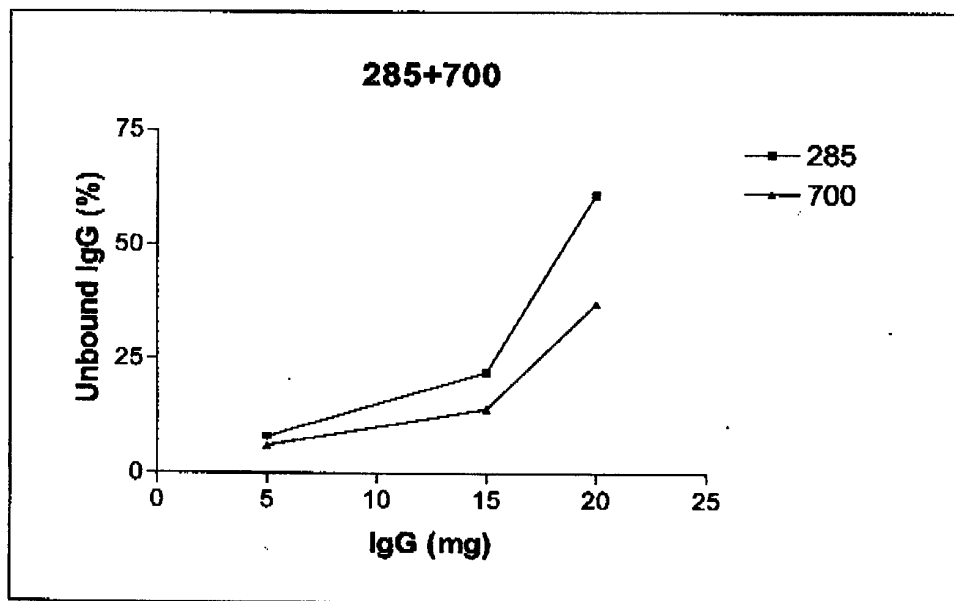
FIGURE 6

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C.



D.

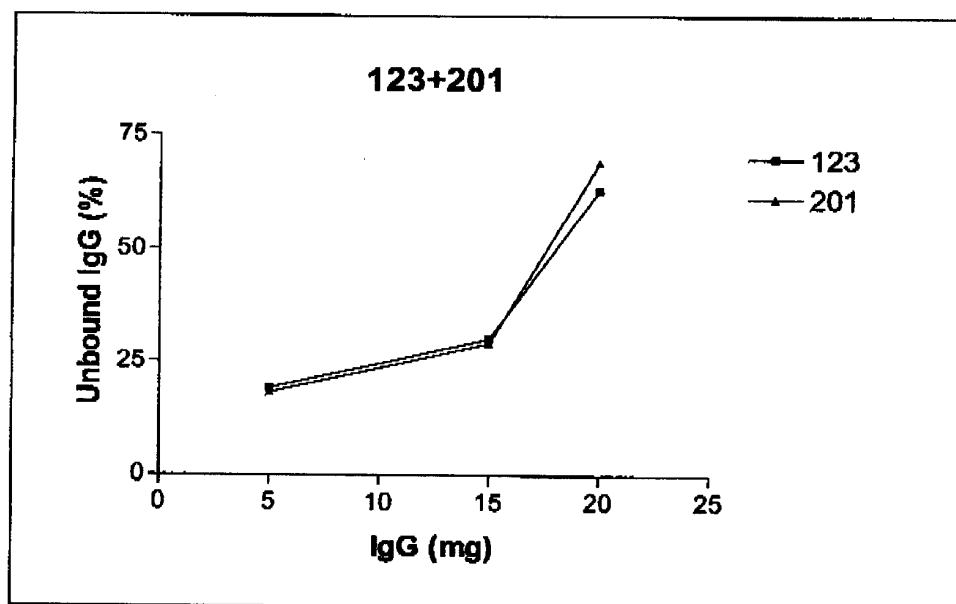


FIGURE 6 (Continued)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/001366

A. CLASSIFICATION OF SUBJECT MATTER IPC: C07K 17/00 (2006.01), B01D 15/08 (2006.01), C07K 17/06 (2006.01), C07K 5/083 (2006.01), C07K 7/06 (2006.01), C12N 15/63 (2006.01) (more IPCs on the last page) According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols) IPC: C07K, C12N, B01D, C40B, G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used) Databases: PubMed, Genome Quest, Canadian Patent Database, Scopus, TotalPatent. Keywords: dilysine, serine, threonine, glycine, alanine, fusion, anchor, linker, spacer, epoxy, immobil*, serine, coupl*, conjugate, solid support, substrate, ATK, GASK, ATKGATK,		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KUMADA, Y. et al, Development of a one-step ELISA method using an affinity peptide tag specific to a hydrophilic polystyrene surface. J. Biotechnol. 2007, Vol.127, No.2, pages 288-299, ISSN:0168-1656, the whole document.	1, 2, 4-6, 13-18, 20-22, 29-35, 37-39, 46-50, 54-56, 58, 65-76
X	ALLARD, L. et al, Versatile method for production and controlled polymer-immobilization of biologically active recombinant proteins. Biotechnol. Bioeng. 2002, Vol.80, No.3, pages 341-348, ISSN: 0006-3592, the whole document.	1-4, 6-8, 13-20, 22-24, 29-37, 39-41, 46-56, 58-60, 65-76
---		---
Y		9-12, 25-28, 42-45, 61-64
X	ZHU, J. et al, Design and synthesis of biomimetic hydrogel scaffolds with controlled organization of cyclic RGD peptides. Bioconj. Chem. 2009, Vol.20, No.2, pages 333-339, ISSN:1043-1802, the whole document.	1-3, 6, 13-19, 22, 29-36, 39, 46-55, 58, 65-76
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.		
* Special categories of cited documents :	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "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	
"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		
Date of the actual completion of the international search 16 April 2012 (16-04-2012)	Date of mailing of the international search report 10 May 2012 (10-05-2012)	
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Riad Qanbar (819) 934-7937	

INTERNATIONAL SEARCH REPORT

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
 - ☒ on paper
 - ☒ in electronic form
 - b. (time)
 - ☒ in the international application as filed
 - ☐ together with the international application in electronic form
 - ☐ subsequently to this Authority for the purposes of search
2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments :

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Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of the 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. ☐ Claim Nos. :
because they relate to subject matter not required to be searched by this Authority, namely :

2. ☐ Claim 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. ☐ Claim 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 :

The claims are directed to a plurality of inventive concepts as follows:

Group 1 - Claims 1, 7-12, 17, 19, 23-28, 34, 36, 40-45, 54, 59-64 (partially) and claims 2, 4-6, 13-16, 18, 20-22, 29-33, 35, 37-39, 46-53, 55-58, 65-76 (completely) featuring an anchor peptide comprising one "neutral polar" residue and one basic polar residue.

Continued in supplementary box

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 claim 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 claim 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.

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Continuation of Box No. III (Unity of invention is lacking)

Group 2 - Claims 1, 3, 19, 36 (all partially) featuring an anchor peptide of the formula XX, wherein one X is K and the other is S or T.
Group 3 - Claims 1, 7, 23, 40, 59 (all partially) featuring an anchor peptide of the formula XX-UU-XX wherein one X is K, S, or T and U is any amino acid.
Group 4 - Claims 1, 8, 24, 41, 60 (all partially) featuring an anchor peptide of the formula XX-UU-XX wherein one X is A, K, S, or T and U is any amino acid.
Group 5 - Claims 1, 9, 25, 42, 61 (partially) featuring an anchor peptide of the formula XX-GA-XX wherein one X is K, S, or T .
Group 6 - Claims 1, 10, 26, 43, 62 (all partially) featuring an anchor peptide comprising the sequence ATK.
Group 7 - Claims 1, 10, 26, 43, 62 (all partially) featuring an anchor peptide comprising the sequence ASK.
Group 8 - Claims 1, 11, 27, 44, 63 (all partially) featuring an anchor peptide comprising the sequence ATKASK.
Group 9 - Claims 1, 12, 28, 45, 64 (all partially) featuring an anchor peptide comprising the sequence ATKGATK.

The subject matter of groups 1-9 relates to peptides that are useful for linking a protein to a solid substrate. The structural feature that links these groups is the presence of the amino acid lysine in combination with either serine or threonine. This feature, however, was known in the art on the relevant date (see references cited on pages 4 and 5 above).

Additionally, the subject matter of each of groups 1 to 5 comprises multiple sequences that do not have a novel and inventive feature in common and therefore could be further divided into subgroups, each corresponding to a specific, fully defined sequence.

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CA2011/001366

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	WO 2008/150101 A2 (GIM, Y.S. et al) 11 December 2008 (11-12-2008), the whole document, particularly SEQ ID NOS: 27-30 and 32.	1, 2, 4-6, 13-19, 22, 29-36, 39, 46-55, 58, 65-76 --- 7-12, 25-28, 42-45, 61-64
X	BURZIO, V.A. et al, Mussel adhesive enhances the immobilization of human chorionic gonadotrophin to a solid support. Anal. Biochem. 1996, Vol.241, No.2, pages 190-194, ISSN: 0003-2697, the whole document	1-5, 13
X --- Y	BURZIO, L.A. et al, The adhesive protein of <i>Choromytilus chorus</i> (Molina, 1782) and <i>Aulacomya ater</i> (Molina, 1782): a proline-rich and a glycine-rich polyphenolic protein. Biochim. Biophys. Acta. 2000, Vol.1479, No.1-2, pages 315-320, ISSN: 0006-3002, the whole document, particularly the third full paragraph of the Discussion.	1-6 --- 7-12, 25-28, 42-45, 61-64
X --- Y	RZEPECKI, L.M. et al, Molecular diversity of marine glues: polyphenolic proteins from five mussel species. Mol. Mar. Biol. Biotechnol. 1991, Vol.1, No.1, pages 78-88, ISSN: 1053-6426, the whole document.	1-8 --- 7-12, 25-28, 42-45, 61-64
X	MATSUNAGA, T. et al, Hypothetical protein [<i>Magnetospirillum magneticum</i> AMB-1]. GenBank, Accession Number: BAE51773, first seen at NCBI on 1 December 2005 (01-12-2010).	1-11
X	MARDANI, A. et al, Knob-associated histidine-rich protein, partial [<i>Plasmodium falciparum</i>]. GenBank, Accession Number: ADM31373, first seen at NCBI on 29 August 2010 (29-08-2010).	1-10, 12
A	MATEO, C. et al, Advances in the design of new epoxy supports for enzyme immobilization-stabilization. Biochem. Soc. Trans. 2007, Vol.35, No.6, pages1593-1601, ISSN: 0300-5127.	1-76

INTERNATIONAL SEARCH REPORT

International application No.
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C40B 30/04 (2006.01), *C40B 50/18* (2006.01), *C40B 80/00* (2006.01), *G01N 33/53* (2006.01),
G01N 33/564 (2006.01), *C07K 1/22* (2006.01)

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(74) Agents: **WILLIS, Michael, A.** et al.; Wilson Sonsini Goodrich & Rosati, 650 Page Mill Road, Palo Alto, CA 94304-1050 (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, 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.

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Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))



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(54) Title: METHODS AND COMPOSITIONS FOR CONSUMABLES

(57) Abstract: Provided herein are methods and compositions related to plant based meat substitutes which have properties similar to meat.

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METHODS AND COMPOSITIONS FOR CONSUMABLES

This application claims priority to U.S. application Ser. No. 61/572,205 filed July 12, 2011, which is incorporated herein in its entirety by reference.

BACKGROUND OF THE INVENTION

[0001] Animal farming has a profound negative environmental impact. Currently it is estimated that 30% of Earth's land surface is dedicated to animal farming and that livestock account for 20% of total terrestrial animal biomass. Due to this massive scale animal farming accounts for more than 18% of net greenhouse gas emissions. Animal farming may be the largest human source of water pollution, and animal farming is by far the world's largest threat to biodiversity. It has been estimated that if the world's human population could shift from a meat containing diet to a diet free of animal products, 26% of Earth's land surface would be freed for other uses. Furthermore the shift to a vegetarian diet would massively reduce water and energy consumption.

[0002] The consumption of meat has a profound negative impact on human health. The health benefits of a vegetarian diet are well established. If the human population would shift to a vegetarian diet the cost savings in health care would be significant.

[0003] Hunger is a worldwide problem, yet the world's 4 major commodity crops (soybeans, maize, wheat, and rice) already supply more than 100% of the human population's requirements for calories and protein, including every essential amino acid.

[0004] Plant based meat substitutes have largely failed to cause a shift to a vegetarian diet. The current state of the art for meat substitute compositions involves the extrusion of soy/grain mixture, resulting in products which largely fail to replicate the experience of cooking and eating meat. Common limitations of these products are a texture and mouthfeel that are more homogenous than that of equivalent meat products. Furthermore, as the products must largely be sold pre-cooked, with artificial flavors and aromas built in, they fail to replicate aromas, flavors, and other key features associated with cooking meat. As a result, these products appeal mainly to a limited consumer base that is already committed to vegetarianism/veganism, but have failed to appeal to the larger consumer segment accustomed to eating meat.

[0005] Disclosed herein are improved methods and compositions which more accurately replicate the characteristics that consumers value in the preparation and consumption of meat and which overcome the shortcomings and drawbacks of current meat substitutes.

SUMMARY OF THE INVENTION

[0006] In some aspects, the invention provides a meat substitute composition comprising a protein content, wherein one or more isolated and purified proteins accounts for 10% or more of said protein content by weight, wherein said meat substitute composition accurately mimics the taste, texture, or color of a meat product derived from animal sources.

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[0007] In one embodiment, the meat substitute composition accurately mimics the color of said meat product in its raw state and in a cooked state after cooking.

[0008] In another embodiment, the one or more isolated and purified proteins accounts for 25% or more of said protein content by weight.

[0009] In another embodiment, the one or more isolated and purified proteins accounts for 50% or more of said protein content by weight.

[0010] In another embodiment, the one or more isolated and purified proteins accounts for 75% or more of said protein content by weight.

[0011] In another embodiment, the one or more isolated and purified proteins accounts for 90% or more of said protein content by weight.

[0012] In another embodiment, gluten does not account for 10% or more of said protein content by weight.

[0013] In another embodiment, each of said isolated, purified proteins is separately isolated and purified.

[0014] In another embodiment, the meat substitute composition comprises 1-7 isolated and purified proteins.

[0015] In another embodiment, said 1-7 isolated and purified proteins are each isolated from different plant species.

[0016] In some embodiments the meat substitute comprises less than 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 individual proteins isolated from one or more plant species.

[0017] In another embodiment, said protein content comprises no more than trace amounts of any other proteins derived from the one or more plant species.

[0018] In another embodiment, said one or more isolated and purified proteins are selected from the group consisting of leghemoglobin, non-symbiotic hemoglobin, hemoglobin, myoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin.

[0019] In another embodiment, said one or more isolated and purified proteins are not isolated from an animal.

[0020] In another embodiment, said one or more isolated and purified proteins are isolated from a single plant source.

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[0021] In another embodiment, said one or more isolated and purified proteins are isolated from multiple plant sources.

[0022] In another embodiment, wherein said one or more isolated, purified proteins are isolated from a genetically modified organism.

[0023] In some embodiments, said genetically modified organism is a genetically modified bacteria or yeast organism.

[0024] In some embodiments, said isolated, purified protein has been formed into fibers.

[0025] In particular embodiments, said fibers resemble skeletal muscle fibers.

[0026] In yet more particular embodiments, said fibers are asymmetric fibers.

[0027] In some embodiments, the meat substitute composition further comprises one or more isolated and purified iron-containing proteins.

[0028] In some embodiments, said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins,.

[0029] In a particular embodiment, said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1. SEQ ID NO 1:

MVAFTEKQDALVSSSF EAFKANIPQYSVVFYTSILEKAPAAKDLFSFLANGVDPTNP KLTGHA EK
LFALVRDSAGQLKASGTVVADAALG SVHAQKAVTDPQFVVVKEALLKTIKAAVGDKWSDELS
RAWEVAYDELA AAIKKA.

[0030] In a particular embodiment, said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 2. SEQ ID NO 2: MIDQKEKELI KESWKRIEPN
KNEIGLLFYA NLFKEEPTVS VLFQNPISQ SRKLMQVLGI LVQGIDNLEG LIPTLQDLGR
RHKQYGVVDS HYPLVGDCLL KSIQEYLGQG FTEEAKAAWT KVGIAAQVM TAE. In some
embodiments said iron-containing protein comprises an amino acid sequence with at least 80% homology
to SEQ ID NO 2. In some embodiments said iron-containing protein comprises an amino acid sequence
with at least 90% homology to SEQ ID NO 2. In some embodiments said iron-containing protein
comprises an amino acid sequence with at least 98% homology to SEQ ID NO 2.

[0031] In a particular embodiment, said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 3. SEQ ID NO 3: MRKQPTVF EK LGGQAAMHAA
VPLFYKKVLA DDRVKHYFKN TNMEHQAKQQ EDFLTMLLG PNHYKGNMA
EAHKGMNLQN SHFDAIENL AATLKELGVS DQIIGEAAKV IEHTRKDCLG K. In some
embodiments said iron-containing protein comprises an amino acid sequence with at least 80% homology
to SEQ ID NO 3. In some embodiments said iron-containing protein comprises an amino acid sequence
with at least 90% homology to SEQ ID NO 3. In some embodiments said iron-containing protein
comprises an amino acid sequence with at least 98% homology to SEQ ID NO 3.

[0032] In some embodiments, the isolated and purified proteins are assembled into one or more gels.

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- [0033] In some embodiments, the meat substitute composition further comprises one or more fats.
- [0034] In particular embodiments, said one or more fats are derived from a plant source.
- [0035] In another aspect, the invention provides a meat substitute product that comprises an indicator that indicates cooking progression from a raw state to a cooked state, wherein said meat substitute product is derived from non-animal sources.
- [0036] In some embodiments, said indicator is a visual indicator that accurately mimics the color transition of a meat product during said cooking progression.
- [0037] In one embodiment, said color transition is from red to brown.
- [0038] In one embodiment, said color transition is from pink to white or tan.
- [0039] In one embodiment, said visual indicator transitions from a translucent to opaque color during said cooking progression.
- [0040] In some embodiments, the indicator is an olfactory indicator that indicates cooking progression.
- [0041] In one embodiment, said olfactory indicator is one or more volatile odorants released during cooking.
- [0042] In some embodiments, said indicator comprises one or more isolated, purified iron-containing proteins.
- [0043] In particular embodiments, said one or more isolated, purified iron-containing proteins is in a reduced state before cooking.
- [0044] In one embodiment, said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins.
- [0045] In a particular embodiment, said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1. SEQ ID NO 1:
MVAFTEKQDALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLANGVDPTNPKLTGHAEK
LFALVRDSAGQLKASGTVVADAALGSVHAQKA VTD PQFVVVKEALLKTIKAAVGDKWSDELS
RAWEVAYDELA AAIKKA.
- [0046] In some embodiments, said one or more isolated and purified iron-containing proteins are not isolated from an animal. In some embodiments compositions of the invention do not contain any proteins from an animal.
- [0047] In particular embodiments, said one or more isolated and purified iron-containing proteins are isolated from one or more plant sources.
- [0048] In yet more particular embodiments, said one or more isolated, purified proteins are isolated from the root nodules, roots, seeds, leaves, or stems of said one or more plant sources.
- [0049] In other particular embodiments, said one or more plant sources are soy or pea plants.
- [0050] In one embodiment, said one or more plant sources comprises one or more plants of the legume family.

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[0051] In some embodiments, said one or more isolated and purified iron carrying proteins in a reduced or oxidized state has a similar UV-VIS profile to a myoglobin protein derived from an animal source when in an equivalent reduced or oxidized state.

[0052] In a particular embodiment, the difference between the peak absorbance wavelength of said one or more isolated and purified iron-containing proteins and the peak absorbance wavelength of myoglobin derived from an animal source is less than 5%.

[0053] In some embodiments, said one or more isolated, purified proteins are isolated from a genetically modified organism.

[0054] In one embodiment, said genetically modified organism is a genetically modified bacteria or yeast organism.

[0055] In some embodiments, the meat substitute product contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.

[0056] In particular embodiments, the meat substitute product additionally contains less than 1% wheat gluten.

[0057] In a more particular embodiment, said meat substitute product contains no wheat gluten.

[0058] In other particular embodiments, said meat substitute product contains no soy protein isolate.

[0059] In other particular embodiments, said meat substitute product contains no soy protein concentrate.

[0060] In other particular embodiments, said meat substitute product contains no soy protein.

[0061] In a more particular embodiment, said meat substitute product contains less than 5% carbohydrates.

[0062] In other particular embodiments, said meat substitute product contains no tofu.

[0063] In some embodiments, said meat substitute product contains no tofu, and no wheat gluten.

[0064] In some embodiments, said meat substitute product contains no soy protein, and no wheat gluten.

[0065] In some embodiments, said meat substitute product contains no animal products and less than 5% carbohydrates.

[0066] In some embodiments, said meat substitute product contains less than 1% cellulose.

[0067] In some embodiments, said meat substitute product contains less than 5% insoluble carbohydrates.

[0068] In some embodiments, said meat substitute product contains no soy protein, and less than 1% cellulose.

[0069] In some embodiments, said meat substitute product contains no soy protein, and less than 5% insoluble carbohydrates.

[0070] In some embodiments, said meat substitute product contains no wheat gluten, and less than 1% cellulose.

[0071] In some embodiments, said meat substitute product contains no wheat gluten, and less than 5% insoluble carbohydrates.

[0072] In another aspect, the invention provides a muscle tissue replica comprising a protein content, wherein said protein content comprises one or more isolated and purified proteins, wherein said muscle

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tissue replica approximates the taste, texture, or color of an equivalent muscle tissue derived from an animal source.

[0073] In some embodiments, said one or more isolated and purified proteins accounts for at least 50% of said protein content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 40% of said protein content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 30% of said protein content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 20% of said protein content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 10% of said protein content by weight.

[0074] In some embodiments, said one or more isolated and purified proteins accounts for at least 50% of said composition content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 40% of said composition content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 30% of said composition content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 20% of said composition content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 10% of said composition content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 5% of said composition content by weight. In some embodiments, said one or more isolated and purified proteins accounts for at least 1% of said composition content by weight.

[0075] In some embodiments, said protein content is derived from one or more non-animal sources.

[0076] In particular embodiments, said one or more non-animal sources is a plant source.

[0077] In other particular embodiments, said one or more non-animal sources is a genetically modified yeast or bacteria.

[0078] In some embodiments, each of said one or more isolated proteins is isolated and purified separately.

[0079] In some embodiments, said one or more isolated proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobins, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin. .

[0080] In one embodiment, said seed storage protein is moong bean 8S protein.

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- [0081] In some embodiments, said protein content is suspended in a gel.
- [0082] In some embodiments, said protein content is in the form of a gel.
- [0083] In one embodiment, said gel comprises an isolated, purified cross-linking enzyme.
- [0084] In some embodiments, said isolated, purified cross-linking enzyme is selected from the group consisting of transglutaminase, lysyl oxidases, and amine oxidases.
- [0085] In a particular embodiment, said isolated, purified cross-linking enzyme is transglutaminase.
- [0086] In some embodiments, said protein content has been assembled into fibers.
- [0087] In particular embodiments, said fibers are arranged isotropically.
- [0088] In one embodiment, said fibers are asymmetric fibers.
- [0089] In some embodiments, the muscle tissue replica further comprises one or more isolated and purified iron-containing proteins.
- [0090] In some embodiments, said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins.
- [0091] In a particular embodiment, said one or more isolated and purified iron-containing proteins comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1. SEQ ID NO 1: MVAFTEKQDALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLANGVDPTNPKLGTGHAEL LFALVRDSAGQLKASGTVVADAALGSVHAQKAVTDPQFVVVKEALLKTIKAAVGDKWSDELS RAWEVAYDELA AIIKKA. In a particular embodiment, said one or more isolated and purified iron-containing proteins comprises an amino acid sequence with at least 80% homology to SEQ ID NO 1. In a particular embodiment, said one or more isolated and purified iron-containing proteins comprises an amino acid sequence with at least 90% homology to SEQ ID NO 1. In a particular embodiment, said one or more isolated and purified iron-containing proteins comprises an amino acid sequence with at least 95% homology to SEQ ID NO 1. In a particular embodiment, said one or more isolated and purified iron-containing proteins comprises an amino acid sequence with at least 98% homology to SEQ ID NO 1.
- [0092] In particular embodiments, the muscle tissue replica comprises a protein content, wherein (i) one isolated and purified protein that is not an iron-containing protein accounts for 40-95% of said protein content, (ii) one or more isolated and purified iron-containing proteins accounts for 1-20% of said protein content, and (iii) one or more cross-linking reagents accounts for 0.1-35% of said protein content.
- [0093] In one embodiment, said protein content accounts for 5-50% of said replica by weight or by weight/volume.
- [0094] In one embodiment, said one isolated and purified protein is moong bean 8S protein.
- [0095] In one embodiment, said one or more isolated and purified iron-containing proteins is leghemoglobin.
- [0096] In one embodiment, said one or more cross-linking reagents is transglutaminase.

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[0097] In some embodiments, the muscle tissue replica contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.

[0098] In particular embodiments, the muscle tissue replica additionally contains less than 1% wheat gluten. In particular embodiments, the muscle tissue replica additionally contains less than 5% wheat gluten. In particular embodiments, the muscle tissue replica additionally contains less than 10% wheat gluten. In particular embodiments, the muscle tissue replica additionally contains less than 0.1% wheat gluten.

[0099] In a more particular embodiment, said muscle tissue replica contains no wheat gluten.

[00100] In other particular embodiments, said muscle tissue replica contains no soy protein isolate.

[00101] In other particular embodiments, said muscle tissue replica contains no soy protein concentrate.

[00102] In other particular embodiments, said muscle tissue replica contains no soy protein.

[00103] In a more particular embodiment, said muscle tissue replica contains less than 5% carbohydrates.

[00104] In other particular embodiments, said muscle tissue replica contains no tofu.

[00105] In some embodiments, said muscle tissue replica contains no tofu, and no wheat gluten.

[00106] In some embodiments, said muscle tissue replica contains no soy protein, and no wheat gluten.

[00107] In some embodiments, said muscle tissue replica contains no animal products and less than 5% carbohydrates.

[00108] In some embodiments, said muscle tissue replica contains less than 1% cellulose.

[00109] In some embodiments, said muscle tissue replica contains less than 5% insoluble carbohydrates.

[00110] In some embodiments, said muscle tissue replica contains no soy protein, and less than 1% cellulose.

[00111] In some embodiments, said muscle tissue replica contains no soy protein, and less than 5% insoluble carbohydrates.

[00112] In some embodiments, said muscle tissue replica contains no wheat gluten, and less than 1% cellulose.

[00113] In some embodiments, said muscle tissue replica contains no wheat gluten, and less than 5% insoluble carbohydrates.

[00114] In some embodiments, the muscle tissue replica contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.

[00115] In particular embodiments, the meat replica contains less than 1% wheat gluten. In particular embodiments, the meat replica contains less than 5% wheat gluten. In particular embodiments, the meat replica contains less than 10% wheat gluten. In particular embodiments, the meat replica contains less than 0.1% wheat gluten.

[00116] In a more particular embodiment, the meat replica contains no wheat gluten.

[00117] In other particular embodiments, the meat replica contains no soy protein isolate.

[00118] In other particular embodiments, the meat replica contains no soy protein concentrate.

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[00119] In other particular embodiments, the meat replica contains no soy protein.

[00120] In a more particular embodiment, the meat replica contains less than 5% carbohydrates.

[00121] In other particular embodiments, the meat replica contains no tofu.

[00122] In some embodiments, the meat replica contains no tofu, and no wheat gluten.

[00123] In some embodiments, the meat replica contains no soy protein, and no wheat gluten.

[00124] In some embodiments, the meat replica contains no animal products and less than 5% carbohydrates.

[00125] In some embodiments, the meat replica contains less than 1% cellulose. In some embodiments, the meat replica contains less than 0.1% cellulose. In some embodiments, the meat replica contains less than 10% cellulose. In some embodiments, the meat replica contains less than 5% cellulose.

[00126] In some embodiments, the meat replica contains less than 5% insoluble carbohydrates.

[00127] In some embodiments, the meat replica contains no soy protein, and less than 1% cellulose.

[00128] In some embodiments, the meat replica contains no soy protein, and less than 5% insoluble carbohydrates.

[00129] In some embodiments, the meat replica contains no wheat gluten, and less than 1% cellulose.

[00130] In some embodiments, the meat replica contains no wheat gluten, and less than 5% insoluble carbohydrates.

[00131] In another aspect, the invention provides a fat tissue replica comprising a gelled emulsion, said gelled emulsion comprising a protein solution with fat droplets suspended therein.

[00132] In some embodiments, said fat droplets are derived from a non-animal source.

[00133] In some embodiments, said fat droplets are comprised of one or more plant oils.

[00134] In some embodiments, said one or more plant oils is selected from the group consisting of corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, algal oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, rice bran oil, oils produced by bacteria, algae, archaea or fungi or genetically engineered bacteria, algae, archaea or fungi, triglycerides, monoglycerides, diglycerides, sphingosides, glycolipids, lecithin, lysolecithin, phosphatidic acids, lysophosphatidic acids, oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, conjugated oleic acid, or esters of: oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or glycerol esters of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or triglyceride derivatives of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid,

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undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid.

[00135] In one embodiment, said one or more plant oils is rice bran oil or canola oil.

[00136] In some embodiments, said protein solution comprises one or more isolated, purified proteins.

[00137] In some embodiments, said one or more isolated, purified proteins accounts for 75% or more of the protein in said protein solution.

[00138] In some embodiments, said one or more isolated, purified proteins are derived from a non-animal source.

[00139] In some embodiments, said non-animal source is a plant source.

[00140] In some embodiments, said non-animal source is a genetically modified yeast or bacteria.

[00141] In some embodiments, each of said one or more isolated proteins is isolated and purified separately.

[00142] In some embodiments, said one or more isolated proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin. .

[00143] In some embodiments, said one or more isolated, purified proteins is an albumin protein, a seed storage protein, or pea globulin protein.

[00144] In particular embodiments, said albumin protein is isolated pea albumin protein.

[00145] In some embodiments, said seed storage protein is moong bean 8S protein.

[00146] In some embodiments, said gelled emulsion comprises a protein solution comprising 1-3 isolated and purified proteins, wherein said solution accounts for 30-70% of the volume of said emulsion; a plant oil, wherein said plant oil accounts for 30-70% of the volume of said emulsion; and an isolated, purified cross-linking enzyme, wherein said cross-linking enzyme accounts for 0.5-5% of said emulsion by wt/volume; wherein said plant oil is emulsified in said protein solution, wherein said emulsion is formed into a gel by said cross-linking enzyme.

[00147] In other embodiments said gelled emulsion comprises a protein solution comprising 1-3 isolated and purified proteins, wherein said solution accounts for 1-30% of the volume of said emulsion; a plant oil, wherein said plant oil accounts for 70-99% of the volume of said emulsion; and an isolated, purified cross-linking enzyme, wherein said cross-linking enzyme accounts for 0.5-5% of said emulsion by

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wt/volume; wherein said plant oil is emulsified in said protein solution, wherein said emulsion is formed into a gel by said cross-linking enzyme.

[00148] In some embodiments, the fat replica further comprises a cross-linking enzyme.

[00149] In some embodiments, said cross-linking enzyme is transglutaminase.

[00150] In some embodiments, one of said 1-3 isolated and purified proteins is moong bean 8S protein, pea albumin protein, or pea globulin protein.

[00151] In particular embodiments, said plant oil is rice bran oil or canola oil.

[00152] In some embodiments, the fat tissue replica contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.

[00153] In particular embodiments, the fat tissue replica additionally contains less than 1% wheat gluten.

[00154] In a more particular embodiment, said fat tissue replica contains no wheat gluten.

[00155] In other particular embodiments, said fat tissue replica contains no soy protein isolate.

[00156] In other particular embodiments, said fat tissue replica contains no soy protein concentrate.

[00157] In other particular embodiments, said fat tissue replica contains no soy protein.

[00158] In a more particular embodiment, said fat tissue replica contains less than 5% carbohydrates.

[00159] In other particular embodiments, said fat tissue replica contains no tofu.

[00160] In some embodiments, said fat tissue replica contains no tofu, and no wheat gluten.

[00161] In some embodiments, said fat tissue replica contains no soy protein, and no wheat gluten.

[00162] In some embodiments, said fat tissue replica contains no animal products and less than 5% carbohydrates.

[00163] In some embodiments, said fat tissue replica contains less than 1% cellulose.

[00164] In some embodiments, said fat tissue replica contains less than 5% insoluble carbohydrates.

[00165] In some embodiments, said fat tissue replica contains no soy protein, and less than 1% cellulose.

[00166] In some embodiments, said fat tissue replica contains no soy protein, and less than 5% insoluble carbohydrates.

[00167] In some embodiments, said fat tissue replica contains no wheat gluten, and less than 1% cellulose.

[00168] In some embodiments, said fat tissue replica contains no wheat gluten, and less than 5% insoluble carbohydrates.

[00169] In another aspect, the invention provides a connective tissue replica, comprising a protein content comprising one or more isolated, purified proteins, wherein said protein content has been assembled into structures approximating the texture and visual appearance of connective tissue or skin.

[00170] In some embodiments, said protein content is derived from non-animal source.

[00171] In some embodiments, said non-animal source is a plant source.

[00172] In some embodiments, said non-animal source is a genetically modified yeast or bacteria.

[00173] In some embodiments, said one or more isolated proteins account for 50% or more of said protein content by weight.

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[00174] In some embodiments, said one isolated and purified protein accounts for 90% or more of said protein content by weight.

[00175] In some embodiments, each of said one or more isolated proteins is isolated and purified separately.

[00176] In some embodiments, said one or more isolated proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocruorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin.

[00177] In some embodiments, said one or more isolated and purified proteins is a prolamin family protein.

[00178] In some embodiments, said one or more isolated and purified proteins is zein.

[00179] In some embodiments, said protein content is suspended in a gel.

[00180] In some embodiments, said gel comprises an isolated, purified cross-linking enzyme.

[00181] In some embodiments, said isolated, purified cross-linking enzyme is selected from the group consisting of transglutaminase, lysyl oxidases, and amine oxidases.

[00182] In some embodiments, said isolated, purified cross-linking enzyme is transglutaminase.

[00183] In some embodiments, said protein content is formed into a fiber.

[00184] In some embodiments, said fiber is produced by an extrusion process.

[00185] In some embodiments, said fiber is stabilized by protein crosslinks.

[00186] In some embodiments, fiber contains an isolated, purified cross-linking enzyme.

[00187] In some embodiments, said isolated, purified cross-linking enzyme is selected from the group consisting of transglutaminase, lysyl oxidases, and amine oxidases.

[00188] In some embodiments, said isolated, purified cross-linking enzyme is transglutaminase.

[00189] In another aspect, the invention provides a meat substitute product, comprising a muscle replica; a fat tissue replica; and a connective tissue replica; wherein said muscle replica, fat tissue replica, and/or connective tissue replica are assembled in a manner that approximates the physical organization of meat.

[00190] In some embodiments, the meat substitute product comprises two or more of said muscle replica, fat tissue replica, and connective tissue replica.

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[00191] In some embodiments of the meat substitute product, said muscle replica accounts for 40-90% of said product by weight, said fat tissue replica accounts for 1-60% of said product by weight, and said connective tissue replica accounts for 1-30% of said product by weight.

[00192] In some embodiments, the meat substitute product comprises 60-90% water; 5-30% protein content; and 1-20% of a fat or fat replica; wherein said protein content comprises one or more isolated, purified plant proteins.

[00193] In some embodiments, said protein content is derived from non-animal source.

[00194] In some embodiments, non-animal source is a plant source.

[00195] In some embodiments, said non-animal source is a genetically modified yeast or bacteria.

[00196] In some embodiments, 50% or more of said protein content by weight are one or more isolated purified proteins.

[00197] In some embodiments, each of said one or more isolated proteins is isolated and purified separately from different plant species.

[00198] In some embodiments, one or more of said isolated proteins is selected from the group consisting of: hemoglobin, myoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin. .

[00199] In some embodiments, the meat substitute product further comprises one or more isolated and purified iron-containing proteins.

[00200] In some embodiments, said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins,. In some embodiments, said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1. [SEQ ID NO 1: MVAFTEKQDALVSSSFEAFKANIPQYSVVFYTSILEKAPAAKDLFSFLANGVDPTNPKLTGHAEL LFALVRDSAGQLKASGTVVADAALGSVHAQKAVTDPQFVVVKEALLKTIKAAVGDKWSDELS RAWEVAYDELA AAIKKA]

[00201] In some embodiments, the meat substitute product contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.

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[00202] In particular embodiments, the meat substitute product additionally contains less than 1% wheat gluten.

[00203] In a more particular embodiment, said meat substitute product contains no wheat gluten.

[00204] In other particular embodiments, said meat substitute product contains no soy protein isolate.

[00205] In other particular embodiments, said meat substitute product contains no soy protein concentrate.

[00206] In other particular embodiments, said meat substitute product contains no soy protein.

[00207] In a more particular embodiment, said meat substitute product contains less than 5% carbohydrates.

[00208] In other particular embodiments, said meat substitute product contains no tofu.

[00209] In some embodiments, said meat substitute product contains no tofu, and no wheat gluten.

[00210] In some embodiments, said meat substitute product contains no soy protein, and no wheat gluten.

[00211] In some embodiments, said meat substitute product contains no animal products and less than 5% carbohydrates.

[00212] In some embodiments, said meat substitute product contains less than 1% cellulose.

[00213] In some embodiments, said meat substitute product contains less than 5% insoluble carbohydrates.

[00214] In some embodiments, said meat substitute product contains no soy protein, and less than 1% cellulose.

[00215] In some embodiments, said meat substitute product contains no soy protein, and less than 5% insoluble carbohydrates.

[00216] In some embodiments, said meat substitute product contains no wheat gluten, and less than 1% cellulose.

[00217] In some embodiments, said meat substitute product contains no wheat gluten, and less than 5% insoluble carbohydrates.

[00218] In another aspect, the invention provides a food product comprising one or more isolated, purified iron-containing proteins, wherein said food product is configured for consumption by an animal.

[00219] In some embodiments, said one or more isolated, purified iron-containing proteins is derived from a non-animal source.

[00220] In some embodiments, said non-animal source is a plant source.

[00221] In some embodiments, said plant source comprises one or more plants of the legume family.

[00222] In some embodiments, said one or more plants of the legume family is a soy or pea plant.

[00223] In some embodiments, said non-animal source is a genetically modified yeast or bacteria.

[00224] In some embodiments, said iron-containing protein is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins.

[00225] In one embodiment, said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1. [SEQ ID NO 1:

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MVAFTEKQDALVSSSF EAFKANIPQYSVVFYTSILEKAPAAKDLFSFLANGVDPTNP KLTGHA EK
LFALVRDSAGQLKASGTVVADAALG SVHAQKAVTDPQFVVVKEALLKTIKAAVGDKWSDELS
RAWEVAYDELA AAIKKA]

[00226] In some embodiments, the food product contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.

[00227] In particular embodiments, the food product additionally contains less than 1% wheat gluten.

[00228] In a more particular embodiment, said food product contains no wheat gluten.

[00229] In other particular embodiments, said food product contains no soy protein isolate.

[00230] In other particular embodiments, said food product contains no soy protein concentrate.

[00231] In other particular embodiments, said food product contains no soy protein.

[00232] In a more particular embodiment, said food product contains less than 5% carbohydrates.

[00233] In other particular embodiments, said food product contains no tofu.

[00234] In some embodiments, said food product contains no tofu, and no wheat gluten.

[00235] In some embodiments, said food product contains no soy protein, and no wheat gluten.

[00236] In some embodiments, said food product contains no animal products and less than 5% carbohydrates.

[00237] In some embodiments, said food product contains less than 1% cellulose.

[00238] In some embodiments, said food product contains less than 5% insoluble carbohydrates.

[00239] In some embodiments, said food product contains no soy protein, and less than 1% cellulose.

[00240] In some embodiments, said food product contains no soy protein, and less than 5% insoluble carbohydrates.

[00241] In some embodiments, said food product contains no wheat gluten, and less than 1% cellulose.

[00242] In some embodiments, said food product contains no wheat gluten, and less than 5% insoluble carbohydrates.

[00243] In another aspect, the invention provides a method of making a meat substitute composition, comprising isolating and purifying one or more proteins; and assembling said one or more proteins into a physical organization that approximates the physical organization of meat.

[00244] In another aspect, the invention provides a method of making a muscle tissue replica, comprising isolating and purifying one or more proteins; and assembling said one or more proteins into a physical organization that approximates the physical organization of skeletal muscle.

[00245] In another aspect, the invention provides a method of making a fat tissue replica, comprising isolating and purifying one or more proteins; preparing a solution comprising one or more proteins; emulsifying one or more fats in said solution; and stabilizing said solution into a gelled emulsification with one or more cross-linking reagents.

[00246] In another aspect, the invention provides a method of making a connective tissue replica, comprising isolating and purifying one or more proteins; and precipitating said one or more proteins, wherein said precipitating results in said one or more proteins forming physical structures approximating the physical organization of connective tissue.

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[00247] In some embodiments, said precipitating comprises solubilizing said one or more proteins in a first solution; and extruding said first solution into a second solution, wherein said one or more proteins is insoluble in said second solution, wherein said extruding induces precipitation of said one or more proteins.

[00248] In another aspect, the invention provides a food product comprising one or more isolated, purified iron-containing proteins, wherein said food product is configured for consumption by an animal.

[00249] In another aspect, the invention provides a food product comprising one or more isolated, purified iron-containing proteins, wherein said food product is configured for consumption by humans.

[00250] In another aspect, the invention provides a food product comprising one or more isolated, purified iron-containing proteins, wherein said food product is configured for consumption by an animal. In another aspect, the invention provides a food product comprising one or more isolated, purified iron-containing proteins, wherein said food product is configured for consumption by humans.

[00251] All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[00252] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[00253] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are utilized, and the accompanying drawings of which:

[00254] **Figure 1** depicts a comparison of aligned GCMS profiles (in mirrored orientations) of otherwise identical muscle-replica samples cooked in the presence of leghemoglobin (LHb, Right side profile) or Ferric ion (Left side profile). In this illustration, the vertical axis represents retention time in the gas chromatography separation step, and the horizontal axis represents m/z ratios of ions produced by fragmentation of the corresponding separated volatile compounds. Selected compounds differentially represented in the two samples are labeled on the Right of the figure.

[00255] **Figure 2** depicts a portion of the root of a pea plant (*Pisum sativum*) with the root nodules sliced open to demonstrate the red color conferred by leghemoglobin contained therein. The sliced open root nodule appears red.

[00256] **Figure 3** depicts leghemoglobin isolated from 1 oz of pea roots. The red color commonly attributed to meat is evident in the color photo.

[00257] **Figure 4** shows that leghemoglobins from different species are homologs and have similar color properties. In Figure 4, panel A shows an SDS-PAGE gels of lysed root-nodules of three legume plant species (1) Fava bean (2) English Pea (3) Soybean. Arrows mark respective leghemoglobins. Panel B

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shows the similarity of UV-VIS spectral profile of leghemoglobins from two different plant species (Favabean and Soybean).

[00258] Figure 5 shows a comparison of reduced (heme iron 2+) and oxidized (heme iron 3+) soybean leghemoglobin (Figure 5 panel A) and equine heart muscle myoglobin (Figure 5 panel B) showing similarity of UV-VIS absorption profiles of two proteins. We purified soybean leghemoglobin from soybean root-nodules using here described protocol. Purified equine myoglobin was purchased from SigmaAldrich. Soybean leghemoglobin (Figure 5 panel A) and equine myoglobin (Figure 5 panel B) were reduced with 1mm sodium hydrosulfite. Shown are UV-VIS absorption spectra of heme Fe³⁺ (blue line – the higher peak in figures 5 and 6) and heme Fe²⁺ (red line) of soybean leghemoglobin (Figure 5 panel A) and equine myoglobin (Figure 5 panel B). Insets show a zoom-in of UV-VIS spectra in 450nm to 700nm region. (Figure 5 panel C) Images of 10ul liquid droplet of a 40 mg/ml solution of soybean leghemoglobin in the heme-Fe³⁺ state (left droplet) showing characteristic rusty red color and a 40mg/ml solution of soybean leghemoglobin solution in the heme-Fe²⁺ state (right droplet) showing characteristic red color of and (right image) corresponding samples of equine myoglobin.

[00259] Figure 6 depicts examples of successful reduction of leghemoglobin heme iron with sodium hydrosulfite and titanium citrate. In Figure 6 the UV-VIS spectrogram of purified soybean leghemoglobin in which the heme iron is in the oxidized (+3) state is represented by the blue curves in each panel (the blue curves have the higher peaks on the main graphs). The red curves in each panel represent the UV-VIS spectra of the same leghemoglobin species after reduction to the (+2) state (red lines) by addition of (Panel A) 1mM sodium hydrosulfite or (Panel B) 0.24 % (wt/v) titanium citrate in 20mM potassium phosphate pH 7.3, 100mM sodium chloride buffer. The Insets show a zoom-in of UV-VIS spectra in 450 -700 nm region. For this example, leghemoglobin was purified from soybean root nodules using 60/90% ammonium sulfate fractionation and exchanged into 20mM potassium phosphate pH 7.4, 100mM sodium chloride buffer. Sodium hydrosulfite stock solution was prepared by dissolving 100mM sodium hydrosulfite in 1mM sodium hydroxide in water. Titanium citrate stock solution was prepared from 20 % (wt/v) Ti-chloride in hydrochloric acid by mixing it with 0.2M sodium citrate (1:10 v/v). pH was adjusted using sodium carbonate to pH 7.0.

[00260] Figure 7 depicts an example of the leghemoglobin purification flow from soybean root nodules. The figure shows SDS-PAGE fractionation of different soybean leghemoglobin purification steps (Lane 1) Soybean root-nodule lysate; (Lane 2) Soybean root-nodule lysate purified by 60/90 % (wt/v) ammonium sulfate fractionation. Shown is the protein content of 90% ammonium sulfate fractionated protein pellet resuspended in 20mm potassium phosphate pH 7.4, 100mM sodium chloride, 1mM EDTA buffer; Proteins from 90% ammonium sulfate pellet were further purified by anion-exchange chromatography (FFQ GE Healthcare) in 20mM potassium phosphate pH 7.4, 100mM sodium chloride. Leghemoglobin collected in the flowthrough is shown in Lane 3. Anion-exchange flowthrough was fractionated using size-exclusion chromatography (Sephacryl S-100 GE Healthcare) and resulting leghemoglobin fraction is shown in Lane 4. Leghemoglobin content at different purification steps was determined by determining the fraction of leghemoglobin band on SDS-PAGE gel in a respective sample

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using ImageDoc analysis software (BioRad). Purity (partial abundance) of leghemoglobin at respective steps in the purification steps was: lysate: 32.7% (lane 1), 60/90 % (wt/v) ammonium sulfate fractionation 78% (lane 2), anion-exchange chromatography ~ 83% (lane 3), and size-exclusion chromatography to ~ 95% (lane 4).

[00261] Figure 8 shows stained SDS-PAGE gel analysis of (A) soybean leghemoglobin expressed and purified using recombinant protein technology and (B) soybean leghemoglobin purified from soybean root nodules. (A) Recombinant Soybean leghemoglobin A carrying His-tag and TEV protease His-tag removal site was expressed in E.coli BL21 strain and purified using His-tag affinity chromatography (Talon resin, CloneTech). The left lane contains molecular weight standards, the right lane contains purified recombinant soybean leghemoglobin A (arrow). Expected molecular weight of the recombinant soybean leghemoglobin A is 17.1kDa. (B) SDS-PAGE gel of purified Soybean leghemoglobin from root nodules. The left lane contains molecular weight standards, the right lane contains purified soybean leghemoglobin A (arrow). Mass spectrometry analysis of purified material determined that all four soybean leghemoglobin isoforms are present, and are full length (data not shown). Expected molecular weights (MW) of soybean leghemoglobin isoforms range from MW15.4 to 15.8kDa.

[00262] Figure 9 shows an example of 6 cubes of a commercial meat analog (Quorn chicken analog), about 1cm on a side, 4 of which (Left and lower right) have been soaked in a solution of about 10 mg/ml soybean leghemoglobin in 20 mM Potassium phosphate pH 7.4 and 100 mM NaCl; the remaining two (Upper right) were soaked in the same buffer without leghemoglobin. A deep pink color of the leghemoglobin-infused cubes is apparent in color photos contrasting the pale tan color of the un-infused cubes.

[00263] Figure 10 shows the 4 leghemoglobin-infused cubes of Quorn chicken analog in the process of cooking in a pan at 350°C. The two lower cubes have been turned over to expose the grilled surface, which has turned brown. In the upper two cubes that the heated portion has turned grey-brown, while the cooler top surface retains its pink color. In some embodiments the consumable is injected with a heme containing solution, for instance a leghemoglobin solution, until the consumable is the color of uncooked meat.

[00264] Figure 11 depicts 43 ml of moong bean protein solution (150 mg/ml in dialysis buffer) were mixed with 37 ml of leghemoglobin solution (46.5 mg/ml leghemoglobin and 20mg/ml of other soybean root nodule protein) in 20 mM potassium phosphate, 100 mM NaCl, pH 7.3). 20 ml of transglutaminase solution (20% w/w) were added, solutions thoroughly mixed, divided into two 50 ml Falcon tubes and incubated overnight at room temperature. Final protein concentrations were 65 mg/ml for moong bean protein, 18 mg/ml of leghemoglobin, 91 mg/ml total protein.

[00265] Figure 12 depicts “White” muscle analog prepared by mixing 43ml moong bean protein solution (150mg/ml) with 45ml of 11.7 mg/ml solution of leghemoglobin and 0.8 % (wt/v) of transglutaminase solution. Final protein concentrations were 63 mg/ml for moong bean protein, 5.2 mg/ml of leghemoglobin, 68 mg/ml total protein.

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[00266] Figure 13 depicts a fat tissue analog based on moong beans and prepared in eppendorf tubes formed an opaque gel of off-white color, smooth uniform texture, with no visible discernible liquid that was not incorporated into the gel. The gel was freely standing, elastic and springy. The gel has a slight, pleasant aroma and a mild and pleasant flavor. The taste is mildly salty.

[00267] Figure 14 depicts a tissue analog based on pea globulin and prepared in eppendorf tubes very similar to moong bean-based fat analog, except that it gave up a little of oil upon compression.

[00268] Figure 15 shows connective-tissue analog strands that were created using a 1:3 ratio in 70% ethanol, loaded into a syringe with a 23 gauge needle (ID 0.337 mm). The solution was slowly extruded from the bottom of a 5 inch-high vessel into an excess of 5 M NaCl solution. The ethanol-zein solution being less dense than the NaCl solution, floated upward, drawing out a fibrous strand of solidifying zein. The NaCl was constantly stirred as the strands began to develop to assist in the strand lengthening. The strands bunch together and become a hard, dense mass.

[00269] Figure 16 depicts a ground beef prototype patty was made by combining 62% (wt/wt) muscle analog (62% (wt/wt) “dark muscle analog” and 38% (wt/wt) “white muscle analog”), 29% (wt/wt) fat tissue analog (from pea globulin and canola oil), 5% (wt/wt) connective tissue analog (Figure 16 panel A). A ground beef prototype patty was made by combining 62% muscle analog (62% “dark muscle analog” and 38% “white muscle analog), 29% fat tissue analog (from moong bean seed 8S protein and rice bran oil), 5% connective tissue analog (Figure 16 panel B). A ground beef prototype patty was made by combining 71 % (wt/wt) muscle tissue analog (composed of 60% “white” muscle analog, 40 % “dark” muscle analog), 23% fat tissue (from pea seed globulin proteins and canola oil) (Figure 16 panel C). A ground beef prototype patty was made by combining 67% “White” muscle analog, with 28% fat tissue analog (from pea globulins and rice bran oil), (Figure 16, panel D)

[00270] Figure 17 depicts a ground beef patty analog was made by combining 62% (wt/wt) muscle tissue analog (62% (wt/wt) “dark muscle analog” and 38% (wt/wt) “muscle analog”), 29% (wt/wt) fat tissue analog (from pea globulin and canola oil), 5% (wt/wt) connective tissue analog. The panel on the left shows the patty before cooking and the panel on the right shows the same patty after cooking for about 2 minutes. Observers described the aroma of the cooking ground beef replica as distinctly “beefy”.

DETAILED DESCRIPTION OF THE INVENTION

[00271] Methods and compositions for the production of consumables are described herein. The consumables can be for animal consumption. For example the consumable can be food fit for human consumption. The consumable can be approved by suitable regulatory authorities. The consumables can be sold in grocery stores or prepared in restaurants, schools, hospitals, military facilities, prisons, shelters, long-term care facilities, similar to already existing human foods. The consumables could also be food for domestic animals. For instance, dog food could be produced according to the present inventions. The consumables may also be food for wild animals. For instance, the consumables could be provided to non-domesticated predatory animals.

[00272] The consumables of the present invention can compete with, supplement or replace animal based foods. For instance the consumables can be meat replicas made entirely from plant sources. The

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consumables can be made to mimic the cut or appearance of meat as it is currently sold. For instance a consumable may be visually similar to or indistinguishable from ground beef or a particular cut of beef. Alternatively, the consumables can be made with a unique look or appearance. For instance the consumable could contain patterns or lettering that is based upon the structure of the consumable. In some instances the consumables look like traditional meat products after they are prepared. For example a consumable may be produced which is larger than a traditional cut of beef but which, after the consumable is sliced and cooked appears the same as a traditional cooked meat. In some embodiments the consumable may resemble a traditional meat shape in two dimensions, but not in a third. For example the consumable may resemble a cut of meat in two dimensions (for example when viewed from the top), but may be much longer (or thicker) than the traditional cut. So in some embodiments a composition that can be cut repeatedly into traditionally meat shaped products is provided.

[00273] The consumable may be made entirely from plant based sources. In some instances the consumable can be made from organic sources. The consumables may also be made from a combination of plant based sources and animal based sources. For instance, the consumable may be a ground beef product supplemented with plant based products of the invention.

[00274] The consumables can be made from local products. For instance the consumables can be made from plants grown within a certain radius of the eventual consumer. That radius could be 1, 10, 100, or 1000 miles for example. So, in some embodiments, the invention provides a method for producing a meat replica which does not contain products which have been shipped over 1, 10, 100, or 1000 miles prior to producing the meat replica.

[00275] The present invention provides methods for producing consistent properties from the consumables when they are produced from various sources. So, for example, a plant based meat replica produced from local plants in Iowa, USA, will have substantially similar taste, odor, and texture as a plant based meat replica produced from local plants in Lorraine, France. This consistency allows for methods for advertising locally grown foods with consistent properties. The consistency can arise from the concentration or purification of similar components at different locations. These components can be combined in predetermined ratios to insure consistency. In some embodiments a high degree of characteristic consistency is possible using components (e.g. isolated or concentrated proteins and fats) which come from the same plant species. In some embodiments a high degree of characteristic consistency is possible using components (e.g. isolated or concentrated proteins and fats) which come from the different plant species. In some embodiments the same proteins can be isolated from different plant species. In some embodiments the invention provides for a method comprising isolating similar plant constituents from plant sources in different locations, assembling in both locations compositions provided herein, and selling the compositions, wherein the compositions assembled and sold at different the geographic locations have consistent physical and chemical properties. In some embodiments the isolated constituents are from different plant populations in different locations. In some embodiments one or more of the isolated constituents are shipped to the separate geographic locations.

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[00276] The consumables may require fewer resources to produce than consumables produced from domesticated animals. Accordingly, the present invention provides for meat replicates which require less water or energy to produce than meat. For example a consumable can require less than about 10, 50, 100, 200, 300, 500, or 1000 gallons of water per pound of consumable. For comparison beef can require over 2000 gallons of water per pound of meat.

[00277] The consumable may require less land area to produce than a meat product with similar protein content. For example the consumable may require 30% or less of the land area required to produce a meat product with similar protein content.

[00278] The consumable may have health benefits compared to an animal product it replaces in the diet. For example it may have less cholesterol or lower levels of saturated fats than comparable meat products.

[00279] The consumable may have animal welfare benefits compared to an animal product it replaces in the diet. For instance it may be produced without requiring confinement, forced feeding, premature weaning, disruption of maternal-offspring interactions, or slaughter of animals for their meat.

[00280] The consumable may have a smaller “carbon footprint” than the meat products they replace. For example the consumable may result in net greenhouse gas emissions of 1%, 5%, 10%, 25%, 50% or 75% of the greenhouse gas emissions attributable to the animal product it replaces.

[00281] The consumable may provide alternatives to animal products or combinations of animal products whose consumption is forbidden by religious beliefs. For example, the consumable may be a kosher pork chop.

[00282] The consumable can also be shipped in components and produced or assembled at a different location. When available local components can be used for production of the consumable. These can be supplemented with components which are not locally available. This allows for methods of producing consumables, for instance meat replicates, using less energy in shipment than is required for meat. For example, local water can be used in combination with a kit which provides other components of the consumable. Using local water will reduce shipping weight thereby reducing cost and environmental impact.

[00283] The consumables can be produced or assembled wholly or in part in areas where animal farming is not practical or is not allowed. The consumable can be produced or assembled within an urban environment. For example a kit may be provided to a user to enable the user to produce the consumable. The user could use local water or use plants from a rooftop garden, for instance in Shanghai. In another example, the consumables could be produced aboard a space craft, space station, or lunar base. Accordingly, the present invention provides methods and systems for the production of meat replicas for use in space travel or for training for the same. For instance the present invention could be used in earth based training for space travel. The consumables could also be produced on an island or upon a manmade platform at sea where the keeping of livestock is difficult or prohibited.

[00284] The consumables are, in some embodiments, designed to replicate the experience of eating meat. The look, texture, and taste of the consumable can be such that it is similar or indistinguishable from

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meat. The invention therefore provides in certain embodiments methods for determining whether an animal or human can distinguish the consumable from meat.

[00285] One method to determine whether the consumable is comparable to meat is to a) define the properties of meat and b) determine whether the consumable has similar properties. Properties of meat that can be tested include mechanical properties such as hardness, cohesiveness, brittleness, chewiness, gumminess, viscosity, elasticity, and adhesiveness. Properties of meat that can be tested also include geometric properties such as particle size and shape, and particle shape and orientation. Additional properties can include moisture content and fat content. These properties can be described using terms such as “soft,” “firm” or “hard” describe hardness; “crumbly,” “crunchy,” “brittle,” “chewy,” “tender,” “tough,” “short,” “mealy,” “pasty,” or “gummy,” to describe cohesiveness; “thin” or “viscous” to describe viscosity; “plastic” or “elastic” to describe elasticity; “sticky,” “tacky” or “goopy” to describe adhesiveness; “gritty,” “grainy” or “course” to describe particle shape and size; “fibrous,” “cellular” or “crystalline” to describe particle shape and orientation, “dry,” “moist,” “wet,” or “watery” to describe moisture content; or “oily” or “greasy” to describe fat content. So, in one embodiment a group of people can be asked to rate a certain meat, for instance ground beef, according to properties which describe the meat. These ratings can be used as an indication of the properties of the meat. The consumables of the present invention can then be compared to the meat to determine how similar the consumable is to the meat. In some instances the properties of the consumables are then altered to make the consumable more similar to the meat. So, in some embodiments, the consumable is rated similar to meat according to human evaluation. In some embodiments the consumable is indistinguishable from real meat to a human.

[00286] In some embodiments, subjects asked to identify the consumable identify it as a form of meat. In some embodiments one property of the compositions of the invention is that an animal, for example a human, will identify the composition as a meat. In some embodiments the human identifies the composition of the invention as having properties equivalent to meat. In some embodiments one or more properties of meat are equivalent according to a human’s perception. Such properties include the properties that can be tested. In some embodiments a human identifies a consumable of the present invention as more meat like than meat substitutes found in the art.

[00287] In embodiments an experiment can demonstrate that consumables acceptable to consumers. A panel can be used to screen a variety of consumables described herein. A number of human panelists can tested multiple consumable samples, namely, natural meats vs. the consumable compositions described herein. Variables such as fat content can be standardized for example to 20% fat using lean and fat meat mixes. Fat content can be determined using the Babcock for meat method (S. S. Nielson, Introduction to the Chemical Analysis of Foods (Jones & Bartlett Publishers, Boston, 1994)). Mixtures of ground beef and consumables of the invention prepared according to the procedure described herein can be formulated.

[00288] Panelists can be served samples in booths, under red lights or under white light, in an open consumer panel. Samples can be assigned random three-digit numbers and rotated in ballot position to prevent bias. Panelists can be asked to evaluate samples for tenderness, juiciness, texture, flavor, and

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overall acceptability using a hedonic scale from 1=dislike extremely, to 9=like extremely, with a median of 5=neither like nor, dislike. Panelists can be encouraged to rinse their mouths with water between samples, and given opportunity to comment on each sample.

[00289] The results of this experiment can indicate significant differences ($p < 0.05$) or similarities between the traditional meats and the compositions of the invention.

[00290] These results will demonstrate that the compositions of the invention are judged as acceptably equivalent to real meat products. Additionally these results can demonstrate that compositions of the invention are preferred by panelist over other commercially available meat substitutes. So, in some embodiments the present invention provides for consumables that are significantly similar to traditional meats.

[00291] Consumables of the invention can also have similar physical characteristics as traditional meat. In one embodiment the force required to pierce a 1 inch thick structure (e.g. a patty) made of a consumable of the invention with a fixed diameter steel rod is not significantly different than the force required to pierce a 1 inch thick similar meat structure (e.g. a ground beef patty) with a similar fixed diameter steel rod. Accordingly, the invention provides for consumables with similar physical strength characteristics to meat.

[00292] In some embodiments composition of the invention have a similar cook loss characteristic as meat. In one embodiment a consumable of the invention with a similar fat and protein content as ground beef has the same reduction in size when cooked as real ground beef. Similar similarities in size loss profiles can be achieved for various compositions of consumables described herein matched to various meats.

[00293] In some embodiments the consumable is compared to real meat based upon olfactometer readings. In various embodiments the olfactometer can be used to assess odor concentration and odor thresholds, odor suprathresholds with comparison to a reference gas, hedonic scale scores to determine the degree of appreciation, or relative intensity of odors. In some embodiments the olfactometer allows the training and automatic evaluation of expert panels. So in some embodiments the consumable is a product that causes similar or identical olfactometer readings. In some embodiments the similarity is sufficient to be beyond the detection threshold of human perception.

[00294] Gas chromatography–mass spectrometry (GCMS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to separate and identify different substances within a test sample. GCMS can, in some embodiments, be used to evaluate the properties of a consumable. For example volatile chemicals can be isolated from the head space around meat. These chemicals can be identified using GCMS. A profile of the volatile chemicals in the headspace around meat is thereby created. In some instances each peak of the GCMS can be further evaluated. For instance, a human could rate the experience of smelling the chemical responsible for a certain peak. This information could be used to further refine the profile. GCMS could then be used to evaluate the properties of the consumable. The GCMS profile could be used to refine the consumable.

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[00295] Characteristic flavor and fragrance components are mostly produced during the cooking process by chemical reactions molecules including amino acids, fats and sugars which are found in plants as well as meat. Therefore in some embodiments the consumable is tested for similarity to meat during or after cooking. In some embodiments human ratings, human evaluation, olfactometer readings, or GCMS measurements, or combinations thereof, are used to create an olfactory map of cooked meat. Similarly, an olfactory map of the consumable, for instance a meat replica, can be created. These maps can be compared to assess how similar the cooked consumable is to meat. In some embodiments the olfactory map of the consumable during or after cooking is similar to or indistinguishable from that of cooked or cooking meat. In some embodiments the similarity is sufficient to be beyond the detection threshold of human perception.

[00296] In one aspect, the invention provides a meat substitute product (alternatively referred to herein as “consumable”) that is substantially or entirely composed of ingredients derived from non-animal sources, yet recapitulates key features associated with the cooking and consumption of an equivalent meat product derived from animals. The equivalent meat product can be a white meat or a dark meat. The equivalent meat product can be derived from any animal. Non-limiting examples of animals used to derive the equivalent meat product include farmed animals such as, e.g., cattle, sheep, pig, chicken, turkey, goose, duck, horse, dog or game animals (whether wild or farmed) such as, e.g., rabbit, deer, bison, buffalo, boar, snake, pheasant, quail, bear, elk, antelope, pigeon, dove, grouse, fox, wild pig, goat, kangaroo, emu, alligator, crocodile, turtle, groundhog, marmot, possum, partridge, squirrel, raccoon, whale, seal, ostrich, capybara, nutria, guinea pig, rat, mice, vole, any variety of insect or other arthropod, seafood such as, e.g., fish, crab, lobster, oyster, muscle, scallop, abalone, squid, octopus, sea urchin, tunicate and others. Many meat products are typically derived from skeletal muscle of an animal but it is understood that meat can also come from other muscles or organs of the animal. In some embodiments, the equivalent meat product is a cut of meat derived from skeletal muscle. In other embodiments, the equivalent meat product is an organ such as, e.g., a kidney, heart, liver, gallbladder, intestine, stomach, bone marrow, brain, thymus, lung, tongue. Accordingly, in some embodiments the compositions of the present invention are consumables similar to skeletal muscle or organs.

[00297] In some aspects, the present invention provides meat substitute products comprising one or more of a first composition comprising a muscle tissue replica, a second composition comprising an adipose tissue replica, and/or a third composition comprising a connective tissue replica, wherein the one or more compositions are combined in a manner that recapitulates the physical organization of meat. In other aspects, the present invention provides compositions for a muscle tissue replica (herein referred to as “muscle replica”), an adipose tissue replica (herein referred to as “fat replica”), and a connective tissue replica (herein referred to as “connective tissue replica”). In some embodiments, the compositions and meat substitute products are principally or entirely composed of ingredients derived from non-animal sources. In alternative embodiments, the muscle, fat, and/or connective tissue replica, or the meat substitute products comprising one or more of said replicas, are partially derived from animal sources but supplemented with ingredients derived from non-animal sources. In yet other alternative embodiments,

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the invention provides meat products substantially derived from animal sources but which are supplemented with one or more of a muscle tissue replica, a fat replica, and/or a connective tissue replica, wherein said replicas are derived substantially or entirely from non-animal sources. A non-limiting example of such a meat product is an ultra-lean ground beef product supplemented with a non-animal derived fat replica which improves texture and mouthfeel while preserving the health benefits of a consumable low in animal fat. Such alternative embodiments result in products with properties that more closely recapitulate key features associated with preparing and consuming meat but which are less costly and associated with a lesser environmental impact, less animal welfare impact, or improved health benefits for the consumer.

[00298] The physical organization of the meat substitute product can be manipulated by controlling the localization, organization, assembly, or orientation of the muscle, fat, and/or connective tissue replicas described herein. In some embodiments the product is designed in such a way that the replicas described herein are associated with one another as in meat. In some embodiments the consumable is designed so that after cooking the replicas described herein are associated with one another as in cooked meat. In some embodiments, one or more of the muscle, fat, and/or connective tissue replicas are combined in a manner that recapitulate the physical organization of different cuts or preparations of meat. In an example embodiment, the replicas are combined in a manner that approximates the physical organization of natural ground meat. In other embodiments, the replicas are combined in a manner that approximates different cuts of beef, such as, e.g., ribeye, filet mignon, London broil, among others.

PROTEINS AND PROTEIN SOURCES

[00299] In some embodiments, any of the meat substitute products, muscle tissue replica, fat replica, or connective tissue replica, comprise one or more isolated, purified proteins. In some embodiments, the meat substitute products are comprised of one or more of a muscle replica, a fat replica, and/or connective tissue replica which comprise one or more isolated, purified proteins. In other embodiments, the muscle replica, fat replica, and/or connective tissue replica comprises one or more isolated, purified proteins. In some embodiments, about 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more of the protein component is comprised of one or more isolated, purified proteins. For the purposes of this document, “purified protein” will refer to a preparation in which the cumulative abundance by mass of protein components other than the specified protein, which can be a single monomeric or multimeric protein species, is reduced by a factor of 2 or more, 3 or more, 5 or more, 10 or more, 20 or more, 50 or more, 100 or more or 1000 or more relative to the source material from which the specified protein was isolated.

[00300] In some embodiments, the one or more isolated, purified proteins are derived from non-animal sources. Non-limiting examples of non-animal sources include plants, fungi, bacteria, archaea, genetically modified organisms such as genetically modified bacteria or yeast, chemical or in vitro synthesis. In particular embodiments, the one or more isolated, purified proteins are derived from plant

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sources. Non-limiting examples of plant sources include grains such as, e.g., corn, maize, rice, wheat, barley, rye, triticale, teff, oilseeds including cottonseed, sunflower seed, safflower seed, rapeseed, leafy greens such as, e.g., lettuce, spinach, kale, collard greens, turnip greens, chard, mustard greens, dandelion greens, broccoli, cabbage, green matter not ordinarily consumed by humans, including biomass crops, including switchgrass, miscanthus, sorghum, other grasses, alfalfa, corn stover, green matter ordinarily discarded from harvested plants, sugar cane leaves, leaves of trees, root crops such as cassava, sweet potato, potato, carrots, beets, turnips, plants from the legume family, such as, e.g., clover, peas such as cowpeas, english peas, yellow peas, green peas, beans such as, e.g., soybeans, fava beans, lima beans, kidney beans, garbanzo beans, mung beans, pinto beans, lentils, lupins, mesquite, carob, soy, and peanuts, vetch (vicia), stylo (stylosanthes), arachis, indigofera, acacia, leucaena, cyamopsis, and sesbania. One of skill in the art will understand that proteins that can be isolated from any organism in the plant kingdom may be used in the present invention.

[00301] Proteins that are abundant in plants can be isolated in large quantities from one or more source plants and thus are an economical choice for use in any of the muscle, fat, connective tissue replicas, or meat substitute products. Accordingly, in some embodiments, the one or more isolated proteins comprises an abundant protein found in high levels in a plant and capable of being isolated and purified in large quantities. In some embodiments, the abundant protein comprises about 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70% of the total protein content of the source plant. In some embodiments, the abundant protein comprises about 0.5-10%, about 5-40%, about 10-50%, about 20-60%, or about 30-70% of the total protein content of the source plant. In some embodiments, the abundant protein comprises about 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% of the total weight of the dry matter of the source plant. In some embodiments, the abundant protein comprises about 0.5-5%, about 1-10%, about 5-20%, about 10-30%, about 15-40%, about 20-50% of the total weight of the dry matter of the source plant.

[00302] In particular embodiments, the one or more isolated proteins comprises an abundant protein that is found in high levels in the leaves of plants. In some embodiments, the abundant protein comprises about 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80% of the total protein content of the leaves of the source plant. In some embodiments, the abundant protein comprises about 0.5-10%, about 5%-40%, about 10%-60%, about 20%-60%, or about 30-70% of the total protein content of the leaves of the source plant. In particular embodiments, the one or more isolated proteins comprise ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco activase). Rubisco is a particularly useful protein for meat replicas because of its high solubility and an amino acid composition with close to the optimum proportions of essential amino acids for human nutrition. In particular embodiments, the one or more isolated proteins comprise ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase). In particular embodiments, the one or more isolated proteins comprise a vegetative storage protein (VSP).

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[00303] In some embodiments, the one or more isolated proteins includes an abundant protein that is found in high levels in the seeds of plants. In some embodiments, the abundant protein comprises about 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85% or 90% or more of the total protein content of the seeds of the source plant. In some embodiments, the abundant protein comprises about 0.5-10%, about 5%-40%, about 10%-60%, about 20%-60%, or about 30-70% or >70% of the total protein content of the seeds of the source plant. Non-limiting examples of proteins found in high levels in the seeds of plants are seed storage proteins, e.g., albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins

[00304] In some embodiments, the one or more isolated proteins includes proteins that interact with lipids and help stabilize lipids in a structure. Without wishing to be bound by a particular theory, such proteins may improve the integration of lipids and/or fat replicas with other components of the meat substitute product, resulting in improved mouthfeel and texture of the final product. A non-limiting example of a lipid-interacting plant protein is the oleosin family of proteins. Oleosins are lipid-interacting proteins that are found in oil bodies of plants. Other non-limiting examples of plant proteins that can stabilize emulsions include seed storage proteins from Great Northern Beans, albumins from peas, globulins from peas, 8S globulins from Moong bean, 8S globulins from Kidney bean.

MUSCLE REPLICAS

[00305] A large number of meat products comprise a high proportion of skeletal muscle. Accordingly, the present invention provides a composition derived from non-animal sources which replicates or approximates key features of animal skeletal muscle. In another aspect, the present invention provides a meat substitute product that comprises a composition derived from non-animal sources which replicates or approximates animal skeletal muscle. Such a composition will be labeled herein as “muscle replica”. In some embodiments, the muscle replica and/or meat substitute product comprising the muscle replica are partially derived from animal sources. In some embodiments, the muscle replica and/or meat substitute product comprising the muscle replica are entirely derived from non-animal sources.

[00306] Many meat products comprise a high proportion of striated skeletal muscle in which individual muscle fibers are organized mainly in an isotropic fashion. Accordingly, in some embodiments the muscle replica comprises fibers that are to some extent organized isotropically. In some embodiments the fibers comprise a protein component. In some embodiments, the fibers comprise about 1%, about 2%, about 5%, about 10%, about 15%, about 20%, about 30%, about 40%, about 50%, about 60%, about 70%, about 80%, about 90%, about 95%, about 99% or more of a protein component.

[00307] In some embodiments, the protein component comprises one or more isolated, purified proteins. For example the one or more isolated, purified protein can comprise the 8S globulin from Moong bean seeds, or the albumin or globulin fraction of pea seeds. These proteins provide examples of proteins with favorable properties for constructing meat replicas because of their ability to form gels with textures

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similar to animal muscle or fat tissue. Examples and embodiments of the one or more isolated, purified proteins are described herein. The list of potential candidates here is essentially open and may include Rubisco, any major seed storage proteins, proteins isolated from fungi, bacteria, archaea, viruses, or genetically engineered microorganisms, or synthesized in vitro. The proteins may be artificially designed to emulate physical properties of animal muscle tissue. The proteins may be artificially designed to emulate physical properties of animal muscle tissue. In some embodiments, one or more isolated, purified proteins accounts for about 0.1%, 0.2%, 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99% or more of the protein component by weight.

[00308] Skeletal muscle of animals such as beef cattle typically contain substantial quantities of glycogen, which can comprise on the order of 1% of the mass of the muscle tissue at the time of slaughter. After slaughter, a fraction of this glycogen continues to be metabolized yielding products including lactic acid, which contributes to lowering the pH of the muscle tissue, a desirable quality in meat. Glycogen is a branched polymer of glucose linked together by alpha (1->4) glycosidic bonds in linear chains, with branch points comprising alpha (1->6) glycosidic bonds. Starches from plants, particularly amylopectins are also branched polymers of glucose linked together by alpha (1->4) glycosidic bonds in linear chains, with branch points comprising alpha (1->6) glycosidic bonds and can therefore be used as an analog of glycogen in constructing meat replicas. Thus in some embodiments, the muscle or meat replica includes a starch or pectin.

[00309] Additional components of animal muscle tissue include sodium, potassium, calcium, magnesium, other metal ions, lactic acid, other organic acids, free amino acids, peptides, nucleotides and sulfur compounds. Thus in some embodiments, the muscle replica can include sodium, potassium, calcium, magnesium, other metal ions, lactic acid, other organic acids, free amino acids, peptides, nucleotides and sulfur compounds. In some embodiments the concentration of sodium, potassium, calcium, magnesium, other metal ions, lactic acid, other organic acids, free amino acids, peptides, nucleotides and/or sulfur compounds in the muscle replica or consumable are within 10% of the concentrations found in a muscle or meat being replicated.

[00310] In another aspect, the invention provides methods for making a muscle replica. In some embodiments, the composition is formed into asymmetric fibers prior to incorporation into the consumable. In some embodiments these fibers replicate muscle fibers. In some embodiments the fibers are spun fibers. In other embodiments the fibers are extruded fibers. Accordingly, the present invention provides for methods for producing asymmetric or spun protein fibers. In some embodiments, the fibers are formed by extrusion of the protein component through an extruder. Methods of extrusion are well known in the art, and are described in US6379738, US3693533, US20120093994, which are herein incorporated by reference.

[00311] In some embodiments extrusion can be conducted using an MPF19 twin-screw extruder (APV Baker, Grand Rapids, Mich.) with a cooling die. The cooling die can cool the extrudate prior to return of the extrudate to atmospheric pressure, thus substantially inhibiting expansion or puffing of the final

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product. In the MPF19 apparatus, dry feed and liquid can be added separately and mixed in the barrel. Extrusion parameters can be, for example: screw speed of 200 rpm, product temperature at the die of 150 C., feed rate of 23 g/min, and water-flow rate of 11 g/min. Product temperature can be measured during extrusion by a thermocouple at the end of the extrusion barrel. Observations can be made on color, opacity, structure, and texture for each collected sample. Collected samples can be optionally dried at room temperature overnight, then ground to a fine powder (<60 mesh) using a Braun food grinder. The pH of samples can be measured in duplicate using 10% (w/v) slurries of powdered sample in distilled water.

[00312] FAT REPLICA

[00313] Animal fat is important for the experience of eating cooked meat. Accordingly, the present invention provides a composition derived from non-animal sources which recapitulates key features of animal fat. In another aspect, the present invention provides a meat substitute product that comprises a composition derived from non-animal sources which recapitulates animal fat. Such a composition will be labeled herein as a “fat replica”. In some embodiments, the fat replica and/or meat substitute product comprising the fat replica are partially derived from animal sources.

[00314] In some embodiments the meat substitute product has a fat component. In some embodiments the fat content of the consumable is 1%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, or 60% fat. In some embodiments, the fat replica comprises a gel with droplets of fat suspended therein. In some embodiments, the gel is a soft, elastic gel comprising proteins and optionally carbohydrates. In particular embodiments, the proteins used in the gel are plant or microbial proteins. In some embodiments, the proteins used in the fat replica might include Rubisco, any major seed storage proteins, proteins isolated from fungi, bacteria, archaea, viruses, or genetically engineered microorganisms, or synthesized in vitro. The proteins may be artificially designed to emulate physical properties of animal fat. The proteins may be artificially designed to emulate physical properties of animal fat.

[00315] The fat droplets used in some embodiments of the present invention can be from a variety of sources. In some embodiments, the sources are non-animal sources. In particular embodiments, the sources are plant sources. Non-limiting examples of oils include corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, algal oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, rice bran oil, oils produced by bacteria, algae, archaea or fungi or genetically engineered bacteria, algae, archaea or fungi, triglycerides, monoglycerides, diglycerides, sphingosides, glycolipids, lecithin, lysolecithin, phosphatidic acids, lysophosphatidic acids, oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, conjugated oleic acid, or esters of: oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid,

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eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or glycerol esters of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or triglyceride derivatives of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid.

[00316] In some embodiments, fat droplets are derived from pulp or seed oil. In other embodiments, the source may be yeast or mold. For instance, in one embodiment the fat droplets comprise triglycerides derived from *Mortierella isabellina*.

[00317] In some embodiments plant oils are modified to resemble animal fats. The plant oils can be modified with flavoring or other agents to recapitulate the taste and smell of meat during and after cooking. Accordingly, some aspects of the invention involve methods for testing the qualitative similarity between the cooking properties of animal fat and the cooking properties of plant oils in the consumable.

[00318] In some embodiments, the fat replica comprises a protein component comprising one or more isolated, purified proteins. The purified proteins contribute to the taste and texture of the meat replica. In some embodiments purified proteins can stabilize emulsified fats. In some embodiments the purified proteins can form gels upon denaturation or enzymatic crosslinking, which replicate the appearance and texture of animal fat. Examples and embodiments of the one or more isolated, purified proteins are described herein. In particular embodiments, the one or more isolated proteins comprise a protein isolated from the legume family of plants. Non-limiting examples of legume plants are described herein, although variations with other legumes are possible. In some embodiments, the legume plant is a pea plant. In some embodiments the isolated purified proteins stabilize emulsions. In some embodiments the isolated purified proteins form gels upon crosslinking or enzymatic crosslinking. In some embodiments, the isolated, purified proteins comprise seed storage proteins. In some embodiments, the isolated, purified proteins comprise albumin. In some embodiments, the isolated, purified proteins comprise globulin. In a particular embodiment, the isolated, purified protein is a purified pea albumin protein. In another particular embodiment, the isolated, purified protein is a purified pea globulin protein. In another particular embodiment the isolate purified protein is a Moong bean 8S globulin. In another particular embodiment, the isolated, purified protein is an oleosin. In another particular embodiment, the isolated, purified protein is a caloleosin. In another particular embodiment, the isolated, purified protein is Rubisco. In some embodiments, the protein component comprises about 0.1%, 0.5%, 1%, 2%, 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or more of the fat replica by dry weight or total weight. In some embodiments, the protein component comprises about 0.1-5%, about 0.5-10%, about 1-20%, about 5-30%, about 10-50%, about 20-70%, or

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about 30-90% or more of the fat replica by dry weight or total weight. In some embodiments, the protein component comprises a solution containing one or more isolated, purified proteins.

[00319] In some embodiments, the fat replica comprises cross-linking enzymes that catalyze reactions leading to covalent crosslinks between proteins. Cross-linking enzymes can be used to create or stabilize the desired structure and texture of the adipose tissue replica, to mimic the desired texture of an equivalent desired animal fat. Non-limiting examples of cross-linking enzymes include, e.g., transglutaminase, lysyl oxidases, or other amine oxidases (e.g. *Pichia pastoris* lysyl oxidase). In some embodiments, the cross-linking enzymes are isolated and purified from a non-animal source, examples and embodiments of which are described herein. In some embodiments, the fat replica comprises at least 0.0001%, or at least 0.001%, or at least 0.01%, or at least 0.1%, or at least 1% (wt/vol) of a cross-linking enzyme. In particular embodiments, the cross-linking enzyme is transglutaminase.

[00320] In another aspect, the invention provides methods for making a fat replica. In some embodiments, the fat droplets are suspended in a gel. In some embodiments the present invention provides for methods for producing droplets of fat suspended in the gel. The fat can be isolated and homogenized. For example an organic solvent mixture can be used to help mix a lipid. The solvent can then be removed. At this point the lipid can be frozen, lyophilized, or stored. So in some aspects the invention provides for a method for isolating and storing a lipid which has been selected to have characteristics similar to animal fat. The lipid film or cake can then be hydrated. The hydration can utilize agitation or temperature changes. The hydration can occur in a precursor solution to a gel. After hydration the lipid suspension can be sonicated or extruded to further alter the properties of the lipid in the solution.

[00321] In some embodiments, the fat replica is assembled to approximate the organization of adipose tissue in meat. In some embodiments some or all of the components of the fat replica are suspended in a gel. In various embodiments the gel can be a proteinaceous gel, a hydrogel, an organogel, or a xerogel. In some embodiments, the gel can be thickened to a desired consistency using an agent based on polysaccharides or proteins. For example fucula, arrowroot, cornstarch, katakuri starch, potato starch, sago, tapioca, alginin, guar gum, locust bean gum, xanthan gum, collagen, egg whites, furcellaran, gelatin, agar, carrageenan, cellulose, methylcellulose, hydroxymethylcellulose, acacia gum, konjac, starch, pectin, amylopectin or proteins derived from legumes, grains, nuts, other seeds, leaves, algae, bacteria, or fungi can be used alone or in combination to thicken the gel, forming an architecture or structure for the consumable.

[00322] In particular embodiments, the fat replica is an emulsion comprising a solution of one or more proteins and one or more fats suspended therein as droplets. In some embodiments, the emulsion is stabilized by one or more cross-linking enzymes into a gel. In more particular embodiments, the one or more proteins in solution are isolated, purified proteins. In yet more particular embodiments, the isolated, purified proteins comprise a purified pea albumin enriched fraction. In other more particular embodiments, the isolated, purified proteins comprise a purified pea globulin enriched fraction. In other more particular embodiments, the isolated, purified proteins comprise a purified Moong bean 8S globulin

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enriched fraction. In yet more particular embodiments, the isolated, purified proteins comprise a Rubisco enriched fraction. In other particular embodiments, the one or more fats are derived from plant-based oils. In more particular embodiments, the one or more fats are derived from one or more of: corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, algal oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, rice bran oil, oils produced by bacteria, algae, archaea or fungi or genetically engineered bacteria, algae, archaea or fungi, triglycerides, monoglycerides, diglycerides, sphingosides, glycolipids, lecithin, lysolecithin, phosphatidic acids, lysophosphatidic acids, oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, conjugated oleic acid, or esters of: oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or glycerol esters of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or triglyceride derivatives of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid. In yet even more particular embodiments, the one or more fats is a rice bran oil. In another particular embodiment, the one or more fats is a canola oil. In other particular embodiments, the cross-linking enzyme is transglutaminase, lysyl oxidase, or other amine oxidase. In yet even more particular embodiments, the cross-linking enzyme is transglutaminase. In particular embodiments, the fat replica is a high fat emulsion comprising a protein solution of purified pea albumin emulsified with 40-80% rice bran oil, stabilized with 0.5-5% (wt/vol) transglutaminase into a gel. In particular embodiments, the fat replica is a high fat emulsion comprising a protein solution of partially-purified moong bean 8S globulin emulsified with 40-80% rice bran oil, stabilized with 0.5-5% (wt/vol) transglutaminase into a gel. In particular embodiments, the fat replica is a high fat emulsion comprising a protein solution of partially-purified moong bean 8S globulin emulsified with 40-80% canola oil, stabilized with 0.5-5% (wt/vol) transglutaminase into a gel. In particular embodiments, the fat replica is a high fat emulsion comprising a protein solution of purified pea albumin emulsified with 40-80% rice bran oil, stabilized with 0.0001-1% (wt/vol) transglutaminase into a gel. In particular embodiments, the fat replica is a high fat emulsion comprising a protein solution of partially-purified moong bean 8S globulin emulsified with 40-80% rice bran oil, stabilized with 0.0001-1% (wt/vol) transglutaminase into a gel. In particular embodiments, the fat replica is a high fat emulsion comprising a protein solution of partially-purified moong bean 8S globulin emulsified with 40-80% canola oil, stabilized with 0.0001-1% (wt/vol) transglutaminase into a gel.

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[00323] CONNECTIVE TISSUE REPLICA

[00324] Animal connective tissue provides key textural features that are an important component of the experience of eating meat. Accordingly, the present invention provides a composition derived from non-animal sources which recapitulates key features of animal connective tissue. In another aspect, the present invention provides a meat substitute product that comprises a composition derived from non-animal sources which recapitulates important textural and visual features of animal connective tissue. Such a composition will be labeled herein as “connective tissue replica”. In some embodiments, the connective tissue replica and/or meat substitute product comprising the connective tissue replica are partially derived from animal sources.

[00325] Animal connective tissue can generally be divided into fascia-type and cartilage-type tissue. Fascia-type tissue is highly fibrous, resistant against extension (has high elastic modulus), and has a high protein content, a moderate water content (ca. 50%), and low-to-none fat and polysaccharide content. Accordingly, the present invention provides a connective tissue replica that recapitulates key features of fascia type tissue. In some embodiments, the connective tissue replica comprises about 50% protein by total weight, about 50% by liquid weight, and has a low fat and polysaccharide component.

[00326] The protein content of most fascia-type connective tissue is comprised mainly of collagen. Collagen is characterized by a high fraction of proline and alanine, and also is assembled into characteristic elongated fibrils or rod-like, flexible structures. Prolamins are one family of proteins found in non-animal sources, such as plant sources. Prolamins are highly abundant in plants and are similar in amino acid composition to collagen. Among proteins we tested for this purpose, prolamins were particularly favorable because of their low cost and their ability to readily form fibers or sheets when spun or extruded. Non-limiting examples of prolamin family proteins include, e.g., zein (found in corn), these include hordein from barley, gliadin from wheat, secalin, extensins from rye, kafirin from sorghum, avenin from oats. In fascia-type connective tissue, the prolamin family of proteins, individually or combinations thereof, demonstrates suitability for the protein component because they are highly abundant, similar in global amino acid composition to collagen (high fraction of proline and alanine), and amenable to processing into films and fibers. In addition to zein (found in corn), these include hordein from barley, gliadin from wheat, secalin, extensins from rye, kafirin from sorghum, avenin from oats. Other proteins may be necessary to supplement prolamins in order to achieve target specifications for physicochemical and nutritional properties. The list of potential candidates here is essentially open and may include Rubisco, any major seed storage proteins, proteins isolated from fungi, bacteria, archaea, viruses, or genetically engineered microorganisms, or synthesized in vitro. The proteins may be artificially designed to emulate physical properties of animal connective tissue. animal-derived or recombinant collagen, extensins (hydroxyproline-rich glycoproteins abundant in cell walls e.g. *Arabidopsis thaliana*, monomers of which are “collagen-like” rod-like flexible molecules). The proteins may be artificially designed to emulate physical properties of animal connective tissue. .

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[00327] Methods for forming fascia-type connective tissue will be as those practiced in the art with a bias towards methods producing fibrous or fibrous-like structures by biological, chemical, or physical means, individually or in combination, serially or in parallel, before final forming. These methods may include extrusion or spinning.

[00328] Cartilage-type tissue is macroscopically homogenous, resistant against compression, has higher water content (up to 80%), lower protein (collagen) content, and higher polysaccharide (proteoglycans) contents (ca. 10% each).

[00329] Compositionally, cartilage-type connective tissue will be very similar to fascia-type tissue with the relative ratios of each adjusted to more closely mimic ‘meat’ connective tissue.

[00330] Methods for forming cartilage-type connective tissue will be similar to those for fascia-type connective tissue, but with a bias towards methods producing isotropically homogenous structures.

[00331] The fat can be suspended in a gel. In some embodiments the present invention provides for methods for producing droplets of fat suspended in the proteinaceous gel. The fat can be isolated from plant tissues and emulsified. The emulsification can utilize high-speed blending, homogenization, agitation or temperature changes. The lipid suspension can be sonicated or extruded to further alter the properties of the lipid in the solution. At this point, in some embodiments other components of the consumable are added to the solution followed by a gelling agent. In some embodiments crosslinking agents (e.g. transglutaminase or lysyl oxidase) are added to bind the components of the consumable. In other embodiments the gelling agent is added and the lipid/gel suspension is later combined with additional components of the consumable. In fascia-type connective tissue, the prolamin family of proteins, individually or combinations thereof, demonstrates suitability for the protein component because they are highly abundant, similar in global amino acid composition to collagen (high fraction of proline and alanine), and amenable to processing into films. In addition to zein (found in corn), these include hordein from barley, gliadin from wheat, secalin, extensions from rye, kafirin from sorghum, avenin from oats. Other proteins may be necessary to supplement prolamins in order to achieve targets specifications for physicochemical and nutritional properties. The list of potential candidates here is essentially open and may include any major seed storage proteins, animal-derived or recombinant collagen, extensins (hydroxyproline-rich glycoproteins abundant in cell walls e.g. *Arabidopsis thaliana*, monomers of which are “collagen-like” rod-like flexible molecules).

[00332] In some embodiments some or all of the components of the consumable are suspended in a gel. In various embodiments the gel can be a hydrogel, an organogel, or a xerogel, The gel can be made thick using an agent based on polysaccharides or proteins. For example fecula, arrowroot, cornstarch, katakuri starch, potato starch, sago, tapioca, alginin, guar gum, locust bean gum, xanthan gum, collagen, egg whites, furcellaran, gelatin, agar, carrageenan, cellulose, methylcellulose, hydroxymethylcellulose, acacia gum, konjac, starch, pectin, amylopectin or proteins derived from legumes, grains, nuts, other seeds, leaves, algae, bacteria, of fungi can be used alone or in combination to thicken the gel, forming an architecture or structure for the consumable. Enzymes that catalyze reactions leading to covalent crosslinks between proteins can also be used alone or in combination to form an architecture or structure

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for the consumable. For example transglutaminase, lysyl oxidases, or other amine oxidases (e.g. *Pichia pastoris* lysyl oxidase (PPLLO)) can be used alone or in combination to form an architecture or structure for the consumable. In some embodiments multiple gels with different components are combined to form the consumable. For example a gel containing a plant-based protein can be associated with a gel containing a plant-based fat. In some embodiments fibers or strings of proteins are oriented parallel to one another and then held in place by the application of a gel containing plant based fats.

[00333] The compositions of the invention can be puffed or expanded by heating, such as frying, baking, microwave heating, heating in a forced air system, heating in an air tunnel, and the like, according to methods well known in the art.

[00334] In some embodiments multiple gels with different components are combined to form the consumable. For example a gel containing a plant-based protein can be associated with a gel containing a plant-based fat. In some embodiments fibers or strings of proteins are oriented parallel to one another and then held in place by the application of a gel containing plant based fats.

[00335] In some embodiments the meat replica contains no animal products, less than 1% wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum.

[00336] In some embodiments the meat replica contains no animal products, no wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum.

[00337] In some embodiments the meat replica contains no animal products, no soy protein isolate, no wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum.

[00338] In some embodiments the meat replica contains no animal products, no soy protein concentrate, no wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum.

[00339] In some embodiments the meat replica contains no animal products, no soy protein, no wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum.

[00340] In some embodiments the meat replica contains no animal products, no tofu, no wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum.

[00341] In some embodiments the meat replica contains no animal products, no tofu, and no wheat gluten.

[00342] In some embodiments the meat replica contains no animal products, no soy protein, and no wheat gluten.

[00343] In some embodiments the meat replica contains no methylcellulose, no carrageenan, no caramel color, no Konjac flour, no gum Arabic, and no acacia gum.

[00344] In some embodiments the meat replica contains no animal products and less than 5% carbohydrates.

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[00345] In some embodiments the meat replica contains no animal products, no soy protein, no wheat gluten, no methylcellulose, no carrageenan, no caramel color and no Konjac flour, no gum Arabic, and no acacia gum and less than 5% carbohydrates.

[00346] In some embodiments the meat replica contains no animal products, and less than 1% cellulose.

[00347] In some embodiments the meat replica contains no animal products, and less than 5% insoluble carbohydrates.

[00348] In some embodiments the meat replica contains no animal products, no soy protein, and less than 1% cellulose.

[00349] In some embodiments the meat replica contains no animal products, no soy protein, and less than 5% insoluble carbohydrates.

[00350] In some embodiments the meat replica contains no animal products, no wheat gluten, and less than 1% cellulose.

[00351] In some embodiments the meat replica contains no animal products, no wheat gluten, and less than 5% insoluble carbohydrates.

[00352] The percentage of different components may also be controlled. For example non-animal-based substitutes for muscle, fat tissue, connective tissue, and blood components can be combined in different ratios and physical organizations to best approximate the look and feel of meat. The various can also components can be arranged to insure consistency between bites of the consumable. The components can be arranged to insure that no waste is generated from the consumable. For example, while a traditional cut of meat may have portions that are not typically eaten, a meat replicate can improve upon meat by not including these inedible portions. Such an improvement allows for all of the product made or shipped to be consumed, which cuts down on waste and shipping costs. Alternatively, a meat replica may include inedible portions to mimic the experience of meat consumption. Such portions can include bone, cartilage, connective tissue, or other materials commonly referred to as gristle, or materials included simulating these components. In some embodiments the consumable may contain simulated inedible portions of meat products which are designed to serve secondary functions. For example a simulated bone can be designed to disperse heat during cooking, making the cooking of the consumable faster or more uniform than meat. In other embodiments a simulated bone may also serve to keep the consumable at a constant temperature during shipping. In other embodiments, the simulated inedible portions may be biodegradable.

[00353] In some embodiments the meat substitute compositions contains no animal protein, comprising between 10-30% protein, between 5-80% water, between 5-70% fat, comprising one or more isolated purified proteins. In particular embodiments, the meat substitute compositions comprise transglutaminase.

[00354] In some embodiments the consumable contains components to replicate the components of meat. The main component of meat is typically skeletal muscle. Skeletal muscle typically consists of roughly 75 percent water, 19 percent protein, 2.5 percent intramuscular fat, 1.2 percent carbohydrates and 2.3 percent other soluble non-protein substances. These include organic acids, sulfur compounds, nitrogenous

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compounds, such as amino acids and nucleotides, and inorganic substances such as minerals.

Accordingly, some embodiments of the present invention provide for replicating approximations of this composition for the consumable. For example, in some embodiments the consumable is a plant-based meat replica can comprise roughly 75% water, 19% protein, 2.5% fat, 1.2% carbohydrates; and 2.3 percent other soluble non-protein substances. In some embodiments the consumable is a plant-based meat replica comprising between 60-90% water, 10-30% protein, 1-20% fat, 0.1-5% carbohydrates; and 1-10 percent other soluble non-protein substances. In some embodiments the consumable is a plant-based meat replica comprising between 60-90% water, 5-10% protein, 1-20% fat, 0.1-5% carbohydrates; and 1-10 percent other soluble non-protein substances. In some embodiments the consumable is a plant-based meat replica comprising between 0-50% water, 5-30% protein, 20-80% fat, 0.1-5% carbohydrates; and 1-10 percent other soluble non-protein substances. In some embodiments, the replica contains between 0.01% and 5% by weight of a heme protein. In some embodiments, the replica contains between 0.01% and 5% by weight of leghemoglobin. Some meat also contains myoglobin, a heme protein, which accounts for most of the red color and iron content of some meat. In some embodiments, the replica contains between 0.01% and 5% by weight of a heme protein. In some embodiments, the replica contains between 0.01% and 5% by weight of leghemoglobin. It is understood that these percentages can vary in meat and the meat replicas can be produced to approximate the natural variation in meat. Additionally, in some instances, the present invention provides for improved meat replicas, which comprise these components in typically unnatural percentages. For example a meat replica can be produced with a higher than typical average fat content. The percentages of these components may also be altered to increase other desirable properties.

[00355] In some instances a meat replica is designed so that, when cooked, the percentages of components are similar to cooked meat. So, in some embodiments, the uncooked consumable has different percentages of components than uncooked meat, but when cooked the consumable is similar to cooked meat. For example, a meat replica may be made with a higher than typical water content for raw meat, but when cooked in a microwave the resulting product has percentages of components similar to meat cooked over a fire.

[00356] In some embodiments the consumable is a meat replica with a lower than typical water content for meat. In some embodiments the invention provides for methods for hydrating a meat replica to cause the meat replica to have a content similar to meat. For example a meat replica with a water content that would be low for meat, for example 1%, 10%, 20%, 30%, 40% or 50% water, is hydrated to roughly 75% water. Once hydrated, in some embodiments, the meat replica is then cooked for human consumption.

[00357] The consumable can have a protein component. In some embodiments the protein content of the consumable is 10%, 20%, 30%, or 40%. In some embodiments the protein content of the consumable is similar to meat. In some embodiments the protein content in the consumable is greater than that of meat. In some embodiments the consumable has less protein than meat.

[00358] The protein in the consumable can come from a variety or combination of sources. Non-animal sources can provide some or all of the protein in the consumable. Non-animal sources can include

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vegetables, fruits, nuts, grains, algae, bacteria, or fungi. The protein can be isolated or concentrated from one or more of these sources. In some embodiments the consumable is a meat replica comprising protein only obtained from non-animal sources.

[00359] In some embodiments protein is formed into asymmetric fibers for incorporation into the consumable. In some embodiments these fibers replicate muscle fibers. In some embodiments the protein are spun fibers. Accordingly, the present invention provides for methods for producing asymmetric or spun protein fibers. In some embodiments the consumable contains a protein or proteins that have all of the amino acids found in proteins that are essential for human nutrition. In some embodiments the proteins added to the consumable are supplemented with amino acids.

[00360] INDICATORS OF COOKING MEAT

[00361] The release of odorants upon cooking is an important aspect of meat consumption. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking beef. In some embodiments, the consumable when cooked generates an aroma recognizable by humans as typical of cooking pork. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking bacon. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking chicken. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking lamb. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking fish. In some embodiments, the consumable is a meat replica entirely composed of non-animal products that when cooked generates an aroma recognizable by humans as typical of cooking turkey. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with an odorant that is released upon cooking. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with an odorant that is produced by chemical reactions that take place upon cooking. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, containing mixtures of proteins, peptides, amino acids, nucleotides, sugars and polysaccharides and fats in combinations and spatial arrangements that enable these compounds to undergo chemical reactions during cooking to produce odorants and flavor-producing compounds. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with a volatile or labile odorant that is released upon cooking. In some embodiments the consumable is a method for preparing a meat replica where meat replicas principally or entirely composed of ingredients derived from non-animal sources are heated to release a volatile or labile odorant.

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[00362] Odorants released during cooking of meat are generated by reactions that can involve as reactants fats, protein, amino acids, peptides, nucleotides, organic acids, sulfur compounds, sugars and other carbohydrates. In some embodiments the odorants that combine during the cooking of meat are identified and located near one another in the consumable, such that upon cooking of the consumable the odorants combine. So, in some embodiments, the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions involving amino acids, fats and sugars found in plants as well as meat. So, in some embodiments, the characteristic flavor and fragrance components are mostly produced during the cooking process by chemical reactions involving one or more amino acids, fats, peptides, nucleotides, organic acids, sulfur compounds, sugars and other carbohydrates found in plants as well as meat.

[00363] Some reactions that generate odorants released during cooking of meat can be catalyzed by iron, in particular the heme iron of myoglobin. Thus in some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by iron. In some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by heme. . In some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by the heme iron in leghemoglobin. In some embodiments, some of the characteristic flavor and fragrance components are produced during the cooking process by chemical reactions catalyzed by the heme iron in a heme protein.

[00364] Evidence that the presence of leghemoglobin contributes favorably to aroma of meat replicas: A muscle replica comprising pea flour, sunflower oil, and glucose was heated for 10 minutes at 140C in the presence of either reduced leghemoglobin (LHb) or a mixture of iron (Fe^{3+}), sodium and EDTA (EFS) in sealed containers carrying solid phase microextraction (SPME) fibers. These fibers contain polydimethylsiloxane (PDMS) which adsorbs volatile compounds for analysis by GC-MS. Analysis of GC-MS data from multiple replicas reveal consistent differences between the LHb and EFS samples. Non-limiting examples of compounds found exclusively or more abundantly in the LHb samples are: 2-octanone, 2-methyl furan, which are often associated with the aroma of cooked meat, and many other unidentified compounds. For example, Figure 1 shows a comparison of aligned GCMS profiles (in mirrored orientations) of otherwise identical muscle-replica samples cooked in the presence of leghemoglobin (LHb, Right side profile) or Ferric ion (Left side profile). In this illustration, the vertical axis represents retention time in the gas chromatography separation step, and the horizontal axis represents m/z ratios of ions produced by fragmentation of the corresponding separated volatile compounds. Selected compounds differentially represented in the two samples are labeled on the Right of the figure.

[00365] COLOR INDICATORS

[00366] The color of meat is an important part the experience of cooking and eating meat. For instance, cuts of beef are of a characteristic red color in a raw state and gradually transition to a brown color during

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cooking. As another example, white meats such as chicken or pork have a characteristic pink color in their raw state and gradually transition to a white or brownish color during cooking. The amount of the color transition is used to indicate the cooking progression of beef and titrate the cooking time and temperature to produce the desired state of done-ness. In some aspects, the invention provides a non-meat based meat substitute product that provides a visual indicator of cooking progression. In some embodiments, the visual indicator is a color indicator that undergoes a color transition during cooking. In particular embodiments, the color indicator recapitulates the color transition of a cut of meat as the meat progresses from a raw to a cooked state. In more particular embodiments, the color indicator colors the meat substitute product a red color before cooking to indicate a raw state and causes the meat substitute product to transition to a brown color during cooking progression. In other particular embodiments, the color indicator colors the meat substitute product a pink color before cooking to indicate a raw state and causes the meat substitute product to transition to a white or brown color during cooking progression.

[00367] The main determinant of the nutritional definition of the color of meat is the concentration of iron carrying proteins in the meat. In the skeletal muscle component of meat products, one of the main iron-carrying proteins is myoglobin. It is estimated that the white meat of chicken has under 0.05%; pork and veal have 0.1-0.3%; young beef has 0.4-1.0%; and old beef has 1.5-2.0%. So, in some embodiments, the consumable is a meat replica which comprises an iron-carrying protein. In some embodiments, the meat replica comprises about 0.05%, about 0.1%, about 0.2%, about 0.3%, about 0.4%, about 0.5%, about 0.6%, about 0.7%, about 0.8%, about 0.9%, about 1%, about 1.1%, about 1.2%, about 1.3%, about 1.4%, about 1.5%, about 1.6%, about 1.7%, about 1.8%, about 1.9%, about 2%, or more than about 2% of an iron-carrying protein by dry weight or total weight. In some cases, the iron carrying protein has been isolated and purified from a source. In other cases, the iron carrying protein has not been isolated and purified. In some cases, the source of the iron-carrying protein is an animal source, or a non-animal source such as a plant, fungus, or genetically modified organisms such as, e.g., bacteria or yeast. In some cases, the iron-carrying protein is myoglobin. In some embodiments the consumable is a plant based meat replica that has animal myoglobin added. So, for example a replica of young beef can have about 0.4-1% myoglobin. In some cases, the iron-carrying protein is leghemoglobin. In some embodiments the consumable is a plant based meat replica that has leghemoglobin added. So, for example a replica of young beef can have about 0.4-1% leghemoglobin. In some cases, the iron-carrying protein is a cytochrome. In some embodiments the consumable is a plant based meat replica that has a cytochrome added. So, for example a replica of young beef can have about 0.4-1% of a cytochrome.

[00368] Another example of iron-carrying proteins is hemoglobin, the iron-containing oxygen-binding protein in the red blood cells of vertebrates. Hemoglobin is similar in color to myoglobin. In some embodiments the invention provides methods of saving and recycling blood from animal farming to supplement the color of a consumable. For example blood is saved from a slaughter house, hemoglobin from the blood is used to enhance the color of a consumable. In some aspects the consumable is a plant-based meat replica containing hemoglobin.

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[00369] Additional iron containing proteins exist in nature. In some embodiments the consumable comprises an iron containing protein that is not myoglobin. In some embodiments the consumable does not contain myoglobin. In some embodiments the consumable does not contain hemoglobin. In some embodiments the consumable is a meat replica that comprises an iron containing protein other than myoglobin or hemoglobin.

[00370] Examples of iron containing proteins include hemoglobin, myoglobin, neuroglobin, cytoglobin, leghemoglobin, non-symbiotic hemoglobin, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins. In various embodiments these iron containing proteins are added to the consumable to alter the visual characteristics or iron content of the consumable. In some embodiments the consumable comprises a hemoprotein (e.g. hemoglobin, myoglobin, neuroglobin, cytoglobin, leghemoglobin, non-symbiotic hemoglobin, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins.).

[00371] Leghemoglobin, similar in structure and physical properties to myoglobin, is readily available as an unused by-product of commodity legume crops (eg., soybean, pea). The leghemoglobin in the roots of these crops in the US exceeds the myoglobin content of all the red meat consumed in the US. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, including a muscle tissue replica, an adipose tissue replica, a connective tissue replica, and leghemoglobin. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, containing a heme protein. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, containing a leghemoglobin. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, containing a member of the globin protein family. In some embodiments the consumable is a meat replica principally or entirely composed of ingredients derived from non-animal sources, with a high iron content from a heme protein. In some embodiments the iron content is similar to meat. In some embodiments the consumable has the distinctive red color of meat, such color provided by leghemoglobin.

[00372] Leghemoglobin is, in some embodiments, used as an indicator that the consumable is finished cooking. So, one embodiment of the invention is a method for cooking a consumable comprising detecting leghemoglobin which has migrated from the interior of the consumable to the surface when the product is cooked. Another embodiment of the invention is a method for cooking a consumable comprising detecting the change in color of from red to brown when the product is cooked.

[00373] A heme protein is, in some embodiments, used as an indicator that the consumable is finished cooking. So, one embodiment of the invention is a method for cooking a consumable comprising detecting leghemoglobin which has migrated from the interior of the consumable to the surface when the product is cooked. Another embodiment of the invention is a method for cooking a consumable comprising detecting the change in color of from red to brown when the product is cooked.

[00374] A heme protein from the group of: Hemoglobin, myoglobin, neuroglobin, cytoglobin, leghemoglobin, non-symbiotic hemoglobin, Hell's gate globin I, bacterial hemoglobins, ciliate

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myoglobins, flavohemoglobins, is, in some embodiments, used as an indicator that the consumable is finished cooking. So, one embodiment of the invention is a method for cooking a consumable comprising detecting leghemoglobin which has migrated from the interior of the consumable to the surface when the product is cooked. Another embodiment of the invention is a method for cooking a consumable comprising detecting the change in color of from red to brown when the product is cooked.

Food products comprising isolated, purified leghemoglobin

[00375] In some embodiments leghemoglobin is added to meat to enhance the properties of meat. For example, a leghemoglobin containing solution can be injected into raw or cooked meat. In another example a leghemoglobin solution is dripped over meat or a consumable of the invention to enhance appearance. In one embodiment advertising, photography, or videography of food products such as meat or a meat substitute is enhanced with leghemoglobin.

Sources of leghemoglobin

[00376] In some embodiments the present invention provides methods for obtaining leghemoglobin from plants. Leghemoglobin can be obtained from a variety of plants. Various legumes species and their varieties, for example, Soybean, Fava bean, Lima bean, Cowpeas, English peas, Yellow peas, Lupine, Kidney bean, Garbanzo beans, Peanut, Alfalfa, Vetch hay, Clover, Lespedeza and Pinto bean, contain nitrogen-fixing root nodules in which leghemoglobin has a key role in controlling oxygen concentrations (for example root nodules from a pea plant, Figure 2). Figure 3 shows 100 mls of leghemoglobin solution isolated from 30 grams of pea root nodules. Leghemoglobins from different species are homologs and have similar color properties (Figure 4). In Figure 4, panel A shows an SDS_PAGE gels of lysed root-nodules of three legume plant species (1) Fava bean (2) English Pea (3) Soybean. Arrows mark respective leghemoglobins. Note that leghemoglobin is the most abundant soluble protein in each lysate. Panel B shows the similarity of UV-VIS spectral profile of leghemoglobins from two different plant species (Favabean and Soybean). We purified leghemoglobin from fava bean (green curve) and Soybean (red curve) root nodules using the protocol described elsewhere in the specification. UV-VIS spectra of both proteins shows that the heme iron is in the reduced (+2) state. Note that they are almost perfectly superimposed, consistent with their visually identical red color. The heme iron in the respective leghemoglobins was reduced to the +2 oxidation state by incubating Fava bean and Soybean leghemoglobin with 10mM sodium hydrosulfite in 20mM potassium-phosphate pH 7.4, 100mM sodium chloride buffer. Sodium hydrosulfite was then removed from the leghemoglobin solution using gel-exclusion chromatography. Inset shows a zoom-in of UV-VIS spectra in 450nm to 700nm region. Some plant species express several leghemoglobin isoforms (for example soybean has four leghemoglobin isoforms). Minor variations in precise amino acid sequence can modify overall charge of the protein at a particular pH and can modify precise structural conformation of iron containing heme group in leghemoglobin. Differences in structural conformation of heme group of different leghemoglobins can

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influence oxidation and reduction rates of the heme iron. These differences may contribute to color and flavor generation properties of different leghemoglobins.

[00377] Leghemoglobin has a virtually identical absorbance spectrum and visual appearance to myoglobin from animal muscle. Figure 5 shows a comparison of reduced (heme iron 2+) and oxidized (heme iron 3+) soybean leghemoglobin (Figure 5 panel A) and equine heart muscle myoglobin (Figure 5 panel B) showing similarity of UV-VIS absorption profiles of two proteins. We purified soybean leghemoglobin from soybean root-nodules using here described protocol. Purified equine myoglobin was purchased from SigmaAldrich. Soybean leghemoglobin (Figure 5 panel A) and equine myoglobin (Figure 5 panel B) were reduced with 1mm sodium hydrosulfite. Shown are UV-VIS absorption spectra of heme Fe3+ (blue line) and heme Fe2+ (red line) of soybean leghemoglobin (Figure 5 panel A) and equine myoglobin (Figure 5 panel B). Insets show a zoom-in of UV-VIS spectra in 450nm to 700nm region. (Figure 5 panel C) Images of 10ul liquid droplet of a 40 mg/ml solution of soybean leghemoglobin in the heme-Fe3+ state (left droplet) showing characteristic rusty red color and a 40mg/ml solution of soybean leghemoglobin solution in the heme-Fe2+ state (right droplet) showing characteristic red color of and (right image) corresponding samples of equine myoglobin.

[00378] In other embodiments, leghemoglobin can be sourced from non-plant sources, such as from organisms such as bacteria or yeast which have been genetically modified to express high levels of leghemoglobin.

[00379] The oxidation state of the iron ion in leghemoglobin is important for its color. Leghemoglobin with the heme iron in the +2 oxidation state appears vivid red in color, while leghemoglobin with the heme iron in the +3 oxidation state appears brownish red. Thus, in using leghemoglobin as a source of red color in a meat replica, it is desirable to reduce the heme iron from the +3 state to the +2 state. Heme iron in leghemoglobin can be switched from oxidized (+3) state to reduced (+2) state with reducing reagents. Examples of successful reduction of leghemoglobin heme iron with sodium hydrosulfite and titanium citrate are illustrated in Figure 6. In Figure 6 the UV-VIS spectrogram of purified soybean leghemoglobin in which the heme iron is in the oxidized (+3) state is represented by the blue curves in each panel. The red curves in each panel represent the UV-VIS spectra of the same leghemoglobin species after reduction to the (+2) state (red lines) by addition of (Panel A) 1mM sodium hydrosulfite or (Panel B) 0.24 % (wt/v) titanium citrate in 20mM potassium phosphate pH 7.3, 100mM sodium chloride buffer. The Insets show a zoom-in of UV-VIS spectra in 450 -700 nm region. For this example, leghemoglobin was purified from soybean root nodules using 60/90% ammonium sulfate fractionation and exchanged into 20mM potassium phosphate pH 7.4, 100mM sodium chloride buffer. Sodium hydrosulfite stock solution was prepared by dissolving 100mM sodium hydrosulfite in 1mM sodium hydroxide in water. Titanium citrate stock solution was prepared from 20 % (wt/v) Ti-chloride in hydrochloric acid by mixing it with 0.2M sodium citrate (1:10 v/v). pH was adjusted using sodium carbonate to pH 7.0.

[00380] Leghemoglobin can be purified from legume root nodules, such as the root nodules of peas or soybeans (Figure 1 shows pea root nodules, Figure 2 shows Leghemoglobin isolated from same). Root

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nodules from soy beans were thoroughly cleaned to remove soil and extraneous root tissues prior to root nodule lysis in 20mM potassium phosphate pH 7.4, 100mM sodium chloride, 1mM EDTA and 1mM ascorbic acid. Root nodules were lysed by grinding root-nodules using a Vitamix blender. For some samples Polyvinylpyrrolidone polymer was added at 30% wt/v to aid in removal of plant phenolic small molecules that mediate oxidation of leghemoglobin heme-iron. Root nodule lysate was fractionated using ammonium sulfate in two steps, first ammonium sulfate was added to 60% wt/v. Pellet was discarded and supernatant brought to 90% wt/v. ammonium sulfate. Leghemoglobin was collected as a precipitated pellet in 90% ammonium sulfate fraction. Ammonium sulfate precipitated leghemoglobin was resuspended in 20mM potassium phosphate, 1mM EDTA, 50mM sodium chloride and ammonium sulfate was removed using dialysis or size-exclusion chromatography in the same buffer. In some instances this was the last purification step, while in other instances leghemoglobin was further purified using anion-exchange chromatography (FFQ GE Healthcare), which was sometimes followed by size-exclusion chromatography (Sephacryl S-100, GE Healthcare). Soybean leghemoglobin from 90% ammonium sulfate pellet was loaded on anion exchange columns (FFQ or DEAE, GE Healthcare) in different buffers (20mM potassium phosphate pH 7.4, containing 0 to 100mM sodium chloride, 20mM Tris pH 8 containing 0 to 100mM sodium chloride, 20mM sodium borax pH 9.8, 20mM sodium chloride, 20mM sodium carbonate pH 9, 20mM sodium chloride) and purified either in flow-through or using sodium chloride (0-1M salt gradient). An example of the leghemoglobin purification flow from soybean root nodules is represented in Figure 7. The figure shows SDS-PAGE fractionation of different soybean leghemoglobin purification steps (Lane 1) Soybean root-nodule lysate; (Lane 2) Soybean root-nodule lysate purified by 60/90 % (wt/v) ammonium sulfate fractionation. Shown is the protein content of 90% ammonium sulfate fractionated protein pellet resuspended in 20mm potassium phosphate pH 7.4, 100mM sodium chloride, 1mM EDTA buffer; Proteins from 90% ammonium sulfate pellet were further purified by anion-exchange chromatography (FFQ GE Healthcare) in 20mM potassium phosphate pH 7.4, 100mM sodium chloride. Leghemoglobin collected in the flowthrough is shown in Lane 3. Anion-exchange flowthrough was fractionated using size-exclusion chromatography (Sephacryl S-100 GE Healthcare) and resulting leghemoglobin fraction is shown in Lane 4. Leghemoglobin content at different purification steps was determined by determining the fraction of leghemoglobin band on SDS-PAGE gel in a respective sample using ImageDoc analysis software (BioRad). Purity (partial abundance) of leghemoglobin at respective steps in the purification steps was: lysate: 32.7% (lane 1), 60/90 % (wt/v) ammonium sulfate fractionation 78% (lane 2), anion-exchange chromatography ~ 83% (lane 3), and size-exclusion chromatography to ~ 95% (lane 4).

[00381] Leghemoglobin can also be produced by genetically engineering a bacterium or fungus to produce it. One illustrative example is shown in Figure 8. Figure 8 shows stained SDS-PAGE gel analysis of (A) soybean leghemoglobin expressed and purified using recombinant protein technology and (B) soybean leghemoglobin purified from soybean root nodules. (A) Recombinant Soybean leghemoglobin A carrying His-tag and TEV protease His-tag removal site was expressed in E.coli BL21 strain and purified using His-tag affinity chromatography (Talon resin, CloneTech). The left lane

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contains molecular weight standards, the right lane contains purified recombinant soybean leghemoglobin A (arrow). Expected molecular weight of the recombinant soybean leghemoglobin A is 17.1kDa. (B) SDS-PAGE gel of purified Soybean leghemoglobin from root nodules. The left lane contains molecular weight standards, the right lane contains purified soybean leghemoglobin A (arrow). Mass spectrometry analysis of purified material determined that all four soybean leghemoglobin isoforms are present, and are full length (data not shown). Expected molecular weights (MW) of soybean leghemoglobin isoforms range from MW15.4 to 15.8kDa.

[00382] Leghemoglobin purified from soybean and fava root nodules, respectively was tasted by a panel of volunteers and in each case described as tasting like blood.

[00383] Leghemoglobin can be isolated from the roots nodules of legumes such as soy beans, fava beans, cow peas, lima beans, garbanzo beans, peas, lupine, lotus japonicum or other legumes. The root nodule (for example root nodules from a pea plant, Figure 1) is obtained and homogenized in an aqueous solution, soluble proteins including leghemoglobin are recovered after insoluble matter is removed by precipitation or filtration. Leghemoglobin can be purified by selective precipitation and/or chromatography and/or the use of molecules with specific affinity for leghemoglobin. (Figure 2, showing 100 mls of solution of leghemoglobin isolated from 30 grams of pea root nodules).

[00384] Heme proteins, for example leghemoglobin, can be combined with other plant based meat replica components. In some embodiments the heme proteins are captured in a gel which contains other components, for example lipids and or proteins. In some aspects a multiple gels are combined with non-gel based heme proteins. In some embodiments the combination of the heme proteins and the other compounds of the consumable are done to insure that the heme proteins are able to diffuse through the consumable. In some embodiments the consumable is ed in a heme-protein containing solution, for instance a leghemoglobin solution,. In some embodiments the consumable is soaked in a heme protein containing solution, for instance a leghemoglobin solution for 1, 5, 10, 15, 20 or 30 hours. In some embodiments the consumable is soaked in a heme containing solution, for instance a leghemoglobin solution for 1, 5, 10, 15, 30, or 45 minutes.

[00385] Figure 9 shows an example of 6 cubes of a commercial meat analog (Quorn chicken analog), about 1cm on a side, 4 of which (Left and lower right) have been soaked in a solution of about 10 mg/ml soybean leghemoglobin in 20 mM Potassium phosphate pH 7.4 and 100 mM NaCl; the remaining two (Upper right) were soaked in the same buffer without leghemoglobin. Note the deep pink color of the leghemoglobin-infused cubes in contrast to the pale tan color of the un-infused cubes.

[00386] Figure 10 shows the 4 leghemoglobin-infused cubes of Quorn chicken analog in the process of cooking in a pan at 350°C. The two lower cubes have been turned over to expose the grilled surface, which has turned brown. Note in the upper two cubes that the heated portion has turned grey-brown, while the cooler top surface retains its pink color. In some embodiments the consumable is injected with a heme containing solution, for instance a leghemoglobin solution, until the consumable is the color of uncooked meat.

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[00387] Given the usefulness of heme proteins for coloring consumables it will be useful to detect whether a product contains a particular heme protein. Accordingly the present invention includes in some embodiments methods to determine whether a product contains a heme protein. Methods for detecting proteins are well known in the art. For example an ELISA or proximity-ligation assay or lunex assay or western blot analysis can be performed to determine whether leghemoglobin is present in a food product such as meat or a meat replica. In one embodiment the detection methods are performed to determine whether meat has been altered with leghemoglobin.

Examples

[00388] An exemplary muscle replica composition comprising one or more isolated, purified plant proteins is described herein.

[00389] Protein purification for components of the replica

[00390] Moong bean seeds, Green Pea dry seed were purchased as milled flour and used for purification of respective seed storage proteins. Rubisco was purified from fresh alfalfa plant. Protein composition at individual fractionation steps was monitored by SDS-PAGE and protein concentrations were measured by standard UV-VIS and Pierce assay methods.

[00391] Moong bean 8S globulins: Moong bean flour was resuspended in 50mM potassium phosphate buffer pH 7 and 0.5M NaCl at 1:4 (wt/v) ratio, and mixture was incubated for 1hr. Insoluble material was separated by centrifugation and proteins in the supernatant were fractionated by addition of ammonium sulfate in 2 steps: 50% (wt/v) followed by 90% (wt/v). Protein precipitated in 90% fraction contained the moong bean 8S globulins and was stored at -20C until further use.

[00392] Pea-albumins: Green pea dry seed flour was resuspended at 1: 10 (wt/v) ratio in 50mM sodium acetate buffer pH 5 and incubated for 1hr. Insoluble material was separated by centrifugation and proteins in the supernatant were fractionated by ammonium sulfate precipitation in two steps: 50% (wt/v) followed by 90% (wt/v). Ammonium sulfate solutions were stirred for 1 hour and ammonium sulfate precipitated proteins removed by centrifugation. Proteins of interest precipitated in 90% (wt/v) ammonium sulfate. Pellet was stored at -20C until further use.

[00393] Pea-globulins: Green pea dry seed flour was resuspended at 1: 10 (wt/v) ratio in 20mM potassium phosphate buffer pH 8, 0.4M sodium chloride and stirred for 1hr. After centrifugation, the supernatant was subjected to ammonium sulfate fractionation. First, supernatant was brought to 50% (wt/v) ammonium sulfate, and precipitated proteins removed. Second, 50% (wt/v) ammonium sulfate supernatant was brought to 80% (wt/v) ammonium sulfate saturation. The 80% (wt/v) ammonium sulfate pelleted protein contained globulins of interest. Pellet was stored at -20°C until further use.

[00394] RuBisCO: RuBisCO was fractionated from alfalfa greens (or other green plants eg soybean plants, spinach etc) by first grinding leaves with 4 volumes of cold 50mM KPhosphate buffer pH 7.4 buffer (with (in lab) or without (in field) 0.5M NaCl + 2mM DTT + 1mM EDTA) in a blender. The resulting slurry was centrifuged to remove debris, and the supernatant (crude lysate) was used in further

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purification steps. Proteins in the crude lysate were fractionated by addition of ammonium sulfate to 30% (wt/v) saturation. The solution was stirred for 1hr and then centrifuged. The pellet from this step was discarded and additional ammonium sulfate was added to the supernatant to 50 % (wt/v) ammonium sulfate saturation. The solution was centrifuged again after stirring for 1hr. The pellet from this step contains RuBisCO, and was kept at -20C until used.

[00395] Obtaining plant proteins.

Moong bean seed 8S protein was purified by ammonium sulfate fractionation as described. Pellet was resuspended in 20mM potassium phosphate pH 7.4 and 0.5M sodium chloride and ammonium sulfate removed by dialysis against the same buffer. Any precipitate was removed by centrifugation at 16 000g, 10min and protein concentrated to desired concentration. Pea globulins purified by ammonium sulfate fractionation as described. Protein pellet was resuspended in 20mM potassium phosphate pH 7.4 and 0.4M sodium chloride and ammonium sulfate removed by dialysis against the same buffer. Any precipitate was removed by centrifugation at 16 000g, 10min and protein concentrated to desired concentration. Pea albumin purified by ammonium sulfate fractionation as described. Protein pellet was resuspended in 20mM potassium phosphate pH 7.4 and 0.1M sodium chloride and ammonium sulfate removed by dialysis against the same buffer. Any precipitate was removed by centrifugation at 16 000g, 10min and protein concentrated to desired concentration.

[00396] Constructing a Muscle Tissue Analog

[00397] Moong bean seed 8S protein was purified by ammonium sulfate fractionation as described above, For preparation of gels, 200 g of pellet was dissolved in 400 ml of dialysis buffer (20 mM potassium phosphate, 400 mM NaCl, pH 7.3) and the resulting solution dialyzed for 6 hours against 5 l of dialysis buffer, replaced twice with fresh buffer. Protein solution was centrifuged at 12,000 g for 15 min to remove debris. Protein was concentrated by dialyzing for 36 hours against 5 l of 30% w/w solution of PEG 8000 (polyethylene glycol, molecular weight 8000) in dialysis buffer. Final protein concentration was 150 mg/ml.

[00398] Leghemoglobin was purified from soybean root nodules. Legume root nodules were cleaned to remove soil and extraneous root tissues prior to root nodule lysis in 20mM potassium phosphate pH 7.4, 100mM sodium chloride, 1mM EDTA and 1mM ascorbic acid. Root nodules were lysed by grinding root-nodules using juicer blender. Insoluble material was separated by centrifugation. Root nodule lysate was fractionated using ammonium sulfate in two steps, first ammonium sulfate was added to 60% wt/v and solution incubated for 1hr, 4°C. Pellet was discarded and supernatant brought to 90% wt/v ammonium sulfate and incubated for 12hr, 4°C. Leghemoglobin was collected as a precipitated pellet in 90% ammonium sulfate fraction and resuspended in 20mM potassium phosphate, 1mM EDTA, 100mM sodium chloride. SDS-PAGE gel analysis determined that protein solution contains 70% leghemoglobin and 30% other root nodule proteins. Ammonium sulfate was removed using size-exclusion chromatography in the same buffer. Leghemoglobin was concentrated by dialyzing for 48hr against 30% PEG 8000 (polyethylene glycol, molecular weight 8000) in 20mM potassium phosphate pH 7.3, 100mM

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sodium chloride. Total protein concentration was 57mg/ml. UV-VIS spectra suggested that leghemoglobin was in heme-iron oxidized state. Thus, leghemoglobin was incubated with 5mM sodium hydrosulfite for 5min and sodium hydrosulfite was removed using size-exclusion chromatography in 20mM potassium phosphate, 100mM sodium chloride buffer. Leghemoglobin was further concentrated to 35.4 mg/ml. UV-VIS spectra analysis confirmed that leghemoglobin is in heme-iron reduced state.

[00399] Transglutaminase was obtained commercially from (Activa TI, Ajimoto). Stock solution (20% wt/v) was made in 20mM potassium phosphate pH 7.3, 100mM sodium chloride buffer.

[00400] To prepare “dark” muscle tissue analog (Figure 11), 43 ml of moong bean protein solution (150 mg/ml in dialysis buffer) were mixed with 37 ml of leghemoglobin solution (46.5 mg/ml leghemoglobin and 20mg/ml of other soybean root nodule protein) in 20 mM potassium phosphate, 100 mM NaCl, pH 7.3). 20 ml of transglutaminase solution (20% w/w) were added, solutions thoroughly mixed, divided into two 50 ml Falcon tubes and incubated overnight at room temperature. Final protein concentrations were 65 mg/ml for moong bean protein, 18 mg/ml of leghemoglobin, 91 mg/ml total protein.

[00401] “White” muscle analog (Figure 12) was prepared by mixing 43ml moong bean protein solution (150mg/ml) with 45ml of 11.7 mg/ml solution of leghemoglobin and 0.8 % (wt/v) of transglutaminase solution. Final protein concentrations were 63 mg/ml for moong bean protein, 5.2 mg/ml of leghemoglobin, 68 mg/ml total protein.

[00402] The “dark” muscle tissue analog formed an opaque gel of dark chocolate color, smooth uniform texture, with glistening surface, and a small amount (< 1 ml) of dark red, venous blood colored liquid on top. The gel was freely standing, elastic but fragile, similar in appearance to thin Jell-O. The gel has a medium aroma with notes of beans and blood clearly discernible. The flavor is dominated by notes of beans and iron/blood, with weaker grassy and medicinal/chemical flavors. The taste is salty, with a long aftertaste of blood.

[00403] The “white” muscle tissue analog was very similar, but with much lighter, cappuccino-like, color. It was also more fragile, 2-3-fold less strong against compression.

[00404] Fat Tissue Analog

[00405] Fat tissue analog using moong bean 8S globulin fraction was prepared as follows: 15 ml of moong bean protein solution (150 mg/ml in dialysis buffer) were mixed with 15 ml of rice bran oil. 6 ml of transglutaminase solution (20% w/w) were added, solutions thoroughly emulsified using a homogenizer (VWR) at speed #2. Emulsion was aliquoted into 1.6 eppendorf tubes and incubated overnight at room temperature. After that, tubes were heated at 95 °C for 5 min in a heat block, and allowed to cool down to room temperature on a bench. Final concentrations were 75 mg/ml for moong bean protein, 50% w/w oil.

[00406] Fat tissue analog using pea globulin (100 mg/ml) was prepared by the same method. Additionally, fat tissue analog was prepared from pea globulin, and either rice bran or canola oil, in bulk by the same method, but without aliquoting emulsions into eppendorf tubes. Instead, emulsions in 50 ml Falcon tubes were rotated overnight on a nutator, and were subsequently incubated at 90 °C for 30 min.

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[00407] Fat tissue analog based on moong beans (Figure 13) and prepared in eppendorf tubes formed an opaque gel of off-white color, smooth uniform texture, with no visible discernible liquid that was not incorporated into the gel. The gel was freely standing, elastic and springy. The gel has a slight, pleasant aroma and a mild and pleasant flavor. The taste is mildly salty.

[00408] Fat tissue analog based on pea globulin (Figure 14) and prepared in eppendorf tubes was very similar to moong bean-based fat analog, except that it gave up a little of oil upon compression.

Fat tissue analog prepared in 50 ml Falcon tubes were similar in appearance, texture and aromas, but substantially softer (2-fold softer for canola oil, and 3-fold softer for rice bran oil, according to compressibility measurements).

[00409] Connective Tissue Analog

Connective tissue analog prototypes were developed using zein protein sourced from 100% yellow corn gluten meal, or from commercial sources, such as Amazein (Prairie Gold, Bloomington, IL). Zein proteins were solubilized in 70 - 90% ethanol with desired ratios at 1:3 to 1:5 (solids:solution). By precipitating zein proteins, for example by a change in pH, in a controlled manner, large zein structures result with physicochemical properties that can be manipulated as desired. For example, Figure 15 shows connective-tissue analog strands that were created using a 1:3 ratio in 70% ethanol, loaded into a syringe with a 23 gauge needle (ID 0.337 mm). The solution was slowly extruded from the bottom of a 5 inch-high vessel into an excess of 5 M NaCl solution. The ethanol-zein solution being less dense than the NaCl solution, floated upward, drawing out a fibrous stand of solidifying zein. The NaCl was constantly stirred as the strands began to develop to assist in the strand lengthening. The strands bunch together and become a hard, dense mass.

[00410] Ground beef replica prototypes made from gels of plant proteins and plant oils.

[00411] A ground beef prototype patty was made by combining 62% (wt/wt) muscle analog (62% (wt/wt) “dark muscle analog” and 38% (wt/wt) “white muscle analog”), 29% (wt/wt) fat tissue analog (from pea globulin and canola oil), 5% (wt/wt) connective tissue analog (Figure 16 panel A). A ground beef prototype patty was made by combining 62% muscle analog (62% “dark muscle analog” and 38% “white muscle analog), 29% fat tissue analog (from moong bean seed 8S protein and rice bran oil) , 5% connective tissue analog (Figure 16 panel B). A ground beef prototype patty was made by combining 71 % (wt/wt) muscle tissue analog (composed of 60% “white” muscle analog, 40 % “dark” muscle analog) , 23% fat tissue (from pea seed globulin proteins and canola oil) (Figure 16 panel C). A ground beef prototype patty was made by combining 67% “White” muscle analog, with 28% fat tissue analog (from pea globulins and rice bran oil), (Figure 16, panel D)

[00412] In a further test, the effect of cooking the ground beef replica patties was evaluated by grilling on a 350°F pan. A ground beef patty analog was made by combining 62% (wt/wt) muscle tissue analog (62% (wt/wt) “dark muscle analog” and 38% (wt/wt) “muscle analog”), 29% (wt/wt) fat tissue analog (from pea globulin and canola oil), 5% (wt/wt) connective tissue analog (Figure 17). The panel on the

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left shows the patty before cooking and the panel on the right shows the same patty after cooking for about 2 minutes. Observers described the aroma of the cooking ground beef replica as distinctly “beefy”.

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CLAIMS

WHAT IS CLAIMED IS:

1. A meat substitute composition comprising a protein content, wherein one or more isolated and purified proteins accounts for 10% or more of said protein content by weight, wherein said meat substitute composition accurately mimics the taste, texture, or color of a meat product derived from animal sources.
2. The meat substitute composition of claim 1, wherein said composition accurately mimics the color of said meat product in its raw state and in a cooked state after cooking.
3. The meat substitute composition of claim 1, wherein said one or more isolated and purified proteins accounts for 25% or more of said protein content by weight.
4. The meat substitute composition of claim 3, wherein said one or more isolated and purified proteins accounts for 50% or more of said protein content by weight.
5. The meat substitute composition of claim 4, wherein said one or more isolated and purified proteins accounts for 75% or more of said protein content by weight.
6. The meat substitute composition of claim 5, wherein said one or more isolated and purified proteins accounts for 90% or more of said protein content by weight.
7. The meat substitute composition of claim 1, wherein gluten does not account for 10% or more of said protein content by weight.
8. The meat substitute composition of claim 1, wherein each of said isolated, purified proteins is separately isolated and purified.
9. The meat substitute composition of claim 1, comprising 1-7 isolated and purified proteins.
10. The meat substitute composition of claim 9, wherein said 1-7 isolated and purified proteins are each isolated from different plant species.
11. The meat substitute composition of claim 10, wherein said protein content comprises no more than trace amounts of any other proteins derived from the different plant species.
12. The meat substitute composition of any claim 1, wherein said one or more isolated and purified proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose bisphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or

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other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin.

13. The meat substitute composition of claim 1, wherein said one or more isolated and purified proteins are not isolated from an animal.
14. The meat substitute composition of claim 13, wherein said one or more isolated and purified proteins are isolated from a single plant source.
15. The meat substitute composition of claim 14, wherein said one or more isolated and purified proteins are isolated from multiple plant sources.
16. The meat substitute composition of claim 13, wherein said one or more isolated, purified proteins are isolated from a genetically modified organism.
17. The meat substitute composition of claim 16, wherein said genetically modified organism is a genetically modified bacteria or yeast organism.
18. The meat substitute composition of any of the preceding claims, wherein said isolated, purified protein has been formed into fibers.
19. The meat substitute composition of claim 18, wherein said fibers resemble skeletal muscle fibers.
20. The meat substitute composition of claim 19, wherein said fibers are asymmetric fibers.
21. The meat substitute composition of any of the preceding claims, further comprising one or more isolated and purified iron-containing proteins.
22. The meat substitute composition of claim 21, wherein said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, and flavohemoglobins.
23. The meat substitute composition of claim 22, wherein said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1.
24. The meat substitute composition of any of the preceding claims, wherein said isolated and purified proteins are assembled into one or more gels.
25. The meat substitute composition of any of the preceding claims, further comprising one or more fats.
26. The meat substitute composition of claim 25, wherein said one or more fats are derived from a plant source.
27. A meat substitute product that comprises an indicator that indicates cooking progression from a raw state to a cooked state, wherein said meat substitute product is derived from non-animal sources.
28. The meat substitute product of claim 27, wherein said indicator is a visual indicator that accurately mimics the color transition of a meat product during said cooking progression.
29. The meat substitute product of claim 28, wherein said color transition is from red to brown.

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30. The meat substitute product of claim 28, wherein said color transition is from pink to white or tan.
31. The meat substitute product of claim 28, wherein said visual indicator transitions from a translucent to opaque color during said cooking progression.
32. A meat substitute product of claim 27 wherein the indicator is an olfactory indicator that indicates cooking progression.
33. The meat substitute product of claim 32, wherein said olfactory indicator is one or more volatile odorants released during cooking.
34. The meat substitute product of any of claims 32, wherein said indicator comprises one or more isolated, purified iron-containing proteins.
35. The meat substitute product of claim 34, wherein said one or more isolated, purified iron-containing proteins is in a reduced state before cooking.
36. The meat substitute product of 34, wherein said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocruorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, and flavohemoglobins.
37. The meat substitute product of 36, wherein said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1.
38. The meat substitute product of claim 34, wherein said one or more isolated and purified iron-containing proteins are not isolated from an animal.
39. The meat substitute product of claim 34, wherein said one or more isolated and purified iron-containing proteins are isolated from one or more plant sources.
40. The meat substitute product of claim 39, wherein said one or more isolated, purified proteins are isolated from the root nodules, roots, seeds, leaves, or stems of said one or more plant sources.
41. The meat substitute product of claim 40, wherein said one or more plant sources are soy or pea plants.
42. The meat substitute composition of claim 39, wherein said one or more plant sources comprises one or more plants of the legume family.
43. The meat substitute product of claim 38, wherein said one or more isolated and purified iron carrying proteins in a reduced or oxidized state has a similar UV-VIS profile to a myoglobin protein derived from an animal source when in an equivalent reduced or oxidized state.
44. The meat substitute product of claim 43, wherein the difference between the peak absorbance wavelength of said one or more isolated and purified iron-containing proteins and the peak absorbance wavelength of myoglobin derived from an animal source is less than 5%.
45. The meat substitute product of claim 38, wherein said one or more isolated, purified proteins are isolated from a genetically modified organism.

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46. The meat substitute product of claim 45, wherein said genetically modified organism is a genetically modified bacteria or yeast organism.
47. The meat substitute product of any of claims 1-46 wherein said meat substitute product contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.
48. The meat substitute product of claim 47, wherein said meat substitute product contains less than 1% wheat gluten.
49. The meat substitute product of claim 48, wherein said meat substitute product contains no wheat gluten.
50. The meat substitute product of claim 49, wherein said meat substitute product contains no soy protein isolate.
51. The meat substitute product of claim 49, wherein said meat substitute product contains no soy protein concentrate.
52. The meat substitute product of claim 49, wherein said meat substitute product contains no soy protein.
53. The meat substitute product of claim 49, wherein said meat substitute product contains less than 5% carbohydrates.
54. The meat substitute product of claim 49, wherein said meat substitute product contains no tofu.
55. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no tofu, and no wheat gluten.
56. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no soy protein, and no wheat gluten.
57. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no animal products and less than 5% carbohydrates.
58. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains less than 1% cellulose.
59. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains less than 5% insoluble carbohydrates.
60. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no soy protein, and less than 1% cellulose.
61. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no soy protein, and less than 5% insoluble carbohydrates.
62. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no wheat gluten, and less than 1% cellulose.
63. The meat substitute product of any of claims 1-46, wherein said meat substitute product contains no wheat gluten, and less than 5% insoluble carbohydrates.

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64. A muscle tissue replica comprising a protein content, wherein said protein content comprises one or more isolated and purified proteins, wherein said muscle tissue replica approximates the taste, texture, or color of an equivalent muscle tissue derived from an animal source.
65. The muscle tissue replica of claim 64, wherein said one or more isolated and purified proteins accounts for at least 50% of said protein content by weight.
66. The muscle tissue replica of claim 64, wherein said protein content is derived from one or more non-animal sources.
67. The muscle tissue replica of claim 66, wherein said one or more non-animal sources is a plant source.
68. The muscle tissue replica of claim 66, wherein said one or more non-animal sources is a genetically modified yeast or bacteria.
69. The muscle tissue replica of claim 64, wherein each of said one or more isolated proteins is isolated and purified separately.
70. The muscle tissue replica of any of the preceding claims, wherein said one or more isolated proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocruorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamins, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, and moong seed storage 8S globulin.
71. The muscle tissue replica of claim 70, wherein said seed storage protein is moong bean 8S protein.
72. The muscle tissue replica of claim 64, wherein said protein content is suspended in a gel.
73. The muscle tissue replica of claim 64, wherein said protein content is in the form of a gel.
74. The muscle tissue replica of claim 72 or 73, wherein said gel comprises an isolated, purified cross-linking enzyme.
75. The muscle tissue replica of claim 74, wherein said isolated, purified cross-linking enzyme is selected from the group consisting of transglutaminase, lysyl oxidases, and amine oxidases.
76. The muscle tissue replica of claim 75, wherein said isolated, purified cross-linking enzyme is transglutaminase.

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77. The muscle tissue replica of claim 64, wherein said protein content has been assembled into fibers.
78. The muscle tissue replica of claim 77, wherein said fibers are arranged isotropically.
79. The muscle tissue replica of claim 78, wherein said fibers are asymmetric fibers.
80. The muscle tissue replica of claim 64, further comprising one or more isolated and purified iron-containing proteins.
81. The muscle tissue replica of claim 80, wherein said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins.
82. The muscle tissue replica of claim 80, wherein said one or more isolated and purified iron-containing proteins comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1.
83. The muscle tissue replica of claim 64, comprising a protein content, wherein (i) one isolated and purified protein that is not an iron-containing protein accounts for 40-95% of said protein content, (ii) one or more isolated and purified iron-containing proteins accounts for 1-20% of said protein content, and (iii) one or more cross-linking reagents accounts for 0.1-35% of said protein content.
84. The muscle tissue replica of claim 64, wherein said protein content accounts for 5-50% of said replica by weight or by weight/volume.
85. The muscle tissue replica of claim 83, wherein said one isolated and purified protein is moong bean 8S protein.
86. The muscle tissue replica of claim 83, wherein said one or more isolated and purified iron-containing proteins is leghemoglobin.
87. The muscle tissue replica of claim 83, wherein said one or more cross-linking reagents is transglutaminase.
88. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.
89. The muscle tissue replica of claim 88, wherein said muscle tissue replica contains less than 1% wheat gluten.
90. The muscle tissue replica of claim 89, wherein said muscle tissue replica contains no wheat gluten.
91. The muscle tissue replica of claim 90, wherein said muscle tissue replica contains no soy protein isolate.
92. The muscle tissue replica of claim 90, wherein said muscle tissue replica contains no soy protein concentrate.
93. The muscle tissue replica of claim 90, wherein said muscle tissue replica contains no soy protein.

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94. The muscle tissue replica of claim 93, wherein said muscle tissue replica contains less than 5% carbohydrates.
95. The muscle tissue replica of claim 90, wherein said muscle tissue replica contains no tofu.
96. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no tofu, and no wheat gluten.
97. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no soy protein, and no wheat gluten.
98. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no animal products and less than 5% carbohydrates.
99. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains less than 1% cellulose.
100. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains less than 5% insoluble carbohydrates.
101. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no soy protein, and less than 1% cellulose.
102. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no soy protein, and less than 5% insoluble carbohydrates.
103. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no wheat gluten, and less than 1% cellulose.
104. The muscle tissue replica of any of claims 64-87, wherein said muscle tissue replica contains no wheat gluten, and less than 5% insoluble carbohydrates.
105. A fat tissue replica comprising a gelled emulsion, said gelled emulsion comprising a protein solution with fat droplets suspended therein.
106. The fat tissue replica of claim 105, wherein said fat droplets are derived from a non-animal source.
107. The fat tissue replica of claim 105, wherein said fat droplets are comprised of one or more plant oils.
108. The fat tissue replica of claim 107, wherein said one or more plant oils is selected from the group consisting of corn oil, olive oil, soy oil, peanut oil, walnut oil, almond oil, sesame oil, cottonseed oil, rapeseed oil, canola oil, safflower oil, sunflower oil, flax seed oil, algal oil, palm oil, palm kernel oil, coconut oil, babassu oil, shea butter, mango butter, cocoa butter, wheat germ oil, rice bran oil, oils produced by bacteria, algae, archaea or fungi or genetically engineered bacteria, algae, archaea or fungi, triglycerides, monoglycerides, diglycerides, sphingosides, glycolipids, lecithin, lysolecithin, phosphatidic acids, lysophosphatidic acids, oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, conjugated oleic acid, or esters of: oleic acid, palmitoleic acid, palmitic acid, myristic acid,

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lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or glycerol esters of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid, or triglyceride derivatives of oleic acid, palmitoleic acid, palmitic acid, myristic acid, lauric acid, myristoleic acid, caproic acid, capric acid, caprylic acid, pelargonic acid, undecanoic acid, linoleic acid, 20:1 eicosanoic acid, arachidonic acid, eicosapentanoic acid, docosohexanoic acid, 18:2 conjugated linoleic acid, or conjugated oleic acid.

109. The fat tissue replica of claim 108, wherein said one or more plant oils is rice bran oil or canola oil.
110. The fat tissue replica of claim 105, wherein said protein solution comprises one or more isolated, purified proteins.
111. The fat tissue replica of claim 110, wherein said one or more isolated, purified proteins accounts for 75% or more of the protein in said protein solution.
112. The fat tissue replica of claim 112, wherein said one or more isolated, purified proteins are derived from a non-animal source.
113. The fat tissue replica of claim 112 wherein said non-animal source is a plant source.
114. The fat tissue replica of claim 110, wherein said non-animal source is a genetically modified yeast or bacteria.
115. The fat tissue replica of claim 110, wherein each of said one or more isolated proteins is isolated and purified separately.
116. The fat tissue replica of claim 110 wherein said one or more isolated proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocruorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose bisphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin.

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117. The fat tissue replica of claim 116 wherein said one or more isolated, purified proteins is an albumin protein, a seed storage protein, or pea globulin protein.
118. The fat tissue replica of claim 116 wherein said albumin protein is isolated pea albumin protein.
119. The fat tissue replica of claim 116, wherein said seed storage protein is moong bean 8S protein.
120. The fat tissue replica of claim 105, wherein said gelled emulsion comprises
 - a) a protein solution comprising 1-3 isolated and purified proteins, wherein said solution accounts for 30-70% of the volume of said emulsion;
 - b) a plant oil, wherein said plant oil accounts for 30-70% of the volume of said emulsion; and
 - c) an isolated, purified cross-linking enzyme, wherein said cross-linking enzyme accounts for 0.0001-1% of said emulsion by wt/volume;wherein said plant oil is emulsified in said protein solution, wherein said emulsion is formed into a gel by said cross-linking enzyme.
121. The fat tissue replica of claim 105, wherein said gelled emulsion comprises
 - a) a protein solution comprising 1-3 isolated and purified proteins, wherein said solution accounts for 1-30% of the volume of said emulsion;
 - b) a plant oil, wherein said plant oil accounts for 70-99% of the volume of said emulsion; and
 - c) an isolated, purified cross-linking enzyme, wherein said cross-linking enzyme accounts for 0.5-0.0001-1% of said emulsion by wt/volume;wherein said plant oil is emulsified in said protein solution, wherein said emulsion is formed into a gel by said cross-linking enzyme.
122. The fat tissue replica of claim 105, further comprising a cross-linking enzyme.
123. The fat tissue replica of claim 122, wherein said cross-linking enzyme is transglutaminase.
124. The fat tissue replica of claim 120 or 121, wherein one of said 1-3 isolated and purified proteins is moong bean 8S protein, pea albumin protein, or pea globulin protein.
125. The fat tissue replica of claim 120 or 121, wherein said plant oil is rice bran oil or canola oil.
126. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.
127. The fat tissue replica of claim 126, wherein said fat tissue replica contains less than 1% wheat gluten.
128. The fat tissue replica of claim 127, wherein said fat tissue replica contains no wheat gluten.
129. The fat tissue replica of claim 128, wherein said fat tissue replica contains no soy protein isolate.
130. The fat tissue replica of claim 128, wherein said fat tissue replica contains no soy protein concentrate.
131. The fat tissue replica of claim 128, wherein said fat tissue replica contains no soy protein.
132. The fat tissue replica of claim 131, wherein said fat tissue replica contains less than 5% carbohydrates.
133. The fat tissue replica of claim 128, wherein said fat tissue replica contains no tofu.

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134. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no tofu, and no wheat gluten.
135. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no soy protein, and no wheat gluten.
136. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no animal products and less than 5% carbohydrates.
137. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains less than 1% cellulose.
138. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains less than 5% insoluble carbohydrates.
139. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no soy protein, and less than 1% cellulose.
140. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no soy protein, and less than 5% insoluble carbohydrates.
141. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no wheat gluten, and less than 1% cellulose.
142. The fat tissue replica of any of claims 105-123, wherein said fat tissue replica contains no wheat gluten, and less than 5% insoluble carbohydrates.
143. A connective tissue replica, comprising a protein content comprising one or more isolated, purified proteins, wherein said protein content has been assembled into structures approximating the texture and visual appearance of connective tissue or skin.
144. The connective tissue replica of claim 143, wherein said protein content is derived from non-animal source.
145. The connective tissue replica of claim 143, wherein said non-animal source is a plant source.
146. The connective tissue replica of claim 143, wherein said non-animal source is a genetically modified yeast or bacteria.
147. The connective tissue replica of claim 143, wherein said one or more isolated proteins account for 50% or more of said protein content by weight.
148. The connective tissue replica of claim 143, wherein said one isolated and purified protein accounts for 90% or more of said protein content by weight.
149. The connective tissue replica of claim 143, wherein each of said one or more isolated proteins is isolated and purified separately.
150. The connective tissue replica of claim 143, wherein said one or more isolated proteins are selected from the group consisting of hemoglobin, myoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate

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mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamin, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin.

151. The connective tissue replica of claim 150, wherein said one or more isolated and purified proteins is a prolamin family protein.
152. The connective tissue replica of claim 150, wherein said one or more isolated and purified proteins is zein.
153. The connective tissue replica of claim 143, wherein said protein content is suspended in a gel.
154. The connective tissue replica of claim 153, wherein said gel comprises an isolated, purified cross-linking enzyme.
155. The connective tissue replica of claim 154, wherein said isolated, purified cross-linking enzyme is selected from the group consisting of transglutaminase, lysyl oxidases, and amine oxidases.
156. The connective tissue replica of claim 155, wherein said isolated, purified cross-linking enzyme is transglutaminase.
157. The connective tissue replica of claim 143, wherein said protein content is formed into a fiber.
158. The connective tissue replica of claim 157, wherein said fiber is produced by an extrusion process.
159. The connective tissue replica of claim 157, wherein said fiber is stabilized by protein crosslinks.
160. The connective tissue replica of claim 157, wherein said fiber contains an isolated, purified cross-linking enzyme.
161. The connective tissue replica of claim 160, wherein said isolated, purified cross-linking enzyme is selected from the group consisting of transglutaminase, lysyl oxidases, and amine oxidases.
162. The connective tissue replica of claim 161, wherein said isolated, purified cross-linking enzyme is transglutaminase.
163. A meat substitute product, comprising:
 - a) a muscle replica;
 - b) a fat tissue replica; and
 - c) a connective tissue replica;wherein said muscle replica, fat tissue replica, and/or connective tissue replica are assembled in a manner that approximates the physical organization of meat.
164. The meat substitute product of claim 163, comprising two or more of said muscle replica, fat tissue replica, and connective tissue replica.

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165. The meat substitute product of claim 163, wherein (i) said muscle replica accounts for 40-90% of said product by weight, (ii) said fat tissue replica accounts for 1-60% of said product by weight, and (iii) said connective tissue replica accounts for 1-30% of said product by weight.
166. The meat substitute product of claim 163, comprising:
 - a) 60-90% water;
 - b) 5-30% protein content;
 - c) 1-20% of a fat or fat replica;wherein said protein content comprises one or more isolated, purified plant proteins.
167. The meat substitute product of claim 166, wherein said protein content is derived from non-animal source.
168. The meat substitute product of claim 167, wherein said non-animal source is a plant source.
169. The meat substitute product of claim 167, wherein said non-animal source is a genetically modified yeast or bacteria.
170. The meat substitute product of claim 163, wherein 50% or more of said protein content by weight are one or more isolated purified proteins.
171. The meat substitute product of claim 170, wherein each of said one or more isolated proteins is isolated and purified separately from different plant species.
172. The meat substitute product of claim 170, wherein one or more of said isolated proteins is selected from the group consisting of: hemoglobin, myoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins, ribosomal proteins, actin, hexokinase, lactate dehydrogenase, fructose biphosphate aldolase, phosphofructokinases, triose phosphate isomerases, phosphoglycerate kinases, phosphoglycerate mutases, enolases, pyruvate kinases, glyceraldehyde-3-phosphate dehydrogenases, pyruvate decarboxylases, actins, translation elongation factors, ribulose-1,5-bisphosphate carboxylase oxygenase (rubisco), ribulose-1,5-bisphosphate carboxylase oxygenase activase (rubisco activase), albumins, glycinins, conglycinins, globulins, vicilins, conalbumin, gliadin, glutelin, gluten, glutenin, hordein, prolamins, phaseolin (protein), proteinoplast, secalin, extensins, triticeae gluten, zein, any seed storage protein, oleosins, caloleosins, steroleosins or other oil body proteins, vegetative storage protein A, vegetative storage protein B, moong seed storage 8S globulin.
173. The meat substitute product of claim 163, further comprising one or more isolated and purified iron-containing proteins.
174. The meat substitute product of claim 173, wherein said one or more isolated and purified iron-containing proteins is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrurorin, neuroglobin, cytoglobin, protoglobin, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, and flavohemoglobins.

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175. The meat substitute composition of claim 174 wherein said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1.
176. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum.
177. The meat substitute product of claim 176, wherein said meat substitute product contains less than 1% wheat gluten.
178. The meat substitute product of claim 177, wherein said meat substitute product contains no wheat gluten.
179. The meat substitute product of claim 178, wherein said meat substitute product contains no soy protein isolate.
180. The meat substitute product of claim 178, wherein said meat substitute product contains no soy protein concentrate.
181. The meat substitute product of claim 178, wherein said meat substitute product contains no soy protein.
182. The meat substitute product of claim 181, wherein said meat substitute product contains less than 5% carbohydrates.
183. The meat substitute product of claim 178, wherein said meat substitute product contains no tofu.
184. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no tofu, and no wheat gluten.
185. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no soy protein, and no wheat gluten.
186. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no animal products and less than 5% carbohydrates.
187. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains less than 1% cellulose.
188. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains less than 5% insoluble carbohydrates.
189. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no soy protein, and less than 1% cellulose.
190. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no soy protein, and less than 5% insoluble carbohydrates.
191. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no wheat gluten, and less than 1% cellulose.
192. The meat substitute product of any of claims 163-175, wherein said meat substitute product contains no wheat gluten, and less than 5% insoluble carbohydrates.
193. A food product comprising one or more isolated, purified iron-containing proteins, wherein said food product is configured for consumption by an animal.

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194. The food product of claim 193, wherein said one or more isolated, purified iron-containing proteins is derived from a non-animal source.
195. The food product of claim 194, wherein said non-animal source is a plant source.
196. The food product of claim 195, wherein said plant source comprises one or more plants of the legume family.
197. The meat substitute product of claim 196, wherein said one or more plants of the legume family is a soy or pea plant.
198. The food product of claim 194, wherein said non-animal source is a genetically modified yeast or bacteria.
199. The food product of claim 193, wherein said iron-containing protein is selected from the group consisting of hemoglobin, myoglobin, leghemoglobin, non-symbiotic hemoglobin, chlorocruorin, erythrocrucorin, neuroglobin, cytoglobin, protoglobins, truncated 2/2 globin, HbN, cyanoglobin, HbO, Glb3, and cytochromes, Hell's gate globin I, bacterial hemoglobins, ciliate myoglobins, flavohemoglobins.
200. The food product of claim 193, wherein said iron-containing protein comprises an amino acid sequence with at least 70% homology to SEQ ID NO 1.
201. The food product of any of claims 193-200, wherein said product contains no methylcellulose, no carrageenan, no caramel color, no konjac flour, no gum arabic, and no acacia gum
202. The food product of claim 201, wherein said food product contains less than 1% wheat gluten.
203. The food product of claim 202, wherein said food product contains no wheat gluten.
204. The food product of claim 203, wherein said food product contains no soy protein isolate.
205. The food product of claim 203, wherein said food product contains no soy protein concentrate.
206. The food product of claim 203, wherein said food product contains no soy protein.
207. The food product of claim 206, wherein said food product contains less than 5% carbohydrates.
208. The food product of claim 203, wherein said food product contains no tofu.
209. The food product of any of claims 193-200, wherein said food product contains no tofu, and no wheat gluten.
210. The food product of any of claims 193-200, wherein said food product contains no soy protein, and no wheat gluten.
211. The food product of any of claims 193-200, wherein said food product contains no animal products and less than 5% carbohydrates.
212. The food product of any of claims 193-200, wherein said food product contains less than 1% cellulose.
213. The food product of any of claims 193-200, wherein said food product contains less than 5% insoluble carbohydrates.
214. The food product of any of claims 193-200, wherein said food product contains no soy protein, and less than 1% cellulose.

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215. The food product of any of claims 193-200, wherein said food product contains no soy protein, and less than 5% insoluble carbohydrates.
216. The food product of any of claims 193-200, wherein said food product contains no wheat gluten, and less than 1% cellulose.
217. The food product of any of claims 193-200, wherein said food product contains no wheat gluten, and less than 5% insoluble carbohydrates.
218. A method of making a meat substitute composition, comprising:
- a) isolating and purifying one or more proteins; and
 - b) assembling said one or more proteins into a physical organization that approximates the physical organization of meat.
219. A method of making a muscle tissue replica, comprising:
- a) isolating and purifying one or more proteins; and
 - b) assembling said one or more proteins into a physical organization that approximates the physical organization of skeletal muscle.
220. A method of making a fat tissue replica, comprising:
- a) isolating and purifying one or more proteins;
 - b) preparing a solution comprising one or more proteins;
 - c) emulsifying one or more fats in said solution; and
 - d) stabilizing said solution into a gelled emulsification with one or more cross-linking reagents.
221. A method of making a connective tissue replica comprising:
- a) isolating and purifying one or more proteins; and
 - b) precipitating said one or more proteins,
- wherein said precipitating results in said one or more proteins forming physical structures approximating the physical organization of connective tissue.
222. The method of claim 221, wherein said precipitating comprises
- a) solubilizing said one or more proteins in a first solution; and
 - b) extruding said first solution into a second solution,
 - c) wherein said one or more proteins is insoluble in said second solution, wherein said extruding induces precipitation of said one or more proteins.

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Figure 1

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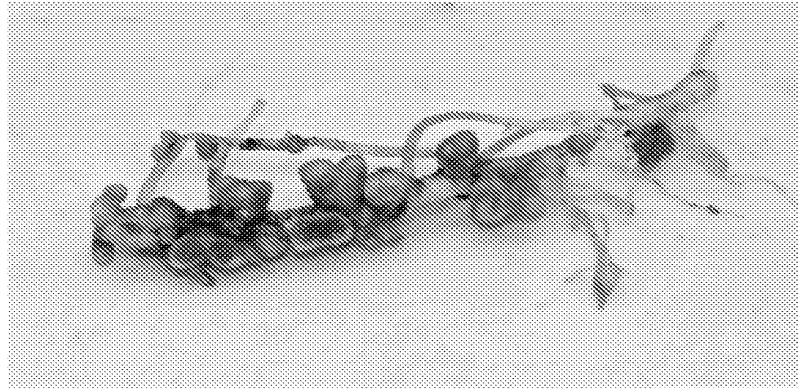


Figure 2.

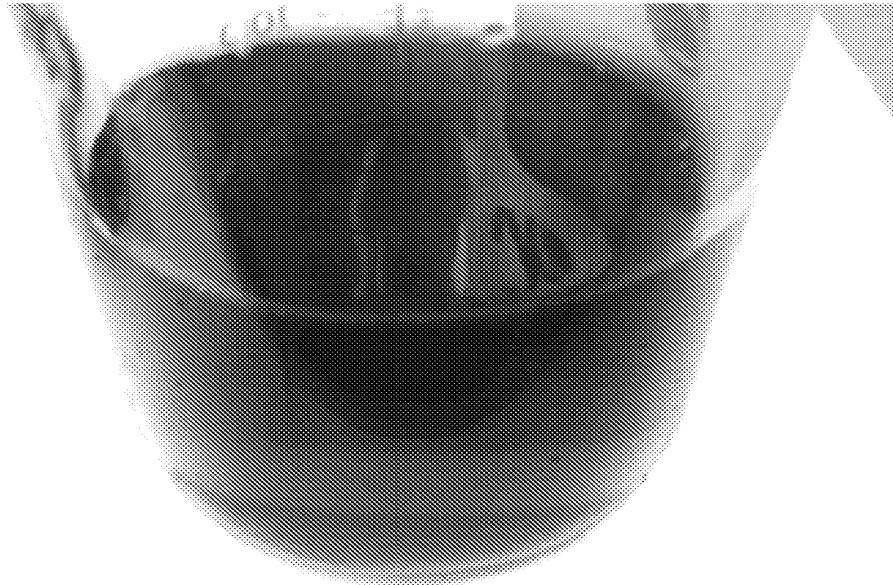


Figure 3.

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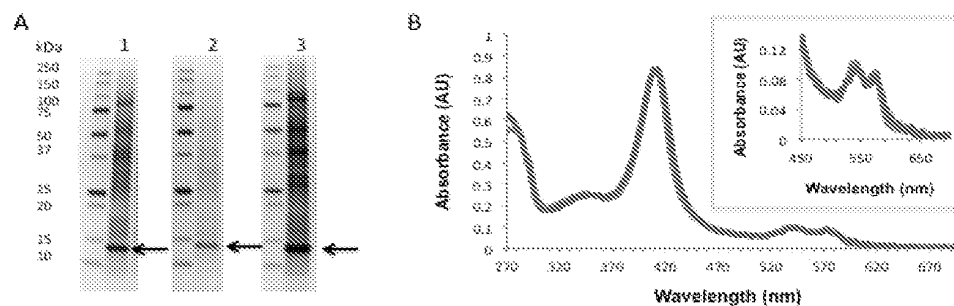


Figure 4

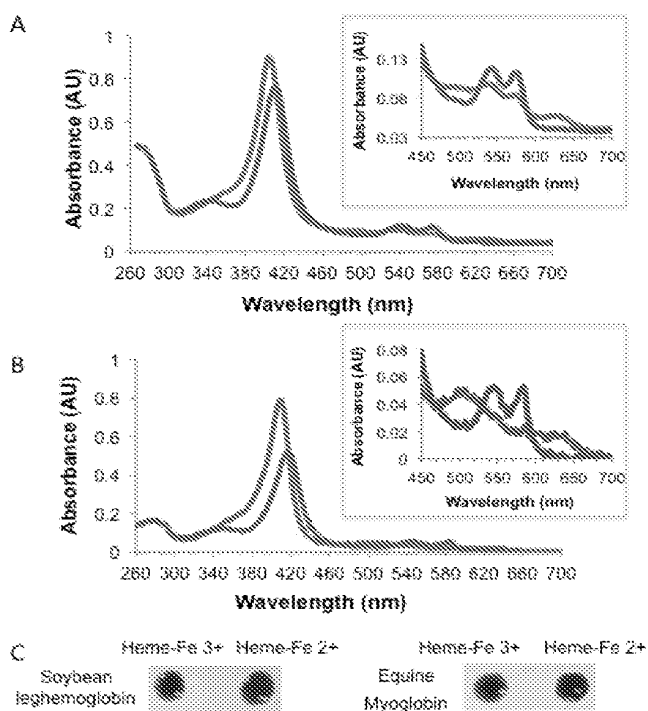


Figure 5

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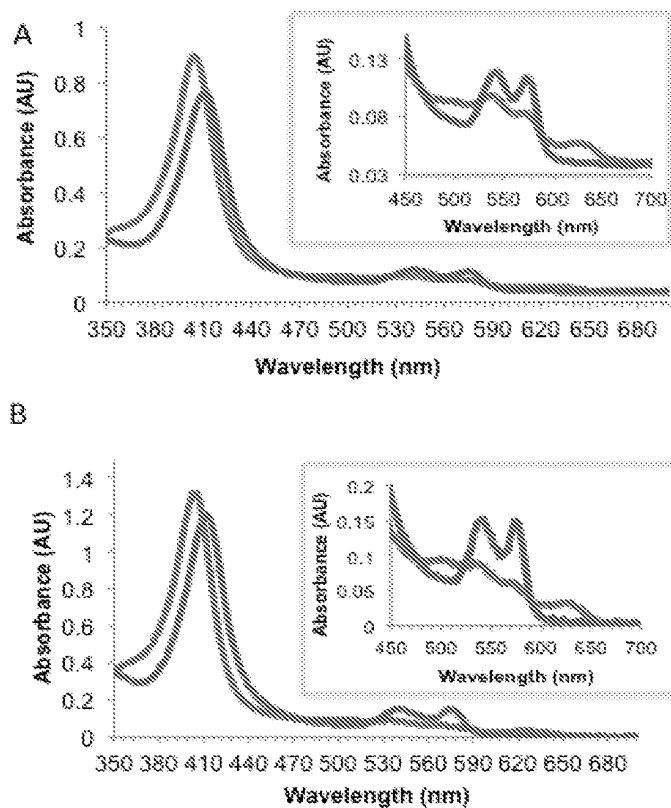


Figure 6

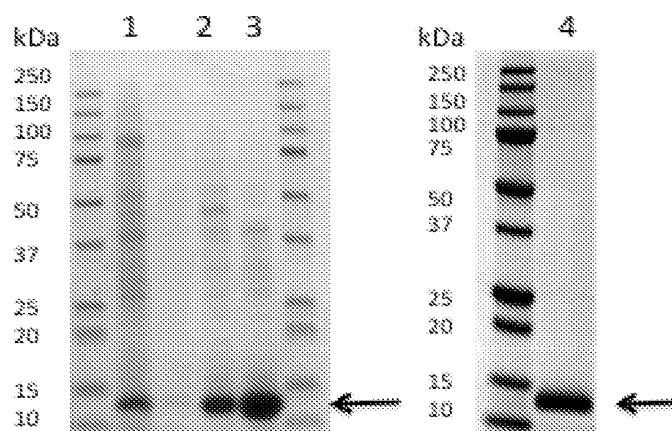


Figure 7

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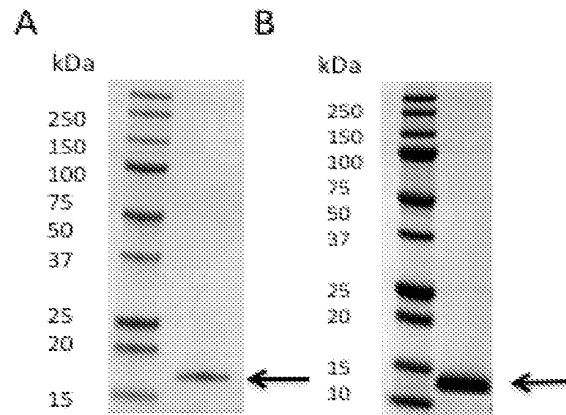


Figure 8

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Figure 9

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Figure 10

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Figure 11

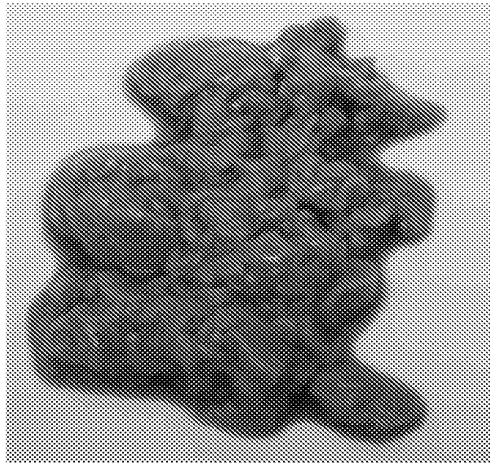


Figure 12

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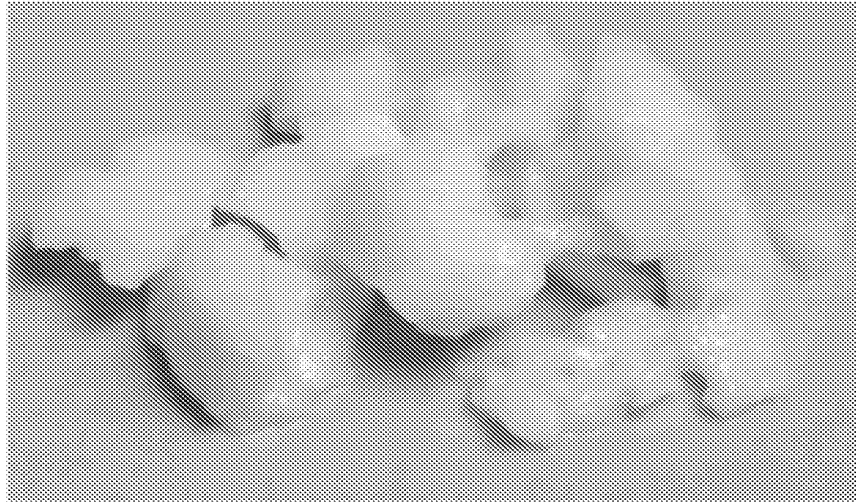


Figure 13

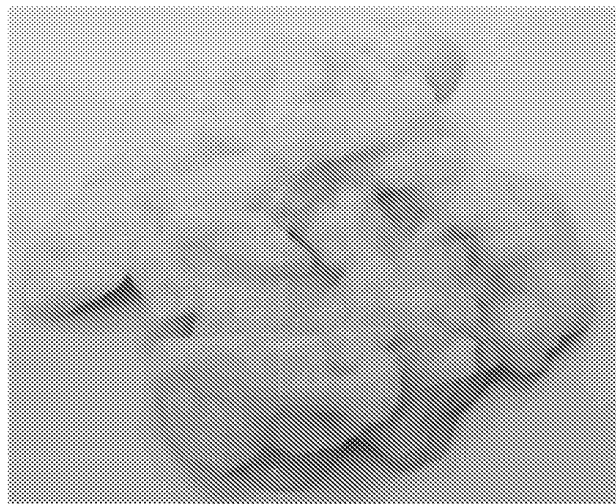


Figure 14

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Figure 15

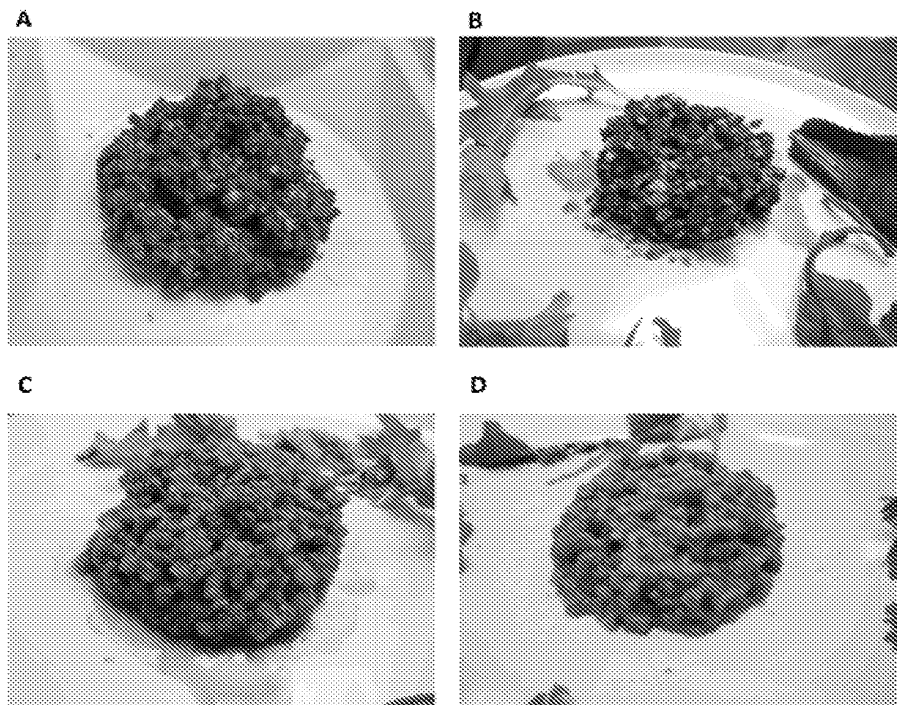


Figure 16

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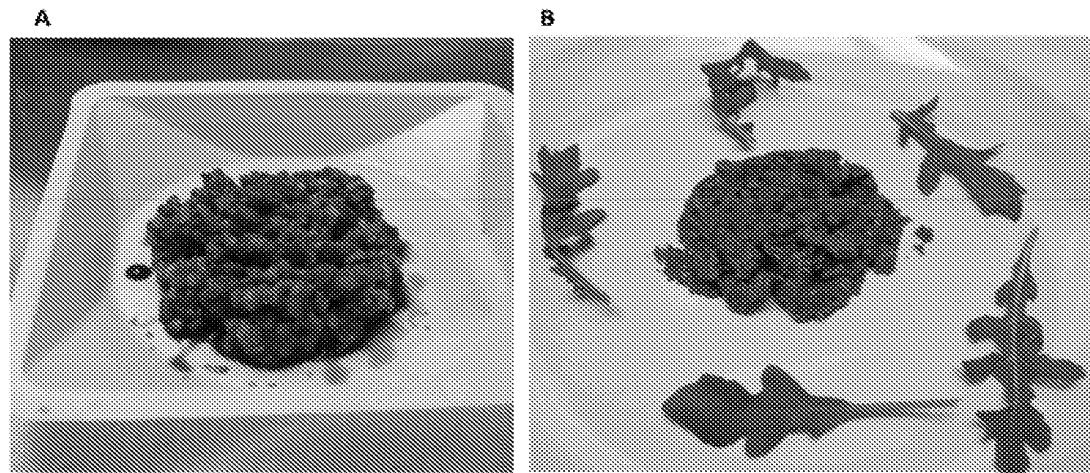


Figure 17

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/46560

A. CLASSIFICATION OF SUBJECT MATTER IPC(8) - A23J 1/00 (2012.01) USPC - 426/656 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) USPC: 426/656 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched USPC: 426/656, 574, 573, 531, 615 (keyword limited; terms below) Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) PatBase; Google; PubMed Search terms: meat, substitute, isolated, purified, protein, gluten, raw, cook, recombinant, yeast												
C. DOCUMENTS CONSIDERED TO BE RELEVANT												
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.										
X	US 2006/0035003 A1 (MCMINDES et al.) 16 February 2006 (16.02.2006) para [0004], [0033], [0039], [0047], [0050], [0052]-[0054], [0058], [0065], [0110], [0118]	1-20										
Y	US 2007/0269571 A1 (AKITA et al.) 22 November 2007 (22.11.2007) entire document.	1-20										
Y	US 2010/0074998 A1 (ESPELETA VEGA et al.) 25 March 2010 (25.03.2010) entire document.	1-20										
Y	US 2004/0161513 A1 (AKASHE et al.) 19 August 2004 (19.08.2004) entire document.	1-20										
Y	US 2011/0008502 A1 (HOSOMI et al.) 13 January 2011 (13.01.2011) entire document.	1-20										
☐ Further documents are listed in the continuation of Box C. ☐												
<p>* Special categories of cited documents:</p> <table border="0"><tr><td>"A" document defining the general state of the art which is not considered to be of particular relevance</td><td>"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</td></tr><tr><td>"E" earlier application or patent but published on or after the international filing date</td><td>"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</td></tr><tr><td>"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)</td><td>"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</td></tr><tr><td>"O" document referring to an oral disclosure, use, exhibition or other means</td><td>"&" document member of the same patent family</td></tr><tr><td>"P" document published prior to the international filing date but later than the priority date claimed</td><td></td></tr></table>			"A" document defining the general state of the art which is not considered to be of particular relevance	"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	"E" earlier application or patent but published on or after the international filing date	"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	"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)	"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	"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family	"P" document published prior to the international filing date but later than the priority date claimed	
"A" document defining the general state of the art which is not considered to be of particular relevance	"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											
"E" earlier application or patent but published on or after the international filing date	"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											
"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)	"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											
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family											
"P" document published prior to the international filing date but later than the priority date claimed												
Date of the actual completion of the international search 05 December 2012 (05.12.2012)		Date of mailing of the international search report 14 DEC 2012										
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201		Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774										

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 12/46560

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.: 21-26, 47-63, 70-71, and 88-104
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:
This application contains the following inventions or groups of inventions which are not so linked to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I claims 1-20 directed to a meat substitute composition comprising a protein content.

Group II claims 27-46 directed to a meat substitute product that comprises an indicator that indicates cooking progression from a raw state to a cooked state, wherein said meat substitute product is derived from non-animal sources.

Group III claims 64-69 and 72-87 directed to a muscle tissue replica.

Group IV claims 105-142 directed to a fat tissue replica.

----- (Continued on extra sheet) -----

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-20

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/US 12/46560

Continuation of Box III - observations where unity of invention is lacking:

Group V claims 143-162 directed to a connective tissue replica.

Group VI claims 163-192 directed to a meat substitute product.

Group VII claims 193-217 directed to a food product comprising one or more isolated, purified iron-containing proteins.

Group VIII claim 218 directed to a method of making a meat substitute composition.

Group IX claim 219 directed to a method of making a muscle tissue replica.

Group X claim 220 directed to a method of making a fat tissue replica.

Group XI claims 221 and 222 directed to a method of making a connective tissue replica.

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

The special technical features of the Groups are delineated as above. Groups I, II, VI, and VIII share the special technical feature of a meat substitute product. Groups I, III, V, and VII-XI share the special technical feature of an isolated and purified protein or isolating and purifying a protein. However, this is not an improvement over the prior art of US 2010/0074998 A1 to ESPELETA VEGA et al. (hereinafter Espeleta Vega) that teaches a meat substitute composition comprising a protein content comprising one or more isolated and purified proteins (abstract; para [0037] - vegetable protein isolate), wherein said meat substitute composition accurately mimics the taste or color of a meat product derived from animal sources (para [0047]). Groups III, VI, and IX share the special technical feature of a muscle tissue replica. Likewise, this is not an improvement over the prior art of US 2004/0161513 A1 to AKASHE et al. (hereinafter Akashe) that teaches a muscle replica (para [0065] - "the deflavored soy materials are texturized prior to incorporation into the meat or meat analog product in order to develop meat-like texture (i.e., similar to fibers found in meat)"). Groups IV, VI and X share the special technical feature of a fat tissue replica. Likewise this is not an improvement over the prior art of US 2011/0008502 A1 to HOSOMI et al. (hereinafter Hosomi) that teaches a fat tissue replica for use in processed meat products in order to impart a fatty tissue-specific mouthfeel and juiciness (para [0277]). Groups V, VI and XI share the special technical feature of a connective tissue replica. Likewise, this is not an improvement over the prior art of Akashe that teaches a connective tissue replica (para [0065] - "Preferred processing includes adding water and carrying out further size reduction by grinding, for example, so as to provide thin protein filaments. In the meat and meat analog products of this invention, these filaments provide texture properties similar to those of connective tissue proteins such as collagen normally present in meat products."); wherein said muscle replica and connective tissue replica are assembled in a manner that approximates the physical organization of meat ("In the meat and meat analog products of this invention, these filaments provide texture properties similar to those of connective tissue proteins such as collagen normally present in meat products."). Further, Group II requires a meat substitute product that comprises an indicator that indicates cooking progression from a raw state to a cooked state, wherein said meat substitute product is derived from non-animal sources which is not required by any of the other Groups. Group III further requires the muscle tissue replica approximates the taste, texture, or color of an equivalent muscle tissue derived from an animal source which is not required by any of the other Groups. Group IV further requires the fat tissue replica comprising a gelled emulsion, said gelled emulsion comprising a protein solution with fat droplets suspended therein which is not required by any of the other Groups. Group V further requires the connective tissue replica has been assembled into structures approximating the texture and visual appearance of connective tissue or skin which is not required by any of the other Groups. Group VI further requires the muscle replica, fat tissue replica, and/or connective tissue replica be assembled in a manner that approximates the physical organization of meat which is not required by any of the other Groups. Group VII further requires purified iron-containing proteins, wherein configured for consumption by an animal which is not required by any of the other Groups. Group VIII further requires assembling said one or more proteins into a physical organization that approximates the physical organization of meat which is not required by any of the other Groups. Group IX further requires assembling said one or more proteins into a physical organization that approximates the physical organization of skeletal muscle which is not required by any of the other groups. Group X further requires preparing a solution comprising one or more proteins, emulsifying one or more fats in said solution, and stabilizing said solution into a gelled emulsification with one or more cross-linking reagents which is not required by any of the other Groups. Group XI further requires solubilizing one or more proteins in a first solution, extruding said first solution into a second solution, wherein said one or more proteins is insoluble in said second solution, wherein said extruding induces precipitation of said one or more proteins which is not required by any of the other Groups.

Therefore the inventions listed as Groups I-XI do not relate to a single general inventive concept under PCT Rule 13.1 because they do not share a same or corresponding special technical feature. This ISA will establish the ISR for the first Group mentioned, specifically, Group I claims 1-20 without additional fees. In order for all inventions to be examined, the appropriate examination fees must be paid.

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- (84) 指定国 (除另有指明, 要求每一种可提供的国家保护): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, 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, PA, 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。
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- 本国际公布:
— 包括国际检索报告(条约第 21 条(3))。
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(54) Title: METHOD OF ELIMINATING DEPENDENCE OF METHANOL INDUCED PROMOTER ON SINGLE METHANOL CARBON SOURCE

(54) 发明名称: 消除甲醇诱导型启动子单一甲醇碳源依赖性的方法

(57) Abstract: Provided is a method of eliminating the dependence of methanol induced promoter on a single methanol carbon source for expressing foreign polypeptide. The method comprises activating the expression of the promoter requiring methanol induction by increasing the expression quantity of Mit1 polypeptide in cells of methylotrophic yeast, such that the promoter originally depending on methanol induction no longer depends on single methanol and can also express foreign polypeptide.

(57) 摘要: 提供了一种消除甲醇诱导型启动子单一甲醇碳源依赖性而表达外源多肽的方法。该方法通过在甲醇营养型酵母细胞内增加 Mit1 多肽的表达量, 激活需要甲醇诱导的启动子的表达, 使得原本依赖甲醇诱导的启动子不再依赖单一性甲醇也可以表达外源多肽。

消除甲醇诱导型启动子单一甲醇碳源依赖性的方法

技术领域

本发明属于生物工程领域；更具体地，本发明涉及一种消除甲醇诱导型启动子单一甲醇碳源依赖性而表达外源多肽的方法。

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背景技术

甲醇营养型酵母(包括 *Pichia*, *Hansenula*, *Candida*, *Torulopsis* 等)表达系统由于其高效的外源多肽表达能力已经在工业生产、制药等领域被非常广泛的运用。其特点在于，它们拥有能够被甲醇高效诱导的启动子(*AOX1* 启动子、*DHAS* 启动子、*FDH* 启动子、*MOX* 启动子、*AOX2* 启动子、*ZZA1*、*PEX5*、*PEX8*、*PEX14* 启动子等)，并且这些启动子严格依赖甲醇，其它碳源(如葡萄糖，甘油等)会抑制这些启动子的表达。

随着石油危机的爆发，利用甲醇生产单细胞蛋白，成本变高。之所以利用甲基营养型酵母表达系统表达如此之多的外源多肽，是因为其具有其他表达系统不可比拟的优势：基因操作简单，外源多肽表达量高，既可以胞内表达，也可以分泌表达；外源多肽基因遗传稳定；作为真核表达系统，具有真核生物的亚细胞结构，具有糖基化、脂肪酰化、蛋白磷酸化等翻译后修饰加工功能；

由于被利用的范围越来越广，在实际的发酵放大过程中遇到很多问题：(1) 表达所用的启动子需要甲醇的诱导，甲醇有毒易燃，在大规模工业发酵中需要进行特别的防暴设计；(2) 甲醇发酵强耗氧，一般靠增大空气的通气量和提高转速很难满足氧气的需求而需要通纯氧，甲醇代谢需要氧气的量是葡萄糖为碳源所需氧气量的三至四倍。消耗的甲醇越多需要的纯氧越多，这给实际的工业化生产带来很大的麻烦。另外消耗的甲醇越多所产的热量也越大，所需设备的冷却能力要求越高；(3) 甲醇作为一种石油化学产品，不适合于一些食品领域添加剂的生产；(4) 甲醇代谢会产生 H_2O_2 ，会导致所表达多肽的水解。

因此，如果得到一种能够利用非甲醇诱导甲醇诱导型启动子表达的方法，使原本依赖甲醇诱导的启动子的高效转录不依赖于甲醇，而可用其他碳源来诱导这些启动子的表达，这对于实现工业上利用非甲醇高效表达外源多肽具有积极意义。

发明内容

本发明的目的在于提供一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱

动甲醇诱导型启动子表达外源多肽的方法。

本发明的另一目的还在于提供重组的甲醇营养型酵母，其可以利用消除甲醇诱导型启动子单一甲醇碳源依赖性来表达外源多肽。

在本发明的第一方面，提供一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法，包括：

(1) 提供甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其表达外源的 Mit1 多肽；以及

表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因；

(2) 在无甲醇条件或非单一甲醇碳源条件下培养(1)的甲醇营养型酵母。

10 在一个优选例中，所述的 Mit1 多肽是转录激活相关因子，使得原本依赖甲醇诱导的启动子不再依赖甲醇也可表达外源多肽。

在另一优选例中，所述的 Mit1 多肽的功能为：激活甲醇代谢中需要甲醇诱导的启动子表达。

在另一优选例中，所述的 Mit1 是 PpMit1。

15 在另一优选例中，所述表达盒 2 在甲醇营养型酵母中以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。

在另一优选例中，所述的甲醇诱导型启动子包括(但不限于)：AOX1 启动子、DHAS 启动子、DAS 启动子、FDH 启动子、FMDH 启动子、MOX 启动子、AOX2 启动子、ZZA1、PEX5-、PEX8-、PEX14-启动子、PMP20 启动子、PMP47 启动子、20 AOD1 启动子、AOD2 启动子。

在另一优选例中，所述的甲醇营养型酵母包括(但不限于)：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括(但不限于)：GS115，MIG1 基因和 MIG2 基因不表达的毕赤酵母($\Delta mig1 \Delta mig2$)，NRG1 基因、MIG1 基因和 MIG2 基因不表达的毕赤酵母25 ($\Delta mig1 \Delta mig2 \Delta nrg1$)，或 HXS1 基因不表达的毕赤酵母菌株。

在另一优选例中，述的 Mit1 多肽选自下组：

(a) SEQ ID NO: 2 所示氨基酸序列的多肽；或

(b) 将 SEQ ID NO: 2 所示氨基酸序列经过一个或多个(如 1-30 个，更佳地 1-20 个，更佳地 1-15 个，更佳地 1-10 个，更佳地 1-5 个或 1-3 个)氨基酸残基的取代、30 缺失或添加而形成的，且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽；

(c) 与(a)限定的序列在具有 70%以上(较佳地 80%以上, 更佳地 90%以上, 更佳地 95%以上, 更佳地 98%以上, 如 99%或更高)的序列相同性的, 且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽。

在另一优选例中, 所述的 Mit1 多肽的编码基因的 NCBI Reference Sequence 为
5 XM_002493021.1。

在另一优选例中, 所述的 Mit1 多肽的编码基因具有 SEQ ID NO: 1 所示的核苷酸序列。

在另一优选例中, 所述的 Mit1 多肽的编码基因在甲醇存在的情况下诱导表达, 在甘油、葡萄糖等碳源存在的情况下不表达。

10 在另一优选例中, 所述的表达盒 1 中, 包括启动子以及与之操作性连接的 Mit1 多肽的编码基因; 较佳地, 该启动子包括(但不限于): 组成型启动子, 诱导型启动子, 组织或器官特异性启动子, 时空特异性表达启动子。

在另一优选例中, 表达盒 1 中, 所述启动子包括(但不限于): *GAP* 启动子、*PGK1* 启动子、*MIT1* 启动子等任意能使 *MIT1* 过表达的启动子。

15 在另一优选例中, 所述的表达盒 1 在甲醇营养型酵母中以单拷贝或多拷贝存在, 例如 1-20 拷贝, 如 15, 10, 8, 6, 5, 3, 2 拷贝。

在另一优选例中, 步骤(2)中, 采用存在甘油和/或葡萄糖的酵母培养基。

在本发明的另一方面, 提供 Mit1 多肽或其编码基因的用途, 用于消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达。

20 在本发明的另一方面, 提供一种重组的甲醇营养型酵母, 所述甲醇营养型酵母含有:

表达盒 1, 其能够表达外源的 Mit1 多肽; 以及

表达盒 2, 包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因。

25 在一个优选例中, 所述的甲醇营养型酵母包括 (但不限于): 毕赤酵母(*Pichia*), 汉逊酵母(*Hansenula*), 假丝酵母(*Candida*), 球拟酵母(*Torulopsis*); 较佳地, 所述的毕赤酵母(*Pichia*)包括(但不限于): GS115, *MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母($\Delta mig1 \Delta mig2$), *NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母($\Delta mig1 \Delta mig2 \Delta nrg1$), 或 *HXS1* 基因不表达的毕赤酵母菌株。

30 在一个优选例中, 所述的所述的 *MIG1* 基因的核苷酸序列如 SEQ ID NO: 9 所示;

所述的 *MIG2* 基因的核苷酸序列如 SEQ ID NO: 10 所示; 或

所述的 *HXS1* 基因的核苷酸序列如 SEQ ID NO: 3 所示。

所述的 *NRG1* 基因的核苷酸序列如 SEQ ID NO:8 所示(NCBI Reference Sequence: XM_002493138.1)。

本发明的其它方面由于本文的公开内容，对本领域的技术人员而言是显而易见的。

附图说明

图 1、pGAPZαA 的质粒图谱示意图。

图 2、载体 pAG32 的质粒图谱示意图。

10 图 3、野生型及 GS115-MIT1 分别在非甲醇或含甲醇(甘油+甲醇)的培养基中培养，并进行 Aox 显色反应的结果。

图 4、野生型及 GS115-MIT1 分别在非甲醇或含甲醇的培养基中培养，并进行 Aox 酶活测定的结果。

15 图 5、菌株 GS115-MIT1-GFP 在 YND 液体培养基中过夜预培养，然后分别转移至非甲醇或含甲醇的培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

图 6、野生型及 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，生长到对数生长期后取样 1ml，在离心后的菌体中加入 Aox 酶活显色液显色。

20 图 7、野生型及 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox 酶活的测定。

图 8、野生型及 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

25 图 9、载体 pPIC3.5K 的质粒图谱示意图。

图 10、载体 pUC18 的质粒图谱示意图

图 11、野生型及 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox 酶活的测定。

30 图 12、野生型及 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 菌株分别在非甲醇或含甲醇的培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

具体实施方式

为了克服目前甲醇诱导型启动子在发酵时对于甲醇诱导的依赖性，本发明人经过深入的研究，揭示了一种消除甲醇诱导型启动子单一甲醇碳源依赖性的方法，
5 通过在甲醇营养型酵母细胞内增加 Mit1(甲醇诱导的转录因子 1; Methanol Induced Transcription Factor 1)多肽的表达量，激活甲醇代谢中需要单一甲醇诱导的启动子表达，以此使得原本依赖甲醇诱导的启动子不再依赖甲醇也可表达外源多肽。

术语

10 如本文所用，所述的“启动子”是指一种核酸序列，其通常存在于目的基因编码序列的上游(5'端)，能够引导核酸序列转录为 mRNA。一般地，启动子或启动子区提供 RNA 聚合酶和正确起始转录所必需的其它因子的识别位点。在本文中，所述的启动子或启动子区包括启动子的活性变异体，该变异体可以是天然发生的等位变异体或非天然发生的变异体。所述变异体包括取代变异体、缺失变异体
15 体和插入变异体。

如本文所用，所述的“甲醇诱导型启动子”是与甲醇代谢有关的酶的启动子。在现有技术中，这些启动子通过向生长培养基中添加甲醇，可以控制外源多肽由所述启动子的表达。所述的“甲醇诱导型启动子”可以由本领域技术人员使用常规技术从酵母中分离获得。

20 如本文所用，所述的“单一甲醇(碳源)诱导”是指启动子需要以甲醇为唯一碳源进行诱导来驱动与之操作性连接的基因的表达，在非甲醇为唯一碳源(如甲醇+葡萄糖)的条件下不能驱动与之操作性连接的基因的表达。所述的“消除单一甲醇诱导”是指使得启动子在不以甲醇为唯一碳源(如甲醇+葡萄糖，或葡萄糖，或甘油)的条件下就可驱动与之操作性连接的基因的表达。“非单一甲醇(碳源)诱导”
25 是指除了甲醇碳源外，还存在至少一种非甲醇的碳源。

如本文所用，所述的“组成型启动子”是指在其调控下不同组织器官和发育阶段的基因表达没有明显差异的一类启动子。

如本文所用，所述的“诱导型启动子”可根据需要在特定细胞生长阶段或特定生长环境下，快速诱导基因转录的“开”与“关”或者“高”与“低”。根据
30 来源，可将诱导型启动子分为天然存在的启动子和人工构建的启动子。

如本文所用，所述的“组织或器官特异性启动子”是指基因转录一般只发生在某些特定器官或组织中的启动子。

如本文所用，“外源的”或“异源的”是指来自不同来源的两条或多条核酸或蛋白质序列之间的关系。例如，如果启动子与目的基因序列的组合通常不是天然存在的，则启动子对于该目的基因来说是外源的。特定序列对于其所插入的细胞或生物体来说是“外源的”。

如本文所用，所述的“表达盒”是指包含有表达目的多肽(本发明中为外源多肽或 Mit1 多肽)所需的所有必要元件的基因表达系统，通常其包括一下元件：启动子、编码多肽的基因序列，终止子；此外还可选择性包括信号肽编码序列等。这些元件是操作性相连的。

如本文所用，所述的“甲醇营养型酵母”是指可利用甲醇作为唯一碳源的酵母。包括来自汉逊酵母属(*Hansenula*)，毕赤酵母属(*Pichia*)，球拟酵母属(*Torulopsis*)，假丝酵母属(*Candida*)等的酵母。

如本文所用，所述的“可操作性连接”是指两个或多个核酸区域或核酸序列的功能性的空间排列。例如：启动子区被置于相对于目的基因核酸序列的特定位置，使得核酸序列的转录受到该启动子区域的引导，从而，启动子区域被“可操作地连接”到该核酸序列上。

如本文所用，术语“严格条件”是指：(1)在较低离子强度和较高温度下的杂交和洗脱，如 $0.2 \times \text{SSC}$ ，0.1%SDS，60℃；或(2)杂交时加有变性剂，如 50%(v/v) 甲酰胺，0.1%小牛血清/0.1%Ficoll，42℃等；或(3)仅在两条序列之间的相同性至少在 50%，优选 55%以上、60%以上、65%以上、70%以上、75%以上、80%以上、85%以上或 90%以上，更优选是 95%以上时才发生杂交。

如本文所用，所述的“含有”，“具有”或“包括”包括了“包含”、“主要由……构成”、“基本上由……构成”、和“由……构成”；“主要由……构成”、“基本上由……构成”和“由……构成”属于“含有”、“具有”或“包括”的下位概念。

Mit1 多肽

本发明揭示了 Mit1 多肽在消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达中的应用。在对依赖甲醇控制外源多肽表达的启动子(甲醇诱导型启动子)的研究工作中，本发明人意外发现 Mit1 多肽可以解除甲醇诱导

型启动子对于甲醇的依赖性，使其可利用非单一甲醇碳源或非甲醇碳源进行外源多肽的表达。

本发明还包括所述的 Mit1 多肽的片段、衍生物和类似物。如本文所用，术语“片段”、“衍生物”和“类似物”是指基本上保持本发明的 MIT1 多肽相同的生物学功能或活性的多肽。本发明的多肽片段、衍生物或类似物可以是(i)有一个或多个保守或非保守性氨基酸残基(优选保守性氨基酸残基)被取代的多肽，而这样的取代的氨基酸残基可以是也可以不是由遗传密码编码的，或(ii)在一个或多个氨基酸残基中具有取代基团的多肽，或(iii)成熟多肽与另一个化合物(比如延长多肽半衰期的化合物，例如聚乙二醇)融合所形成的多肽，或(iv)附加的氨基酸序列融合到此多肽序列而形成的多肽(如前导序列或分泌序列或用来纯化此多肽的序列或多肽原序列，或融合多肽)。根据本文的定义这些片段、衍生物和类似物属于本领域熟练技术人员公知的范围。

在本发明中，术语“Mit1 多肽”指具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的 SEQ ID NO: 2 序列的多肽。该术语还包括具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽功能的、SEQ ID NO: 2 序列的变异形式。这些变异形式包括(但并不限于)：若干个(通常为 1-50 个，较佳地 1-30 个，更佳地 1-20 个，最佳地 1-10 个，还更佳如 1-8 个或 1-5 个)氨基酸的缺失、插入和/或取代，以及在 C 末端和/或 N 末端添加或缺失一个或数个(通常为 20 个以内，较佳地为 10 个以内，更佳地为 5 个以内)氨基酸。例如，在本领域中，用性能相近或相似的氨基酸进行取代时，通常不会改变蛋白质的功能。又比如，在 C 末端和/或 N 末端添加或减少一个或数个氨基酸通常也不会改变蛋白质的功能。

多肽的变异形式包括：同源序列、保守性变异体、等位变异体、天然突变体、诱导突变体、在高或低的严紧度条件下能与编码 Mit1 多肽的 DNA 杂交的 DNA 所编码的多肽、以及利用抗 Mit1 多肽的抗血清获得的多肽或蛋白。本发明还提供了其他多肽，如包含 Mit1 多肽或其片段的融合多肽。

本发明还提供 Mit1 多肽或多肽的类似物。这些类似物与天然 Mit1 多肽的差别可以是氨基酸序列上的差异，也可以是不影响序列的修饰形式上的差异，或者兼而有之。这些多肽包括天然或诱导的遗传变异体。诱导变异体可以通过各种技术得到，如通过辐射或暴露于诱变剂而产生随机诱变，还可通过定点诱变法或其他已知分子生物学的技术。类似物还包括具有不同于天然 L-氨基酸的残基(如 D-氨基酸)的类似物，以及具有非天然存在的或合成的氨基酸(如 β 、 γ -氨基酸)的类

似物。应理解，本发明的多肽并不限于上述例举的代表性的多肽。

本发明还提供了编码本发明 Mit1 多肽或其保守性变异多肽的多核苷酸序列。

本发明的多核苷酸可以是 DNA 形式或 RNA 形式。DNA 形式包括 cDNA、基因组 DNA 或人工合成的 DNA。DNA 可以是单链的或是双链的。DNA 可以是编
5 码链或非编码链。编码成熟多肽的编码区序列可以与 SEQ ID NO: 1 序列相同或者是简并的变异体。如本文所用，“简并的变异体”在本发明中是指编码具有 SEQ ID NO: 2 序列或其变异形式的蛋白，但与 SEQ ID NO: 1 中的编码区序列有差别的核酸序列。

本发明还涉及上述多核苷酸的变异体，其编码与本发明有相同的氨基酸序列
10 的多肽或多肽的片段、类似物和衍生物。此多核苷酸的变异体可以是天然发生的等位变异体或非天然发生的变异体。这些核苷酸变异体包括取代变异体、缺失变异体和插入变异体。如本领域所知的，等位变异体是一个多核苷酸的替换形式，它可能是一个或多个核苷酸的取代、缺失或插入，但不会从实质上改变其编码的多肽的功能。

15 本发明还涉及与上述多核苷酸同源的多核苷酸，它们是与上述多核苷酸具有至少50%，较佳地至少60%，更佳地至少70%，更佳地至少80%相，更佳地至少90%；更佳地至少95%；更佳地至少98%或99%相同性的多核苷酸。这些多核苷酸所编码的蛋白也具有与前述多核苷酸所编码的多肽相同的利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽的功能。

20 本发明还涉及与上述的序列杂交且两个序列之间具有至少 50%，较佳地至少 70%，更佳地至少 80%相同性的多核苷酸。本发明特别涉及在严格条件下与本发明所述多核苷酸可杂交的多核苷酸。

本发明的 Mit1 多肽核苷酸全长序列或其片段通常可以用 PCR 扩增法、重组法或人工合成的方法获得。对于 PCR 扩增法，可根据本发明所公开的有关核苷酸
25 序列，尤其是开放阅读框序列来设计引物，并用市售的 cDNA 库或按本领域技术人员已知的常规方法所制备的 cDNA 库作为模板，扩增而得有关序列。当序列较长时，可进行两次或多次 PCR 扩增，然后再将各次扩增出的片段按正确次序拼接在一起。

30 本发明中，编码 Mit1 多肽的多核苷酸序列可插入到重组表达载体中。术语“重组表达载体”指本领域熟知的细菌质粒、噬菌体、酵母质粒、植物细胞病毒、哺乳动物细胞病毒或其他载体。总之，只要能在宿主体内复制和稳定，任何质粒和

载体都可以用。表达载体的一个重要特征是通常含有复制起点、启动子、标记基因和翻译控制元件。

本领域的技术人员熟知的方法能用于构建含 Mit1 多肽编码 DNA 序列和合适的转录/翻译控制信号的表达载体。这些方法包括体外重组 DNA 技术、DNA 合成技术、体内重组技术等。所述的 DNA 序列可有效连接到表达载体中的适当启动子上，以指导 mRNA 合成。表达载体还包括翻译起始用的核糖体结合位点和转录终止子。

此外，表达载体优选地包含一个或多个选择性标记基因，以提供用于选择转化的宿主细胞的表型性状，如真核细胞培养用的二氢叶酸还原酶、新霉素抗性以及绿色荧光蛋白(GFP)。

包含上述的适当 DNA 序列以及适当启动子或者控制序列的载体，可以用于转化适当的宿主细胞，以使其能够表达蛋白质。

增加 Mit1 多肽表达的方法是本领域周知的。例如，可通过转入携带 Mit1 多肽编码基因的表达构建物使酵母细胞过表达 Mit1 多肽；或可通过用强启动子驱动从而增强 Mit1 多肽的表达；或者通过增强子来增强该 Mit1 多肽的表达。

甲醇诱导型启动子

在现有常规技术中，所述的甲醇诱导型启动子依赖于在培养基中添加甲醇，从而控制外源多肽由所述启动子的表达。而本发明人利用 Mit1 多肽解除了甲醇诱导型启动子对于甲醇的依赖。

本领域技术人员熟悉甲醇依赖型启动子，可使用常规技术从酵母中分离获得。由于甲醇依赖型启动子具有基本相同的工作机制和工作原理，本发明对于甲醇依赖型启动子的种类没有特别的限制。例如，所述的甲醇诱导型启动子包括但不限于：*AOX1* 启动子、*DHAS* 启动子(或者 *DAS* 启动子)、*FDH* 启动子(或者 *FMDH* 启动子)、*MOX* 启动子、*AOX2* 启动子、*ZZA1*、*PEX5*-、*PEX8*-、*PEX14*-启动子、*PMP20* 启动子、*PMP47* 启动子、*AOD1* 启动子、*AOD2* 启动子。

本发明还包括与上述启动子的多核苷酸序列具有至少 70%，更佳地至少 80% (例如 85%、90%、95%、96%、97%、98%、或 99%) 相同性的启动子。这些启动子在引发转录的必要位点及转录起始点的位置上是严格保守的。本发明特别涉及在严格条件下与本发明所述甲醇依赖型启动子的核苷酸序列可杂交的多核苷酸，且该多核苷酸也具有野生型甲醇依赖型启动子的功能。

消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法

5 本发明还提供了一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法，包括：

(1) 提供甲醇营养型酵母，所述甲醇营养型酵母含有：

表达盒 1，其能够表达外源的 Mit1 多肽；以及表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因；

(2) 在无甲醇条件或非单一甲醇碳源条件培养(1)的甲醇营养型酵母。

10 所述的表达盒 1 和表达盒 2 可以存在于同一表达载体中，也可以存在于不同的表达载体中。

所述表达盒 1 在甲醇营养型酵母中以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。所述的表达盒 2 在甲醇营养型酵母中也可以单拷贝或多拷贝存在，例如 1-20 拷贝，如 15，10，8，6，5，3，2 拷贝。

15 所述的表达盒 2 中，包括启动子以及与之操作性连接的 Mit1 多肽的编码基因。应理解，任何可使得 Mit1 多肽在甲醇营养型酵母中重组表达的启动子都可用于本发明。不限于组成型启动子、诱导型启动子或特异性启动子。本领域技术人员可以根据所需达到的目的进行合适的选择，例如当希望通过一定的条件干预来控制 Mit1 多肽的表达或不表达，则可以选择诱导型启动子。较佳地，表达盒 2 中，所述启动子包括：*GAP* 启动子、*PGK1* 启动子、*MIT1* 启动子等任意能够使得 *MIT1* 过表达的启动子。

25 步骤(2)中，“无甲醇的条件”是本领域技术人员易于建立的，也即，在常规应用的甲醇营养型酵母培养基中，不加入甲醇作为碳源，取代以其它类型的碳源，常用的碳源是本领域技术人员熟知的，例如但不限于：甘油、葡萄糖、淀粉(含淀粉水解液，木薯淀粉，玉米淀粉，纤维素类水解液等)、蔗糖、麦芽糖等。较佳地，采用以甘油和/或葡萄糖为碳源的酵母培养基。类似地，“非单一甲醇碳源条件”也是本领域技术人员易于建立的。

重组甲醇营养型酵母

30 基于本发明的新发现，还提供了一种重组的甲醇营养型酵母，所述甲醇营养型酵母含有：表达盒 1，其能够表达外源的 MIT1 多肽；以及表达盒 2，包括甲醇

诱导型启动子及与之操作性连接的外源多肽编码基因。附加条件是，所述的表达盒 1 和表达盒 2 可以存在于同一表达载体中，也可以存在于不同的表达载体中。

任何甲醇营养型酵母均可被应用于本发明中，以构建上述重组的甲醇营养型酵母。例如，所述的甲醇营养型酵母包括但不限于：毕赤酵母(*Pichia*)，汉逊酵母
5 (*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括：GS115，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母($\Delta mig1\Delta mig2$)，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母($\Delta mig1\Delta mig2\Delta nrg1$)，或基因 *HXS1* 不表达的毕赤酵母菌株。

在本发明的具体实施例中，提供了四株能够利用非甲醇碳源诱导 *AOX1* 启动子表达外源多肽的菌株，GS115-MIT1、 $\Delta mig1\Delta mig2$ -MIT1、 $\Delta hxs1$ -MIT1 和
10 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1。在毕赤酵母野生菌株 GS115、毕赤酵母双缺失菌株 $\Delta mig1\Delta mig2$ 、毕赤酵母缺失菌株 $\Delta hxs1$ 和毕赤酵母三缺失菌株 $\Delta mig1\Delta mig2\Delta nrg1$ 中分别过表达了转录激活相关基因 *MIT1*，构建了基因过表达菌株 GS115-MIT1、 $\Delta mig1\Delta mig2$ -MIT1、 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 和 $\Delta hxs1$ -MIT1。经 Aox1 酶活检测
15 发现，甘油可以诱导 GS115-MIT1 的 *AOX1* 启动子的表达，甘油或者葡萄糖均能诱导 $\Delta mig1\Delta mig2$ -MIT1、 $\Delta hxs1$ -MIT1 和 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 的 *AOX1* 启动子表达。并且用流式细胞仪检测了绿色荧光蛋白 GFP 以 *AOX1* 为启动子时在非甲醇碳源中的诱导表达量。

所述的 $\Delta hxs1$ 菌株中，与酿酒酵母己糖感应体 Snf3/Rgt2 有很高相似性的一个基因 *HXS1*(GenBank 登录号: 8197942)的编码区缺失。
20

下面结合具体实施例，进一步阐述本发明。应理解，这些实施例仅用于说明本发明而不适用于限制本发明的范围。下列实施例中未注明具体条件的实验方法，通常按照常规条件如 J.萨姆布鲁克等编著，分子克隆实验指南，科学出版社，2002
25 中所述的条件，或按照制造厂商所建议的条件。除非另外说明，否则百分比和份数按重量计算。

材料

毕赤酵母总蛋白提取技术参照冷泉港公司提供的酵母蛋白提取手册。

所使用的工具酶均购自 TaKaRa 生物公司(大连，中国)，具体的反应条件和使用的
30 方法均参考商品说明书。

下面的商品化质粒和菌株用于基因克隆和蛋白表达：质粒 pGAPZαA、质粒 pPIC3.5k、大肠杆菌 Top10、毕赤酵母菌株 GS115 均购自 Invitrogen 公司。

质粒 pAG32 由质粒 pRDM054 在酶切位点 *Bgl* II 处切除 *P_{AOX1}*-BFP-SKL 表达体系得到；质粒 pRDM05 获自加州大学圣地亚哥分校。

- 5 YPD 培养基：2% 蛋白胨，1% 酵母粉，2% 葡萄糖，2% 琼脂粉；YNB 培养基：0.67% YNB；MGY 培养基：1% 甘油，0.67% YNB； YND 液体培养基：1% 葡萄糖，0.67% YNB。

配制以上培养基时，葡萄糖 115°C 高压灭菌 20 min，甲醇在使用时添加。其它成分 121°C 高压灭菌 20 min。固体培养基加 2% 琼脂粉。

10

实施例 1、甘油可诱导 *AOX1* 启动子表达的毕赤酵母菌株 GS115-MIT1

1. *PpMIT1* 过表达质粒的构建

- 采用 PCR，酶切连接的方法，以 pGAPZαA(图 1)为载体在 GAP 启动子下游的 *Asu* II/*Sal* I 两个酶切位点插入 *PpMIT1* 基因(SEQ ID NO: 1，基因全长 2667bp)，得到的重组质粒称为 pGM 质粒。

以 M1-GAP5 和 M1-AOX1TT 为引物(表 1)，通过 PCR 的方法从 pGM 质粒上扩增得到 pGAP-*PpMIT1*-*AOX1TT* 的表达体系(全长 3615 bp)，以 pAG32(图 2)为载体在 *Sac* I/*Spe* I 处插入该表达体系。得到重组质粒为 pGMhph。

表 1. 引物列表

引物	SEQ ID NO:	序列
M1-GAP5	4	5'-CGAGCTCAGATCTTTTTTGTAGAAATGTC-3'
M1-AOX1TT	5	5'- GACTAGTGCACAAACGAAGGTCTC-3'
HXS1-C-5	6	5- CCGGAATTCTAGTCACGGATTGCTTC -3
HXS1-C-3	7	5- ACATGCATGCGTTCCCAATAGATTTACAA -3

20

2. 电转毕赤酵母和 GS115-MIT1 菌株的筛选

- 将 *PpMIT1* 过表达质粒(pGMhph)电转毕赤酵母菌株 GS115，涂于 4 块加潮霉素的 YPD 固体培养基上，放在 30°C 培养箱培养 48-72 小时。将平板上长出的单克隆挑至 10 ml YPD + 潮霉素液体培养基中，30°C 摇床培养后，提基因组用 PCR 验证。
- 25 把 PCR 检验及测序验证后正确的毕赤酵母菌株命名为 GS115-MIT1。

3. Aox 显色反应

将各菌株分别在 0.5% (v/v) 甲醇, 1% 甘油, 1% 甘油+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养, 生长到对数生长期后取样 1 ml, 在离心后的菌体中加入 Aox1 酶活显色液显色 (参见 Stasyk O. V., T. Y. Nazarko, and A. A. Sibirny.

2008. Methods of Plate Pexophagy Monitoring and Positive Selection for *ATG* Gene Cloning in Yeasts. *Methods in enzymology*. **451**:229-239。显色底物为 o-dianisidine)。

结果如图 3, 可以看到在含有甘油的培养基中生长后, 只有菌株 GS115-MIT1 中有 Aox 酶活, 野生菌株中均没有酶活。

4. Aox 酶活的测定

10 将各菌株在 MGY 液体培养基中过夜预培养, 然后分别转移到含有 0.5% (v/v) 甲醇, 1% 甘油, 1% 甘油 + 0.5% (v/v) 甲醇, 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中, 培养 10 小时后取样提取总蛋白, 经 Bradford 法蛋白定量后进行 Aox1 酶活的测定(方法参见 Verduyn, C., J. P. van Dijken, and W. A. Scheffers. 1984. Colorimetric alcohol assays with alcohol oxidase. *J. Microbiol. Methods* **2**:15-25。显色底物为 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate), ABTS)。

由图 4 可知, 菌株 GS115-MIT1 在含有甘油的培养基中均可以诱导 Aox1 的表达, 野生菌株均不能诱导 Aox 的表达。

5、毕赤酵母 GFP 单拷贝表达菌株的筛选

20 在载体 pPIC3.5k (图 9) 的 *AOX1* 启动子下游的 *SnaB* I 酶切位点处插入 GFP 基因(全长 714 bp), 得到 GFP 表达载体 pP-GFP。

6、毕赤酵母 GFP 单拷贝表达菌株的筛选

25 分别将表达载体 pP-GFP 电转 GS115-MIT1 菌株, 涂于不含组氨酸的 YND 平板, 放在 30℃ 培养箱培养 48-72 小时。将平板上长出的单克隆挑至液体培养基中, 30℃ 摇床培养后提基因组, 用 real-time PCR 验证 GFP 拷贝数(方法参见 Xuan, Y. J., X. S. Zhou, W. W. Zhang, X. Zhang, Z. W. Song, and Y. X. Zhang. 2009. An upstream activation sequence controls the expression of *AOX1* gene in *Pichia pastoris*. *FEMS Yeast Res.* **9**: 1271-1282)。把 real-time PCR 检验为单拷贝的毕赤酵母 GFP 表达菌株分别命名为 GS115-MIT1-GFP。

同理构建了表达菌株 $\Delta mig1 \Delta mig2$ -MIT1-GFP 和 $\Delta hxs1$ -MIT1-GFP。

30 表达菌株 $\Delta mig1 \Delta mig2$ -MIT1-GFP 构建方法具体如下: 将表达载体 pP-GFP 和表达载体 pGMhph 共转 $\Delta mig1 \Delta mig2$ 菌株 (参见中国公开专利 CN101857845A)。

表达菌株 $\Delta hxs1$ -MIT1-GFP 构建方法具体如下：将表达载体 pP-GFP 和表达载体 pGMhph 共转 $\Delta hxs1$ -MIT1 菌株。

7、流式细胞仪检测 GFP 表达量

将菌株 GS115-MIT1-GFP 在 YND 液体培养基中过夜预培养，然后分别转移含有 0.5% (v/v) 甲醇，1%甘油，1%甘油+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

如图 5 所示，GS115-MIT1 可以在甘油存在的情况下诱导外源多肽 GFP 表达。

实施例 2、甘油或者葡萄糖可诱导 AOX1 启动子表达的毕赤酵母菌株

1. HXS1 基因敲除质粒的构建

用 BamH I 和 Sal I 酶将 Zeocin 抗性基因 *Sh ble* 片段从质粒 pGAPZ α A 上切下，以 pUC18 质粒(图 10)为载体，在 BamH I 和 Sal I 处酶切连接插入了 Zeocin 抗性基因 *Sh ble* 片段，命名为 pUC18-*ble* 质粒。以 GS115 基因组为模板，首先使用引物 HS1-5F/HS1-5R 扩增 PpHXS1 基因的 5'端外围启动子区域(起始密码子 ATG 上游)，扩增产物的大小为 728 bp。引物 HS1-3F/HS1-3R 用于扩增 PpHXS1 基因的 3'端的区域(终止密码子下游)，扩增产物的大小为 1011 bp，分别切胶回收目的大小片段。

将回收的 PpHXS1 基因的 5'端外围启动子 DNA 片段，用 EcoRI 和 BamHI 双酶切后，回收片段。pUC18-*ble* 质粒、用 EcoRI 和 BamHI 双酶切，回收片段。将回收的两片段连接过夜，转化大肠杆菌，PCR 验证阳性克隆，命名为 pUC18-(*HS1 5'-ble*)。

将上一步构建好质粒 SalI 和 SphI 双酶切，回收片段。PpHXS1 基因的 3'端区域 DNA 片段也用 SalI 和 SphI 双酶切。两片段连接过夜，转化大肠杆菌，PCR 阳性菌株，阳性菌株命名为 pUC18-(*HS1 5'-ble-HS1 3'*)。

表 2、PpHXS1 敲除使用的引物

引物名称	SEQ ID NO:	序列
HS1-5F	11	5'-CCGGAATTCTAGTCACGGATTGCTTC-3'
HS1-5R	12	5'-CGCGGATCCAGTCTGAAATAGGCTCAGACAT-3'
HS1-3F	13	5'-ACGCGTCGACATGCCTATTGAACAAGACTATT-3'
HS1-3R	14	5'-ACATGCATGCGTTCCCAATAGATTTACAA-3'

2. $\Delta hxs1$ 基因缺失菌株的构建

将 pUC18-(*HS1* 5'-*ble*-*HS1* 3') 质粒用 *Eco*RI 和 *Sph*I 双酶切, 胶回收大小为 3350 bp 的 Pp*HXS1* 敲除片段 *HS1* 5'-*ble*-*HS1* 3'。

将回收的 20 μ l Pp*HXS1* 敲除片段 *HS1* 5'-*ble*-*HS1* 3' (浓度大于 50 ng/ μ l) 与 80 μ l 毕赤酵母 GS115 感受态在冰上混匀, 按照实验步骤提供的方法进行电转化。将 50 μ l 复苏的电转化菌液涂于添加 Zeocin 抗生素的 YP+Glycerol 固体培养基平板, 将筛选平板倒置于 30°C 培养箱培养 2~3 天, 当有肉眼可见菌落长出时, 将菌落挑至含 Zeocin 的 YP+Glycerol 液体培养基中培养 2~3 天。PCR 验证 Pp*HXS1* 敲除的阳性菌株, 命名为 $\Delta hxs1$ 。

3. $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 的构建

将质粒 pGMhph 电转毕赤酵母菌株 $\Delta mig1\Delta mig2$ 和 $\Delta hxs1$, 涂于 4 块加潮霉素的 YPD 平板, 放在 30°C 培养箱培养 48-72 小时。将平板上长出的单克隆挑至 10 ml YPD+潮霉素液体培养基中, 30°C 摇床培养后, 抽提基因组用 PCR 验证。把 PCR 检验及测序验证后正确的毕赤酵母菌株分别命名为 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1。

4. Aox 显色反应

将 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 分别在 1% 甘油, 1% 甘油+0.5% (v/v) 甲醇, 1% 葡萄糖, 1% 葡萄糖+0.5% (v/v) 甲醇和 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养, 生长到对数生长期后取样 1ml, 在离心后的菌体中加入 Aox1 酶活显色液显色。

结果如图 6, 可以看到在含有甘油或者葡萄糖的培养基中, 只有菌株 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 中有酶活。

5. Aox 酶活的测定

将各菌株分别在 1% 甘油, 1% 甘油+0.5% (v/v) 甲醇, 1% 葡萄糖, 1% 葡萄糖+0.5% (v/v) 甲醇和 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养, 生长到对数生长期后取样提取总蛋白, 经 Bradford 法蛋白定量后进行 Aox1 酶活的测定。

由图 7 可知, 菌株 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 在含有甘油或者葡萄糖的培养基中均可以诱导 Aox1 的表达, 野生菌株均不能诱导 Aox 的表达。

6. 流式细胞仪检测 $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 的 GFP 表达量

将 $\Delta mig1\Delta mig2$ -MIT1-GFP 和 $\Delta hxs1$ -MIT1-GFP 在液体培养基中过夜预培养, 然后分别转移到含有 0.5% (v/v) 甲醇, 1% 甘油, 1% 甘油+0.5% (v/v) 甲醇, 1%

葡萄糖和 1% 葡萄糖+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中, 取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

如图 8 所示, $\Delta mig1\Delta mig2$ -MIT1 和 $\Delta hxs1$ -MIT1 可以在甘油或者葡萄糖存在的情况下诱导外源多肽 GFP 表达。

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实施例 5、甘油或者葡萄糖可诱导 *AOX1* 启动子表达的毕赤酵母菌株 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1

1. *NRG1* 基因敲除质粒的构建

以 GS115 基因组为模板, 使用引物 5'NRG1-F/5'NRG1-R 扩增 *PpNIG1* 基因的 5'端外围区域得到大小为 320 bp 片段, 将扩增产物命名为 5'NRG1, 胶回收 5'NRG1 片段。以上所得到的 5'NRG1 片段用 *Sac* I 和 *Sma* I 双酶切, 与同样 *Sac* I 和 *Sma* I 双酶切的 pUC18 质粒连接, 构成 pUC18(*Sac*I-5'NRG1-*Sma*I)。载体 pUC18(*Sac*I-5'NRG1-*Sma*I)转化大肠杆菌感受态, 菌落 PCR 筛选阳性克隆。

然后, 使用引物 HYG-F 和 HYG-R, 以质粒 pRDM054 为模板, 扩增潮霉素 B 抗性基因 *HPH*, 大小为 1648 bp。以上所得 *HPH* 片段用 *Sma* I 和 *Xba* I 双酶切, 与同样 *Sma* I 和 *Xba* I 双酶切的载体 pUC18(*Sac*I-5'NRG1-*Sma*I)相连, 构成载体 pUC18(*Sac*I-5'NRG1-*Sma*I-*HPH*-*Xba*I)。pUC18(*Sac*I-5'NRG1-*Sma*I-*HPH*-*Xba*I)转化入大肠杆菌, 菌落 PCR 筛选阳性克隆。

用引物 5'NRG1-F/NRG1-A1 以质粒 pUC18(*Sac*I-5'NRG1-*Sma*I-*HPH*-*Xba*I)为模板扩增得到片段 Overlap-A。用引物 NRG1-B2/3'NRG1-R, 以 GS115 基因组为模板, 扩增得到片段 Overlap-B。然后通过 Overlap PCR 的方法链接片段 A、B, 得到 5'NRG1-*HPH*-3'NRG1 片段。将该片段连接到 pMD19-T 载体上, 得到敲除质粒 pMD19-T(*Sac*I-5'NRG1-*Sma*I-*HPH*-3'NRG1-*Sph*I)。

2. $\Delta mig1\Delta mig2\Delta nrg1$ 菌株的构建

将得到的 *Sac*I-5'NRG1-*Sma*I-*HPH*-3'NRG1-*Sph*I 片段与 $\Delta mig1\Delta mig2$ 双缺失菌株感受态共转, 电转后迅速加入 700 μ l 预冷的 1 mol/L 山梨醇, 然后转移至 EP 管中, 并加入 700 μ l YPD 液体培养基, 30°C 和 200 r/min 摇床复苏培养 1~2 h。最后将菌液涂于添加 Zeocin、G418 和 Hygromycin 抗生素的 YPD 固体平板。30°C 培养箱倒置培养 2~3 天。将阳性转化子提基因组 PCR 验证, 得到的阳性菌株命名为 $\Delta mig1\Delta mig2\Delta nrg1$ 。

30

3. 三缺失菌株中 MIT1 过表达质粒的构建

以 pPIC3.5k 为载体在 *Sac* I/*Bam*H I 两个酶切位点插入 GAP 启动子得到 pPG 质粒。再于 *Bam*H I/*Not* I 两个酶切位点间插入 PpMPP1 基因序列，得到的重组质粒称为 pPGPP1 质粒。

4. $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 的构建

5 将质粒 pPGPP1 电转毕赤酵母菌株 $\Delta mig1\Delta mig2\Delta nrg1$ ，涂于 4 块不加 his 的 YND 平板，放在 30°C 培养箱培养 48-72 小时。将平板上长出的单克隆挑至 10 ml YND 液体培养基中，30°C 摇床培养后，抽提基因组用 PCR 验证。把 PCR 检验及测序验证后正确的毕赤酵母菌株分别命名为 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1。

5. 毕赤酵母 GFP 单拷贝表达菌株的筛选

10 分别将表达载体 pP-GFP 电转 GS11+-MIT1 菌株，涂于不含组氨酸的 YND 平板，放在 30°C 培养箱培养 48-72 小时。将平板上长出的单克隆挑至 MGY 液体培养基中，在荧光显微镜下观察是否有绿色荧光，有荧光的转化子 30°C 摇床培养后提基因组，用 real-time PCR 验证 GFP 拷贝数(方法参见 Xuan, Y. J., X. S. Zhou, W. W. Zhang, X. Zhang, Z. W. Song, and Y. X. Zhang. 2009. An upstream activation
15 sequence controls the expression of *AOX1* gene in *Pichia pastoris*. FEMS Yeast Res. 9: 1271-1282)。把 real-time PCR 检验为单拷贝的毕赤酵母 GFP 表达菌株命名为 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1-GFP。

6. Aox 酶活的测定

20 将 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 菌株分别在 1% 甘油，1%甘油+0.5% (v/v) 甲醇，1% 葡萄糖，1% 葡萄糖+0.5% (v/v) 甲醇和 0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中培养，生长到对数生长期后取样提取总蛋白，经 Bradford 法蛋白定量后进行 Aox1 酶活的测定。

由图 11 可知，菌株 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 在含有甘油或者葡萄糖的培养基中均可以诱导 Aox1 的表达，野生菌株均不能诱导 Aox 的表达。

25 7. 流式细胞仪检测 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 的 GFP 表达量

将 $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1-GFP 在液体培养基中过夜预培养，然后分别转移到含有 0.5% (v/v) 甲醇，1% 甘油，1% 甘油+0.5% (v/v) 甲醇，1% 葡萄糖和 1% 葡萄糖+0.5% (v/v) 甲醇为碳源的 YNB 液体培养基中，取样后用流式细胞仪检测样品中 GFP 的几何平均荧光强度。

30 如图 12 所示， $\Delta mig1\Delta mig2\Delta nrg1$ -MIT1 可以在甘油或者葡萄糖存在的情况下诱导外源多肽 GFP 表达。

表 3、*PpNIG1* 敲除使用的引物

引物名称	SEQ ID NO:	序列
5'NRG1-F	15	5'- CGAGCTCCTGTGCCTATTACCCCCCTT -3'
5'NRG1-R	16	5'- TCCCCCGGGAACAGATAACCAAAACGGACG -3'
3'NRG1-F	17	5- GCTCTAGAGTATTTATTTACGGATTGGA -3'
3'NRG1-R	18	5'- ACATGCATGCCACCACTTTTTGAATCTCGG -3'
HYG-F	19	5'- TCCCCCGGGAGCTTGCCTTGTCCCCGCCG -3'
HYG-R	20	5'- GCTCTAGATCGACACTGGATGGCGGCGT -3'
NRG1-A1	21	5'-TCCAATCCGTAAATAAATACTCGACACTGGATGGCGGCGT-3'
NRG1-B2	22	5'-ACGCCGCCATCCAGTGTCTCGAGTATTTATTTACGGATTGGA-3'

- 在本发明提及的所有文献都在本申请中引用作为参考，就如同每一篇文献被单独引用作为参考那样。此外应理解，在阅读了本发明的上述讲授内容之后，本
- 5 领域技术人员可以对本发明作各种改动或修改，这些等价形式同样落于本申请所附权利要求书所限定的范围。

权 利 要 求

1. 一种消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达的方法，包括：

- 5 (1) 提供甲醇营养型酵母，所述甲醇营养型酵母含有：
表达盒 1，其表达外源的 Mit1 多肽；以及
表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因；
(2) 在无甲醇条件或非单一甲醇碳源条件下培养(1)的甲醇营养型酵母。
- 10 2. 如权利要求 1 所述的方法，其特征在于，所述的甲醇诱导型启动子包括：
AOX1 启动子、*DHAS* 启动子、*DAS* 启动子、*FDH* 启动子、*FMDH* 启动子、*MOX*
启动子、*AOX2* 启动子、*ZZA1*、*PEX5*-、*PEX8*-、*PEX14*-启动子、*PMP20* 启动子、
PMP47 启动子、*AOD1* 启动子、*AOD2* 启动子。
- 15 3. 如权利要求 1 所述的方法，其特征在于，所述的甲醇营养型酵母包括：毕
赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；
较佳地，所述的毕赤酵母包括：*GS115*，*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵
母，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母，或 *HXS1* 基因不表
达的毕赤酵母菌株。
- 20 4. 如权利要求 1 所述的方法，其特征在于，所述的 Mit1 多肽选自下组：
(a) SEQ ID NO: 2 所示氨基酸序列的多肽；或
(b) 将 SEQ ID NO: 2 所示氨基酸序列经过一个或多个氨基酸残基的取代、缺
失或添加而形成的，且具有利用非甲醇碳源诱导甲醇诱导型启动子表达外源多肽
25 功能的由(a)衍生的多肽；
(c) 与(a)限定的序列在具有 70%以上的序列相同性的，且具有利用非甲醇碳
源诱导甲醇诱导型启动子表达外源多肽功能的由(a)衍生的多肽。
- 30 5. 如权利要求 1 所述的方法，其特征在于，所述的表达盒 1 中，包括启动子
以及与之操作性连接的 Mit1 多肽的编码基因；较佳地，该启动子包括：组成型启
动子，诱导型启动子，组织或器官特异性启动子，时空特异性表达启动子。

6. 如权利要求 1 所述的方法，其特征在于，步骤(2)中，采用存在甘油和/或葡萄糖的酵母培养基。

5 7. Mit1 多肽或其编码基因的用途，用于消除甲醇诱导型启动子单一甲醇碳源依赖性而驱动外源多肽编码基因表达。

8. 一种重组的甲醇营养型酵母，所述甲醇营养型酵母含有：
表达盒 1，其能够表达外源的 Mit1 多肽；以及
10 表达盒 2，包括甲醇诱导型启动子及与之操作性连接的外源多肽编码基因。

9. 如权利要求 8 所述的重组的甲醇营养型酵母，其特征在于，所述的甲醇营养型酵母包括：毕赤酵母(*Pichia*)，汉逊酵母(*Hansenula*)，假丝酵母(*Candida*)，球拟酵母(*Torulopsis*)；较佳地，所述的毕赤酵母(*Pichia*)包括：GS115，*MIG1* 基因
15 和 *MIG2* 基因不表达的毕赤酵母，*NRG1* 基因、*MIG1* 基因和 *MIG2* 基因不表达的毕赤酵母，或 *HXS1* 基因不表达的毕赤酵母菌株。

10. 如权利要求 9 所述的方法，其特征在于，所述的 *MIG1* 基因的核苷酸序列如 SEQ ID NO: 9 所示；
20 所述的 *MIG2* 基因的核苷酸序列如 SEQ ID NO: 10 所示；或
所述的 *HXS1* 基因的核苷酸序列如 SEQ ID NO: 3 所示。
所述的 *NRG1* 基因的核苷酸序列如 SEQ ID NO:8 所示。

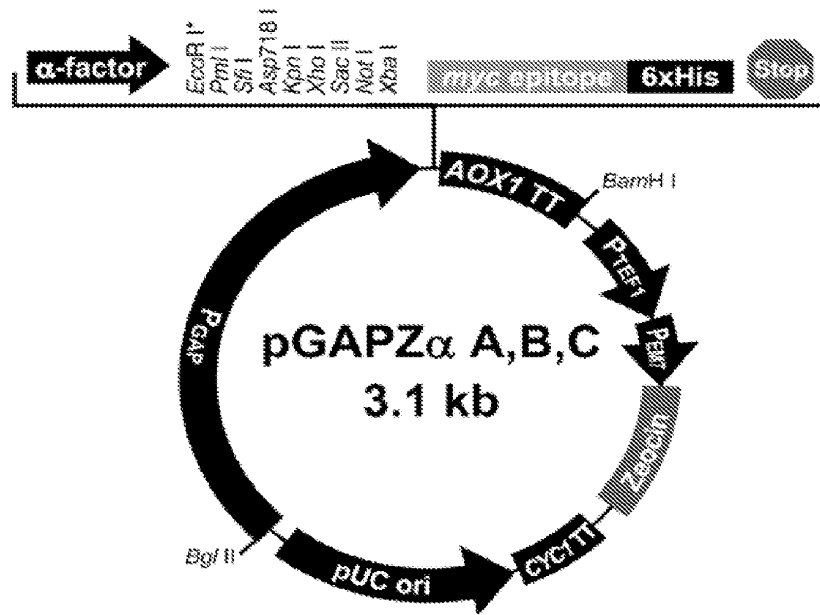


图 1

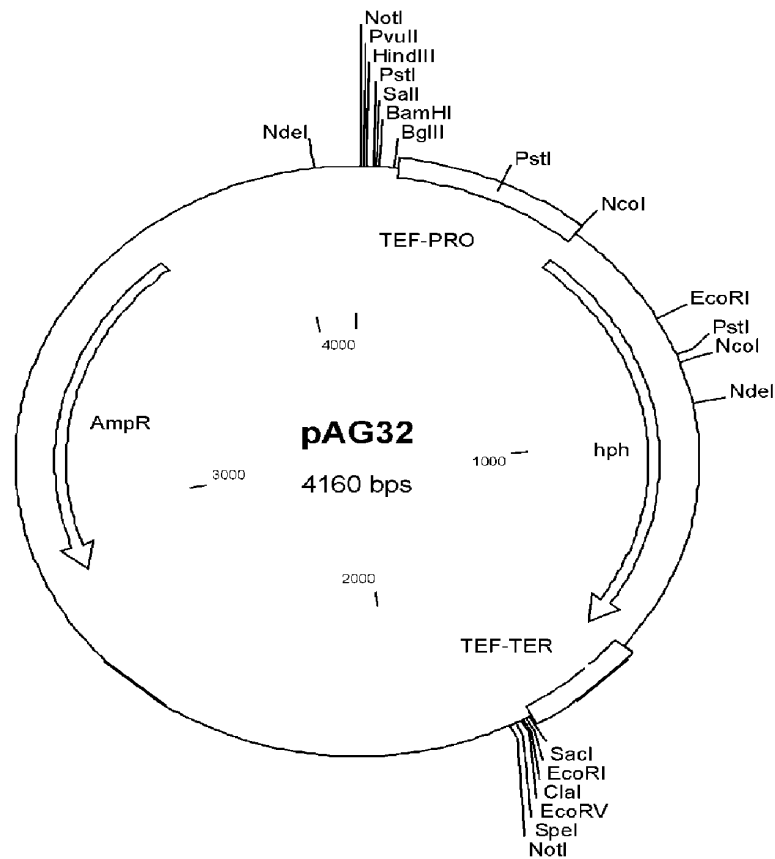


图 2

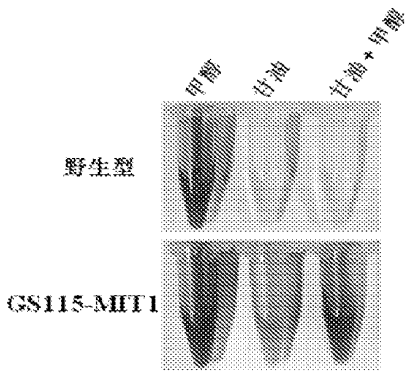


图 3

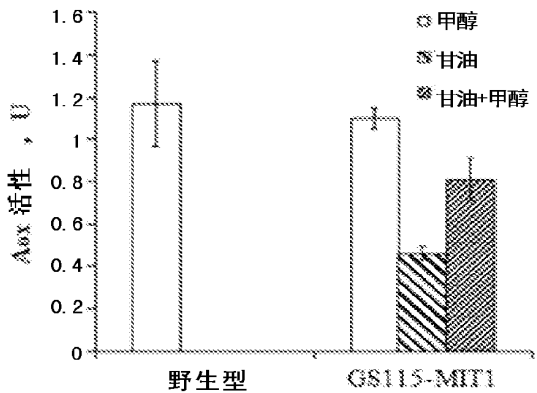


图 4

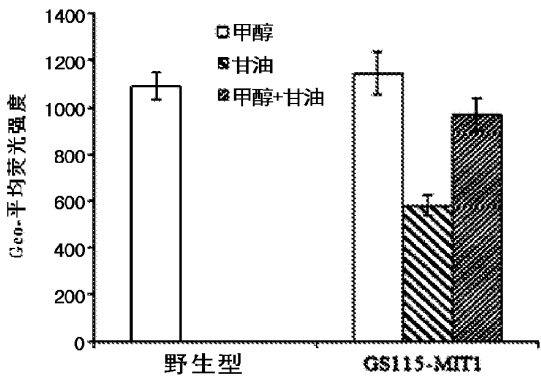


图 5

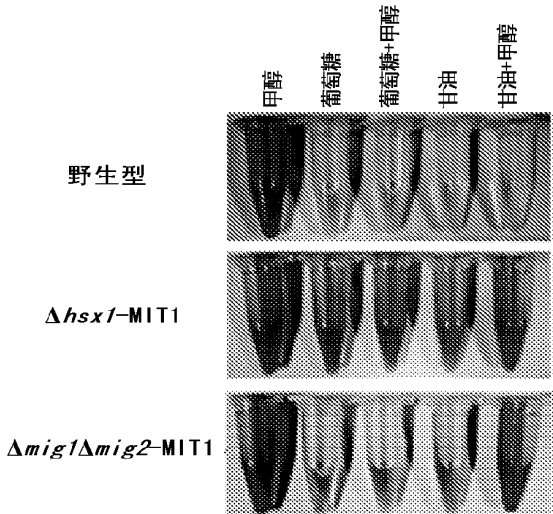


图 6

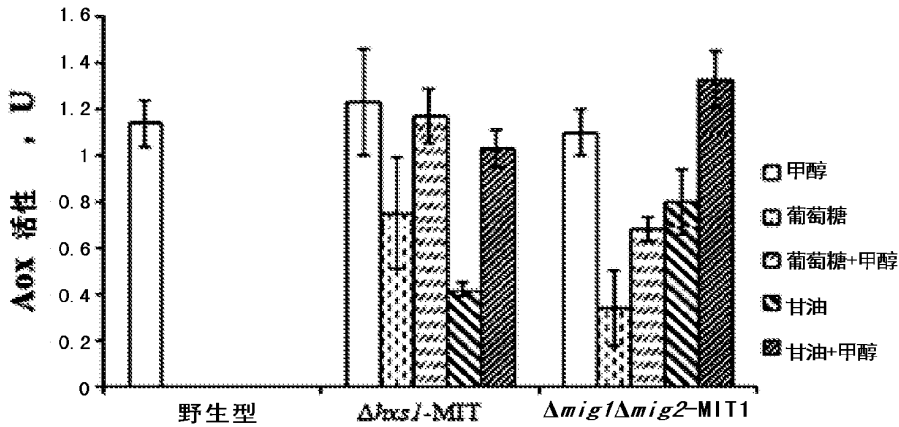


图 7

WO 2014/008729

PCT/CN2012/084642

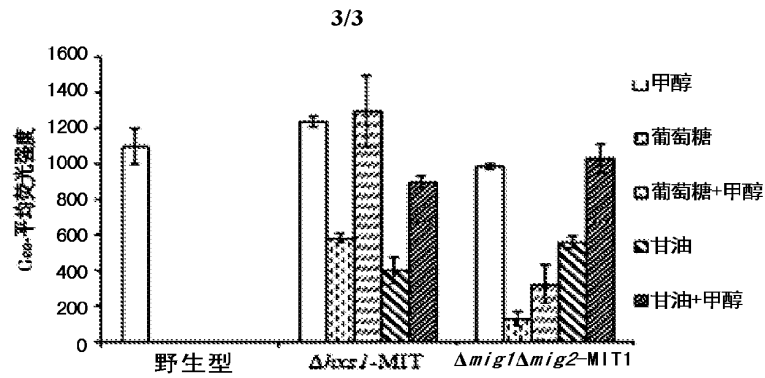


图 8

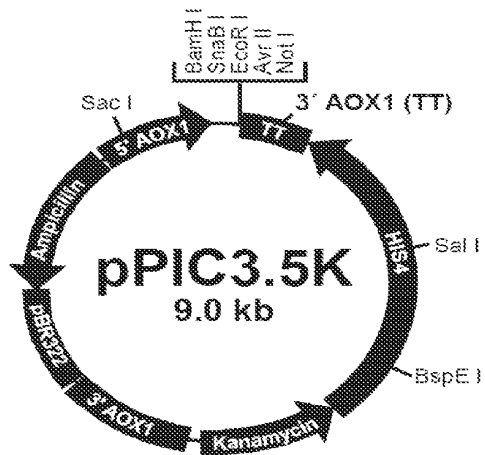


图 9

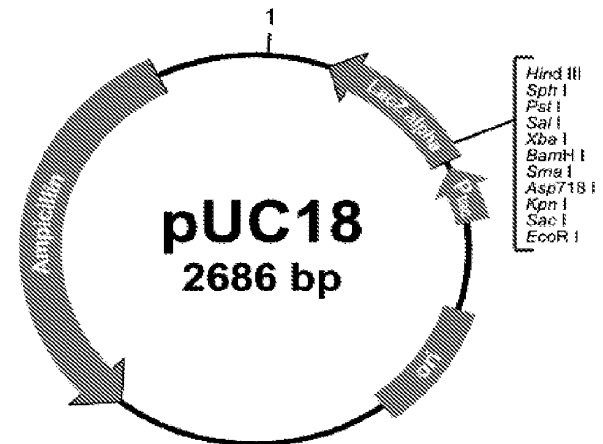


图 10

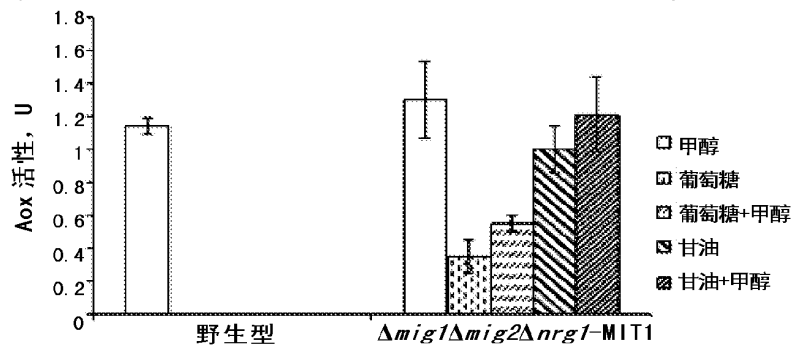


图 11

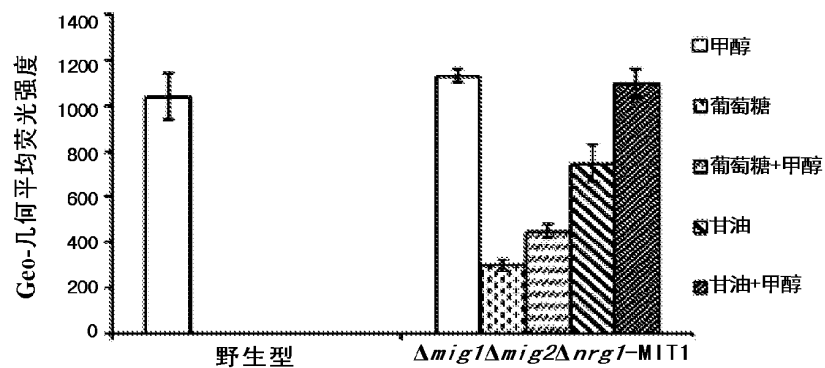


图 12

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/CN2012/084642**A. CLASSIFICATION OF SUBJECT MATTER**

See the extra sheet

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: C12N, C12R

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)

Data bases: CNABS, CPRSABS, DWPI, CPEA, SIPOABS, CNTXT, EPTXT, USTXT, WOTXT, CJFD, CNKI, ISI WEB OF KNOWLEDGE, GenBANK+EMBL+DDBJ

Search terms: transcription activating related factor, containment, carbon source, mitl, methanol, yeast, Pichia, methanol induced transcription factor, induc+, promoter, glycerol, methanol inducible promoter, eliminate+, replac+, substitute+, remov+, sequence search on sequences 1 and 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CN 102757976 A (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY), 31 October 2012 (31.10.2012), see claims 1-10	1-10
A	CN 101589146 A (NOVOZYMES A/S), 25 November 2009 (25.11.2009), see claims 1-19, and description, pages 9 and 18	1-10
A	CN 101857845 A (EAST CHINA UNIVERSITY OF SCIENCE AND TECHNOLOGY), 13 October 2010 (13.10.2010), see the whole document	1-10

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

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

Date of the actual completion of the international search
07 April 2013 (07.04.2013)Date of mailing of the international search report
18 April 2013 (18.04.2013)Name and mailing address of the ISA/CN:
State Intellectual Property Office of the P. R. China
No. 6, Xitucheng Road, Jimenqiao
Haidian District, Beijing 100088, China
Facsimile No.: (86-10) 62019451Authorized officer
WANG, Huimei
Telephone No.: (86-10) **62411090**

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2012/084642

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
 - a. (means)
 - ☐ on paper
 - ☒ in electronic form
 - b. (time)
 - ☒ in the international application as filed
 - ☐ together with the international application in electronic form
 - ☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2012/084642

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIN-CEREGHINO, G.P. et al., "Mxrlp, a key regulator of the methanol Utilization pathway and peroxisomal genes in pichia pastoris", MOLECULAR AND CELLULAR BIOLOGY, vol. 26, no. 3, pages 883-897, February 2006, see the whole document	1-10
A	DE, S.K., "Pichia pastoris GS115 hypothetical protein (PAS_chr3_0836) mRNA, complete cds", GenBank accession number: XM_002493021, 22 July 2009 (22.07.2009), see the whole document	4
A	CAI, Mengnan, "Glycerol-limitation-induced production of recombinant proteins in Hansenula polymorpha", MASTER'S DEGREE THESIS OF INSTITUTE OF MICROBIOLOGY AND BIOCHEMISTRY, NATIONAL TAIWAN UNIVERSITY, 11 May 2010 (11.05.2010), see the whole document	1-10
A	YING, Guoxin; "Screening of the Differentially Expressed Genes and Cystatin C Expression in the Deafferented Hippocampus", CHINA DOCTORAL DISSERTATIONS FULL-TEXT DATABASE (BASIC SCIENCES), ISSN 1671-6779, 2003, no. 2, 20 July 2003 (20.07.2003), see page 32	1-10

Form PCT/ISA/210 (continuation of second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2012/084642

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 102757976 A	31.10.2012	None	
CN 101589146 A	25.11.2009	US 2012142053 A1	07.06.2012
		US 2009311749 A1	17.12.2009
		WO 2008090211 A1	31.07.2008
		JP 2010516268 A	20.05.2010
		US 8143023 B2	27.03.2012
		US 8236528 B2	07.08.2012
		IN 200904439 P4	02.10.2009
		EP 2106447 A1	07.10.2009
CN 101857845 A	13.10.2010	None	

Form PCT/ISA/210 (patent family annex) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/CN2012/084642

A. CLASSIFICATION OF SUBJECT MATTER:

IPC:

C12N 15/81 (2006.01) i

C12N 1/19 (2006.01) i

C12R 1/84 (2006.01) n

C12R 1/78 (2006.01) n

C12R 1/72 (2006.01) n

C12R 1/88 (2006.01) n

国际检索报告		国际申请号 PCT/CN2012/084642
A. 主题的分类 <p style="text-align: center;">参见附加页</p> 按照国际专利分类(IPC)或者同时按照国家分类和 IPC 两种分类		
B. 检索领域 检索的最低限度文献(标明分类系统和分类号) <p style="text-align: center;">IPC: C12N, C12R</p>		
包含在检索领域中的除最低限度文献以外的检索文献 		
在国际检索时查阅的电子数据库(数据库的名称, 和使用的检索词 (如使用)) 数据库: CNABS, CPRSABS, DWPI, CPEA, SIPOABS, CNTXT, EPTXT, USTXT, WOTXT, CJFD, CNKI, ISI WEB OF KNOWLEDGE, GenBANK +EMBL+DDBJ 检索词: 甲醇, 酵母, 毕赤酵母, 转录激活相关因子, 甲醇诱导的转录因子, 甘油, 启动子, 诱导, 消除, 遏制, 取代, 代替, 碳源, 甲醇诱导型启动子, mit1, methanol, yeast, Pichia, methanol induced transcription factor, induc+, promoter, glycerol, methanol inducible promoter, eliminate+, replac+, substitute+, remov+, 关于序列 1 和 2 的序列检索		
C. 相关文件		
类 型*	引用文件, 必要时, 指明相关段落	相关的权利要求
PX	CN102757976A (华东理工大学), 31.10 月 2012 (31.10.2012), 参见权利要求 1-10	1-10
A	CN101589146A (诺维信公司), 25.11 月 2009 (25.11.2009), 参见权利要求 1-19, 说明书第 9 和 18 页	1-10
A	CN101857845A (华东理工大学), 13.10 月 2010 (13.10.2010), 参见全文	1-10
☒ 其余文件在 C 栏的续页中列出。 ☒ 见同族专利附件。		
* 引用文件的具体类型: “A” 认为不特别相关的表示了现有技术一般状态的文件 “E” 在国际申请日的当天或之后公布的在先申请或专利 “L” 可能对优先权要求构成怀疑的文件, 或为确定另一篇引用文件的公布日而引用的或者因其他特殊理由而引用的文件 (如具体说明的) “O” 涉及口头公开、使用、展览或其他方式公开的文件 “P” 公布日先于国际申请日但迟于所要求的优先权日的文件		
“T” 在申请日或优先权日之后公布, 与申请不相抵触, 但为了理解发明之理论或原理的在后文件 “X” 特别相关的文件, 单独考虑该文件, 认定要求保护的发明不是新颖的或不具有创造性 “Y” 特别相关的文件, 当该文件与另一篇或者多篇该类文件结合并且这种结合对于本领域技术人员为显而易见时, 要求保护的发明不具有创造性 “&” 同族专利的文件		
国际检索实际完成的日期 <p style="text-align: center;">07.4 月 2013 (07.04.2013)</p>		国际检索报告邮寄日期 <p style="text-align: center;">18.4 月 2013 (18.04.2013)</p>
ISA/CN 的名称和邮寄地址: 中华人民共和国国家知识产权局 中国北京市海淀区蓟门桥西土城路 6 号 100088 传真号: (86-10)62019451		受权官员 <p style="text-align: center;">王慧梅</p> 电话号码: (86-10) 62411090

PCT/ISA/210 表(第 2 页) (2009 年 7 月)

国际检索报告

国际申请号
PCT/CN2012/084642

第I栏 核苷酸和/或氨基酸序列(续第 1 页第 1.c 项)

1、关于国际申请中所公开的任何对要求保护的发明必要的核苷酸和/或氨基酸序列, 国际检索是在下列基础上进行的:

a. 序列表的提交或提供

☐ 纸件形式

☒ 电子形式

b. 提交或提供时间

☒ 包含在申请提交时的国际申请中

☐ 以电子形式与国际申请一起提交

☐ 为检索之用随后提交本单位

2、☐ 另外, 在提交/提供了多个版本或副本的序列表的情况下, 提供了关于随后提交的或附加的副本中的信息与申请时提交的申请中的信息相同或未超出申请时提交的申请中的信息范围(如适用)的所需声明。

3. 补充意见

国际检索报告

国际申请号
PCT/CN2012/084642

C(续). 相关文件

类 型	引用文件，必要时，指明相关段落	相关的权利要求
A	Geoffrey Paul Lin-Cereghino 等， “Mxr1p, a key regulator of the methanol Utilization pathway and peroxisomal genes in pichia pastoris” , Molecular AND CELLULAR BIOLOGY, 第 26 卷, 第 3 期, 第 883-897 页, 2006 年 2 月, 参见全文	1-10
A	De Schutter,K., “Pichia pastoris GS115 hypothetical protein (PAS_chr3_0836) mRNA, complete cds” , GenBank 登录号: XM_002493021, 22.7 月 2009 (22.07.2009) , 参见全文	4
A	蔡孟男, “利用甘油受限诱导汉逊氏酵母菌表现重组蛋白质”, 台湾大学微生物与生化学研究所硕士论文, 11.5 月 2010 (11.05.2010) , 参见全文	1-10
A	应国新, “去传入海马的差异表达基因的筛选和 Cystatin C 表达”, 中国优秀博士学位论文全文数据库 (基础科学辑), ISSN 1671-6779, 2003 年第 2 期, 20.7 月 2003 (20.07.2003) , 参见第 32 页	1-10

PCT/ISA/210 表(第 2 页续页) (2009 年 7 月)

国际检索报告
关于同族专利的信息

国际申请号
PCT/CN2012/084642

检索报告中引用的 专利文件	公布日期	同族专利	公布日期
CN102757976A	31.10.2012	无	
CN101589146A	25.11.2009	US2012142053 A1	07.06.2012
		US2009311749 A1	17.12.2009
		WO2008090211 A1	31.07.2008
		JP2010516268 A	20.05.2010
		US8143023 B2	27.03.2012
		US8236528 B2	07.08.2012
		IN200904439P4	02.10.2009
		EP2106447 A1	07.10.2009
CN101857845A	13.10.2010	无	

PCT/ISA/210 表(同族专利附件) (2009 年 7 月)

国际检索报告

国际申请号
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A. 主题的分类

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(54) **Title:** METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES

(57) **Abstract:** This document relates to food products containing highly conjugated heterocyclic rings complexed to an iron ion and one or more flavor precursors, and using such food products to modulate the flavor and/or aroma profile of other foods. The food products described herein can be prepared in various ways and can be formulated to be free of animal products.

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METHODS AND COMPOSITIONS FOR AFFECTING THE FLAVOR AND AROMA PROFILE OF CONSUMABLES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority to U.S. Application Serial No. 13/941,211, filed July 12, 2013, U.S. Application Serial No. 61/908,634, filed November 25, 2013, and to U.S. Application Serial No. 61/751,816, filed January 11, 2013, and is related to the
5 following co-pending patent applications: Application Serial No. PCT/US12/46560; Application Serial No PCT/US12/46552; Application Serial No. 61,876,676, filed September 11, 2013; and Application Serial No. 61/751,818, filed January 11, 2013, all of which are incorporated herein by reference.

10

TECHNICAL FIELD

This invention relates to food products and more particularly, to food products that include a highly conjugated heterocyclic ring complexed to iron such as a heme-cofactor and one or more flavor precursor molecules.

15

BACKGROUND

Food is any substance that is either eaten or drunk by any animal, including humans, for nutrition or pleasure. It is usually of plant or animal origin, and can contain essential nutrients, such as carbohydrates, fats, proteins, vitamins, or minerals. The substance is ingested by an organism and assimilated by the organism's cells in an effort
20 to produce energy, maintain life, or stimulate growth.

Food typically has its origin in a photosynthetic organism, such as a plant. Some food is obtained directly from plants, but even animals that are used as food sources are raised by feeding them food which is typically derived from plants.

In most cases, the plant or animal food source is fractionated into a variety of
25 different portions, depending upon the purpose of the food. Often, certain portions of the plant, such as the seeds or fruits, are more highly prized by humans than others and these

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are selected for human consumption, while other less desirable portions, such as the stalks of grasses, are typically used for feeding animals.

Current plant-based meat substitutes have largely failed to cause a shift to a vegetarian diet. Meat substitute compositions are typically extruded soy/grain mixtures which largely fail to replicate the experience of cooking and eating meat. Common limitations of plant-based meat substitute products are a texture and mouth-feel that are more homogenous than that of equivalent meat products. Furthermore, as these products must largely be sold pre-cooked, with artificial flavors and aromas pre-incorporated, they fail to replicate the aromas, flavors, and other key features, such as texture and mouth-feel, associated with cooking or cooked meat. As a result, these products appeal largely to a limited consumer base that is already committed to vegetarianism/veganism, but have failed to appeal to the larger consumer segment accustomed to eating meat. It would be useful to have improved plant-based meat substitutes which better replicate the aromas and flavors of meat, particularly during and/or after cooking.

15

SUMMARY

Provided herein are methods and compositions for modulating the flavor and/or aroma profile of consumable food products, including animal- or non-animal (e.g., plant) based food products, or mixtures of animal- and non-animal-based food products. In some embodiments, the methods and compositions are useful for modulating the flavor and/or aroma profile of a consumable food product during and/or after the cooking process. In some embodiments, the methods and compositions are used to generate one or more chemical compounds that modulate the flavor and/or aroma profile of the consumable food product during and/or after the cooking process.

As provided herein, and without being bound by theory, certain characteristic meaty flavors and/or aromas (e.g., beefy, bacony, umami, savory, bloody, brothy, gravy, metallic, bouillon-like; see Tables 2, 7, and 11), including one or more specific chemical compounds associated with the same (see Tables 3, 8, 9, 12, 14, 16, or 17), are believed to be produced during the cooking process of a consumable food product by chemical reaction of one or more flavor precursor molecules or compositions catalyzed by the

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presence of a highly conjugated heterocyclic ring complexed to an iron ion (e.g., a heme moiety; or a porphyrin; a porphyrinogen; a corrin; a corrinoid; a chlorin; a bacteriochlorophyll; a corphin; a chlorophyllin; a bacteriochlorin; or an isobacteriochlorin moiety complexed to an iron ion). Such highly conjugated heterocyclic moieties

5 include heterocyclic aromatic rings composed of one or more (2, 3, or 4 more) pyrrole, pyrrole-like, and/or pyrroline subunits. The highly conjugated heterocyclic ring complexed to an iron ion is referred to herein as an iron complex. In some embodiments, the heme moiety can be a heme cofactor such as a heme moiety bound to a protein; a heme moiety bound to a non-proteinaceous polymer; a heme moiety bound to a solid

10 support; or a heme moiety encapsulated in a liposome. In some embodiments, the flavors and/or aromas are not generated in the absence of the iron complex (e.g., in the absence of a ferrous chlorin) or are not generated in the absence of a heme-cofactor (e.g., in the absence of a heme-containing protein). Accordingly, as described herein, the iron complexes such as isolated chlorin-iron complexes or heme-cofactors (e.g., heme-

15 containing proteins) can be used to generate meaty flavors and/or aromas in a variety of food products, such as during the cooking process.

Combining one or more iron complexes such as a heme-cofactor (e.g., a heme-containing protein, including, for example a plant-derived heme protein such as a plant leghemoglobin (legH)), with one or more flavor precursor molecules or compositions

20 (see, e.g., Table 1 or Table 13) can generate or provide a range of savory and meaty aromas and tastes (see, e.g., Tables 2, 7, and/or 11) in a cooked consumable food product. Flavor precursor molecules or compositions can be added to the uncooked food product in purified form and/or can be derived from ingredients in the uncooked consumable food product that contain and/or are enriched with one or more of the particular flavor

25 precursors or compositions, including, for example, yeast extract, vegetable oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, canola oil, sunflower oil, coconut oil, mango oil, or an algal oil. The resultant flavor and/or aroma profile can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the type and

30 amount of iron complex (e.g., a heme cofactor such as a heme-containing protein), the

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temperature of the reaction, and the amount of water activity in the product, among other factors.

One or more flavor precursor molecules or compositions can be added along with a iron complex (e.g., ferrous chlorophyllin or a heme cofactor such as a heme-containing protein), to an uncooked food product, before and/or during the cooking process, to give the cooked consumable food product a particular meaty taste and smell, for example, the taste and smell of beef, bacon, pork, lamb, or chicken. Consumable food products can be animal or non-animal based (e.g., plant) food products, or combinations of an animal and non-animal based food product. For example, a plant based veggie burger or an animal-based burger, such as a chicken burger, can be modified with the compositions and methods of the present disclosure to result in a burger having a cooked flavor and/or aroma profile that is more meat like, e.g., beef-like, lamb-like, pork-like, turkey-like, duck-like, deer-like, yak-like, bison-like or other desirable meat flavor.

Food products for use in the present disclosure include those that have an iron-complex (e.g., a heme cofactor such as a heme-containing protein), and one or more flavor precursor molecules included therein. The iron-complex such as a heme cofactor (e.g., a heme-containing protein) and the one or more flavor precursor molecules can be homogenously or heterogeneously included in the food products. A heme protein can be isolated and purified prior to inclusion in the food product. Non-limiting examples of consumable food products which can include an iron complex such as a heme-cofactor (e.g., a heme-containing protein) and one or more flavor precursor molecules include animal-based or non-animal (e.g., plant-based), or combinations of animal-based and non-animal-based, food products in the form of hot dogs, burgers, ground meat, sausages, steaks, filets, roasts, breasts, thighs, wings, meatballs, meatloaf, bacon, strips, fingers, nuggets, cutlets, or cubes.

Consumable food products for use in the present disclosure can be flavor additive compositions, e.g., for addition to another consumable food product before, during, or after its cooking process. A flavor additive composition can include an iron complex such as a heme-cofactor (e.g., a heme-containing protein), and one or more flavor precursors.

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A flavor additive composition can include a heme protein, e.g., an isolated and purified heme protein; such a flavor additive composition can be used to modulate the flavor and/or aroma profile of a consumable food product that comprises one or more flavor precursor molecules or compositions. A flavor additive composition can include
5 one or more flavor precursor molecules or compositions; such a flavor additive composition can be used to modulate the flavor and/or aroma profile of a consumable food product that comprises the heme protein, e.g., an isolated and purified heme protein.

A flavor additive composition can be in the form, of but not limited to, soup or stew bases, bouillon, e.g., powder or cubes, flavor packets, or seasoning packets or
10 shakers. Such flavor additive compositions can be used to modulate the flavor and/or aroma profile for a variety of consumable food products, and can be added to a consumable food product before, during, or after cooking of the consumable food product.

In some embodiments, a flavor additive composition such as one including an
15 iron complex (e.g., ferrous chlorin or a heme protein) and one or more flavor precursors can be reacted (e.g., *in vitro*) with heating to generate a particular flavor and/or aroma profile of interest and the resultant product mixture can be added to the consumable food product of interest, which can then be eaten as-is or can be additionally modified, e.g., by additional cooking. In some embodiments, the iron complex can be removed from the
20 resultant product mixture before adding the product mixture to the consumable food product of interest. For example, the iron complex can be removed from the product mixture using chromatographic techniques such as column chromatography, e.g., a column containing heme or iron-chlorin.

In some embodiments, the iron complex such as a heme-cofactor, e.g., a heme-
25 protein, and the one or more flavor precursor flavor additive compositions can be soy-free, wheat-free, yeast-free, MSG-free, and free of protein hydrolysis products, and can taste meaty, highly savory, and without off odors or flavors.

In one aspect, this document features a food product that includes an iron complex such as a heme moiety, or a porphyrin, a porphyrinogen, a corrin, a corrinoid, a chlorin, a
30 bacteriochlorophyll, a corphin, a chlorophyllin, a bacteriochlorin, or an isobacteriochlorin

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moiety complexed to an iron ion and one or more flavor precursor molecules selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, inosine monophosphate (IMP), guanosine monophosphate (GMP), pyrazine, adenosine monophosphate (AMP), lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, sunflower oil, canola oil, olive oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, a protein hydrolysate, a malt extract, a yeast extract, and a peptone. The heme moiety can be a heme-containing protein, a heme moiety bound to a non-peptidic polymer; or a heme moiety bound to a solid support. The heme-containing protein can be a plant, mammalian, a yeast or filamentous fungi, or bacterial heme-containing protein. The food product can include two to one hundred, two to fifty flavor precursors, two to forty flavor precursors, two to thirty-five flavor precursors, two to ten flavor precursors, or two to six flavor precursors. In some embodiments, the one or more flavor precursor molecules are selected from the group consisting of glucose, ribose, cysteine, a cysteine derivative, thiamine, alanine, methionine, lysine, a lysine derivative, glutamic acid, a glutamic acid derivative, IMP, GMP, lactic acid, maltodextrin, creatine, alanine, arginine, asparagine, aspartate, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, linoleic acid, and mixtures thereof. The heme-containing protein can be a non-symbiotic hemoglobin or a leghemoglobin (e.g., a plant leghemoglobin such as one from soybean, alfalfa, lupin, pea, cow pea, or lupin). The heme-containing protein can include an amino acid sequence having at least 80% sequence identity to a polypeptide set forth in SEQ ID NOs:1-26. The heme-containing protein can be isolated and purified. The food product further can include a food-grade oil, a seasoning agent, a flavoring agent, a protein, a protein concentrate, an emulsifier, a gelling agent, or a fiber. The food product can be a meat

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substitute, a soup base, stew base, snack food, bouillon powder, bouillon cube, a flavor packet, or a frozen food product. Any of the food products can be free of animal products. The food product can be sealed within a packet or shaker.

This document also features a method for producing a flavor compound. The method can include combining an iron complex (e.g., a heme moiety, a porphyrin, a porphyrinogen, a corrin, a corrinoid, a chlorin, a bacteriochlorophyll, a corphin, a chlorophyllin, a bacteriochlorin, or an isobacteriochlorin complexed to an iron) and one or more flavor precursor molecules to form a mixture, the one or more flavor precursor molecules selected from the group consisting of glucose, fructose, arabinose, ribose glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, inosine monophosphate (IMP), guanosine monophosphate (GMP), pyrazine, adenosine monophosphate (AMP), lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, canola oil, olive oil, sunflower oil, flaxseed oil, coconut oil, mango oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, a protein hydrolysate, a malt extract, a yeast extract, and a peptone; and heating the mixture to form one or more flavor compounds selected from the group consisting of phenylacetaldehyde, 1-octen-3-one, 2-n-heptylfuran, 2-thiophenecarboxaldehyde, 3-thiophenecarboxaldehyde, butyrolactone, 2-undecenal, pyrazine, methyl-, furfural, 2-decanone, pyrrole, 1-octen-3-ol, 2-acetylthiazole, (E)-2-octenal, decanal, benzaldehyde, (E)-2-nonenal, pyrazine, 1-hexanol, 1-heptanol, dimethyl trisulfide, 2-nonanone, 2-pentanone, 2-heptanone, 2,3-butanedione, heptanal, nonanal, 2-octanone, 1-octanol, 3-ethylcyclopentanone, 3-octen-2-one, (E,E)-2,4-heptadienal, (Z)-2-heptenal, 2-heptanone, 6-methyl-, (Z)-4-heptenal, (E,Z)-2,6-nonadienal, 3-methyl-2-butenal, 2-pentyl-furan, thiazole, (E, E)-2,4-decadial, hexanoic acid, 1-ethyl-5-methylcyclopentene, (E,E)-2,4-nonadienal, (Z)-2-decenal, dihydro-5-pentyl-2(3H)-furanone, trans-3-nonen-2-one, (E,E)-3,5-octadien-2-

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one, (Z)-2-octen-1-ol, 5-ethyldihydro-2(3H)-furanone, 2-butenal, 1-penten-3-ol, (E)-2-hexenal, formic acid, heptyl ester, 2-pentyl-thiophene, (Z)-2-nonenal, 2-hexyl-thiophene, (E)-2-decenal, 2-ethyl-5-methyl-pyrazine, 3-ethyl-2,5-dimethyl-pyrazine, 2-ethyl-1-hexanol, thiophene, 2-methyl-furan, pyridine, butanal, 2-ethyl-furan, 3-methyl-butanal, trichloromethane, 2-methyl-butanal, methacrolein, 2-methyl-propanal, propanal, acetaldehyde, 2-propyl-furan, dihydro-5-propyl-2(3H)-furanone, 1,3-hexadiene, 4-decyne, pentanal, 1-propanol, heptanoic acid, trimethyl-ethanethiol, 1-butanol, 1-penten-3-one, dimethyl sulfide, 2-ethyl furan, 2-pentyl-thiophene, 2-propenal, 2-tridecen-1-ol, 4-octene, 2-methyl thiazole, methyl-pyrazine, 2-butanone, 2-pentyl-furan, 2-methyl-propanal, butyrolactone, 3-methyl-butanal, methyl-thiirane, 2-hexyl-furan, butanal, 2-methyl-butanal, 2-methyl-furan, furan, octanal, 2-heptenal, 1-octene, formic acid heptyl ester, 3-pentyl-furan, and 4-penten-2-one. The heme moiety can be a heme-containing protein, a heme moiety bound to a non-peptidic polymer; or a heme moiety bound to a solid support. The method can include combining cysteine, ribose, lactic acid, lysine, and/or thiamine with the heme-containing protein.

In another aspect, this document features a method for producing a flavor compound. The method includes combining an iron complex, such as a heme-containing protein, and one or more flavor precursor molecules to form a mixture, the one or more flavor precursor molecules selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, IMP, GMP, pyrazine, AMP, lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, sunflower oil, canola oil, flaxseed oil, coconut oil, mango oil, a free fatty acid, methionine, cysteine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, a protein hydrolysate, a malt extract, a yeast extract, and a peptone; and heating the mixture to form one or more

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flavor compounds set forth in Tables 3, 8, or 9. For example, the flavor precursors can include cysteine, a sugar, and one or more other precursors.

This document also features a method for imparting a meat like flavor (e.g., beef-like, chicken like, pork-like, lamb-like, turkey-like, duck-like, deer-like, or bison-like) to a food product. The method includes contacting the food product with a flavoring composition, the flavoring composition comprising i) an iron complex, such as a heme moiety (e.g., a heme-containing protein); and ii) one or more flavor precursor molecules selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin, inosine, IMP, GMP, pyrazine, AMP, lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, vegetable oil, algal oil, corn oil, soybean oil, palm fruit oil, palm kernel oil, safflower oil, flaxseed oil, rice bran oil, cottonseed oil, olive oil, sunflower oil, canola oil, flaxseed oil, coconut oil, mango oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, a protein hydrolysate, a malt extract, a yeast extract, and a peptone; wherein after heating the food product and the flavoring composition together, a meat like flavor (e.g., beef-like, chicken like, pork-like, lamb-like, turkey-like, duck-like, deer-like, or bison-like) is imparted to the food product. In some embodiments, the iron complex is removed from the food product. The flavoring composition further can include a seasoning agent, a flavoring agent, a protein, a protein concentrate, or an emulsifier. The flavoring composition can be sealed within a packet or shaker.

In another aspect, this document features a method of making a food product. The method includes combining an isolated heme-containing protein and one or more flavor precursor molecules to form a mixture, the one or more flavor precursor molecules selected from the group consisting of glucose, fructose, ribose, arabinose, glucose-6-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, inositol, maltose, sucrose, maltodextrin, glycogen, nucleotide-bound sugars, molasses, a phospholipid, a lecithin,

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inosine, IMP, GMP, pyrazine, AMP, lactic acid, succinic acid, glycolic acid, thiamine, creatine, pyrophosphate, sunflower oil, coconut oil, canola oil, flaxseed oil, mango oil, a free fatty acid, cysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate,
5 glutamine, glycine, proline, serine, tyrosine, glutathione, an amino acid derivative, a protein hydrolysate, a malt extract, a yeast extract, and a peptone; and heating the mixture.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention pertains. Although methods and materials similar or equivalent to those
10 described herein can be used to practice the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials,
15 methods, and examples are illustrative only and not intended to be limiting.

The details of one or more embodiments of the invention are set forth in the accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and drawings, and from the claims. The word “comprising” in the claims may be replaced by “consisting
20 essentially of” or with “consisting of,” according to standard practice in patent law.

DESCRIPTION OF THE DRAWINGS

FIG. 1 contains amino acid sequences of exemplary heme-containing proteins.

FIG. 2 is a bar graph of the beefiness rating of the meat replica with or without the Magic Mix, both samples in triplicate with 1% w/v LegH protein. Tasters rated beefiness
25 on a scale from 1-7, with 1 being not beefy at all and 7 being exactly like ground beef.

DETAILED DESCRIPTION

This document is based on methods and materials for modulating the taste and/or aroma profile of food products. As described herein, compositions containing one or

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more flavor precursors and one or more highly conjugated heterocyclic rings complexed to an iron (referred to herein as an iron complex) can be used to modulate the taste and/or aroma profile of food products. Such iron complexes include heme moieties or other highly conjugated heterocyclic rings complexed to an iron ion (referred to as an iron complex). “Heme” refers to a prosthetic group bound to iron (Fe^{2+} or Fe^{3+}) in the center of a porphyrin ring. Thus, an iron complex can be a heme moiety, or a porphyrin, porphyrinogen, corrin, corrinoid, chlorin, bacteriochlorophyll, corphin, chlorophyllin, bacteriochlorin, or isobacteriochlorin moiety complexed to iron ion. The heme moiety that can be used to modulate the taste and/or aroma profile of food products can be a heme cofactor such as a heme-containing protein; a heme moiety bound to a non-peptidic polymer or other macromolecule such as a liposome, a polyethylene glycol, a carbohydrate, a polysaccharide, a cyclodextrin, a polyethylenimine, a polyacrylate, or derivatives thereof; a siderophore (i.e., an iron chelating compound); or a heme moiety bound to a solid support (e.g., beads) composed of a chromatography resin, cellulose, graphite, charcoal, or diatomaceous earth.

In some embodiments, the iron complexes catalyze some reactions and produce flavor precursors without heating or cooking. In some embodiments, the iron complex destabilizes upon heating or cooking and releases the iron, e.g., the protein is denatured, so flavor precursors can be generated.

Suitable flavor precursors include sugars, sugar alcohols, sugar derivatives, oils (e.g., vegetable oils), free fatty acids, alpha-hydroxy acids, dicarboxylic acids, amino acids and derivatives thereof, nucleosides, nucleotides, vitamins, peptides, protein hydrolysates, extracts, phospholipids, lecithin, and organic molecules. Non-limiting examples of such flavor precursors are provided in Table 1.

TABLE 1**Flavor Precursor Molecules**

Sugars, sugar alcohols, sugar acids, and sugar derivatives: glucose, fructose, ribose, sucrose, arabinose, glucose-6-phosphate, fructose-6-phosphate, fructose 1,6-diphosphate, inositol, maltose, molasses, maltodextrin, glycogen, galactose,

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lactose, ribitol, gluconic acid and glucuronic acid, amylose, amylopectin, or xylose
Oils: coconut oil, mango oil, sunflower oil, cottonseed oil, safflower oil, rice bran oil, cocoa butter, palm fruit oil, palm oil, soybean oil, canola oil, corn oil, sesame oil, walnut oil, flaxseed, jojoba oil, castor, grapeseed oil, peanut oil, olive oil, algal oil, oil from bacteria or fungi
Free fatty acids: caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, palmitoleic acid, stearic, oleic acid, linoleic acid, alpha linolenic acid, gamma linolenic acid, arachidic acid, arachidonic acid, behenic acid, or erucic acid
Amino acids and derivatives thereof: cysteine, cystine, a cysteine sulfoxide, allicin, selenocysteine, methionine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophan, 5-hydroxytryptophan, valine, arginine, histidine, alanine, asparagine, aspartate, glutamate, glutamine, glycine, proline, serine, or tyrosine
Nucleosides and Nucleotides: inosine, inosine monophosphate (IMP), guanosine, guanoside monophosphate (GMP), adenosine, adenosine monophosphate (AMP)
Vitamins: thiamine, vitamin C, Vitamin D, Vitamin B6, or Vitamin E
Misc: phospholipid, lecithin, pyrazine, creatine, pyrophosphate
Acids: acetic acid, alpha hydroxy acids such as lactic acid or glycolic acid, tricarboxylic acids such as citric acid, dicarboxylic acids such as succinic acid or tartaric acid
Peptides and protein hydrolysates: glutathione, vegetable protein hydrolysates, soy protein hydrolysates, yeast protein hydrolysates, algal protein hydrolysates, meat protein hydrolysates
Extracts: a malt extract, a yeast extract, and a peptone

In some embodiments, one flavor precursor or combinations of two to one hundred flavor precursors, two to ninety, two to eighty, two to seventy, two to sixty, or two to fifty flavor precursors are used. For example, combinations of two to forty flavor

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precursors, two to thirty-five flavor precursors, two to ten flavor precursors, or two to six flavor precursors can be used with the one or more iron complexes (e.g., heme co-factors such as a heme-containing proteins). For example, the one or more flavor precursors can be glucose, ribose, cysteine, a cysteine derivative, thiamine, lysine, a lysine derivative, glutamic acid, a glutamic acid derivative, alanine, methionine, IMP, GMP, lactic acid, and mixtures thereof (e.g., glucose and cysteine; cysteine and ribose; cysteine, glucose or ribose, and thiamine; cysteine, glucose or ribose, IMP, and GMP; cysteine, glucose or ribose, and lactic acid). For example, the one or more flavor precursors can be alanine, arginine, asparagine, aspartate, cysteine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine, tryptophan, tyrosine, valine, glucose, ribose, maltodextrin, thiamine, IMP, GMP, lactic acid, and creatine.

As used herein, the term “heme containing protein” can be used interchangeably with “heme containing polypeptide” or “heme protein” or “heme polypeptide” and includes any polypeptide that can covalently or noncovalently bind a heme moiety. In some embodiments, the heme-containing polypeptide is a globin and can include a globin fold, which comprises a series of seven to nine alpha helices. Globin type proteins can be of any class (e.g., class I, class II, or class III), and in some embodiments, can transport or store oxygen. For example, a heme-containing protein can be a non-symbiotic type of hemoglobin or a leghemoglobin. A heme-containing polypeptide can be a monomer, i.e., a single polypeptide chain, or can be a dimer, a trimer, tetramer, and/or higher order oligomers. The life-time of the oxygenated Fe^{2+} state of a heme-containing protein can be similar to that of myoglobin or can exceed it by 10%, 20%, 30% 50%, 100% or more under conditions in which the heme-protein-containing consumable is manufactured, stored, handled or prepared for consumption. The life-time of the unoxygenated Fe^{2+} state of a heme-containing protein can be similar to that of myoglobin or can exceed it by 10%, 20%, 30% 50%, 100% or more under conditions in which the heme-protein-containing consumable is manufactured, stored, handled or prepared for consumption

Non-limiting examples of heme-containing polypeptides can include an androglobin, a cytoglobin, a globin E, a globin X, a globin Y, a hemoglobin, a

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myoglobin, an erythrocrucorin, a beta hemoglobin, an alpha hemoglobin, a protoglobin, a cyanoglobin, a cytoglobin, a histoglobin, a neuroglobins, a chlorocruorin, a truncated hemoglobin (e.g., HbN or HbO), a truncated 2/2 globin, a hemoglobin 3 (e.g., Glb3), a cytochrome, or a peroxidase.

5 Heme-containing proteins that can be used in the compositions and food products described herein can be from mammals (e.g., farm animals such as cows, goats, sheep, pigs, ox, or rabbits), birds, plants, algae, fungi (e.g., yeast or filamentous fungi), ciliates, or bacteria. For example, a heme-containing protein can be from a mammal such as a farm animal (e.g., a cow, goat, sheep, pig, ox, or rabbit) or a bird such as a turkey or
10 chicken. Heme-containing proteins can be from a plant such as *Nicotiana tabacum* or *Nicotiana glauca* (tobacco); *Zea mays* (corn), *Arabidopsis thaliana*, a legume such as *Glycine max* (soybean), *Cicer arietinum* (garbanzo or chick pea), *Pisum sativum* (pea) varieties such as garden peas or sugar snap peas, *Phaseolus vulgaris* varieties of common beans such as green beans, black beans, navy beans, northern beans, or pinto beans,
15 *Vigna unguiculata* varieties (cow peas), *Vigna radiata* (Mung beans), *Lupinus albus* (lupin), or *Medicago sativa* (alfalfa); *Brassica napus* (canola); *Triticum sps.* (wheat, including wheat berries, and spelt); *Gossypium hirsutum* (cotton); *Oryza sativa* (rice); *Zizania sps.* (wild rice); *Helianthus annuus* (sunflower); *Beta vulgaris* (sugarbeet); *Pennisetum glaucum* (pearl millet); *Chenopodium sp.* (quinoa); *Sesamum sp.* (sesame);
20 *Linum usitatissimum* (flax); or *Hordeum vulgare* (barley). Heme-containing proteins can be isolated from fungi such as *Saccharomyces cerevisiae*, *Pichia pastoris*, *Magnaporthe oryzae*, *Fusarium graminearum*, *Aspergillus oryzae*, *Trichoderma reesei*, *Myceliophthora thermophila*, *Kluyvera lactis*, or *Fusarium oxysporum*. Heme-containing proteins can be isolated from bacteria such as *Escherichia coli*, *Bacillus subtilis*, *Bacillus licheniformis*,
25 *Bacillus megaterium*, *Synechocystis sp.*, *Aquifex aeolicus*, *Methylobacterium thermophilum*, or thermophilic bacteria such as *Thermophilus*. The sequences and structure of numerous heme-containing proteins are known. See for example, Reedy, *et al.*, *Nucleic Acids Research*, 2008, Vol. 36, Database issue D307–D313 and the Heme Protein Database available on the world wide web at <http://hemeprotein.info/heme.php>.

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For example, a non-symbiotic hemoglobin can be from a plant selected from the group consisting of soybean, sprouted soybean, alfalfa, golden flax, black bean, black eyed pea, northern, garbanzo, moong bean, cowpeas, pinto beans, pod peas, quinoa, sesame, sunflower, wheat berries, spelt, barley, wild rice, or rice.

5 Any of the heme-containing proteins described herein that can be used for producing food products can have at least 70% (e.g., at least 75%, 80%, 85%, 90%, 95%, 97%, 98%, 99%, or 100%) sequence identity to the amino acid sequence of the corresponding wild-type heme-containing protein or fragments thereof that contain a heme-binding motif. For example, a heme-containing protein can have at least 70%
10 sequence identity to an amino acid sequence set forth in FIG. 1, including a non-symbiotic hemoglobin such as that from *Vigna radiata* (SEQ ID NO:1), *Hordeum vulgare* (SEQ ID NO:5), *Zea mays* (SEQ ID NO:13), *Oryza sativa subsp. japonica* (rice) (SEQ ID NO:14), or *Arabidopsis thaliana* (SEQ ID NO:15), a Hell's gate globin I such as that from *Methylobacterium thermophilum* (SEQ ID NO:2), a flavohemoprotein such as that
15 from *Aquifex aeolicus* (SEQ ID NO:3), a leghemoglobin such as that from *Glycine max* (SEQ ID NO:4), *Pisum sativum* (SEQ ID NO:16), or *Vigna unguiculata* (SEQ ID NO:17), a heme-dependent peroxidase such as from *Magnaporthe oryzae*, (SEQ ID NO:6) or *Fusarium oxysporum* (SEQ ID NO:7), a cytochrome c peroxidase from *Fusarium graminearum* (SEQ ID NO:8), a truncated hemoglobin from *Chlamydomonas*
20 *moewusii* (SEQ ID NO:9), *Tetrahymena pyriformis* (SEQ ID NO:10, group I truncated), *Paramecium caudatum* (SEQ ID NO:11, group I truncated), a hemoglobin from *Aspergillus niger* (SEQ ID NO:12), or a mammalian myoglobin protein such as the *Bos taurus* (SEQ ID NO:18) myoglobin, *Sus scrofa* (SEQ ID NO:19) myoglobin, *Equus caballus* (SEQ ID NO:20) myoglobin, a heme-protein from *Nicotiana benthamiana* (SEQ
25 ID NO:21), *Bacillus subtilis* (SEQ ID NO:22), *Corynebacterium glutamicum* (SEQ ID NO:23), *Synechocystis* PCC6803 (SEQ ID NO:24), *Synechococcus* sp. PCC 7335 (SEQ ID NO:25), or *Nostoc commune* (SEQ ID NO:26).

The percent identity between two amino acid sequences can be determined as follows. First, the amino acid sequences are aligned using the BLAST 2 Sequences
30 (Bl2seq) program from the stand-alone version of BLASTZ containing BLASTP version

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2.0.14. This stand-alone version of BLASTZ can be obtained from Fish & Richardson's web site (e.g., www.fr.com/blast/) or the U.S. government's National Center for Biotechnology Information web site (www.ncbi.nlm.nih.gov). Instructions explaining how to use the Bl2seq program can be found in the readme file accompanying BLASTZ.

5 Bl2seq performs a comparison between two amino acid sequences using the BLASTP algorithm. To compare two amino acid sequences, the options of Bl2seq are set as follows: -i is set to a file containing the first amino acid sequence to be compared (e.g., C:\seq1.txt); -j is set to a file containing the second amino acid sequence to be compared (e.g., C:\seq2.txt); -p is set to blastp; -o is set to any desired file name (e.g., C:\output.txt);

10 and all other options are left at their default setting. For example, the following command can be used to generate an output file containing a comparison between two amino acid sequences: C:\Bl2seq -i c:\seq1.txt -j c:\seq2.txt -p blastp -o c:\output.txt. If the two compared sequences share homology, then the designated output file will present those regions of homology as aligned sequences. If the two compared sequences do not

15 share homology, then the designated output file will not present aligned sequences. Similar procedures can be following for nucleic acid sequences except that blastn is used.

Once aligned, the number of matches is determined by counting the number of positions where an identical amino acid residue is presented in both sequences. The percent identity is determined by dividing the number of matches by the length of the

20 full-length polypeptide amino acid sequence followed by multiplying the resulting value by 100. It is noted that the percent identity value is rounded to the nearest tenth. For example, 78.11, 78.12, 78.13, and 78.14 is rounded down to 78.1, while 78.15, 78.16, 78.17, 78.18, and 78.19 is rounded up to 78.2. It also is noted that the length value will always be an integer.

25 It will be appreciated that a number of nucleic acids can encode a polypeptide having a particular amino acid sequence. The degeneracy of the genetic code is well known to the art; i.e., for many amino acids, there is more than one nucleotide triplet that serves as the codon for the amino acid. For example, codons in the coding sequence for a given enzyme can be modified such that optimal expression in a particular species (e.g.,

30 bacteria or fungus) is obtained, using appropriate codon bias tables for that species.

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Heme-containing proteins can be extracted from the source material (e.g.,
extracted from animal tissue, or plant, fungal, algal, or bacterial biomass, or from the
culture supernatant for secreted proteins) or from a combination of source materials (e.g.,
multiple plant species). Leghemoglobin is readily available as an unused by-product of
5 commodity legume crops (e.g., soybean, alfalfa, or pea). The amount of leghemoglobin
in the roots of these crops in the United States exceeds the myoglobin content of all the
red meat consumed in the United States.

In some embodiments, extracts of heme-containing proteins include one or more
non-heme-containing proteins from the source material (e.g., other animal, plant, fungal,
10 algal, or bacterial proteins) or from a combination of source materials (e.g., different
animal, plant, fungi, algae, or bacteria).

In some embodiments, heme-containing proteins are isolated and purified from
other components of the source material (e.g., other animal, plant, fungal, algal, or
bacterial proteins). As used herein, the term “isolated and purified” indicates that the
15 preparation of heme-containing protein is at least 60% pure, e.g., greater than 65%, 70%,
75%, 80%, 85%, 90%, 95%, or 99% pure. Without being bound by theory, isolating and
purifying proteins can allow the food products to be made with greater consistency and
greater control over the properties of the food product as unwanted material is eliminated.
Proteins can be separated on the basis of their molecular weight, for example, by size
20 exclusion chromatography, ultrafiltration through membranes, or density centrifugation.
In some embodiments, the proteins can be separated based on their surface charge, for
example, by isoelectric precipitation, anion exchange chromatography, or cation
exchange chromatography. Proteins also can be separated on the basis of their solubility,
for example, by ammonium sulfate precipitation, isoelectric precipitation, surfactants,
25 detergents or solvent extraction. Proteins also can be separated by their affinity to
another molecule, using, for example, hydrophobic interaction chromatography, reactive
dyes, or hydroxyapatite. Affinity chromatography also can include using antibodies
having specific binding affinity for the heme-containing protein, nickel NTA for His-
tagged recombinant proteins, lectins to bind to sugar moieties on a glycoprotein, or other
30 molecules which specifically binds the protein.

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Heme-containing proteins also can be recombinantly produced using polypeptide expression techniques (e.g., heterologous expression techniques using bacterial cells, insect cells, fungal cells such as yeast, plant cells such as tobacco, soybean, or *Arabidopsis*, or mammalian cells). In some cases, standard polypeptide synthesis techniques (e.g., liquid-phase polypeptide synthesis techniques or solid-phase polypeptide synthesis techniques) can be used to produce heme-containing proteins synthetically. In some cases, *in vitro* transcription-translation techniques can be used to produce heme-containing proteins.

The protein used in the consumable may be soluble in a solution. In some embodiments, the isolated and purified proteins are soluble in solution at greater than 5, 10, 15, 20, 25, 50, 100, 150, 200, or 250g/L.

In some embodiments, the isolated and purified protein is substantially in its native fold and water soluble. In some embodiments, the isolated and purified protein is more than 50, 60, 70, 80, or 90% in its native fold. In some embodiments, the isolated and purified protein is more than 50, 60, 70, 80, or 90% water soluble.

Modulating Flavor and/or Aroma Profiles

As described herein, different combinations of flavor precursors can be used with one or more iron complexes (e.g., a ferrous chlorin, a chlorin-iron complex, or a heme-cofactor such as a heme-containing protein or heme bound to a non-peptidic polymer such as polyethylene glycol or to a solid support) to produce different flavor and aroma profiles when the flavor precursors and iron complexes are heated together (e.g., during cooking). The resultant flavor and/or aroma profile can be modulated by the type and concentration of the flavor precursors, the pH of the reaction, the length of cooking, the type and amount of iron complex (e.g., a heme-cofactor such as heme-containing protein, heme bound to non-peptidic polymer or macromolecule, or heme bound to a solid support), the temperature of the reaction, and the amount of water activity in the product, among other factors. In embodiments in which a heme moiety is bound to a solid support such as cellulose or a chromatography resin, graphite, charcoal, or diatomaceous earth, the solid support (e.g., beads) can be incubated with sugars and/or one or more other

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flavor precursors to generate flavors, and then the solid support with attached heme moiety can be re-used, i.e., incubated again with sugars and/or one or more other flavor precursors to generate flavors.

5 Table 2 provides non-limiting examples of flavor types that can be generated by combining one or more flavor precursors and one or more heme co-factors (e.g., heme-containing proteins). See also Tables 7 and/or 11.

TABLE 2

Flavor Types	
beef	beef broth
beef dripping	cheesy
cold-cut deli meat	squash
bacon	sharp
meaty	fruity
brothy	floral
ramen	musty
egg	fried food
malty	caramel
bready	barbeque
sulfur	chocolate
fried chicken	sweet
browned	potato
pretzel	french toast
grassy	breadcrust
bloody	mushroom
broccoli	chicken
brothy	cumin
buttery	umami
metallic	raisin

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yeasty	goaty
vegetable broth	

Flavor and aroma profiles are created by different chemical compounds formed by chemical reactions between the heme co-factor (e.g., heme-containing protein) and flavor precursors. Gas chromatography–mass spectrometry (GCMS) can be used to separate and identify the different chemical compounds within a test sample. For example, volatile chemicals can be isolated from the head space after heating a heme-containing protein and one or more flavor precursors.

Table 3 provides non-limiting examples of compounds that can be produced. See also Tables 8, 9, 12, and/or 14.

TABLE 3
Compounds Produced

phenylacetaldehyde	2-butenal, 2-ethyl-	1,3-hexadiene
1-octen-3-one	acetonitrile	4-decyne
2-n-heptylfuran		pentanal
2-thiophenecarboxaldehyde	(E)-2-Hexenal	1-propanol
3-thiophenecarboxaldehyde	4-ethyl-phenol,	heptanoic acid
1-octene	3-octanone	ethanethiol
butyrolactone	styrene	2-methyl-1-heptene
2-undecenal	furan, 3-pentyl-	(E)-4-octene
propyl-cyclopropane	formic acid, heptyl ester	2-methyl-2-heptene
methyl-pyrazine	(E)-2-Heptenal	pentanoic acid
1-hydroxy-propanone	6-methyl-5-hepten-2-one	nonanoic acid
acetic acid	n-caproic acid vinyl ester	1,3-dimethyl-benzene
furfural	2-ethyl-2-hexenal	
2-decanone	1-hepten-3-ol	toluene
pyrrole	1-ethyl-1-methyl-cyclopentane	1-butanol
1-octen-3-ol	3-ethyl-2-methyl-1,3-hexadiene	2,3,3-trimethyl-pentane
2-acetylthiazole	2-pentyl-thiophene	isopropyl alcohol
(E)-2-octenal	(Z)-2-nonenal	2,2,4,6,6-pentamethyl-heptane
decanal	2-n-octylfuran	phenol

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benzaldehyde	2-hexyl-thiophene	1-penten-3-one
(E)-2-Nonenal	4-cyclopentene-1,3-dione	dimethyl sulfide
pyrazine	1-nonanol	thiirane
1-pentanol	(E)-2-decenal	(E)-2-octen-1-ol
trans-2-(2-pentenyl)furan	4-ethyl-benzaldehyde	2,4-dimethyl-1-heptene
1-hexanol	1,7-octadien-3-ol	1,3-bis(1,1-dimethylethyl)-benzene
1-heptanol	octanoic acid	heptane
dimethyl trisulfide	2-ethyl-5-methyl-pyrazine	4,7-dimethyl-undecane
2-nonanone	3-ethyl-2,5-dimethyl-pyrazine	acetophenone
2-pentanone	1,3,5-cycloheptatriene	tridecane
2-heptanone	2-ethyl-1-hexanol	thiophosphoramidate, s-methyl ester
2,3-butanedione	4-methyl-octanoic acid	2-methyl-thiazole
heptanal	m-aminophenylacetylene	3-(1-methylethoxy)-propanenitrile,
nonanal	benzene	2,4-bis(1,1-dimethylethyl)-phenol
2-octanone	thiophene	3-ethyl-2,2-dimethyl-pentane
2-butanone	2-methyl-furan	3-ethyl-pentane
octanal	pyridine	2,3,4-trimethyl-pentane
1-octanol	furan	2,4,6-trimethyl-octane
3-ethylcyclopentanone	butanal	2,6-dimethyl-nonane
8-methyl-1-undecene	2-ethyl-furan	2-hexyl-furan
3-octen-2-one	carbon disulfide	4-methyl-5-thiazoleethanol
2,4-Heptadienal, (E,E)-	Furan, 2-hexyl-:2	4-penten-2-one
(Z)-2-heptenal	3-methyl-butanal	4-methylthiazole
6-methyl-2-heptanone	2-methyl-butanal	2-methyl-3-pentanone
(Z)-4-heptenal	methacrolein	2,3-pentanedione
(E,Z)-2,6-nonadienal	octane	(E)-2-tridecen-1-ol
3-methyl-2-butanal	ethanol	2-thiophenemethanamine
2-pentyl-furan	2-methyl-propanal	(Z)-2-nonenal,
thiazole	acetone	methyl thiolacetate
(E,E)-2,4-decadienal	propanal	methyl ethanoate
hexanoic acid	methyl-thiirane	isothiazole
1-ethyl-5-methylcyclopentene	acetaldehyde	3,3-dimethyl-hexane

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(E,E)-2,4-nonadienal	2-propenal	4-methyl-heptane
(Z)-2-decenal	2-propyl-furan	2,4-dimethyl-heptane
dihydro-5-pentyl-2(3h)-furanone	dihydro-5-propyl-2(3H)-furanone	2,3,4-trimethyl-heptane
trans-3-nonen-2-one	dihydro-3-(2H)-thiophenone	2-methyl-heptane
(E,E)-3,5-octadien-2-one	2,2,6-trimethyl-decane	2-methyl-3-furanthiol
(Z)-2-octen-1-ol	3,3'-dithiobis[2-methyl-furan	4-amino-1,2,5-oxadiazole-3-carbonitrile
5-ethyldihydro-2(3h)-furanone	1-heptene	1,2-benzisothiazol-3(2H)-one
2-butenal	1,3-octadiene	2-acetyl-propen-2-ol,
1-penten-3-ol	1-nonene	1-decen-3-one
1-(ethylthio)-2-(methylthio)-buta-1,3-diene		

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein is heated in the presence of ground chicken, to increase specific volatile flavor and odorant components typically elevated in beef. For example, propanal, butanal, 2-ethyl-furan, heptanal, octanal, trans-2-(2-pentenyl)furan, (Z)-2-heptenal, (E)-2-octenal, pyrrole, 2,4-dodecadialenal, 1-octanal, (Z)-2-decenal, or 2-undecenal can be increased in the presence of the heme-containing protein, which can impart a more beefy flavor to the chicken.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein is heated in the presence of cysteine and glucose or other combinations of flavor precursors to provide a different profile of volatile odorants than when any subset of the three components are used individually. Volatile flavor components that are increased under these conditions include but are not limited to furan, acetone, thiazole, furfural, benzaldehyde, 2-pyridinecarboxaldehyde, 5-methyl-2-thiophenecarboxaldehyde, 3-methyl-2-thiophenecarboxaldehyde, 3-thiophenemethanol and decanol. See, e.g., Tables 8 and 9. Under these conditions, cysteine and glucose alone or in the presence of iron salts such as ferrous glucanate produced a sulfurous, odor, but addition of heme-containing proteins reduced the

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sulfurous odor and replaced it with flavors including but not limited to chicken broth, burnt mushroom, molasses, and bread.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein is heated in the presence of cysteine
5 and ribose to provide a different profile of volatile odorants. Heating in the presence of ribose created some additional compounds as compared to when a heme-containing protein and glucose were heated together. See Tables 8 and 9.

In some embodiments, an iron complex (e.g., a ferrous chlorophyllin or a heme-cofactor such as a heme-containing protein) described herein can be heated in the
10 presence of thiamine and a sugar to affect the formation of 5-Thiazoleethanol, 4-methyl-furan, 3,3'-dithiobis[2-methyl-furan, and/or 4-Methylthiazole. These compounds are known to be present in meat and have beefy, meaty taste notes.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein can be heated in the presence of a
15 nucleotide such as inosine monophosphate and/or guanosine monophosphate to control the formation of flavor compounds such as (E)-4-octene, 2-ethyl-furan, 2-pentanone, 2,3-butanedione, 2-methyl-thiazole, methyl-pyrazine, tridecane, (E)-2-octenal, 2-thiopenecarboxaldehyde, and/or 3-thiopenecarboxaldehyde. These compounds are known to be present in meat and have a beefy, meaty, buttery, and or savory flavor notes.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein can be heated in the presence of
20 lysine, a sugar such as ribose, and cysteine to control the formation of flavor compounds such as dimethyl trisulfide, nonanal, 2-pentyl thiophene, 2-nonenal furfural, 1-octanol, 2-nonenal, thiazole, 2-acetylthiazole, phenylacetaldehyde, and/or 2-acetylthiazole. These
25 compounds are known to be present in meat and some have a beefy, meaty, and or savory flavor.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein can be heated in the presence of
30 lactic acid, a sugar such as ribose, and cysteine to control the formation of the flavor compounds nonanal, thiazole, 2-acetylthiazole, and/or 8-methyl 1-undecene. These

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compounds are known to be present in meat and have beefy, savory, browned, bready, and malty notes.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein can be heated in the presence of amino acids, sugars such as glucose, ribose, and maltodextrin, lactic acid, thiamine, IMP, GMP, creatine, and salts such as potassium chloride and sodium chloride, to control the formation of flavor compounds such as 1,3-bis(1,1-dimethylethyl)-benzene, 2-methyl 3-furanthiol, and/or bis(2-methyl-4,5-dihydro-3-furyl) disulfide. These compounds are known to be present in meat and have beefy notes. See also Table 14.

In some embodiments, a particular type of heme-containing protein is chosen to control the formation of flavor compounds. See, for example, the results of Table 9, which shows that the addition of different types of heme-proteins (LegH, Barley, *B. myoglobin*, or *A. aeolicus*) in flavor reaction mixtures containing one or more flavor precursor compounds results in many of the same key meat flavors, including but not limited to pentanone, 3-methyl butanal, 2-methyl butanal, 2-heptenal, 1-octene, nonanal, 2-propenal, 2-decenal, 2-nonanone, 2-octanone, 2-tridecen-1-ol, 2-octanone, 2-octenal, 4-methyl-2-heptanone, octanal, 2-undecenal, butyrolactone, 1-octen-3-one, 3-methylheptyl acetate, and 2-pentyl-thiophene. These differences in flavor compounds can change the overall taste profile.

In some embodiments, an iron complex (e.g., a ferrous chlorin or a heme-cofactor such as a heme-containing protein) described herein and one or more flavor precursors can be reacted (e.g., *in vitro*) with heating to generate a particular flavor and/or aroma profile of interest and the resultant flavor additive composition can be added to the consumable food product of interest, which can then be eaten as-is or can be additionally modified, e.g., by additional cooking.

In some embodiments, any undesirable flavors can be minimized by deodorizing with activated charcoal or by removing enzymes such as lipoxygenases (LOX), which can be present in trace amounts when using preparations of plant proteins, and which can convert unsaturated triacylglycerides (such as linoleic acid or linolenic acid) into smaller and more volatile molecules. LOX are naturally present in legumes such as peas,

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soybeans, and peanuts, as well as rice, potatoes, and olives. When legume flours are fractionated into separate protein fractions, LOX can act as undesirable “time-bombs” that can cause undesirable flavors on aging or storage. Compositions containing plant proteins (e.g., from ground plant seeds) can be subjected to purification to remove LOX
5 using, for example, an affinity resin that binds to LOX and removes it from the protein sample. The affinity resin can be linoleic acid, linolenic acid, stearic acid, oleic acid, propyl gallate, or epigallocatechin gallate attached to a solid support such as a bead or resin. See, e.g., WO2013138793. In addition, depending on the protein component of the food product, certain combinations of antioxidants and/or LOX inhibitors can be used
10 as effective agents to minimize off-flavor or off-odor generation especially in the presence of fats and oils. Such compounds can include, for example, one or more of β -carotene, α -tocopherol, caffeic acid, propyl gallate, or epigallocatechin gallate.

In some embodiments, specific flavor compounds, such as those described in Tables 3, 8, 9, 12, 14, 16, or 17 can be isolated and purified from the flavor additive
15 composition. These isolated and purified compounds can be used as an ingredient to create flavors useful to the food and fragrance industry.

A flavor additive composition can be in the form, of but not limited to, soup or stew bases, bouillon, e.g., powder or cubes, flavor packets, or seasoning packets or shakers. Such flavor additive compositions can be used to modulate the flavor and/or
20 aroma profile for a variety of food products, and can be added to a consumable food product before, during, or after cooking of the food product.

Food Products

Food products containing one or more flavor precursors and one or more heme-
25 containing proteins can be used as a base for formulating a variety of additional food products, including meat substitutes, soup bases, stew bases, snack foods, bouillon powders, bouillon cubes, flavor packets, or frozen food products. Meat substitutes can be formulated, for example, as hot dogs, burgers, ground meat, sausages, steaks, filets, roasts, breasts, thighs, wings, meatballs, meatloaf, bacon, strips, fingers, nuggets, cutlets,
30 or cubes.

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In addition, food products described herein can be used to modulate the taste and/or aroma profile of other food products (e.g., meat replicas, meat substitutes, tofu, mock duck or other gluten based vegetable product, textured vegetable protein such as textured soy protein, pork, fish, lamb, or poultry products such as chicken or turkey products) and can be applied to the other food product before or during cooking. Using the food products described herein can provide a particular meaty taste and smell, for example, the taste and smell of beef or bacon, to a non-meat product or to a poultry product.

Food products described herein can be packaged in various ways, including being sealed within individual packets or shakers, such that the composition can be sprinkled or spread on top of a food product before or during cooking.

Food products described herein can include additional ingredients including food-grade oils such as canola, corn, sunflower, soybean, olive or coconut oil, seasoning agents such as edible salts (e.g., sodium or potassium chloride) or herbs (e.g., rosemary, thyme, basil, sage, or mint), flavoring agents, proteins (e.g., soy protein isolate, wheat gluten, pea vicilin, and/or pea legumin), protein concentrates (e.g., soy protein concentrate), emulsifiers (e.g., lecithin), gelling agents (e.g., k-carrageenan or gelatin), fibers (e.g., bamboo fiber or inulin), or minerals (e.g., iodine, zinc, and/or calcium).

Food products described herein also can include a natural coloring agent such as turmeric or beet juice, or an artificial coloring agent such as azo dyes, triphenylmethanes, xanthenes, quinines, indigoids, titanium dioxide, red #3, red #40, blue #1, or yellow #5.

Food products described herein also can include meat shelf life extenders such as carbon monoxide, nitrites, sodium metabisulfite, Bombal, vitamin E, rosemary extract, green tea extract, catechins and other anti-oxidants.

Food products described herein can be free of animal products (e.g., animal heme-containing proteins or other animal products).

In some embodiments, the food products can be soy-free, wheat-free, yeast-free, MSG-free, and/or free of protein hydrolysis products, and can taste meaty, highly savory, and without off odors or flavors.

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Assessment of Food Products

Food products described herein can be assessed using trained human panelists. The evaluations can involve eyeing, feeling, chewing, and tasting of the product to judge product appearance, color, integrity, texture, flavor, and mouth feel, etc. Panelists can be served samples under red or under white light. Samples can be assigned random three-digit numbers and rotated in ballot position to prevent bias. Sensory judgments can be scaled for “acceptance” or “likeability” or use special terminology. For example, letter scales (A for excellent, B for good, C for poor) or number scales may be used (1 = dislike, 2 = fair, 3 = good; 4 = very good; 5=excellent). A scale can be used to rate the overall acceptability or quality of the food product or specific quality attributes such as beefiness, texture, and flavor. Panelists can be encouraged to rinse their mouths with water between samples, and given opportunity to comment on each sample.

In some embodiments, a food product described herein can be compared to another food product (e.g., meat or meat substitute) based upon olfactometer readings. In various embodiments, the olfactometer can be used to assess odor concentration and odor thresholds, odor suprathresholds with comparison to a reference gas, hedonic scale scores to determine the degree of appreciation, or relative intensity of odors.

In some embodiments, an olfactometer allows the training and automatic evaluation of expert panels. In some embodiments, a food product described herein causes similar or identical olfactometer readings. In some embodiments, the differences between flavors generated using the methods of the invention and meat are sufficiently small to be below the detection threshold of human perception.

In some embodiments, volatile chemicals identified using GCMS can be evaluated. For example, a human can rate the experience of smelling the chemical responsible for a certain peak. This information could be used to further refine the profile of flavor and aroma compounds produced using a heme-containing protein and one or more flavor precursors.

Characteristic flavor and fragrance components are mostly produced during the cooking process by chemical reactions molecules including amino acids, fats and sugars which are found in plants as well as meat. Therefore, in some embodiments, a food

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product is tested for similarity to meat during or after cooking. In some embodiments human ratings, human evaluation, olfactometer readings, or GCMS measurements, or combinations thereof, are used to create an olfactory map of the food product. Similarly, an olfactory map of the food product, for example, a meat replica, can be created. These
5 maps can be compared to assess how similar the cooked food product is to meat.

In some embodiments, the olfactory map of the food product during or after cooking is similar to or indistinguishable from that of cooked or cooking meat. In some embodiments the similarity is sufficient to be beyond the detection threshold of human perception. The food product can be created so its characteristics are similar to a food
10 product after cooking, but the uncooked food product may have properties that are different from the predicate food product prior to cooking.

These results will demonstrate that the compositions of the invention are judged as acceptably equivalent to real meat products. Additionally, these results can demonstrate that compositions of the invention are preferred by panelist over other
15 commercially available meat substitutes. So, in some embodiments the present invention provides for consumables that are significantly similar to traditional meats and are more meat like than previously known meat alternatives.

The invention will be further described in the following examples, which do not limit the scope of the invention described in the claims.

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EXAMPLES

Example 1: Addition of heme-protein increases beefy qualities of replica burgers

Replica burgers containing the ingredients in Table 4 and the flavor precursors
5 cysteine (10 mM), glutamic acid (10 mM), glucose (10 mM), and thiamine (1 mM) were prepared. Water was added to make up the balance. See, for example, U.S. Provisional Application No. 61/751,816, filed January 11, 2013. Control burgers were prepared as in Table 4 with precursors cysteine (10 mM), glutamic acid (10 mM), glucose (10 mM), and thiamine (1 mM) except LegH was omitted.

10 After cooking for 5 minutes at 150 C, the replica burgers were evaluated by a trained sensory panel. Panelists were served samples under red lights and each panelist individually evaluated the samples. Samples were assigned a random three-digit number and rotated in ballot position to prevent bias. Panelists were asked to evaluate cooked replica burger samples on multiple flavor, aroma, taste, texture and appearance attributes
15 including but not limited to: beefiness, bloody quality, savory quality, and overall acceptability using a 7-point scale from 1=dislike extremely, to 7=like extremely. Panelists were encouraged to rinse their mouths with water between samples, and to fill out a survey to record their evaluation of each sample.

When replica burgers containing the LegH were compared to the control replica
20 burgers without LegH, the samples containing LegH were rated significantly beefier, bloodier, more savory, and overall preferred compared to those that did not include LegH. See Table 5.

TABLE 4
Replica Burger Ingredients

Replica burger	% precooked w/w
Pea vicilin	3.86
Soy protein concentrate (SPC)	2.52
Bamboo fiber	0.34

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NaCl	0.54
Pea legumin	2
Soy Protein Isolate (SPI) (Solae, St. Louis, MO)	4.68
Wheat gluten	4.68
Coconut oil	15
Soy lecithin	0.1
k-carrageenan	1
LegH	1

TABLE 5
Sensory evaluation of replica burger with Heme

Attribute	Beef 20/80	No Heme	1% Heme
Beefyness			
mean	5.33	1.30	3.20
STDEV	1.58	0.67	0.79
Bloody			
mean	4.00	1.10	2.78
STDEV	1.32	0.32	1.64
Savory			
mean	4.67	3.00	5.10
STDEV	1.22	1.63	0.57

5 **Example 2: Replica burgers with a flavor precursor mixture taste beefy and**
bloody

Replica burgers containing a flavor precursor mixture of glucose, cysteine, thiamine, and glutamic acid and 1% LegH pre-cooked w/w (see Table 4) were prepared as described in Example 1, and evaluated by a trained sensory panel after the burgers
10 were cooked for 5 minutes at 150 C. Control burgers included LegH and all other ingredients except for the flavor precursor mixture.

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Panelists were asked to evaluate the samples overall improvement in taste and descriptively analyze each sample using a 5-point scale from 1=dislike extremely, to 5=like extremely. Panelists were encouraged to rinse their mouths with water between samples, and to fill out a survey to record their evaluation of each sample. The replicate burgers which included LegH and the flavor precursor mixture were described as having bouillon, gravy, meaty, bloody, savory, and beefy notes on taste, and were preferred to the same replica burger with LegH but no added flavor precursor mixture. See, Table 6

TABLE 6**Improvement of overall taste with precursors added to LegH burgers**

	with precursors	without precursors
Average	3.5	1.8
STDV	0.6	0.5

10

Example 3: Replica burgers with flavor precursor mixture resulting in a bacon taste

Replica burgers (see Table 4) were cooked with different precursor mixes (see Table 7) and 1% LegH and evaluated by a trained sensory panel after the burgers were cooked for 5 minutes at 150 C. Control burgers contained LegH and all of the other ingredients except for the flavor precursors. Panelists were asked to evaluate each sample and descriptively analyze of each sample. 5-point scale from 1=dislike extremely, to 5=like extremely. Panelists were encouraged to rinse their mouths with water between samples, and to fill out a survey to record their evaluation of each sample. A replica burger with a precursor mixture of 10 mM glucose, 10 mM ribose, 10 mM cysteine, 1 mM thiamine, 1 mM glutamic acid, 1 mM GMP, and LegH was described as having a bacon aroma and taste, and overall meatiness, savory quality, a very umami quality, a brothy quality, and slight beefy notes. See Table 7 for a summary of the flavor description for the various combinations of flavor precursors and heme-containing protein.

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TABLE 7
Flavors generated by addition of precursors to LegH (1 %)

Precursor (concentration)						Flavor Description
	ribose (10 mM)	cysteine (10 mM)				some kind of cold-cut/sliced deli meat
	ribose (10 mM)	cysteine (10 mM)			IMP (2 mM)	bread crust with beef drippings, sweet, grassy, umami
	ribose (10 mM)	cysteine (10 mM)			lactic acid (1 mM)	bready, malty, browned, breadcrust
	ribose (10 mM)	cysteine (10 mM)			lysine (5 mM)	savory, beefy, little grassy, brothy, bread
	ribose (10 mM)	cysteine (10 mM)			alanine (5 mM)	savory, weak beefy, brothy, little metallic
	ribose (10 mM)	cysteine (10 mM)			I+G (2 mM)	savory, weak beefy, brothy, sweet
	ribose (10 mM)	cysteine (10 mM)			methionine	cooked potato
	ribose (10 mM)	cysteine (10 mM)		glutamic acid (5 mM)		little meaty, pretzel, brothy, savory, sweet, chocolate
glucose (10 mM)	ribose (10 mM)	cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)		slight beefy, browned, grassy,
glucose (10 mM)	ribose (10 mM)	cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)	IMP (2 mM)	bacon, very umami, savory, brothy, slight beef
glucose (10 mM)		cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)		beef jerky, bloody, meaty, brothy
glucose (10 mM)		cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)	lactic acid (1 mM)	savory, beefy, bloody, meaty, savory, gravy

glucose (10 mM)		cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)	lysine (5 mM)	roast beef
glucose (10 mM)		cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)	alanine (5 mM)	boiled beef, sweet
glucose (10 mM)		cysteine (10 mM)	thiamine (2 mM)	glutamic acid (5 mM)	I+G (2 mM)	beefy with a sulfury note
glucose (10 mM)		cysteine (10 mM)			I+G (2 mM)	sweet, malty, umami, meaty
glucose (10 mM)					I+G (2 mM)	savory, roast beef, grassy
glucose (10 mM)				glutamic acid (5 mM)		umami, savory, meaty, sweaty, fermented

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Example 4: Type of sugar modulates flavor compounds created in the presence of Hemeprotein

The addition of different sugars to flavor reaction mixtures containing a hemeprotein and one or more flavor precursor compounds resulted in distinct differences in the flavor compounds generated and the overall flavor profile. LegH heme protein at 1% pre-cooked w/w/ was mixed with cysteine (10 mM) and glucose (20 mM) at pH 6 in phosphate buffer to form a flavor reaction mixture and heated to 150 C for 3 minutes; this reaction created flavor compounds known to be present in meat; *see* Table 8. Similarly, a flavor reaction mixture made when LegH heme protein at 1% was mixed with cysteine (10 mM) and ribose (20 mM) at pH 6 and heated to 150 C for 3 minutes created flavor compounds known to be in meat; *see* Table 8.

The characteristic flavor and fragrance components were mostly produced during the cooking process when the flavor precursor molecules reacted with the heme-protein. Gas chromatography–mass spectrometry (GCMS) is a method that combines the features of gas-liquid chromatography and mass spectrometry to separate and identify different substances within a test sample. Samples were evaluated by GCMS to identify the flavor compounds generated after heating and also evaluated for their sensory profiles. Volatile chemicals were isolated from the head space around the flavor reactions. The profile of the volatile chemicals in the headspace around the flavor reaction mixtures is shown in Table 8. In particular, the use of ribose created some additional compounds as compared to glucose, as shown in Table 8.

Notably, the control mixtures of cysteine with ribose or glucose heated in the absence of the LegH heme-protein did not generate the same set of flavor compounds. The flavor reaction mixtures containing LegH also were evaluated by a blinded trained sensory panel, which described the samples with ribose as having beefy, savory, brothy, and gravy-like notes, and the samples with glucose as savory, bloody, metallic, raw meat, and bouillon-like.

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TABLE 8

Flavor compounds generated with cysteine, LegH, and either glucose or ribose in the flavor reaction mixture.

LegH 1%		
Compounds created	cysteine (10 mM), glucose (20 mM)	cysteine (10 mM), ribose (20 mM)
benzaldehyde	X	X
2-butanone	X	X
dimethyl trisulfide	X	X
2-pentyl-furan	X	X
2-methyl-propanal	X	X
thiazole	X	X
butyrolactone	X	X
2-acetylthiazole	X	X
pentanal	X	X
3-methyl-butanal	X	X
methyl-thiirane	X	X
nonanal	X	X
heptanal	X	X
2,3-butanedione	X	X
1,3,5-cycloheptatriene	X	X
propyl-cyclopropane	X	X
2-hexyl-furan	X	X
butanal	X	X
2-methyl-butanal	X	X
2-ethyl-furan		X
2-octanone	X	X
propanal	X	X

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trichloromethane	X	
2-methyl-furan	X	X
furan	X	X
pyrazine	X	X
thiophene	X	X
1,3-dimethyl-benzene	X	X
octane		X
octanal	X	X
thiazole	X	X
2-pentanone		X
furfural	X	X
2-nonanone	X	X
(Z)-2-heptenal	X	X
(E)-2-heptenal	X	X
1-octene	X	X
formic acid, heptyl ester	X	X
2-pentyl-thiophene		X
1-octen-3-one	X	X
3-pentyl-furan	X	X
2-propenal		X
(E)-2-tridecen-1-ol		X
benzene		X
(E)-4-octene		X
1-penten-3-one		X
4-penten-2-one	X	X
2-methyl-thiazole		X
methyl-pyrazine		X
trans-2-(2-pentenyl)furan		X
3-ethylcyclopentanone		X

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pyrrole	X	X
2-thiophenecarboxaldehyde		X
3-thiophenecarboxaldehyde		X

Example 5: Heme-protein in the presence of thiamine affects the production of certain flavor compounds

The addition of thiamine in a flavor reaction mixtures with a heme protein and other flavor precursors affected the formation of 5-Thiazoleethanol, 4-methyl-furan, 3,3'-dithiobis[2-methyl-thiazole, and 4-methylthiazole. These compounds are known to be present in meat and have beefy, meaty taste notes.

Flavor reaction mixtures at pH 6 containing LegH (1%), cysteine (10 mM), thiamine (1 mM), either glucose or ribose (20 mM), and with or without glutamic acid (10 mM) were prepared and subsequently heated to 150 C for 3 minutes. These flavor reaction samples then were evaluated by GCMS for the flavor compounds generated and evaluated by a trained panel for their sensory profiles. Volatile chemicals were isolated from the head space around the flavor reactions. GCMS showed 4-methyl-5-thiazoleethanol, 3,3'-dithiobis[2-methyl]-furan, and 4-methylthiazole compounds were created by a mixture of LegH with thiamine, a sugar (either glucose or ribose), and cysteine. The same flavor reaction mixtures without thiamine did not generate these compounds; additionally these compounds were not generated when heme-proteins were not present in the flavor reaction mixtures.

The flavor reaction samples also were evaluated by a blinded trained sensory panel, which described the samples with the addition of thiamine as more complex in taste and more beefy, meaty, and savory.

Example 6: Heme-proteins with nucleotides controls particular flavor compound production.

The addition of inosine monophosphate and guanosine monophosphate in mixes with heme protein and other precursors controlled the formation of flavor compounds (E)-4-octene, 2-ethyl-furan, 2-pentanone, 2,3-butanedione, 2-methyl-thiazole, , methyl-

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pyrazine, tridecane, (E)-2-octenal, 2-thiophenecarboxaldehyde, and 3-thiophenecarboxaldehyde. These compounds are known to be present in meat and have a beefy, meaty, buttery, and or savory flavor notes.

Reactions containing heme protein at 1% (LegH) with cysteine (10 mM), and glucose (20 mM), 1 mM IMP and 1 mM GMP, at pH 6.0 were prepared and heated to 150C for 3 minutes. Characteristic flavor and fragrance components were mostly produced during the cooking process where precursors reacting heme-protein. These samples were evaluated by GCMS for the flavor compounds generated and evaluated for the sensory experience. Volatile chemicals were isolated from the head space around the flavor reaction and identified using GCMS, creating a profile of the volatile chemicals in the headspace around the flavor reaction mixture. GCMS showed 4-octene, 2-ethyl furan, 2-pentanone, 2,3-butanedione, 2-methyl-thiazole, methyl-pyrazine, tridecane, 2-octenal, 2-thiophenecarboxaldehyde, 3-thiophenecarboxaldehyde compounds were created by a mixture of hemeprotein LegH with IMP, GMP, glucose, and cysteine. The same samples without IMP and GMP did not generate these compounds, additionally these compounds were also not created when heme-proteins were not present, just precursor molecules. Sensory evaluation by blinded trained panelist found the samples with the addition of inosine and guanosine as described as having more complexity in taste and more beefy, meaty, brothly and savory. Figure 2 shows the abundance of the novel flavor compounds created with heme protein at 1% was mixed in a reaction at pH 6, with cysteine (10 mM), and glucose (20 mM), IMP (1 mM) and GMP (1 mM), and detected by solid phase microextraction (SPME) and then detected by GCMS.

Example 7: Flavor generation with the addition of a particular organic acid

The addition of lactic acid in mixes with heme protein, ribose, and cysteine controlled the formation of the flavor compounds nonanal, thiazole, 2-acetylthiazole, and 8-methyl-1-undecene. These compounds are known to be present in meat.

Reactions containing heme protein at 1%, cysteine (10 mM), and ribose (20 mM), and lactic acid (1mM), pH 6.0, were prepared and heated to 150C for 3 minutes. Characteristic flavor and fragrance components were mostly produced during the cooking

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process where precursors reacting heme-protein. These samples were evaluated by GCMS for the flavor compounds generated and evaluated for the sensory experience. Volatile chemicals were isolated from the head space around the flavor reaction and identified using GCMS, creating a profile of the generated compounds. Nonanal, thiazole, 2-acetylthiazole, and 8-methyl-1-undecene compounds were created by a mixture of LegH with lactic acid, ribose, and cysteine. The same samples without lactic acid did not generate these compounds, additionally these compounds were not created in the absence of heme-proteins.

Sensory evaluation by blinded trained panelist found the samples with the addition of lactic acid as described as beefy, savory, browned, bready, and having malty notes. The sample with everything but lactic acid rated lower in browned, bready and malty notes.

Example 8: Flavor generated with the addition of a particular amino acid.

The addition of lysine in mixes with heme protein ribose, and cysteine controlled the formation of flavor compounds dimethyl trisulfide, nonanal, 2-pentyl-thiophene, furfural, 2-nonenal, 1-octanol, 2-nonenal, thiazole, 2-acetylthiazole, phenylacetaldehyde, 2-acetylthiazole. These compounds are known to be present in meat and some have a beefy, meaty, and or savory flavor.

Reactions containing heme protein at 1%, cysteine (10 mM), and ribose (20 mM), and lysine (1mM), at pH 6.0, were prepared and heated to 150C for 3 minutes. These samples were evaluated by GCMS for the flavor compounds generated and evaluated for the sensory experience. Characteristic flavor and fragrance components were mostly produced during the cooking process where precursors could react with the heme-protein. These samples were evaluated by GCMS for the flavor compounds generated and evaluated for the sensory experience. Volatile chemicals were isolated from the head space around the flavor reaction. Dimethyl trisulfide, nonanal, 2-pentyl-thiophene, furfural, 2-nonenal, 1-octanol, 2-nonenal, thiazole, 2-acetylthiazole, phenylacetaldehyde, 2-acetylthiazole compounds were created by a mixture of LegH with lactic acid, ribose, and cysteine. The same samples without lactic acid did not generate these compounds,

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additionally these compounds were not created when heme-proteins were not present, just precursor molecules. Sensory evaluation by blinded trained panelist found the samples with the addition of lysine as described as roast beefy, savory, and browned. The addition of lysine increased the roasted browned notes.

5

Example 9 - Flavor compound production by different Heme-proteins

The addition of different types of heme-proteins (LegH, Barley, *B. myoglobin*, or *A. aeolicus*) in flavor reaction mixtures containing one or more flavor precursor compounds results in many of the same key meat flavors, including but not limited to 2-pentyl-furan, 2,3-Butanedione, Thiophene, 2-methyl-thiazole, Pyrazine, Furan, Pyrrole, 2-methyl-furan and distinct differences in the flavor compounds, including but not limited to 2-pentyl-thiophene, Nonanal, 2-Nonanone, and 1-Octen-3-one. These differences in flavor compounds can change the overall taste profile. The different types of heme-protein were LegH, Barley, *B. myoglobin*, or *A. aeolicus* used at 1% w/w in a reaction mixed with cysteine (10 mM) and ribose (10 mM) at pH 6. The pre- reaction mixture was heated to 150 C for 3 minutes; this reaction created flavor compounds known to be present in meat; see Table 9. The characteristic flavor and fragrance components are mostly produced during the cooking process where the flavor precursor molecules react with the heme-protein. Samples were evaluated by GCMS to identify the flavor compounds generated after heating and also evaluated for their sensory profiles. Volatile chemicals were isolated from the head space around the flavor reactions. Table 9 shows the similarity and differences in volatile flavor compounds created by the different types of heme-proteins.

25

TABLE 9

**Flavor compounds created by different heme-protein
when heated with ribose and cysteine.**

Name	LegH	Barley	<i>B. myoglobin</i>	<i>A. aeolicus</i>
Furan	x	x	x	x
Thiazole	x	x	x	x
benzaldehyde	x	x	x	x
2-acetylthiazole	x	x	x	x

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2-methyl-propanal	x	x	x	x
furfural	x	x	x	x
2,3-butanedione	x	x	x	x
2-pentyl-furan	x	x	x	x
2-pentanone	x	x		
pyrazine	x	x	x	x
dimethyl trisulfide	x	x	x	x
3-methyl-butanal	x	x		x
2-methyl-thiazole	x	x	x	x
pentanal	x	x	x	x
1,3,5-cycloheptatriene	x	x	x	x
methacrolein	x	x	x	x
heptanal	x	x	x	x
2-methyl-butanal	x	x		x
isothiazole	x	x	x	x
thiophene	x	x	x	x
propanal	x	x	x	x
2-heptenal	x		x	x
methyl-pyrazine	x	x	x	x
1-octene	x		x	x
butanal	x	x	x	x
2-acetyl-propen-2-ol	x	x	x	x
pyrrole	x	x	x	x
2-methyl-furan	x	x	x	x
nonanal		x	x	x
2-propenal		x	x	x
2-decenal		x	x	x
2-nonanone		x		x
2-octanone		x	x	x
2-tridecen-1-ol,			x	x
2-octanone			x	
2-octenal			x	x
4-methyl-2-heptanone			x	x
octanal			x	x
2-undecenal				x
butyrolactone				x
1-octen-3-one				x
3-methylheptyl acetate				x

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2-pentyl-thiophene				x
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Example 10 - Generation of Meat flavors from different lipids

Several different samples including oils (canola oil or coconut oil), free fatty acids (FFA) (linoleic acid (C18:2), oleic acid (C18:1), stearic acid (C18:0), or myristic acid (C14:0)) and phospholipids (PL) (beef heart polar lipids extract, Biolipon95 (from Perimond), or NatCholinePC40 (from Perimond)) were tested for their ability to produce beefy flavor in the absence and in the presents of other precursors. Oils, FFAs, and PLs were added to 50 mM potassium phosphate buffer (PPB) pH 6.0 or a Maillard reaction mix (MRM) containing 50 mM potassium phosphate pH 6.0, 5 mM Cysteine, 10 mM Glucose, 0.1 mM Thiamine, and 0.1% (w/v) LegHemoglobin. Lipids in combination with MRM were designed to capture the cross reactions of lipid degradation and Maillard reaction productions while lipids in phosphate buffer functioned as a lipid control. The oils were added at 3% of the total 1 mL volume of solution while FFAs and PLs were added at 1% of the total 1 mL volumes. All samples were cooked at 150°C for 3 mins, cooled to 50°C and then analyzed using GCMS (SPME fiber sampling of headspace). After all samples were analyzed by GCMS the caps were removed and samples were smelled by a trained flavor scientist and aromas recorded.

Table 10**Legend showing components of each sample**

Sample Name	Solution	Additives
MRM None	Maillard Reaction Mix	None
MRM_Linoelic Acid	Maillard Reaction Mix	1% linoleic acid
MRM_Oleic Acid	Maillard Reaction Mix	1% oleic acid
MRM_C14	Maillard Reaction Mix	1% C14:0 free fatty acid
MRM_C18	Maillard Reaction Mix	1% C18:0 free fatty acid
MRM_Canola	Maillard Reaction Mix	3% Canola Oil
MRM_Coconut	Maillard Reaction Mix	3% Coconut Oil
MRM_BeefHeart	Maillard Reaction Mix	1% Beef Heart Polar Lipids Extract
MRM_Biolipon95	Maillard Reaction Mix	1% Biolipon95 (emulsifier)

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MRM_NatCholinePC40	Maillard Reaction Mix	1% NatCholinePC40 (emulsifier)
KPhos6_Linoelic Acid	PPB, pH 6	1% linoelic acid
KPhos6_Oleic Acid	PPB, pH 6	1% oleic acid
KPhos6_C14	PPB, pH 6	1% C14:0 free fatty acid
KPhos6_C18	PPB, pH 6	1% C18:0 free fatty acid
KPhos6_Canola	PPB pH 6	3% Canola Oil
KPhos6_Coconut	PPB, pH 6	3% Coconut Oil
KPhos6_BeefHeart	PPB, pH 6	1% Beef Heart Polar Lipids Extract
KPhos6_Biolipon95	PPB, pH 6	1% Biolipon95 (emulsifier)
KPhos6_NatCholinePC40	PPB, pH 6	1% NatCholinePC40 (emulsifier)

Table 11 contains the aroma descriptions and Table 12 contains the GCMS data from the most interesting samples analyzed. Many of the lipids introduced a “fatty” aroma to MRM that was otherwise absent. The combinations of Linoleic Acid or NatCholinePC40 in MRM produced the greatest abundance of fatty compounds suggesting that these lipids may improve the flavor perception of beef tallow. Linoleic Acid and NatCholinePC40 also showed high abundance of earthy-mushroom aromas. The addition of lipids to MRM significantly increased the abundance of “nutty & roasted” aromas. Less desirable “green” aroma compounds were most prominent in samples with unsaturated free fatty acids (linoleic acid or oleic acid) or phospholipids. In general, the addition of lipids significantly increased the number of target beef compounds made.

TABLE 11**Aroma descriptions of each sample after it was cooked.**

Sample Names	Aroma Descriptions
MRM_Only	brothy, malty, beef stew
KPhos6_BeefHeart	fatty, creamy, beef tallow, slight sweet, slight roasted nutty
MRM_BeefHeart	fatty, beef tallow, old meat, mushroom
KPhos6_Biolipon95	fatty, fresh
MRM_Biolipon95	fatty, brothy, hay, malty green

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KPhos6_NatCholinePC40	light fatty, fresh
MRM_NatCholinePC40	fatty, beef tallow, brothy
K-Phos6_C14	light/faint plastic/waxy
MRM_C14	brothy, beefy, minty, fresh
K-Phos6_C18	light/faint plastic/waxy
MRM_C18	beefy with cucumber &/or pepper aroma
K-Phos6_Canola	fresh, cucumber
MRM_Canola	fatty, brothy, oil, roasted nuts
K-Phos6_Coconut	nothing
MRM_Coconut	brothy, beefy, slight fatty, crackers
K-Phos6_Oleic Acid	fresh, cucumber, camphorous/minty-like
MRM_Oleic Acid	herbal, plastic, slight cheesy, brothy
K-Phos6_Linoelic Acid	light plastic
MRM_Linoelic Acid	fatty, light waxy, brothy, herbal

Table 12

List of aromatic compounds found in Beef by GCMS and a chart showing which were detected in each lipid plus MRM sample.

Compounds in Beef	MRM only	MRM_BeefHeart	MRM_NatCholinePC40	MRM_Linoelic acid
(s)-isopropyl lactate	N	N	N	N
1-ethyl-5-methylcyclopentene	Y	Y	Y	Y
1-heptanol	N	Y	N	N
1-hepten-3-ol	N	Y	Y	Y
1-heptene	N	Y	Y	Y
2-methyl-1-heptene	N	N	N	N
1-hexanol	N	Y	Y	Y
2-ethyl-1-hexanol	N	N	N	N
1-nonanol	N	N	Y	N
1-nonene	N	Y	Y	N
1-octanol	N	Y	Y	N
1-octen-3-ol	N	Y	Y	Y