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(54) Title: PROCESS

(57) Abstract: In one aspect there is provided aprocess for extracting a plant oil, comprising contacting an oil-containing plant material with an organic solvent comprising hexane; obtaining a mixture comprising the organic solvent and a crude plant oil derived from the plant material; and separating the crude plant oil from the organic solvent; wherein an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative is contacted with the plant material and/or crude plant oil in the presence of the organic solvent.

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PROCESS

FIELD

The present invention relates to the field of oil extraction from oil-containing plant materials, such as plant seeds. In particular, the invention relates to a process which may be applied during organic solvent (e.g. hexane) extraction of plant materials, in order to reduce the content of chlorophyll and related compounds in oil obtained from the plant materials.

BACKGROUND

Oil may be extracted from plant seeds using various methods. The oil is usually pressed out of the seed or extracted with organic solvents after crushing of the seed. A combination of pressing and organic extraction may be used, e.g. the oil component remaining in the pressed seeds is extracted with organic solvents after a pressing step. If the pressed seeds are intended to be used as animal feed, organic solvents should not be used. The pressed seed cake preferably has a low residual oil content and a high protein content making it particularly suitable as a feed material.

Oils may be produced from, for example, rapeseed by expeller pressing of the seeds without organic solvent extraction. The pressing method typically results in a relatively low content of phospholipids and coloured pigments such as chlorophyll in the resultant oil. However, the resultant yield of oil from e.g. rapeseed using pressing only is also relatively low.

Extraction using organic solvents is therefore often used to increase the yield of oil from oil-containing plant materials, such as rapeseed. Plant materials with a relatively low oil content (such as soya bean) are not normally pressed, and oil may be extracted using organic extraction alone. However, oil obtained using the extraction method typically contains high amounts of phospholipids, which must be removed by a degumming process before use as an edible oil or biodiesel. Oil obtained by organic solvent extraction also typically contains high amounts of chlorophyll and related pigments, which must also be removed during oil processing.

For example, crude vegetable oils derived from oilseeds such as soybean, palm or rape seed (canola), cotton seed and peanut oil typically contain some chlorophyll. Chlorophyll imparts an undesirable green colour and can induce photooxidation of oil during storage, leading to a deterioration of the oil.

Various methods have been employed in order to remove chlorophyll from vegetable oils. Chlorophyll may be removed during many stages of the oil production process, including the seed crushing, oil extraction, degumming, caustic treatment and bleaching steps. However the bleaching step is usually the most significant for reducing chlorophyll residues to an acceptable level. During bleaching the oil is heated and passed through an adsorbent to remove chlorophyll and other colour-bearing compounds that impact the appearance and/or stability of the finished oil. The adsorbent used in the bleaching step is typically clay.

In the edible oil processing industry, the use of such steps typically reduces chlorophyll levels in processed oil to between 0.02 to 0.05 ppm. However the bleaching step increases processing cost and reduces oil yield due to entrainment in the bleaching clay. The use of clay may remove many desirable compounds such as carotenoids and tocopherol from the oil. Moreover, recent studies have shown that bleaching clay can contaminate oil with chloride ions. This leads to the formation 3-monochloropropane-1,2-diol (3-MCPD), which is very toxic. Also the use of clay is expensive, this is particularly due to the treatment of the used clay (i.e. the waste) which can be difficult, dangerous (prone to self-ignition) and thus costly to handle. Thus attempts have been made to remove chlorophyll from oil by other means, for instance using the enzyme chlorophyllase.

In plants, chlorophyllase (chlase) is thought to be involved in chlorophyll degradation and catalyzes the hydrolysis of an ester bond in chlorophyll to yield chlorophyllide and phytol. WO 2006009676 describes an industrial process in which chlorophyll contamination can be reduced in a composition such as a plant oil by treatment with chlorophyllase. The water-soluble chlorophyllide which is produced in this process is also green in colour but can be removed by an aqueous extraction or silica treatment.

Chlorophyll is often partly degraded in the seeds used for oil production as well as during extraction of the oil from the seeds. One common modification is the loss of the magnesium ion from the porphyrin (chlorin) ring to form the derivative known as pheophytin (see Figure 1). The loss of the highly polar magnesium ion from the porphyrin ring results in significantly different physico-chemical properties of pheophytin compared to chlorophyll. Typically pheophytin is more abundant in the oil during processing than chlorophyll. Pheophytin has a greenish colour and may be removed from the oil by an analogous process to that used for chlorophyll, for instance as described in WO 2006009676 by an esterase reaction catalyzed by an enzyme having a pheophytinase activity. Under certain conditions, some chlorophyllases are capable of hydrolyzing pheophytin as well as chlorophyll, and so are suitable for removing both of these contaminants. The products of pheophytin hydrolysis are the red/brown-colored pheophorbide and phytol. Pheophorbide can also be produced by the loss of a magnesium ion from chlorophyllide, i.e. following hydrolysis of chlorophyll (see Figure 1). WO 2006009676 teaches removal of pheophorbide by an analogous method to chlorophyllide, e.g. by aqueous extraction or silica adsorption.

Pheophytin may be further degraded to pyropheophytin, both by the activity of plant enzymes during harvest and storage of oil seeds or by processing conditions (e.g. heat) during oil refining (see "Behaviour of Chlorophyll Derivatives in Canola Oil Processing", JAOCS, Vol, no. 9 (Sept. 1993) pages 837-841). One possible mechanism is the enzymatic hydrolysis of the methyl ester bond of the isocyclic ring of pheophytin followed by the non-enzymatic conversion of the unstable intermediate to pyropheophytin. A 28-29 kDa enzyme from *Chenopodium album* named pheophorbidase is reportedly capable of catalyzing an analogous reaction on pheophorbide, to produce the phytol-free derivative of pyropheophytin known as pyropheophorbide. Pyropheophorbide is less polar than pheophorbide, resulting in the pyropheophorbide having a decreased water solubility and an increased oil solubility compared with pheophorbide.

Depending on the processing conditions, pyropheophytin can be more abundant than both pheophytin and chlorophyll in vegetable oils during processing (see Table 9 in volume 2.2. of Bailey's Industrial Oil and Fat Products (2005), 6th edition, Ed. by

Fereidoon Shahidi, John Wiley & Sons). This is partly because of the loss of magnesium from chlorophyll during harvest and storage of the plant material. If an extended heat treatment at 90°C or above is used, the amount of pyropheophytin in the oil is likely to increase and could be higher than the amount of pheophytin. Chlorophyll levels are also reduced by heating of oil seeds before pressing and extraction as well as the oil degumming and alkali treatment during the refining process. It has also been observed that phospholipids in the oil can complex with magnesium and thus reduce the amount of chlorophyll. Thus chlorophyll is a relatively minor contaminant compared to pyropheophytin (and pheophytin) in many plant oils.

There is a still a need for an improved process for removing chlorophyll and chlorophyll derivatives such as pheophytin and pyropheophytin from plant oils. In particular, there is a need for a process in which chlorophyll and chlorophyll derivatives are removed with enhanced efficiency, whilst reducing the loss of other desirable compounds from the oil.

SUMMARY

Accordingly, in one aspect the present invention provides a process for extracting a plant oil, comprising (a) contacting an oil-containing plant material with an organic solvent comprising hexane; (b) obtaining a mixture comprising the organic solvent and a crude plant oil derived from the plant material; and (c) separating the crude plant oil from the organic solvent; wherein an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative is contacted with the plant material and/or crude plant oil in the presence of the organic solvent.

In one embodiment, the plant material comprises oil-containing plant seeds. For instance, the seeds may be soya beans, peanuts, cotton seeds, sunflower seeds or rapeseeds. Preferably the seeds comprise soya or rapeseed.

In one embodiment, the plant material (e.g. oil-containing seeds) is flaked, peeled or pressed before contacting with the organic solvent (e.g. hexane).

In one embodiment, the enzyme is added to the plant material and the organic solvent (e.g. hexane), before or during extraction of the crude plant oil from the plant material.

In an alternative embodiment, the enzyme is added to the mixture, after extraction of the crude plant oil from the plant material.

In one embodiment, the enzyme is contacted with the plant material and/or crude plant oil in the presence of 0.5 to 5% by weight water, based on the total weight of (i) the plant material, the organic solvent (e.g. hexane) and water or (ii) the crude plant oil, the organic solvent (e.g. hexane) and water.

In one embodiment, the enzyme is contacted with the plant material and/or crude plant oil in the presence of 10 to 90% by weight of the organic solvent (e.g. hexane), based on the total weight of (i) the plant material, the organic solvent (e.g. hexane) and water or (ii) the crude plant oil, the organic solvent (e.g. hexane) and water.

In one embodiment, the enzyme comprises a polypeptide sequence as defined in any one of SEQ ID NOs: 1, 2, 4, 6 or 8 to 15, or a functional fragment or variant thereof. For instance, the enzyme may comprise a polypeptide sequence having at least 75% sequence identity to any one of SEQ ID NOs: 1, 2, 4, 6 or 8 to 15 over at least 50 amino acid residues.

In one embodiment, the enzyme is contacted with the plant material and/or crude plant oil at a temperature of 50 to 70°C.

In a further aspect, the invention provides a process for producing a refined plant oil, comprising extracting a crude plant oil by a process as defined above, and refining the crude plant oil to obtain a refined plant oil.

In a further aspect, the invention provides a crude or refined plant oil obtainable by a process as defined in any preceding claim.

As described herein, it has surprisingly been found that chlorophyllases and related enzymes can be used to hydrolyze chlorophyll and chlorophyll derivatives during organic solvent (e.g. hexane) extraction of plant oils. It has previously been proposed that chlorophyllases be used at other stages of oil refining processes, e.g. in a water degumming step. However, since some oil refining processes do not include a water degumming step, the method describes herein advantageously enables chlorophyllases

to be used conveniently in such processes with minimum disruption to the existing apparatus and systems.

BRIEF DESCRIPTION OF DRAWINGS

Figure 1 shows the reactions involving chlorophyll and derivatives and enzymes used in the present invention.

Figure 2 shows the amino acid sequence of *Arabidopsis thaliana* chlorophyllase (SEQ ID NO:1).

Figure 3 shows the amino acid sequence of *Triticum aestivum* chlorophyllase (SEQ ID NO:2).

Figure 4 shows a nucleotide sequence encoding *Triticum aestivum* chlorophyllase (SEQ ID NO:3).

Figure 5 shows the amino acid sequence of *Chlamydomonas reinhardtii* chlorophyllase (SEQ ID NO:4).

Figure 6 shows a nucleotide sequence encoding *Chlamydomonas reinhardtii* chlorophyllase (SEQ ID NO:5).

Figure 7 shows the amino acid sequence of a pheophytin pheophorbide hydrolase (PPH) from *Arabidopsis thaliana* (SEQ ID NO:6). A chloroplast transit peptide is shown in bold.

Figure 8 shows the nucleotide sequence of a cDNA from *Arabidopsis thaliana* encoding pheophytin pheophorbide hydrolase (SEQ ID NO:7). The PPH of SEQ ID NO:6 is encoded by residues 173 to 1627 of SEQ ID NO:7.

Figure 9 shows the polypeptide sequence of *Populus trichocarpa* PPH (SEQ ID NO:8).

Figure 10 shows the polypeptide sequence of *Vitis vinifera* PPH (SEQ ID NO:9).

Figure 11 shows the polypeptide sequence of *Ricinus communis* PPH (SEQ ID NO:10).

Figure 12 shows the polypeptide sequence of *Oryza sativa* (japonica cultivar-group) PPH (SEQ ID NO:11).

Figure 13 shows the polypeptide sequence of *Zea mays* PPH (SEQ ID NO:12).

Figure 14 shows the polypeptide sequence of *Nicotiana tabacum* PPH (SEQ ID NO:13).

Figure 15 shows the polypeptide sequence of *Oryza sativa* Japonica Group PPH (SEQ ID NO:14).

Figure 16 shows (a) the polypeptide sequence of *Physcomitrella patens* subsp. patens PPH (SEQ ID NO:15).

Figure 17 shows TLC analysis of oil/hexane samples treated with phospholipases. PE: Phosphatidylethanolamine. PI: Phosphatidylinositol, PA: Phosphatidic acid. PC: Phosphatidylcholine.

DETAILED DESCRIPTION

In one aspect the present invention relates to a process for extracting a plant oil. Typically the process is used to remove chlorophyll and/or chlorophyll derivatives from the resulting oil, or to reduce the level of chlorophyll and/or chlorophyll derivatives in the oil, for instance where the chlorophyll and/or chlorophyll derivatives are present as a contaminant.

Plant materials

The present process may be used to extract oil from any oil-containing plant material, and to remove undesirable contamination by chlorophyll and/or chlorophyll derivatives in the resulting oil. Suitable oil-containing plant materials may be derived from any type of plant, and include, for example, whole plants, leaves, stems, flowers, roots, plant protoplasts, seeds and plant cells and progeny of same. The class of plants from which the plant materials are obtained includes higher plants, including angiosperms (monocotyledonous and dicotyledonous plants), as well as gymnosperms. It includes plants of a variety of ploidy levels, including polyploid, diploid, haploid and hemizygous states.

Oil-containing seeds

In a preferred embodiment, the plant material comprises oil-containing plant seeds. By "oil-containing seeds" it is typically meant any oleaginous plant seeds, including beans, grain (including bran), kernels, fruits, nuts and the like. The seeds may be derived from any type of plant, especially higher plants, including angiosperms (monocotyledonous and dicotyledonous plants), as well as gymnosperms.

For example, the National Sustainable Agriculture Information Service lists the following as sources of oil for food, specialty, or industrial uses: almonds, apricot kernels, avocado, beech nut, bilberry, black currant, borage, brazil nut, calendula, caraway seed, cashew nut, castor seed, citrus seed, clove, cocoa, coffee, copra (dried coconut), coriander, corn seed, cotton seed, elderberry, evening primrose, grape seed, groundnut, hazelnut, hemp seed, jojoba, linseed, macadamia nut, mace, melon seed, mustard seed, neem seed, niger seed, nutmeg, palm kernel, passion fruit, pecan, pistachio, poppy seed, pumpkin seed, rape seed, raspberry seed, red pepper, rose hip, rubber seed, safflower seed, sea buckthorn, sesame seed, soybean, spurge, stinging nettle, sunflower seed, tropho plant, tomato seed, or walnut. Also useful herein are seeds and the like derived from various plants whose oil content is of interest for use as fuel, such as "eco- fuel", biodiesel or the like. Such plants include but are not limited to jatropha (e.g. *Jatropha curcas*, *J. mahafalensis*, and cultivars thereof); *Elaeis guineensis* (e.g. Oil palm), *Aleurites fordii* (rung oil tree or wood oil tree), *Ricinus communis* (castor bean tree), *Copaifera langsdorfii* (diesel tree), and *Pongammia pinnata* (Honge oil tree, or Pongam tree, and cultivars thereof).

Preferred examples of suitable seeds include soya, canola (rape seed), palm, olive, cottonseed, rice bran, corn, palm kernel, coconut, peanut, sesame or sunflower. The process of the invention can be used in conjunction with methods for extracting and processing essential oils, e.g., those from fruit seed oils, e.g. grapeseed, apricot, borage, etc. The process of the invention can be used in conjunction with methods for extracting and processing high phosphorus oils (e.g. soy bean oil).

In a preferred embodiment, the plant material comprises soya beans or rapeseed.

Chlorophyll and chlorophyll derivatives

By "chlorophyll derivative" it is typically meant compounds which comprise both a porphyrin (chlorin) ring and a phytol group (tail), including magnesium-free phytol-containing derivatives such as pheophytin and pyropheophytin. Chlorophyll and (phytol-containing) chlorophyll derivatives are typically greenish is colour, as a result of the porphyrin (chlorin) ring present in the molecule. Loss of magnesium from the porphyrin ring means that pheophytin and pyropheophytin are more brownish in colour than chlorophyll. Thus the presence of chlorophyll and chlorophyll derivatives in an oil, can give such an oil an undesirable green, greenish or brownish colour. In one embodiment, the present process may be performed in order to remove or reduce the green or brown colouring present in oil extracted from the plant material. Accordingly the present process may be referred to as a bleaching or de-colorizing process.

Enzymes used in the process may hydrolyse chlorophyll and phytol-containing chlorophyll derivatives to cleave the phytol tail from the chlorin ring. Hydrolysis of chlorophyll and chlorophyll derivatives typically results in compounds such as chlorophyllide, pheophorbide and pyropheophorbide which are phytol-free derivatives of chlorophyll. These compounds still contain the colour-bearing porphyrin ring, with chlorophyllide being green and pheophorbide and pyropheophorbide a reddish brown colour. In some embodiments, it may also be desirable to remove these phytol-free derivatives and to reduce the green/red/brown colouring in the extracted oil. Thus in one embodiment of the invention, the process may further comprise a step of removing or reducing the level of phytol-free chlorophyll derivatives in the oil extracted from the seeds. The process may involve bleaching or de-colorizing to remove the green and/or red/brown colouring of the extracted oil.

The chlorophyll or chlorophyll derivative may be either a or b forms. Thus as used herein, the term "chlorophyll" includes chlorophyll a and chlorophyll b. In a similar way both a and b forms are covered when referring to pheophytin, pyropheophytin, chlorophyllide, pheophorbide and pyropheophorbide. Chlorophyll a and b and pheophytin a and b each exist as a pair of epimers determined by the stereochemistry of H and $COOCH_3$ around the carbon number 13^2 (numbering according to the IUPAC system). Thus chlorophyll a exists as the pair of epimers chlorophyll *a* and chlorophyll

a', and chlorophyll b comprises *b* and *b'* forms. Likewise pheophytin a comprises the epimer *a* and *a'* pair and pheophytin b comprises *b* and *b'* forms. All such stereoisomers are covered when referring to the respective chlorophyll derivatives.

Chlorophyll and chlorophyll derivatives in plant materials

The chlorophyll and/or chlorophyll derivatives (e.g. chlorophyll, pheophytin and/or pyropheophytin) may be present in the plant material naturally, as a contaminant, or as an undesired component in a processed product. The chlorophyll and/or chlorophyll derivatives (e.g. chlorophyll, pheophytin and/or pyropheophytin) may be present at any level in the plant material. Typically chlorophyll, pheophytin and/or pyropheophytin may be present as a natural contaminant in the plant material at a concentration of 0.001 to 1000 mg/kg (0.001 to 1000 ppm, 10^{-7} to 10^{-1} wt %), based on the total weight of the plant material. In further embodiments, the chlorophyll and/or chlorophyll derivatives may be present in the plant material at a concentration of 0.1 to 100, 0.5 to 50, 1 to 50, 1 to 30 or 1 to 10 mg/kg, based on the total weight of the plant material.

Phytol-free chlorophyll derivatives may also be present in the plant material. For instance, chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present at any level in the plant material. Typically chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present in the plant material, either before or after treatment with an enzyme according to the method of the present invention, at a concentration of 0.001 to 1000 mg/kg (0.001 to 1000 ppm, 10^{-7} to 10^{-1} wt %), based on the total weight of the plant material. In further embodiments, the chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present in the plant material at a concentration of 0.1 to 100, 0.5 to 50, 1 to 50, 1 to 30 or 1 to 10 mg/kg, based on the total weight of the plant material.

Preparation of plant material

In some embodiments, untreated plant material may be contacted with the organic solvent comprising hexane (and optionally the enzyme). Alternatively the plant material (e.g. seeds) may first be subjected to various treatments such as cleaning, conditioning, grinding, pressing and/or flaking, e.g. as described in Bailey's Industrial

Oil and Fat Products (2005), 6th edition, Ed. by Fereidoon Shahidi, John Wiley & Sons, Chapter 2. In one embodiment, the plant material (e.g. seeds) is subjected to a mechanical and/or chemical treatment step which increases the surface area of the plant material and/or facilitates penetration of the organic solvent comprising hexane (and enzyme) into the plant material, in order to increase the extraction of oil from the plant material (and the rate of hydrolysis of chlorophyll and chlorophyll metabolites). For instance, in some embodiments seeds may be crushed, ground, peeled or flaked before contacting with the organic solvent comprising hexane (and the enzyme). Suitable methods are well known in the art for preparing seeds in such a manner.

In one embodiment, the organic solvent comprising hexane (and optionally the enzyme) is contacted with seed flakes. Seeds may be flaked, for example, using smooth-surface rolling mills in one or more stages. The flake thickness may be about 0.1 to 1 mm, preferably 0.1 to 0.5 mm, more preferably 0.2 to 0.4 mm (e.g. about 0.3 mm). In an embodiment of a single stage flaking process, flakes of e.g. rapeseed or soya having a thickness of about 0.3 mm may be produced in a single step. In one embodiment of a two-stage method, suitable for use with e.g. rapeseed, a flake thickness of about 0.4–0.7 mm is produced by a first set of rolls, and then flakes of 0.2–0.3 mm thickness are produced in a second stage. Flaking ruptures the cell walls, which not only releases some of the oil from the seeds but increases penetration of the organic solvent (e.g. hexane) and the enzyme, thereby increasing oil extraction and facilitating hydrolysis of chlorophyll and its metabolites.

In some embodiments, the plant material (e.g. seeds or seed flakes) is pressed before contact with the organic solvent (e.g. hexane). By "pressing" it is intended to refer to any application of mechanical force, which typically results in expulsion of a significant proportion of the oil from the oilseeds. This step may be performed using any suitable apparatus known in the art, e.g. continuous screw presses, expellers, single-screw or twin-screw extruders. Pressing may be performed, for example, using a one-stage or multistage process. Expeller pressing typically reduces the oil content of the seed from e.g. (in the case of rapeseed) about 40% to about 20%. The organic solvent (e.g. hexane) extraction step may then be used to recover the remaining oil from the press-cake.

Further enzyme treatments

In some embodiments, one or more further enzymes may be applied to the plant material (e.g. oilseeds), before the organic solvent (e.g. hexane) extraction step. By "further enzymes" it is here intended to refer to enzymes other than chlorophyllases and related enzymes, i.e. enzymes which are capable of hydrolysing chlorophyll or a chlorophyll derivative. Typically these further enzymes are applied to the plant material in the absence of the organic solvent (e.g. hexane), e.g. in order to prepare the plant material for the organic solvent extraction. Other than in the context of the current section of the description, the term "the enzyme" as used herein generally refers to an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative.

A number of enzymes may be used in order to digest plant seed material and improve the yield of oil from the seeds (see e.g. WO1991/013956, EP0113165, WO2008/088489 and CA2673926). Such further enzymatic treatments are useful for weakening and partially decomposing cell walls (primary and secondary cell walls) as well as the destruction of the membrane envelope surrounding the oil. This facilitates oil release from the plant material and its subsequent recovery.

For instance, the plant material may be treated with an aqueous solution comprising one or more cellulolytic, hemicellulolytic, lipolytic, pectinolytic and/or proteolytic enzymes, e.g. as described in CA2673926. In one embodiment, the solution further comprises one or more cellulases, endoglucanases, cellobiohydrolases, hemicellulases, pectinases, proteases and/or phytases. Such enzymes may be obtained from natural or recombinant sources. These enzymes may be used individually or in combination, depending of the composition of the plant material, e.g., for protein-rich seeds such as soy beans a protease is preferably used. Such further enzyme activities may be present in differing amounts in commercially available products, e.g. Rohalase®OS, a mixture comprising cellulase, beta-glucanase and xylanase activities available from AB Enzymes, Darmstadt, Germany.

Hemicellulolytic and pectinolytic enzymes are preferably used for plant materials (e.g. seeds) that contain an increased amount of these storage substances in the cell walls, typically where cellulases alone would not cause sufficient loosening or perforation of

the cell wall. Pectinases are particularly useful for degrading the protopectine of the middle lamellae, which results in improved paste formation for pressing and facilitates oil release. Galactomannanases are preferred for use with, for example, soy beans. Thermostable forms of such enzymes may be used in some embodiments.

Suitable proteases may be obtained, for example, from microbial sources including *B. amyloliquifaciens*, *B. subtilis*, *B. lichenformis*, *A. niger* or *A. oryzae*. In one embodiment, the protease activity comprises an endopeptidase. Metalloproteases, whether exo- or endo-proteases, may also be used. Many proteases are available commercially, e.g. Fungal Protease 500,000, Protex 6L protease and Fungal Protease Concentrate available from Genencor (Rochester, NY).

Contacting the plant material with an organic solvent comprising hexane

In embodiments of the present invention, the oil-containing plant material is contacted with an organic solvent comprising hexane. Hexane is a saturated hydrocarbon having the formula C_6H_{14}, and is a colourless liquid at room temperature having a boiling point of about 69°C and a density of about 0.65 g/ml. The term "hexane" may refer to any branched or unbranched structural isomer having the above formula or mixtures thereof. Typically the term "hexane" refers to the unbranched isomer, i.e. n-hexane.

The organic solvent used in the majority of oilseed solvent extraction plants is commercial hexane, a mixture of hydrocarbons generally boiling in the temperature range of 65–69°C. Most commercial hexane available contains approximately 65% by weight normal (n-)hexane, with the remaining 35% of the composition consisting of cyclopentane and hexane isomers. Any such organic solvent mixture may be used in embodiments of the present invention, provided that the solvent comprises hexane. Preferably the organic solvent comprises at least 10%, at least 30%, at least 50%, at least 60%, at least 70%, at least 80% or at least 90% by weight hexane, more preferably n-hexane. In one embodiment, the organic solvent comprises at least 90%, 95% or 99% by weight (or consists essentially of) hydrocarbons, e.g. C_5 to C_7 alkanes (including branched, straight chain and/or cyclic alkanes).

The amount of organic solvent (e.g. hexane) used relative to the amount of plant material may vary depending on various factors, such as the type of plant material, the oil content of the plant material and so on. However, typically the amount of organic solvent (e.g. hexane) used may be 10 to 90% by weight, based on the total amount of organic solvent (e.g. hexane) and plant material (e.g. plant seeds). In particular embodiments, the amount of organic solvent (e.g. hexane) may comprise 20 to 80%, 30 to 70%, 40 to 60%, or about 50% by weight, based on the total amount of organic solvent (e.g. hexane) and plant material.

Suitably the oil-containing plant material may be contacted with the organic solvent (e.g. hexane) at a temperature of about 10°C to about 90°C. In particular embodiments, this step may be performed at about 20°C to about 80°C, about 30°C to about 70°C, about 40°C to about 70°C, about 50°C to about 70°C, about 55°C to about 65°C, or about 60°C.

Preferably the temperature of the organic solvent (e.g. hexane) is at the desired reaction temperature when the plant material is admixed therewith. The plant material may be heated and/or cooled to the desired temperature before and/or during hexane addition.

Suitably the extraction time (i.e. the time period for which the organic solvent (e.g. hexane) is incubated with the plant material), preferably with agitation, is for a sufficient period of time to allow extraction of oil from the plant material. For example, the extraction time may be at least about 1 minute, more preferable at least about 5 minutes, more preferably at least about 10 minutes. In some embodiments the extraction time may be between about 15 minutes to about 48 hours, about 30 minutes to 12 hours, about 30 minutes to about 6 hours, about 1 to 4 hours, or about 1 to 2 hours.

Obtaining a mixture of an organic solvent comprising hexane and crude plant oil

Contacting the oil-containing plant material with the organic solvent (e.g. hexane) typically results in extraction of oil from the plant material into an organic phase comprising hexane. Thus at this stage of the process, the mixture comprising the organic solvent (e.g. hexane) and the crude plant oil is still in contact with the remaining plant material (e.g. crushed or flaked seeds) from which the oil has been extracted. The

mixture comprising the organic solvent (e.g. hexane) and the crude plant oil may be separated from the plant residue by any suitable means, e.g. a centrifugal separator.

Typically the mixture obtained at this stage comprises 10 to 90% of the organic solvent (e.g. hexane) by weight, based on the total weight of the mixture. The remaining content of the mixture is primarily the crude plant oil, e.g. the mixture may comprise 10 to 90% by weight of the crude plant oil. In particular embodiments, the mixture may comprise 20 to 80%, 30 to 70%, 40 to 60%, or about 50% by weight of the organic solvent (e.g. hexane), based on the total amount of the organic solvent (e.g. hexane) and crude plant oil.

Separating the crude plant oil from the organic solvent comprising hexane

After the mixture comprising the organic solvent (e.g. hexane) and the crude plant oil is obtained, the method comprises a further step of separating the crude plant oil from the organic solvent (e.g. hexane). Typically this may be done by evaporating the volatile organic solvent (e.g. hexane) component from the mixture. Removal of hexane may be facilitated by elevated temperature (the boiling point of hexane is approximately 69°C) or by application of a vacuum.

Enzymes hydrolysing chlorophyll or a chlorophyll derivative

The process of the present invention comprises a step of contacting the plant material and/or crude plant oil with an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative. Typically "hydrolyzing chlorophyll or a chlorophyll derivative" means hydrolysing an ester bond in chlorophyll or a (phytol-containing) chlorophyll derivative, e.g. to cleave a phytol group from the chlorin ring in the chlorophyll or chlorophyll derivative. Thus the enzyme typically has an esterase or hydrolase activity. Preferably the enzyme has esterase or hydrolase activity in both an oil phase and an aqueous phase.

Thus the enzyme may, for example, be a chlorophyllase, pheophytinase or pyropheophytinase. Preferably, the enzyme is capable of hydrolysing at least one, at least two or all three of chlorophyll, pheophytin and pyropheophytin. In a particularly preferred embodiment, the enzyme has chlorophyllase, pheophytinase and

pyropheophytinase activity. In further embodiments, two or more enzymes may be used in the method, each enzyme having a different substrate specificity. For instance, the method may comprise the combined use of two or three enzymes selected from a chlorophyllase, a pheophytinase and a pyropheophytinase.

Any polypeptide having an activity that can hydrolyse chlorophyll or a chlorophyll derivative can be used as the enzyme in the process of the invention. By "enzyme" it is intended to encompass any polypeptide having hydrolytic activity on chlorophyll or a chlorophyll derivative, including e.g. enzyme fragments, etc. Any isolated, recombinant or synthetic or chimeric (or a combination of synthetic and recombinant) polypeptide can be used.

Enzyme (chlorophyllase, pheophytinase or pyropheophytinase) activity assay

Hydrolytic activity on chlorophyll or a chlorophyll derivative may be detected using any suitable assay technique, for example based on an assay described herein. For example, hydrolytic activity may be detected using fluorescence-based techniques. In one suitable assay, a polypeptide to be tested for hydrolytic activity on chlorophyll or a chlorophyll derivative is incubated in the presence of a substrate, and product or substrate levels are monitored by fluorescence measurement. Suitable substrates include e.g. chlorophyll, pheophytin and/or pyropheophytin. Products which may be detected include chlorophyllide, pheophorbide, pyropheophorbide and/or phytol.

Assay methods for detecting hydrolysis of chlorophyll or a chlorophyll derivative are disclosed in, for example, Ali Khamessan et al. (1994), Journal of Chemical Technology & Biotechnology, 60(1), pages 73 – 81; Klein and Vishniac (1961), J. Biol. Chem. 236: 2544-2547; and Kiani et al. (2006), Analytical Biochemistry 353: 93–98.

Alternatively, a suitable assay may be based on HPLC detection and quantitation of substrate or product levels following addition of a putative enzyme, e.g. based on the techniques described below. In one embodiment, the assay may be performed as described in Hornero-Mendez et al. (2005), Food Research International 38(8-9): 1067-1072. In another embodiment, the following assay may be used:

170 µl 50mM HEPES, pH 7.0 is added to 20 µl 0.3 mM chlorophyll, pheophytin or pyropheophytin dissolved in acetone. The enzyme is dissolved in 50 mM HEPES, pH 7.0. 10 µl enzyme solution is added to 190 µl substrate solution to initiate the reaction and incubated at 40°C for various time periods. The reaction was stopped by addition of 350 µl acetone. Following centrifugation (2 min at 18,000 g) the supernatant was analyzed by HPLC, and the amounts of (i) chlorophyll and chlorophyllide (ii) pheophytin and pheophorbide or (iii) pyropheophytin and pyropheophorbide determined.

In a further embodiment, hydrolytic activity on chlorophyll or a chlorophyll derivative may be determined using a method as described in WO 2011/125028.

One unit of enzyme activity is defined as the amount of enzyme which hydrolyzes one micromole of substrate (e.g. chlorophyll, pheophytin or pyropheophytin) per minute at 40°C, e.g. in an assay method as described herein.

In preferred embodiments, the enzyme used in the present process has chlorophyllase, pheophytinase and/or pyropheophytinase activity of at least 1000 U/g, at least 5000 U/g, at least 10000 U/g, or at least 50000 U/g, based on the units of activity per gram of the purified enzyme, e.g. as determined by an assay method described herein.

Chlorophyllases

In one embodiment, the enzyme is capable of hydrolyzing at least chlorophyll. Any polypeptide that catalyses the hydrolysis of a chlorophyll ester bond to yield chlorophyllide and phytol can be used in the process. For example, a chlorophyllase, chlase or chlorophyll chlorophyllido-hydrolyase or polypeptide having a similar activity (e.g., chlorophyll-chlorophyllido hydrolase 1 or chlase 1, or, chlorophyll-chlorophyllido hydrolase 2 or chlase 2, see, e.g. NCBI P59677-1 and P59678, respectively) can be used in the process.

In one embodiment the enzyme is a chlorophyllase classified under the Enzyme Nomenclature classification (E.C. 3.1.1.14). Any isolated, recombinant or synthetic or chimeric (a combination of synthetic and recombinant) polypeptide (e.g., enzyme or catalytic antibody) can be used, see e.g. Marchler-Bauer (2003) Nucleic Acids Res. 31:

383-387. In one aspect, the chlorophyllase may be an enzyme as described in WO 0229022 or WO 2006009676. For example, the *Arabidopsis thaliana* chlorophyllase can be used as described, e.g. in NCBI entry NM_123753. Thus the chlorophyllase may be a polypeptide comprising the sequence of SEQ ID NO:1 (see Figure 2). In another embodiment, the chlorophyllase is derived from algae, e.g. from *Phaeodactylum tricornutum*.

In another embodiment, the chlorophyllase is derived from wheat, e.g. from *Triticum spp.*, especially from *Triticum aestivum*. For example, the chlorophyllase may be polypeptide comprising the sequence of SEQ ID NO:2 (see Figure 3), or may be encoded by the nucleotide sequence of SEQ ID NO:3 (see Figure 4).

In another embodiment, the chlorophyllase is derived from *Chlamydomonas spp.*, especially from *Chlamydomonas reinhardtii*. For example, the chlorophyllase may be a polypeptide comprising the sequence of SEQ ID NO:4 (see Figure 5), or may be encoded by the nucleotide sequence of SEQ ID NO:5 (see Figure 6).

Pheophytin pheophorbide hydrolase

In one embodiment, the enzyme is capable of hydrolyzing pheophytin and pyropheophytin. For example, the enzyme may be pheophytinase or pheophytin pheophorbide hydrolase (PPH), e.g. an enzyme as described in Schelbert *et al.*, The Plant Cell 21:767-785 (2009).

PPH and related enzymes are capable of hydrolyzing pyropheophytin in addition to pheophytin. However PPH is inactive on chlorophyll. As described in Schelbert *et al.*, PPH orthologs are commonly present in eukaryotic photosynthesizing organisms. PPHs represent a defined sub-group of α/β hydrolases which are phylogenetically distinct from chlorophyllases, the two groups being distinguished in terms of sequence homology and substrates.

In specific embodiments of the invention, the enzyme may be any known PPH derived from any species or a functional variant or fragment thereof or may be derived from any known PPH enzyme. For example, in one embodiment, the enzyme is a PPH from *Arabidopsis thaliana*, e.g. a polypeptide comprising the amino acid sequence of SEQ ID

NO:6 (see Figure 7), or a polypeptide encoded by the nucleotide sequence of SEQ ID NO:7 (see Figure 8, NCBI accession no. NP_196884, GenBank ID No. 15240707), or a functional variant or fragment thereof.

In further embodiments, the enzyme may be a PPH derived from any one of the following species: *Arabidopsis thaliana, Populus trichocarpa, Vitis vinifera, Oryza sativa, Zea mays, Nicotiana tabacum, Ostreococcus lucimarinus, Ostreococcus taurii, Physcomitrella patens, Phaeodactylum tricornutum, Chlamydomonas reinhardtii,* or *Micromonas sp. RCC299*. For example, the enzyme may be a polypeptide comprising an amino acid sequence, or encoded by a nucleotide sequence, defined in one of the following database entries shown in Table 1, or a functional fragment or variant thereof:

Table 1

Organism	**Accession**	**Genbank ID**
Arabidopsis thaliana	NP_196884	15240707
Populus trichocarpa	XP_002314066	224106163
Vitis vinifera	CAO40741	157350650
Oryza sativa (japonica)	NP_001057593	115467988
Zea mays	ACF87407	194706646
Nicotiana tabacum	CAO99125	156763846
Ostreococcus lucimarinus	XP_001415589	145340970
Ostreococcus tauri	CAL50341	116000661
Physcomitrella patens	XP_001761725	168018382
Phaeodactylum tricornutum	XP_002181821	219122997
Chlamydomonas reinhardtii	XP_001702982	159490010
Micromonas sp. RCC299	ACO62405	226516410

For example, the enzyme may be a polypeptide as defined in any of SEQ ID NO:s 8 to 15 (Figures 9 to 16), or a functional fragment or variant thereof.

Variants and fragments

Functional variants and fragments of known sequences which hydrolyse chlorophyll or a chlorophyll derivative may also be employed in the present invention. By "functional" it is meant that the fragment or variant retains a detectable hydrolytic activity on chlorophyll or a chlorophyll derivative. Typically such variants and fragments show homology to a known chlorophyllase, pheophytinase or pyropheophytinase sequence, e.g. at least about 50%, 60%, 70%, 75%, 80%, 85%, 90%,

95%, 96%, 97%, 98%, 99%, or more sequence identity to a known chlorophyllase, pheophytinase or pyropheophytinase amino acid sequence, e.g. to SEQ ID NO:l or any one of SEQ ID NOs: 1, 2, 4, 6 or 8 to 15, e.g. over a region of at least about 10, 20, 30, 50, 100, 200, 300, 500, or 1000 or more residues, or over the entire length of the sequence.

The percentage of sequence identity may be determined by analysis with a sequence comparison algorithm or by a visual inspection. In one aspect, the sequence comparison algorithm is a BLAST algorithm, e.g., a BLAST version 2.2.2 algorithm.

Other enzymes having chlorophyllase, pheophytinase and/or pyropheophytinase activity suitable for use in the process may be identified by determining the presence of conserved sequence motifs present e.g. in known chlorophyllase, pheophytinase or pyropheophytinase sequences. For example, conserved sequence motifs found in PPH enzymes include the following: LPGFGVG (SEQ ID NO:16), DFLGQG (SEQ ID NO:17), GNSLGG (SEQ ID NO:18), LVKGVTLLNATPFW (SEQ ID NO:19), HPAA (SEQ ID NO:20), EDPW (SEQ ID NO:21), and SPAGHCPH (SEQ ID NO:22). In some embodiments, an enzyme for use in the present invention may comprise one or more of these sequences. The GNSLGG (SEQ ID NO:18) motif contains an active site serine residue. Polypeptide sequences having suitable activity may be identified by searching genome databases, e.g. the microbiome metagenome database (JGI-DOE, USA), for the presence of these motifs.

Isolation and production of enzymes

Enzymes for use in the present invention may be isolated from their natural sources or may be, for example, produced using recombinant DNA techniques. Nucleotide sequences encoding polypeptides having chlorophyllase, pheophytinase and/or pyropheophytinase activity may be isolated or constructed and used to produce the corresponding polypeptides.

For example, a genomic DNA and/or cDNA library may be constructed using chromosomal DNA or messenger RNA from the organism producing the polypeptide. If the amino acid sequence of the polypeptide is known, labeled oligonucleotide probes

may be synthesised and used to identify polypeptide-encoding clones from the genomic library prepared from the organism. Alternatively, a labelled oligonucleotide probe containing sequences homologous to another known polypeptide gene could be used to identify polypeptide-encoding clones. In the latter case, hybridisation and washing conditions of lower stringency are used.

Alternatively, polypeptide-encoding clones could be identified by inserting fragments of genomic DNA into an expression vector, such as a plasmid, transforming enzyme-negative bacteria with the resulting genomic DNA library, and then plating the transformed bacteria onto agar containing an enzyme inhibited by the polypeptide, thereby allowing clones expressing the polypeptide to be identified.

In a yet further alternative, the nucleotide sequence encoding the polypeptide may be prepared synthetically by established standard methods, e.g. the phosphoroamidite method described by Beucage S.L. *et al* (1981) Tetrahedron Letters 22, p 1859-1869, or the method described by Matthes *et al* (1984) EMBO J. 3, p 801-805. In the phosphoroamidite method, oligonucleotides are synthesised, e.g. in an automatic DNA synthesiser, purified, annealed, ligated and cloned in appropriate vectors.

The nucleotide sequence may be of mixed genomic and synthetic origin, mixed synthetic and cDNA origin, or mixed genomic and cDNA origin, prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate) in accordance with standard techniques. Each ligated fragment corresponds to various parts of the entire nucleotide sequence. The DNA sequence may also be prepared by polymerase chain reaction (PCR) using specific primers, for instance as described in US 4,683,202 or in Saiki R K *et al* (Science (1988) 239, pp 487-491).

The term "nucleotide sequence" as used herein refers to an oligonucleotide sequence or polynucleotide sequence, and variant, homologues, fragments and derivatives thereof (such as portions thereof). The nucleotide sequence may be of genomic or synthetic or recombinant origin, which may be double-stranded or single-stranded whether representing the sense or antisense strand.

Typically, the nucleotide sequence encoding a polypeptide having chlorophyllase, pheophytinase and/or pyropheophytinase activity is prepared using recombinant DNA techniques. However, in an alternative embodiment of the invention, the nucleotide sequence could be synthesised, in whole or in part, using chemical methods well known in the art (see Caruthers MH *et al* (1980) Nuc Acids Res Symp Ser 215-23 and Horn T *et al* (1980) Nuc Acids Res Symp Ser 225-232).

Modification of enzyme sequences

Once an enzyme-encoding nucleotide sequence has been isolated, or a putative enzyme-encoding nucleotide sequence has been identified, it may be desirable to modify the selected nucleotide sequence, for example it may be desirable to mutate the sequence in order to prepare an enzyme in accordance with the present invention.

Mutations may be introduced using synthetic oligonucleotides. These oligonucleotides contain nucleotide sequences flanking the desired mutation sites. A suitable method is disclosed in Morinaga *et al* (Biotechnology (1984) 2, p646-649). Another method of introducing mutations into enzyme-encoding nucleotide sequences is described in Nelson and Long (Analytical Biochemistry (1989), 180, p 147-151).

Instead of site directed mutagenesis, such as described above, one can introduce mutations randomly for instance using a commercial kit such as the GeneMorph PCR mutagenesis kit from Stratagene, or the Diversify PCR random mutagenesis kit from Clontech. EP 0 583 265 refers to methods of optimising PCR based mutagenesis, which can also be combined with the use of mutagenic DNA analogues such as those described in EP 0 866 796. Error prone PCR technologies are suitable for the production of variants of enzymes which hydrolyse chlorophyll and/or chlorophyll derivatives with preferred characteristics. WO0206457 refers to molecular evolution of lipases.

A third method to obtain novel sequences is to fragment non-identical nucleotide sequences, either by using any number of restriction enzymes or an enzyme such as Dnase I, and reassembling full nucleotide sequences coding for functional proteins. Alternatively one can use one or multiple non-identical nucleotide sequences and

introduce mutations during the reassembly of the full nucleotide sequence. DNA shuffling and family shuffling technologies are suitable for the production of variants of enzymes with preferred characteristics. Suitable methods for performing 'shuffling' can be found in EP0752008, EP1138763, EP1103606. Shuffling can also be combined with other forms of DNA mutagenesis as described in US 6,180,406 and WO 01/34835.

Thus, it is possible to produce numerous site directed or random mutations into a nucleotide sequence, either *in vivo* or *in vitro*, and to subsequently screen for improved functionality of the encoded polypeptide by various means. Using in silico and exo mediated recombination methods (see WO 00/58517, US 6,344,328, US 6,361,974), for example, molecular evolution can be performed where the variant produced retains very low homology to known enzymes or proteins. Such variants thereby obtained may have significant structural analogy to known chlorophyllase, pheophytinase or pyropheophytinase enzymes, but have very low amino acid sequence homology.

As a non-limiting example, in addition, mutations or natural variants of a polynucleotide sequence can be recombined with either the wild type or other mutations or natural variants to produce new variants. Such new variants can also be screened for improved functionality of the encoded polypeptide.

The application of the above-mentioned and similar molecular evolution methods allows the identification and selection of variants of the enzymes of the present invention which have preferred characteristics without any prior knowledge of protein structure or function, and allows the production of non-predictable but beneficial mutations or variants. There are numerous examples of the application of molecular evolution in the art for the optimisation or alteration of enzyme activity, such examples include, but are not limited to one or more of the following: optimised expression and/or activity in a host cell or in vitro, increased enzymatic activity, altered substrate and/or product specificity, increased or decreased enzymatic or structural stability, altered enzymatic activity/specificity in preferred environmental conditions, e.g. temperature, pH, substrate.

As will be apparent to a person skilled in the art, using molecular evolution tools an enzyme may be altered to improve the functionality of the enzyme. Suitably, a

nucleotide sequence encoding an enzyme (e.g. a chlorophyllase, pheophytinase and/or pyropheophytinase) used in the invention may encode a variant enzyme, i.e. the variant enzyme may contain at least one amino acid substitution, deletion or addition, when compared to a parental enzyme. Variant enzymes retain at least 1%, 2%, 3%, 5%, 10%, 15%, 20%, 30%, 40%, 50 %, 60%, 70%, 80%, 90%, 95%, 97%, or 99% identity with the parent enzyme. Suitable parent enzymes may include any enzyme with hydrolytic activity on chlorophyll and/or a chlorophyll derivative.

Polypeptide sequences

The present invention also encompasses the use of amino acid sequences encoded by a nucleotide sequence which encodes a pyropheophytinase for use in any one of the methods and/or uses of the present invention.

As used herein, the term "amino acid sequence" is synonymous with the term "polypeptide" and/or the term "protein". In some instances, the term "amino acid sequence" is synonymous with the term "peptide". The amino acid sequence may be prepared/isolated from a suitable source, or it may be made synthetically or it may be prepared by use of recombinant DNA techniques. Suitably, the amino acid sequences may be obtained from the isolated polypeptides taught herein by standard techniques.

One suitable method for determining amino acid sequences from isolated polypeptides is as follows. Purified polypeptide may be freeze-dried and 100 µg of the freeze-dried material may be dissolved in 50 µl of a mixture of 8 M urea and 0.4 M ammonium hydrogen carbonate, pH 8.4. The dissolved protein may be denatured and reduced for 15 minutes at 50°C following overlay with nitrogen and addition of 5 µl of 45 mM dithiothreitol. After cooling to room temperature, 5 µl of 100 mM iodoacetamide may be added for the cysteine residues to be derivatized for 15 minutes at room temperature in the dark under nitrogen.

135 µl of water and 5 µg of endoproteinase Lys-C in 5 µl of water may be added to the above reaction mixture and the digestion may be carried out at 37°C under nitrogen for 24 hours. The resulting peptides may be separated by reverse phase HPLC on a VYDAC C18 column (0.46x15cm;10µm; The Separation Group, California, USA)

using solvent A: 0.1% TFA in water and solvent B: 0.1% TFA in acetonitrile. Selected peptides may be re-chromatographed on a Develosil C18 column using the same solvent system, prior to N-terminal sequencing. Sequencing may be done using an Applied Biosystems 476A sequencer using pulsed liquid fast cycles according to the manufacturer's instructions (Applied Biosystems, California, USA).

Sequence comparison

Here, the term "homologue" means an entity having a certain homology with the subject amino acid sequences and the subject nucleotide sequences. Here, the term "homology" can be equated with "identity". The homologous amino acid sequence and/or nucleotide sequence should provide and/or encode a polypeptide which retains the functional activity and/or enhances the activity of the enzyme.

In the present context, a homologous sequence is taken to include an amino acid sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to the subject sequence. Typically, the homologues will comprise the same active sites etc. as the subject amino acid sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

In the present context, a homologous sequence is taken to include a nucleotide sequence which may be at least 75, 85 or 90% identical, preferably at least 95 or 98% identical to a nucleotide sequence encoding a polypeptide of the present invention (the subject sequence). Typically, the homologues will comprise the same sequences that code for the active sites etc. as the subject sequence. Although homology can also be considered in terms of similarity (i.e. amino acid residues having similar chemical properties/functions), in the context of the present invention it is preferred to express homology in terms of sequence identity.

Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences. %

homology may be calculated over contiguous sequences, i.e. one sequence is aligned with the other sequence and each amino acid in one sequence is directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an "ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues.

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the Vector NTI Advance™ 11 (Invitrogen Corp.). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see Ausubel et al 1999 Short Protocols in Molecular Biology, 4th Ed - Chapter 18), and FASTA (Altschul et al 1990 J. Mol. Biol. 403-410). Both BLAST and FASTA are available for offline and online searching (see Ausubel et al 1999, pages 7-58 to 7-60). However, for some applications,

it is preferred to use the Vector NTI Advance™ 11 program. A new tool, called BLAST 2 Sequences is also available for comparing protein and nucleotide sequence (see FEMS Microbiol Lett 1999 174(2): 247-50; and FEMS Microbiol Lett 1999 177(1): 187-8.).

Although the final % homology can be measured in terms of identity, the alignment process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. Vector NTI programs generally use either the public default values or a custom symbol comparison table if supplied (see user manual for further details). For some applications, it is preferred to use the default values for the Vector NTI Advance™ 11 package.

Alternatively, percentage homologies may be calculated using the multiple alignment feature in Vector NTI Advance™ 11 (Invitrogen Corp.), based on an algorithm, analogous to CLUSTAL (Higgins DG & Sharp PM (1988), Gene 73(1), 237-244). Once the software has produced an optimal alignment, it is possible to calculate % homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

Should Gap Penalties be used when determining sequence identity, then preferably the default parameters for the programme are used for pairwise alignment. For example, the following parameters are the current default parameters for pairwise alignment for BLAST 2:

FOR BLAST2	DNA	PROTEIN
EXPECT THRESHOLD	10	10
WORD SIZE	11	3
SCORING		

PARAMETERS		
Match/Mismatch Scores	2, -3	n/a
Matrix	n/a	BLOSUM62
Gap Costs	Existence: 5 Extension: 2	Existence: 11 Extension: 1

In one embodiment, preferably the sequence identity for the nucleotide sequences and/or amino acid sequences may be determined using BLAST2 (blastn) with the scoring parameters set as defined above.

For the purposes of the present invention, the degree of identity is based on the number of sequence elements which are the same. The degree of identity in accordance with the present invention for amino acid sequences may be suitably determined by means of computer programs known in the art such as Vector NTI Advance™ 11 (Invitrogen Corp.). For pairwise alignment the scoring parameters used are preferably BLOSUM62 with Gap existence penalty of 11and Gap extension penalty of 1.

Suitably, the degree of identity with regard to a nucleotide sequence is determined over at least 20 contiguous nucleotides, preferably over at least 30 contiguous nucleotides, preferably over at least 40 contiguous nucleotides, preferably over at least 50 contiguous nucleotides, preferably over at least 60 contiguous nucleotides, preferably over at least 100 contiguous nucleotides. Suitably, the degree of identity with regard to a nucleotide sequence may be determined over the whole sequence.

Amino acid mutations

The sequences may also have deletions, insertions or substitutions of amino acid residues which produce a silent change and result in a functionally equivalent substance. Deliberate amino acid substitutions may be made on the basis of similarity in polarity,

charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues as long as the secondary binding activity of the substance is retained. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; and amino acids with uncharged polar head groups having similar hydrophilicity values include leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, and tyrosine.

Conservative substitutions may be made, for example according to the Table below. Amino acids in the same block in the second column and preferably in the same line in the third column may be substituted for each other:

ALIPHATIC	Non-polar	G A P
		I L V
	Polar – uncharged	C S T M
		N Q
	Polar – charged	D E
		K R
AROMATIC		H F W Y

The present invention also encompasses homologous substitution (substitution and replacement are both used herein to mean the interchange of an existing amino acid residue, with an alternative residue) that may occur i.e. like-for-like substitution such as basic for basic, acidic for acidic, polar for polar etc. Non-homologous substitution may also occur i.e. from one class of residue to another or alternatively involving the inclusion of unnatural amino acids such as ornithine (hereinafter referred to as Z),

diaminobutyric acid ornithine (hereinafter referred to as B), norleucine ornithine (hereinafter referred to as O), pyriylalanine, thienylalanine, naphthylalanine and phenylglycine. Replacements may also be made by unnatural amino acids.

Variant amino acid sequences may include suitable spacer groups that may be inserted between any two amino acid residues of the sequence including alkyl groups such as methyl, ethyl or propyl groups in addition to amino acid spacers such as glycine or β-alanine residues. A further form of variation, involves the presence of one or more amino acid residues in peptoid form, will be well understood by those skilled in the art. For the avoidance of doubt, "the peptoid form" is used to refer to variant amino acid residues wherein the α-carbon substituent group is on the residue's nitrogen atom rather than the α-carbon. Processes for preparing peptides in the peptoid form are known in the art, for example Simon RJ et al., PNAS (1992) 89(20), 9367-9371 and Horwell DC, Trends Biotechnol. (1995) 13(4), 132-134.

Nucleotide sequences

Nucleotide sequences for use in the present invention or encoding a polypeptide having the specific properties defined herein may include within them synthetic or modified nucleotides. A number of different types of modification to oligonucleotides are known in the art. These include methylphosphonate and phosphorothioate backbones and/or the addition of acridine or polylysine chains at the 3' and/or 5' ends of the molecule. For the purposes of the present invention, it is to be understood that the nucleotide sequences described herein may be modified by any method available in the art. Such modifications may be carried out in order to enhance the *in vivo* activity or life span of nucleotide sequences.

The present invention also encompasses the use of nucleotide sequences that are complementary to the sequences discussed herein, or any derivative, fragment or derivative thereof. If the sequence is complementary to a fragment thereof then that sequence can be used as a probe to identify similar coding sequences in other organisms etc.

Polynucleotides which are not 100% homologous to the sequences of the present invention but fall within the scope of the invention can be obtained in a number of ways. Other variants of the sequences described herein may be obtained for example by probing DNA libraries made from a range of individuals, for example individuals from different populations. In addition, other viral/bacterial, or cellular homologues particularly cellular homologues found in plant cells, may be obtained and such homologues and fragments thereof in general will be capable of selectively hybridising to the sequences shown in the sequence listing herein. Such sequences may be obtained by probing cDNA libraries made from or genomic DNA libraries from other plant species, and probing such libraries with probes comprising all or part of any one of the sequences in the attached sequence listings under conditions of medium to high stringency. Similar considerations apply to obtaining species homologues and allelic variants of the polypeptide or nucleotide sequences of the invention.

Variants and strain/species homologues may also be obtained using degenerate PCR which will use primers designed to target sequences within the variants and homologues encoding conserved amino acid sequences within the sequences of the present invention. Conserved sequences can be predicted, for example, by aligning the amino acid sequences from several variants/homologues. Sequence alignments can be performed using computer software known in the art. For example the GCG Wisconsin PileUp program is widely used.

The primers used in degenerate PCR will contain one or more degenerate positions and will be used at stringency conditions lower than those used for cloning sequences with single sequence primers against known sequences.

Alternatively, such polynucleotides may be obtained by site directed mutagenesis of characterised sequences. This may be useful where for example silent codon sequence changes are required to optimise codon preferences for a particular host cell in which the polynucleotide sequences are being expressed. Other sequence changes may be desired in order to introduce restriction polypeptide recognition sites, or to alter the property or function of the polypeptides encoded by the polynucleotides.

Polynucleotides (nucleotide sequences) of the invention may be used to produce a primer, e.g. a PCR primer, a primer for an alternative amplification reaction, a probe e.g. labelled with a revealing label by conventional means using radioactive or non-radioactive labels, or the polynucleotides may be cloned into vectors. Such primers, probes and other fragments will be at least 15, preferably at least 20, for example at least 25, 30 or 40 nucleotides in length, and are also encompassed by the term polynucleotides of the invention as used herein.

Polynucleotides such as DNA polynucleotides and probes according to the invention may be produced recombinantly, synthetically, or by any means available to those of skill in the art. They may also be cloned by standard techniques.

In general, primers will be produced by synthetic means, involving a stepwise manufacture of the desired nucleic acid sequence one nucleotide at a time. Techniques for accomplishing this using automated techniques are readily available in the art.

Longer polynucleotides will generally be produced using recombinant means, for example using a PCR (polymerase chain reaction) cloning techniques. This will involve making a pair of primers (e.g. of about 15 to 30 nucleotides) flanking a region of the pyropheophytinase sequence which it is desired to clone, bringing the primers into contact with mRNA or cDNA obtained from a plant cell, performing a polymerase chain reaction under conditions which bring about amplification of the desired region, isolating the amplified fragment (e.g. by purifying the reaction mixture on an agarose gel) and recovering the amplified DNA. The primers may be designed to contain suitable restriction enzyme recognition sites so that the amplified DNA can be cloned into a suitable cloning vector.

Contacting the enzyme with the plant material and/or crude plant oil

In embodiments of the present invention, an enzyme which is capable of hydrolyzing chlorophyll or a chlorophyll derivative is contacted with the plant material and/or crude plant oil in the presence of an organic solvent comprising hexane. By this it is meant that the enzyme may be applied any stage of the process described above, provided that hexane is present at that stage. Thus the enzyme may be applied after the plant material

is contacted with the organic solvent (e.g. hexane) and before the organic solvent is separated from the crude plant oil.

In one embodiment, the enzyme may be applied before or during the organic solvent (e.g. hexane) extraction step. Thus the enzyme may be used during the step of contacting the plant material with an organic solvent (e.g. hexane). As discussed above, in this step the plant material is incubated with the organic solvent (e.g. hexane), resulting in extraction of oil from the plant material into the the organic solvent (e.g. hexane). In particular embodiments, the enzyme may be used under the conditions described above in relation to that step.

In an alternative embodiment, the enzyme may be applied to the mixture comprising the organic solvent (e.g. hexane) and a crude plant oil. Thus the enzyme may be used after the crude plant oil has been extracted from the plant material. In this embodiment, the enzyme is typically applied to the mixture after separation of the mixture from the residue of the plant material, although in some embodiments the enzyme may be applied to the mixture while the residue is still present.

The enzyme may be applied to the plant material and/or crude plant oil in any suitable manner. Typically the enzyme is added in the form of an aqueous solution, e.g. an aqueous enzyme solution recovered from the culture supernatant of a microorganism which produces the enzyme. A suitable purified enzyme solution may be obtained using known purification techniques such as filtration and chromatography. For instance, one purification method might involve separating the bio-mass from the culture liquid, and concentrating the obtained solution by ultra-filtration and disinfection filtration.

Enzymes used in the methods of the invention can be formulated or modified, e.g., chemically modified, to enhance oil solubility, stability, activity or for immobilization. For example, enzymes used in the methods of the invention can be formulated to be amphipathic or more lipophilic. For example, the enzyme may be formulated with surfactants in order to increase the activity of the enzyme. Surfactants such as sorbitan esters, citric acid esters, glycerol or polyglycerol esters, or polyoxyethylene esters may be used. In other embodiments, enzymes used in the methods of the invention can be

encapsulated, e.g., in liposomes or gels, e.g., alginate hydrogels or alginate beads or equivalents. Enzymes used in the methods of the invention can be formulated in micellar systems, e.g., a ternary micellar (TMS) or reverse micellar system (RMS) medium. Enzymes used in the methods of the invention can be formulated as described in Yi (2002) J. of Molecular Catalysis B: Enzymatic, Vol. 19, pgs 319-325.

The enzyme may be applied to the plant material and/or crude plant oil in any suitable amount. For example, the solution may comprise the enzyme at a concentration of about 0.001 to 10U/g, preferably 0.01 to 1 U/g, e.g. 0.01 to 0.1 U/g, based on the total weight of the solution. One unit is defined as the amount of enzyme which hydrolyses 1 μmol of substrate (e.g. chlorophyll, pheophytin and/or pyropheophytin) per minute at 40 °C, e.g. under assay conditions as described in J. Biol. Chem. (1961) 236: 2544-2547.

In one embodiment, the enzyme is contacted with the plant material and/or crude plant oil in the presence of water. Water may be present by virtue of the addition of an aqueous enzyme solution, or additional water may be added separately. Typically the enzyme is contacted with the plant material and/or crude plant oil in the presence of 0.1 to 10% by weight water.

For instance, in an embodiment where the enzyme is contacted with the plant material and the organic solvent (e.g. hexane), the added water content (e.g. the water added together with the enzyme solution or separately) may be 0.1 to 10%, 0.5 to 5% or 1 to 3% by weight, based on the total weight of the plant material, the organic solvent (e.g. hexane) and water. In some embodiments, if the natural water content of the plant material is taken into account, the overall water content may be significantly higher. For instance, oil-containing seeds may typically contain about 4-8% by weight water, based on the mass of the seed. Thus the overall water content may be, for example, about 1 to 20%, about 2 to 10%, or about 2 to 5%, based on the total weight of the plant material, the organic solvent (e.g. hexane) and water.

In an alternative embodiment where the enzyme is contacted with the mixture comprising the organic solvent (e.g. hexane) and a crude plant oil, the water content may be 0.1 to 10%, 0.5 to 5% or 1 to 3% by weight, based on the total weight of the mixture (i.e. the organic solvent (e.g. hexane) and the crude plant oil) and water. In this

embodiment, typically the water content of the mixture obtained from organic solvent (e.g. hexane) extraction of the plant material is very low. Thus the water present during incubation of the enzyme with the mixture is typically added together with the enzyme solution or separately.

The enzyme is contacted with the plant material and/or crude plant oil in the presence of the organic solvent (e.g. hexane). For instance, the enzyme incubation step may be performed in the presence of at least 1%, at least 5%, at least 10%, at least 30%, at least 50% or at least 70% organic solvent (e.g. hexane) by weight, based on the total weight of the composition. Typically the organic solvent (e.g. hexane) content during the enzyme incubation is dependent on the organic solvent (e.g. hexane) content at that stage of the process. For instance, where the enzyme is contacted with the plant material and the organic solvent (e.g. hexane), the the organic solvent (e.g. hexane) content during the enzyme incubation may be about 10 to 90%, about 30 to 70%, or about 40 to 60% by weight based on the total weight of the plant material, the organic solvent (e.g. hexane) and water. In an alternative embodiment where the enzyme is contacted with the mixture comprising the organic solvent (e.g. hexane) and a crude plant oil, the the organic solvent (e.g. hexane) content during the enzyme incubation may be about 10 to 90%, about 30 to 70%, or about 40 to 60% by weight based on the total weight of the mixture (i.e. the crude plant oil and the organic solvent (e.g. hexane)) and water.

The incubation of the enzyme with the plant material and/or crude plant oil may be carried out at any suitable temperature at which the enzyme is active. At higher temperatures pheophytin is decomposed to pyropheophytin, which is generally less preferred because some chlorophyllases are less active on pyropheophytin compared to pheophytin. In addition, the chlorophyllase degradation product of pyropheophytin, pyropheophorbide, is less water soluble compared to pheophorbide and thus more difficult to remove from the oil afterwards. The enzymatic reaction rate is increased at higher temperatures but it is favourable to keep the conversion of pheophytin to pyropheophytin to a minimum.

In view of the above, in particularly preferred embodiments the enzyme is incubated with the plant material and/or crude plant oil at below about 80°C, preferably below

about 70°C, preferably at about 68°C or below, preferably at about 65°C or below, in order to reduce the amount of conversion to pyropheophytin. However, in order to keep a good reaction rate it is preferred to keep the temperature of the reaction as high as possible. It is also preferred to perform the reaction at a temperature which is high enough to inactivate endogenous lipases. Accordingly, preferably the enzyme is contacted with the plant material and/or crude plant oil between about 5°C to and about 80°C, about 10°C to about 80°C, about 15°C to about 75°C, about 20°C to about 70°C, about 30°C to about 70°C, about 40°C to about 70°C, about 50°C to about 70°C or about 55°C to about 65°C.

The enzyme may be contacted with the plant material and/or crude plant oil for any suitable period, preferably with agitation. For example, the reaction time may be at least about 1 minute, at least about 5 minutes, or at least about 10 minutes. In some embodiments the reaction time may be between about 15 minutes to about 48 hours, about 30 minutes to 12 hours, about 30 minutes to about 6 hours, about 1 to 4 hours, or about 1 to 2 hours.

As disclosed in WO 2011/110967, it has been found that the activity of chlorophyllases and related enzymes is dependent on the presence of phospholipids and/or other surfactants. Moreover elevated lysophospholipid levels are associated with reduced activity of chlorophyllases. Oil obtained from seeds by solvent extraction may have a relatively high concentration of phospholipids. Accordingly, it is an advantage that in the present process, the chlorophyllase is active at a stage when phospholipids are still present in the oil. If the chlorophyllase were to be contacted with oil after a degumming step, chlorophyllase activity would be likely to be lower due to the absence or very low levels of phospholipids.

Chlorophyll and/or chlorophyll derivative removal

The process of the present invention involving treating plant materials or a crude plant oil with a chlorophyllase or related enzyme typically reduces the level of chlorophyll and/or chlorophyll derivatives in the extracted oil, compared to an extracted crude oil which has not been enzyme treated. For example, the process may reduce the concentration of chlorophyll, pheophytin and/or pyropheophytin by at least 5%, at least

10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99%, compared to the concentration of chlorophyll, pheophytin and/or pyropheophytin (by weight) present in the oil obtained from untreated plant material. Thus in particular embodiments, the concentration of chlorophyll and/or chlorophyll derivatives in the extracted oil after treatment may be less than 100, less than 50, less than 30, less than 10, less than 5, less than 1, less than 0.5, less than 0.1 mg/kg or less than 0.02 mg/kg, based on the total weight of the oil.

Further processing steps

Following extraction from the plant material and separation from the organic solvent (e.g. hexane), the crude plant oil may optionally be washed with water or organic or inorganic acid such as, e.g., acetic acid, citric acid, phosphoric acid, succinic acid, malic acid and the like, or with salt solutions.

In a typical plant oil processing method, crude oil is obtained using pressing and/or organic solvent (e.g. hexane) extraction, the crude vegetable oil is degummed, optionally caustic neutralized, bleached using, e.g. clay adsorption with subsequent clay disposal, and deodorized to produce refined, bleached and deodorized or RBD oil (see Figure 21). The need for the degumming step depends on phosphorus content and other factors. The process of the present invention can be used in conjunction with processes based on extraction with hexane and/or enzyme assisted oil extraction (see Journal of Americal Oil Chemists' Society (2006), 83 (11), 973-979). In general, the process of the invention may be performed using oil processing steps as described in Bailey's Industrial Oil and Fat Products (2005), 6th edition, Ed. by Fereidoon Shahidi, John Wiley & Sons, and as shown in Figure 21. In some embodiments, the process may comprise a further chlorophyllase treatment at a later stage of the process (i.e. after hexane extraction), e.g. during a water degumming step as described in WO 2011/110967.

Further processing steps, after treatment with the enzyme, may assist in removal of the products of enzymatic hydrolysis of chlorophyll and/or chlorophyll derivatives. For instance, further processing steps may remove chlorophyllide, pheophorbide, pyropheophorbide and/or phytol.

Degumming

The degumming step in oil refining serves to separate phosphatides by the addition of water. The material precipitated by degumming is separated and further processed to mixtures of lecithins. The commercial lecithins, such as soybean lecithin and sunflower lecithin, are semi-solid or very viscous materials. They consist of a mixture of polar lipids, primarily phospholipids such as phosphatidylcholine with a minor component of triglycerides. Thus as used herein, the term "degumming" means the refining of oil by removing phospholipids from the oil. In some embodiments, degumming may comprise a step of converting phosphatides (such as lecithin and phospholipids) into hydratable phosphatides.

The process of the invention can be used with any degumming procedure, including water degumming, ALCON oil degumming (e.g., for soybeans), safinco degumming, "super degumming," UF degumming, TOP degumming, uni-degumming, dry degumming and ENZYMAX^{TM} degumming. See e.g. U.S. Patent Nos. 6,355,693; 6,162,623; 6,103,505; 6,001,640; 5,558,781; 5,264,367, 5,558,781; 5,288,619; 5,264,367; 6,001,640; 6,376,689; WO 0229022; WO 98118912; and the like. Various degumming procedures incorporated by the methods of the invention are described in Bockisch, M. (1998), Fats and Oils Handbook, The extraction of Vegetable Oils (Chapter 5), 345-445, AOCS Press, Champaign, Illinois.

Water degumming typically refers to a step in which the oil is incubated with water (e.g. 1 to 5% by weight) in order to remove phosphatides. Typically water degumming may be performed at elevated temperature, e.g. at 50 to 90°C. The oil/water mixture may be agitated for e.g. 5 to 60 minutes to allow separation of the phosphatides into the water phase, which is then removed from the oil.

Acid degumming may also be performed. For example, oil may be contacted with acid (e.g. 0.1 to 0.5% of a 50% solution of citric or malic acid) at 60 to 70°C, mixed, contacted with 1 to 5% water and cooled to 25 to 45 °C.

Further suitable degumming procedures for use with the process of the present invention are described in WO 2006/008508. Acyltransferases suitable for use in the

degumming step of the process are also described in WO 2004/064537, WO 2004/064987 and WO 2009/024736. Any enzyme having acyltransferase activity (generally classified as E.C.2.3.1) may be used, particularly enzymes comprising the amino acid sequence motif GDSX, wherein X is one or more of the following amino acid residues: L, A, V, I, F, Y, H, Q, T, N, M or S. In one embodiment, acyltransferase is a mutant *Aeromonas salmonicida* mature lipid acyltransferase (GCAT) with a mutation of Asn80Asp, e.g. an acyltransferase comprising the amino acid sequence of SEQ ID NO:23 after undergoing post-translational modification (see Figure 22), or an enzyme having at least 80% sequence identity thereto. In one embodiment the lipid acyltransferase is Lysomax Oil® available from Danisco A/S, Denmark.

In another embodiment, the process comprises a degumming step in which the oil is contacted with a phospholipase. Any enzyme having e.g. a phospholipase A1 (E.C.3.1.1.32) or a phospholipase A2 (E.C.3.1.1.4) activity may be used, for example Lecitase Ultra® or pancreatic phospholipase A2 (Novozymes, Denmark). In one embodiment the process comprises performing an enzymatic degumming step using a phospholipase, for example using a degumming step as described in US 5,264,367, EP 0622446, WO 00/32758 or Clausen (2001) "Enzymatic oil degumming by a novel microbial phospholipase," Eur. J. Lipid Sci. Technol. 103:333-340.

Acid treatment/caustic neutralization

In some embodiments, an acid treatment/caustic neutralization step may be performed in order to further reduce phospholipid levels in the oil after water degumming. In another embodiment, a single degumming step comprising acid treatment/caustic neutralization may be performed. Such methods are typically referred to as total degumming or alkali refining.

It has been found that an acid treatment/caustic neutralization step is particularly effective in removing products of the enzymatic hydrolysis of chlorophyll, e.g. chlorophyllide, pheophorbide and pyropheophorbide. Thus this step may be performed at any stage in the process after the enzyme treatment step. For example, such a step may comprise addition of an acid such as phosphoric acid followed by neutralization with an alkali such as sodium hydroxide. Following an acid/caustic neutralization

treatment compounds such as chlorophyllide, pheophorbide and pyropheophorbide are extracted from the oil in an aqueous phase.

In such methods, the oil is typically first contacted with 0.05 to 0.5% by weight of concentrated phosphoric acid, e.g. at a temperature of 50 to 90°C, and mixed to help precipitate phosphatides. The contact time may be, e.g. 10 seconds to 30 minutes. Subsequently an aqueous solution of an alkali (e.g. 1 to 20% aqueous sodium hydroxide) is added, e.g. at a temperature of 50 to 90°C, followed by incubation and mixing for 10 seconds to 30 minutes. The oil may then be heated to about 90°C and the aqueous soap phase separated from the oil by centrifugation.

Optionally, further wash steps with e.g. sodium hydroxide or water may also be performed.

Chlorophyllide, pheophorbide and pyropheophorbide removal

The method of the present invention may optionally involve a step of removing phytol-free derivatives of chlorophyll such as chlorophyllide, pheophorbide and pyropheophorbide. Such products may be present in the composition due to the hydrolysis of chlorophyll or a chlorophyll derivative by the enzyme of the invention, or may be present naturally, as a contaminant, or as an undesired component in a processed product. Pyropheophorbide may also be present in the composition due to the breakdown of pheophorbide, which may itself be produced by the activity of an enzyme having pheophytinase activity on pheophytin, or pheophorbide may be formed from chlorophyllide following the action of chlorophyllase on chlorophyll (see Figure 1). Processing conditions used in oil refining, in particular heat, may favour the formation of pyropheophorbide as a dominant component, for instance by favouring the conversion of pheophytin to pyropheophytin, which is subsequently hydrolysed to pyropheophorbide.

In one embodiment the process of the present invention reduces the level of chlorophyllide, pheophorbide and/or pyropheophorbide in the oil, compared to either or both of the levels before and after enzyme treatment. Thus in some embodiments the chlorophyllide, pheophorbide and/or pyropheophorbide concentration may increase

after enzyme treatment. Typically the process involves a step of removing chlorophyllide, pheophorbide and/or pyropheophorbide such that the concentration of such products is lower than after enzyme treatment. Preferably the chlorophyllide, pheophorbide and/or pyropheophorbide produced by this enzymatic step is removed from the oil, such that the final level of these products in the oil is lower than before enzyme treatment.

It is an advantage of the present process that reaction products such as chlorophyllide, pheophorbide and/or pyropheophorbide may be simply and easily removed from the oil by a step such as acid treatment/caustic neutralization. Thus in preferred embodiments chlorophyll and chlorophyll derivatives may be substantially removed from the oil without the need for further processing steps such as clay and/or silica treatment and deodorization.

Clay treatment

It is preferred that the process does not comprise a clay treatment step. Avoiding the use of clay is advantageous as this reduces cost, reduces losses of oil through adherence to the clay and the increases retention of useful compounds such as carotenoids and tocopherol.

In some embodiments, the process may be performed with no clay treatment step and no deodorization step, which results in an increased concentration of such useful compounds in the refined oil, compared to a process involving clay treatment.

Silica treatment

Although not always required, in some embodiments the process may comprise a step of silica treatment. For example, the method may comprise use of an adsorbent-free or reduced adsorbent silica refining devices and processes, which are known in the art, e.g., using TriSyl Silica Refining Processes (Grace Davison, Columbia, MD), or, SORBSIL R^{TM} silicas (INEOS Silicas, Joliet, IL).

The silica treatment step may be used to remove any remaining chlorophyllide, pheophorbide and/or pyropheophorbide or other polar components in the oil. For

example, in some embodiments a silica treatment step may be used as an alternative to an acid treatment/caustic neutralization (total degumming or alkali refining) step.

In one embodiment the process comprises a two-stage silica treatment, e.g. comprising two silica treatment steps separated by a separation step in which the silica is removed, e.g. a filtration step. The silica treatment may be performed at elevated temperature, e.g. at above about 30°C, more preferably about 50 to 150°C, about 70 to 110°C, about 80 to 100°C or about 85 to 95°C , most preferably about 90°C.

Deodorization

In some embodiments, the process may comprise a deodorization step, typically as the final refining step in the process. In one embodiment, deodorization refers to steam distillation of the oil, which typically removes volatile odor and flavor compounds, tocopherol, sterols, stanols, carotenoids and other nutrients. Typically the oil is heated to 180°C to 260°C under low pressure (e.g. 0.1 to 1 kPa) to exclude air. Steam (e.g. 1-3% by weight) is blown through the oil to remove volatile compounds, for example for 15 to 120 minutes. The aqueous distillate may be collected.

In another embodiment, deodorization may be performed using an inert gas (e.g. nitrogen) instead of steam. Thus the deodoriztion step may comprise bubble refining or sparging with an inert gas (e.g. nitrogen), for example as described by A. V. Tsiadi *et al.* in "Nitrogen bubble refining of sunflower oil in shallow pools", Journal of the American Oil Chemists' Society (2001), Volume 78 (4), pages 381-385. The gaseous phase which has passed through the oil may be collected and optionally condensed, and/or volatile compounds extracted therefrom into an aqueous phase.

In some embodiments, the process of the present invention is performed with no clay treatment but comprising a deodorization step. Useful compounds (e.g. carotenoids, sterols, stanols and tocopherol) may be at least partially extracted from the oil in a distillate (e.g. an aqueous or nitrogenous distillate) obtained from the deodorization step. This distillate provides a valuable source of compounds such as carotenoids and tocopherol, which may be at least partially lost by entrainment in a process comprising clay treatment.

The loss of tocopherol during bleaching depends on bleaching conditions and the type of clay applied, but 20-40% removal of tocopherol in the bleaching step has been reported (K. Boki, M, Kubo, T. Wada, and T. Tamura, ibid., 69, 323 (1992)). During processing of soy bean oil a loss of 13% tocopherol in the bleaching step has been reported (S. Ramamurthi, A. R. McCurdy, and R. T. Tyler, in S. S. Koseoglu, K. C. Rhee, and R. F. Wilson, eds., Proc. World Conf. Oilseed Edible Oils Process, vol. 1, AOCS Press, Champaign, Illinois, 1998, pp. 130–134).

Carotenoids may be removed from the oil during deodorization in both clay-treated and non-clay-treated oil. Typically the removal of coloured carotenoids is controlled in order to produce an oil having a predetermined colour within a specified range of values. The level of carotenoids and other volatile compounds in the refined oil can be varied by modifying the deodorization step. For instance, in an embodiment where it is desired to retain a higher concentration of carotenoids in the oil, the deodorization step may be performed at a lower temperature (e.g. using steam at 200°C or below). In such embodiments it is particularly preferable to avoid a clay treatment step, since this will result in a higher concentration of carotenoids in the refined oil.

The invention will now be further illustrated with reference to the following non-limiting examples.

EXAMPLE 1

Chlorophyllase treatment of a crude plant oil in hexane

A chlorophyllase from *Arapidopsis thaliana* (SEQ ID NO:1) obtained by expression in *E. coli*, and a chlorophyllase from *Triticum aestivum* (SEQ ID NO:2) obtained by expression in *Trichoderma reesei*, were tested in a mixture of crude rape seed oil (No. 14 available from AarhusKarlshamn, Karlshamn, Sweden), hexane and water according to the recipe shown in Table 1:

Table 1: Recipe for chlorophyllase treatment

Sample number		1	2	3
crude rapeseed oil	g	6	6	6
n-hexane*	ml	4	4	4

water	ml	0.2		0.14
A. thaliana chlorophyllase, 14.8 Units /ml	ml		0.20	
T. aestivum chlorophyllase, 54 Units/ml	ml			0,056
% water		2	2	2
Units chlorophyllase/g oil		0	0.5	0.5

*For conversion of the volume to an equivalent mass, n-hexane has a density of about 0.65 g/ml.

The reactions were incubated at 55°C under magnetic stirring. Samples were taken out after 1 and 4 hrs and the reaction hexane was evaporated by nitrogen purging. The oil phase was collected by centrifugation. The content of chlorophyll components (pheophytin a, pheophytin a', pyropheophytin a and pheophorbide a) in the oil phase was analyzed by liquid chromatography-mass spectrometry (LC-MS). The results are shown in Table 2.

LC-MS analysis of chlorophyll derivatives was performed as follows. The samples are analysed with reversed phase chromatography coupled on-line with triple quadropole mass spectrometry in electrospray positive mode (LC-MS/MS-ESI-positive). In addition UV at 410 nm is measured. A RP C18 column is used and the gradient is based on methanol/water with ammonium acetate and methanol/acetone. The ion traces of $MS>MS_2$ from each component are extracted and the amounts are calculated by using the calibration curves obtained from each component. The results are calculated as the average of three determinations and reported as µg/g oil.

Table 2: LC-MS analysis of chlorophyll components

Sample no.	Hr	Pheophorbide_a	Pheophytin_a	Pheophytin_a'	Pheophtyin a+a'	Pyropheophytin_a
		µg/g= ppm	µg/g= ppm	µg/g= ppm	µg/g= ppm	µg/g= ppm
1	1	1,09	13,25	5,15	18,40	2,19
1	4	0,68	13,24	5,02	18,25	1,78
2	1	8,63	0,72	0,38	1,09	0,32
2	4	6,34	0,04	0,03	0,07	0,02
3	1	6,01	2,99	1,59	4,57	1,71
3	4	5,17	0,25	0,31	0,56	1,29

From the results in Table 2 it is clear that both of the tested chlorophyllases can degrade pheophytin and pyropheophytin components in the hexane miscella to very low residual levels.

EXAMPLE 2

Low dose treatment of crude plant oils with chlorophyllase in hexane

The chlorophyllase derived from *Arapidopsis thaliana* (SEQ ID NO:1) was tested again in hexane at a lower dosage. In this experiment two different crude oils were tested, Nos. 14 and 15 available from AarhusKarlshamn, Karlshamn Sweden. Samples were prepared as described in Table 3:

Table 3: Recipe for chlorophyllase treatment in hexane

Sample no.			1	2	3	4
Hexane		ml	4	4	4	4
Crude oil 14		g	6		6	
Crude oil 15		g		6		6
Water		ml	0,2	0,2	0,152	0,152
Chlorophyllase	12,5 U/ml	ml			0,048	0,048
Chlorophyllase	Units/g oil				0,1	0,1

Incubation was carried out at 55°C under magnetic stirring. 1 ml samples were taken out after 1, 2 and 4 hrs and the reaction terminated by adding 20µl 5% citric acid. The oil was isolated as described previously and analyzed by LC-MS (Table 4).

Table 4: LC-MS analysis of oil

Sample no.	Hr	Pheophorbide_a	Pheophytin_a	Pheophytin_a'	Pheophtyin a+a'	Pyropheophytin_a
		µg/g= ppm	µg/g= ppm	µg/g= ppm	µg/g= ppm	µg/g= ppm
1	1	0,48	11,34	4,26	15,60	1,52
2	1	0,52	4,96	1,84	6,80	1,00
3	1	2,38	5,50	2,50	8,00	1,04
4	1	3,57	1,72	0,94	2,66	0,53
1	2	0,60	10,89	4,49	15,38	1,59
2	2	0,59	5,41	2,03	7,44	1,11
3	2	6,74	3,62	1,82	5,44	0,83
4	2	4,77	0,98	0,65	1,63	0,39
1	4	0,47	9,69	3,82	13,51	1,44
2	4	0,51	5,14	1,89	7,03	1,07
3	4	4,35	0,54	0,31	0,85	0,25
4	4	5,76	0,16	0,17	0,33	0,13

Table 4 shows that in both oils pheophytin and pyropheophytin components are degraded over time in the hexane miscella.

EXAMPLE 3

Chlorophyllase treatment during hexane extraction of oil seeds

In this experiment, chlorophyllases were used to degrade chlorophyll derivatives during hexane extraction of soya and canola (rapeseed) according to the recipe in Table 5:

Table 5: Recipe for chlorophyllase treatment

Sample no.			1	2	3	4	5	6
n-hexane		ml	10	10	10	10	10	10
Soya flakes		g	5	5	5			
Rape seeds		g				5	5	5
Water		ml	0,2	0	0	0,2		
A. thaliana chl'ase, 6 Units/ml		ml	0	0,2			0,2	
T. aestivum chl'ase, 6 Units/ml		ml			0,2			0,2
chlorophyllase, Units/g of flakes or seed			0	0,24	0,24	0	0,24	0,24

The rapeseeds were ground in a coffee grinder to improve the oil extraction. Hexane and soya flakes or ground rapeseeds were transferred to a Wheaton glass and the enzyme or water added. Incubation was carried out at 60°C for 4.5 hrs under magnetic stirring. After the reaction the samples were centrifuged and 3ml of a mixture comprising hexane and the extracted crude oil was taken for analysis. The solvent was evaporated and the oil analyzed by LC-MS (Table 6).

Table 6: LC-MS analysis of oil

Sample no.	Pheophorbide a	Pheophytin a+a´	Pyropheophytin a
	µg/g = ppm	µg/g = ppm	µg/g = ppm
1	0.08	0.60	0.10
2	0.20	0.21	0.04
3	0.11	0.12	0.07
4	0.35	2.13	0.08
5	0.53	0.20	0.02
6	0.58	0.16	0.03

The results show that pheophytin and pyropheophytin are present in oil samples extracted from soybean and rapeseed. When chlorophyllase is added during extraction, the extracted oil contained a lower content of pheophytin and pyropheophytin and an increased level of pheophorbide. These results demonstrate that chlorophyllase can degrade chlorophyll components during hexane extraction.

EXAMPLE 4 (comparative)

Phopholipase/acyltransferase treatment of a crude plant oil in hexane

In this study, the effect of applying other enzymes (phospholipases and acyltransferases) to a crude plant oil in the presence of hexane was tested. *Arapidopsis thaliana* chlorophyllase (SEQ ID NO:1) obtained by expression in *E. coli* was also included in some samples. The enzymes were added to a mixture of crude rape seed oil (No. 14 available from AarhusKarlshamn, Karlshamn, Sweden), hexane and water according to the recipe shown in Table 7:

Table 7: Recipe for phospholipase/acyltransferase treatment

sample number		1	2	3	4	5
hexane	ml	4	4	4	4	4
crude rapeseed oil	g	6	6	6	6	6
water	ml	0.2	0.152	0.092	0.1400	0.1400
A. thaliana chlorophyllase, 12.5 Units /ml	ml		0.048	0.0480	0.0480	0.0480
LysoMax Oil diluted 1:100	ml			0.06		

Lipomod 699L diluted 1:10	ml				0.012	
Purifine diluted 1:10	ml					0.012
chlorophyllase (*A. thaliana*)	Units/g oil		0.1	0.1	0.1	0.1
lipid acyltransferase (LysoMax Oil)	Units/g oil			0.1		
phospholipase (Lipomod 699L)	ppm				200	
phospholipase C (Purifine)	ppm					200

The following phospholipases and acyltransferases were tested (in samples 3 to 5 respectively): LysoMax Oil® (lipid acyltransferase from Danisco A/S), Lipomod® 699L (phospholipase from porcine pancreas from Biocatalysts, Cardiff UK) and a phospholipase C (PLC, Purifine®).

Hexane and oil are heated to 45°C and water and enzyme is added. Incubation was carried out for 4 hrs with magnetic stirring. Samples are taken out for thin layer chromatography and LC-MS analysis after 1, 2 and 4 hours reaction time.

The effect of the phospholipids in the crude oil was analysed by TLC according to the following protocol:

Applicator: CAMAG applicator AST4.

HPTLC plate: 20 x 10 cm (Merck no. 1.05641)

The plate was activated before use by drying on a hot plate at 160°C for 20-30 minutes.

Application: 5.0µl of oil sample (10% solution) dissolved in $CHCl_3$:Methanol (2:1) was applied to the HPTLC plate using AST4 applicator.

Running-buffer 6: Methylacetate:$CHCl_3$:1-propanol:MeOH:0.25 % KCl (25:25:25:10:9)

Elution length: 7 cm

Developing fluid: 6% Cupriacetate in 16% H_3PO_4

After elution the plate was dried on a hot plate at 160°C for 10 minutes, cooled and immersed in the developing fluid (10 sec) and then dried for an additional 6 minutes at 160°C. The plate was evaluated visually and scanned by using a Camag TLC Plate Scanner.

The results from TLC analysis are shown in Fig. 17. Based on the TLC scanning of the plate the relative amount of phosphatidylcholine was calculated with results shown in Table 8:

Sample	Reaction time, hr	%, Relative Phosphatidylcholine
1	2	100
3	2	111
4	2	108
5	2	98
1	4	100
3	4	92
4	4	105
5	4	110

The TLC analysis shown in Fig. 17 and Table 8 indicates that the phospholipase/acyltransferase added to the hexane and oil mixture did not hydrolyse phospholipids in the oil to any significant degree.

LCMS analysis of pheophytin and pyropheophytin in the samples treated chlorophyllase and phospholipases/acyltransferasess are shown in Table 9:

Table 9: LC-MS analysis of pheophytin and pyropheophytin in oil samples treated with enzymes according to table 7.

sample	pheophytin (ppm)			pyropheophytin (ppm)		
	1 hr	2 hr	4 hr	1 hr	2 hr	4 hr
1	15.60	15.38	13.51	1.522	1.59	1.441
2	8.00	5.44	0.85	1.039	0.833	0.253
3	3.71	3.21	0.35	0.573	0.559	0.123
4	6.62	3.71	0.41	0.833	0.63	0.13
5	6.48	4.26	0.63	0.837	0.679	0.226

The results from Table 9 show a clear effect of chlorophyllase added to the crude oil and hexane mixture. The results of these analyses showed no significant hydrolysis of phospholipids by the tested phospholipases/acyltransferases (Table 8), despite the fact that the chlorophyllase was active on pheophytin and pyropheophytin (Table 9) under these conditions.

Conclusion

The results shown above demonstrate that chlorophyllases can degrade chlorophyll components in a matrix composed of oil and hexane, during hexane extraction from oilseeds. Thus chlorophyllases can be applied directly in the hexane extraction step of vegetable oils. Almost all soy bean oil is extracted with hexane, and thus chlorophyllases can advantageously be used in this step in existing oil refining plants. An alternative option would be to treat the oil with chlorophyllase during a water degumming step. However, since not all oil refining processes employ a water degumming step, the activity of chlorophyllases in a hexane extraction step is of particular interest.

All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the present invention. Although the present invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in biochemistry and biotechnology or related fields are intended to be within the scope of the following claims.

CLAIMS

1. A process for extracting a plant oil, comprising:

(a) contacting an oil-containing plant material with an organic solvent comprising hexane;

(b) obtaining a mixture comprising the organic solvent and a crude plant oil derived from the plant material; and

(c) separating the crude plant oil from the organic solvent;

wherein an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative is contacted with the plant material and/or crude plant oil in the presence of the organic solvent.

2. A process according to claim 1, wherein the plant material comprises oil-containing plant seeds.

3. A process according to claim 2, wherein the seeds are selected from soya beans, peanuts, cotton seeds, sunflower seeds and rapeseeds, preferably soya or rapeseed.

4. A process according to any preceding claim, wherein the plant material is flaked, peeled or pressed before contacting with the organic solvent.

5. A process according to any preceding claim, wherein the enzyme is added to the plant material and the organic solvent, before or during extraction of the crude plant oil from the plant material.

6. A process according to any of claims 1 to 4, wherein the enzyme is added to the mixture, after extraction of the crude plant oil from the plant material.

7. A process according to any preceding claim, wherein the enzyme is contacted with the plant material and/or crude plant oil in the presence of 0.5 to 5% by weight water, based on the total weight of (i) the plant material, the organic solvent and water or (ii) the crude plant oil, the organic solvent and water.

8. A process according to any preceding claim, wherein the enzyme is contacted with the plant material and/or crude plant oil in the presence of 10 to 90% by weight organic solvent, based on the total weight of (i) the plant material, organic solvent and water or (ii) the crude plant oil, organic solvent and water.

9. A process according to any preceding claim, wherein the enzyme comprises a polypeptide sequence as defined in any one of SEQ ID NOs: 1, 2, 4, 6 or 8 to 15, or a functional fragment or variant thereof having at least 75% sequence identity to any one of SEQ ID NOs: 1, 2, 4, 6 or 8 to 15 over at least 50 amino acid residues.

10. A process according to any preceding claim, wherein the enzyme is contacted with the plant material and/or crude plant oil at a temperature of 50 to 70°C.

11. A process according to any preceding claim, wherein the organic solvent comprises at least 50% by weight n-hexane.

12. A process according to any preceding claim, wherein the organic solvent comprises at least 90% by weight C_5 to C_7 alkanes.

13. A process for producing a refined plant oil, comprising extracting a crude plant oil by a process as defined in any preceding claim, and refining the crude plant oil to obtain a refined plant oil.

14. A crude or refined plant oil obtainable by a process as defined in any preceding claim.

Figure 1

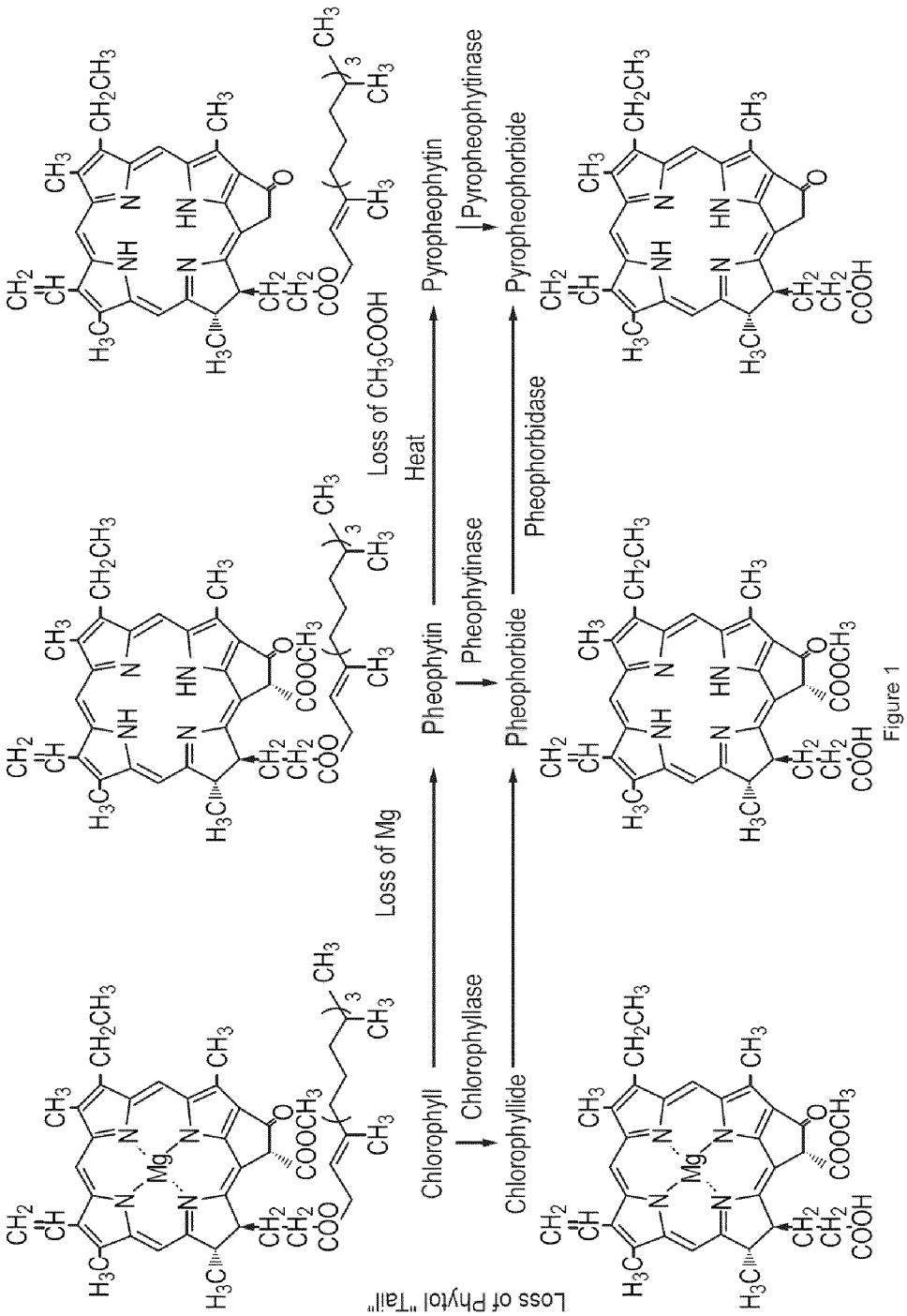


Figure 1

Figure 2

SEQ ID NO:1

```
  1 MSSSSSRNAF EDGKYKSNLL TLDSSSRCCK ITPSSRASPS PPKQLLVATP VEEGDYPVVM
 61 LLHGYLLYNS FYSQLMLHVS SHGFILIAPQ LYSIAGPDTM DEIKSTAEIM DWLSVGLNHF
121 LPAQVTPNLS KFALSGHSRG GKTAFAVALK KFGYSSNLKI STLIGIDPVD GTGKGKQTPP
181 PVLAYLPNSF DLDKTPILVI GSGLGETARN PLFPPCAPPG VNHREFFREC QGPAWHFVAK
241 DYGHLDMLDD DTKGIRGKSS YCLCKNGEER RPMRRFVGGL VVSFLKAYLE GDDRELVKIK
301 DGCHEDVPVE IQEFEVIM
```

Figure 3
SEQ ID NO. 2

```
  1 MAAAAPAETM NKSAAGAEVP EAFTSVFQPG KLAVEAIQVD ENAAPTPPIP VLIVAPKDAG
 61 TYPVAMLLHG FFLHNHFYEH LLRHVASHGF IIVAPQFSIS IIPSGDAEDI AAAAKVADWL
121 PDGLPSVLPK GVEPELSKLA LAGHSRGGHT AFSLALGHAK TQLTFSALIG LDPVAGTGKS
181 SQLQPKILTY EPSSFGMAMP VLVIGTGLGE EKKNIFFPPC APKDVNHAEF YRECRPPCYY
241 FVTKDYGHLD MLDDDAPKFI TCVCKDGNGC KGKMRRCVAG IMVAFLNAAL GEKDADLEAI
301 LRDPAVAPTT LDPVEHRVA
```

Figure 4
SEQ ID No. 3

```
  1 GCGCGCAGGC TGCTGGAAAA ATGGCAGCGG CTGCCCCGGC CGAAACAATG AATAAAAGCG
 61 CAGCGGGTGC CGAAGTTCCT GAAGCATTTA CGTCTGTGTT TCAACCGGGC AAATTGGCTG
121 TCGAAGCCAT TCAGGTAGAC GAAAACGCTG CCCCTACACC GCCTATTCCG GTCCTGATCG
181 TAGCACCTAA AGATGCGGGA ACGTATCCGG TCGCGATGCT GCTTCATGGC TTTTTCCTGC
241 ATAACCATTT TTACGAACAT TTGTTGCGTC ATGTCGCGTC CCATGGATTT ATCATCGTAG
301 CTCCTCAATT TTCAATTAGC ATTATCCCGT CAGGCGACGC GGAAGATATC GCAGCGGCTG
361 CCAAAGTTGC TGACTGGCTG CCGGATGGCC TTCCTAGCGT TTTACCGAAA GGCGTTGAAC
421 CTGAACTTTC TAAACTGGCT CTTGCCGGAC ATTCCCGCGG CGGACATACA GCTTTTTCAT
481 TAGCCTTGGG CCATGCAAAA ACACAGTTAA CGTTTTCTGC CCTGATTGGA CTTGATCCGG
541 TTGCAGGTAC AGGCAAATCA AGCCAATTGC AGCCTAAAAT CCTGACGTAT GAACCGTCTT
601 CCTTTGGCAT GGCTATGCCT GTTCTTGTGA TTGGAACAGG TTTGGGCGAA GAAAAGAAAA
661 ATATTTTCTT TCCGCCGTGC GCCCCGAAAG ATGTTAACCA TGCAGAATTT TATCGTGAAT
721 GCCGGCCGCC TTGTTATTAC TTTGTGACAA AAGACTACGG ACATTTAGAT ATGTTGGATG
781 ACGATGCACC GAAATTTATT ACGTGCGTCT GTAAAGATGG AAATGGTTGC AAAGGTAAAA
841 TGAGACGCTG TGTCGCGGGC ATTATGGTAG CATTTCTGAA CGCAGCGCTG GGCGAAAAAG
901 ACGCGGATCT TGAAGCTATC TTAAGAGACC CGGCAGTTGC GCCGACAACG CTTGATCCGG
961 TTGAACATCG CGTGGCTTAA TTAA
```

Figure 5
SEQ ID No. 4

```
    1 MPSTQFLGAS TLLLFGLRAV MSSDDYIKRG DLPTSKWSGR VTLRVDSAMA VPLDVVITYP
   61 SSGAAAYPVL VMYNGFQAKA PWYRGIVDHV SSWGYTVVQY TNGGLFPIVV DRVELTYLEP
  121 LLTWLETQSA DAKSPLYGRA DVSRLGTMGH SRGGKLAALQ FAGRTDVSGC VLFDPVDGSP
  181 MTPESADYPS ATKALAAAGR SAGLVGAAIT GSCNPVGQNY PKFWGALAPG SWQMVLSQAG
  241 HMQFARTGNP FLDWSLDRLC GRGTMMSSDV ITYSAAFTVA WFEGIFRPAQ SQMGISNFKT
  301 WANTQVAARS ITFDIKPMQS PQ
```

Figure 6
SEQ ID No. 5

```
    1 GCGCGCAGGC TGCTGGAAAA ATGCCTTCTA CACAATTTCT TGGAGCATCC ACGCTGCTTT
   61 TATTTGGTTT ACGTGCGGTC ATGTCAAGCG ATGACTATAT TAAACGGGGT GATTTGCCGA
  121 CATCAAAATG GAGCGGAAGA GTCACGTTGC GCGTAGATTC AGCTATGGCC GTTCCGCTGG
  181 ACGTTGTGAT CACATATCCT TCTTCCGGCG CAGCGGCTTA TCCGGTCCTG GTAATGTACA
  241 ATGGATTTCA GGCAAAAGCG CCGTGGTACA GAGGCATTGT TGATCATGTG TCAAGCTGGG
  301 GATATACAGT CGTACAATAC ACGAACGGCG GACTTTTTCC TATCGTTGTG GACCGCGTCG
  361 AACTTACATA TTTAGAACCG TTGCTGACAT GGTTAGAAAC GCAGTCTGCT GATGCCAAAT
  421 CCCCGTTGTA CGGCAGAGCT GACGTATCAC GCCTGGGCAC AATGGGACAT AGCAGAGGTG
  481 GCAAATTGGC CGCACTGCAA TTTGCGGGCC GCACGGATGT TTCAGGATGC GTGCTTTTTG
  541 ATCCTGTGGA CGGCAGCCCG ATGACACCTG AATCAGCTGA CTATCCGAGC GCTACGAAAG
  601 CACTTGCGGC TGCCGGACGT TCTGCCGGTT TAGTTGGCGC AGCGATTACA GGTTCATGTA
  661 ATCCGGTGGG CCAGAACTAC CCTAAATTTT GGGGAGCATT GGCGCCTGGT TCATGGCAAA
  721 TGGTCCTGAG CCAGGCAGGC CATATGCAAT TTGCGAGAAC AGGAAATCCG TTTTTAGATT
  781 GGAGCCTTGA CCGTTTATGC GGACGGGGTA CGATGATGTC TTCCGATGTC ATCACATATT
  841 CTGCTGCCTT TACGGTAGCT TGGTTTGAAG GCATTTTTCG TCCGGCCCAA TCTCAGATGG
  901 GAATCTCCAA TTTTAAAACA TGGGCAAACA CGCAAGTTGC AGCGCGGTCT ATTACATTTG
  961 ATATCAAACC GATGCAATCC CCTCAGTAAT TAATTAA
```

Figure 7
SEQ ID NO:6

```
  1 MEIISLNVVP QCSVVTWSSK LATKRLVPNR SSLLFSGVKK SRLVIRSGNS DGYVVGENDD
 61 LGRIARRGES TSKVLIPGLP DESNGEIAAR ISHSHCEWKP KLRVHYEKAG CDNLDAPAVL
121 FLPGFGVGSF HYEKQLTDLG RDYRVWAIDF LGQGLSLPTE DPTTMTEETS SSEDKEPFWG
181 FGDKTEPWAD QLVFSLDLWR DQVQYFVEEV IGEPVYIAGN SLGGYVALYF AATHPHLVKG
241 VTLLNATPFW GFFPNPVRSP KLARLFPWPG AFPLPERVKK ITELVWQKIS DPESIAEILK
301 QVYTDHSINV DKVFSRIVEV TQHPAAAASF ASIMLAPGGE LSFSEALSRC KENNVQICLM
361 YGREDPWVRP LWGKKIKKEI PNAPYYEISP AGHCPHDEVP EVVNYLMRGW IKHLESGGFE
421 ALPLLEDTEE DWEESRIGRE IEFPRDGWKK AVNLWLYGSN YTYWRGVRES FRSSFIRVFG
481 GKSA
```

Figure 8
SEQ ID NO:7

```
   1 aacccaattc ttctatttct tcttcacctt tagatttttc ctcgcttaat ttctcaataa
  61 cgctctcaga gagaccattt gatgaagctt ctcgcttctg gaatttgaaa aggatttgat
 121 aagacgagtt catagaagat taccgcaagt tcatcaactt tttgaacttg ttatggagat
 181 aatctcactg aacgttgtgc cccagtgctc tgtggttact tggagtagta aattagcaac
 241 gaaaagattg gtcccaaatc ggtcaagttt gttattctca ggggtcaaaa aatccagact
 301 tgtgattcga agtggaaatt ccgatggtta tgttgttggt gagaatgatg acttgggtcg
 361 tatagccaga agaggagaat caacgtcaaa ggttttgatt cctggtttgc ctgatgaatc
 421 aaatggtgaa attgctgctc gaatcagtca ttctcactgc gagtggaagc ccaagcttag
 481 agtacattat gagaaagccg gttgtgacaa tctcgatgct cctgcggtgt tgtttcttcc
 541 tggctttggc gttggttcat ttcactatga gaagcagctt accgatttgg gaagggatta
 601 tcgagtatgg gctattgatt ttcttggaca gggtttatct ctccctactg aagatcctac
 661 taccatgact gaagaaacca gttcctcgga agataaggaa ccattttggg gatttggtga
 721 caaaactgaa ccgtgggctg atcaacttgt attctctctg gatctctgga gggatcaagt
 781 tcagtatttt gtagaagagg ttatcggtga gcctgtgtac attgcaggga actcacttgg
 841 agggtatgta gctctctact ttgcagcaac ccatcctcac ctggttaagg gtgttacctt
 901 gcttaatgca acacctttct ggggtttctt ccctaatcca gtaagatccc caaagctagc
 961 acgtctcttt ccatggcccg gagcattccc tctgccggaa agagtgaaaa aaatcacaga
1021 attggtgtgg caaaagataa gtgatcctga aagcatagct gagatactta aacaggtcta
1081 cacagaccat tctatcaatg tggataaagt attctcacgt attgtggagg tcacacagca
1141 tccggctgct gcagcatcgt ttgcttcaat catgcttgct cctggtggag agctatcttt
1201 ctccgaagct ttatctaggt gtaaggaaaa caatgttcag atatgtctca tgtatggaag
1261 agaagatcca tgggtgagac cgttatgggg aaagaagata aagaaggaaa tccccaacgc
1321 tccatactac gagatcagcc cagcgggtca ctgcccacac gatgaagtcc ctgaggtggt
1381 gaactatctg atgcgcgggt ggatcaagca cctggagtct ggtggttttg aagcgctccc
1441 gcttttggag gacactgaag aagattggga ggagtccagg attggtagag aaattgagtt
1501 cccgagagat ggttggaaaa aagcagtgaa tctgtggtta tatgggtcaa actatacgta
1561 ctggagagga gttagagaat ctttcagatc cagttttata agggtgtttg gagggaagtc
1621 tgcatagaag aagcatggaa cagtcgtcta gtgtaaatta attgtaatct atgttgcatc
1681 cgatgctagc tatataatgt tgtctgtaga atcaagtttc taaaatgttc aaaaggaaaa
1741 gttagaaaaa tatctacttg atagttagtc acctaaatcg aaggaactcc tttcttgcat
1801 tgttgtatat aatccacagg ttcagattaa tataggaagg cgacattgca ggc
```

Figure 9

SEQ ID NO:8

```
1   MMILAFFLIF MEFYFQLRRR YASYLLINMI LLITADQPFW GMEILTSSTA SCCLVVNLRW
61  KLAENGSNSS QLKLPTSRER KILFARTNQR NGSLRFSSVD KFLKKLNHGK GSRSLDSFGG
121 LKNGNSKVFS GNSSSYVVGG EDDVGSITEN GESPTKVLIP GLPDESNGEY SAPVSSCFWK
181 WKPKLNVHYE KAGCENVNSP PVLFLPGFGV GSFHYEKQLK DLGRDYRVWA IDFLGQGMSL
241 PVENPTLFSK DGAASEGKDS IWGFGDEIEP WANDLVFSMD LWQDQVHNFI EEVIGEPVYI
301 VGNSLGGFVA LYFAARYPHL VKGVTLLNAT PFWGFLPNPI RSPRLARIFP WSGTFPLPAN
361 VRKLIAFFWQ KISDPKSIAE ILKQVYTDHS TNIDKVFSRI LEITQHPAAA ASFASIMFAP
421 QGQLSFRETL ARCKMSDTPI CLVYGKEDPW VKPVWGLQVK QQVPEAPYYE ISPAGHCPHD
481 EVPEAVNYLL RGWIKNLESH GSVALPLHED AEVVENSFAM DLEFVREGSR KSVIVRFFGS
541 RFSIWNSFSS YIKSQFKETT SRILTP
```

Figure 10

SEQ ID NO:9

```
  1  MEILSCHSAP CCKLVNLGGT SVHKSSGSSQ AKLPGSRNNR ILCARIGSKL GSSGYSNLDD
 61  FCTKNFGRHE GSRSLTAFKG SANVNSKALS ESYNGYVIDG KEGVGDISER GDLITQILIP
121  GLPDDSNDDS GAQISSCFWE WKPKLTVHYE KSGCENVNSP PVLFLPGFGV GSFHYEKQLK
181  DLGRDFRVWA VDFLGQGMSL PFEDPAPQSK KELDSERNDF SWGFGDETEP WANELVYSID
241  LWQDQVRYFI EQVIGEPVYI VGNSLGGFVA LYFAACNPQL VKGVTLLNAT PFWGFLPNPS
301  RSPSLARIFP WAGTFPLPAF VRKLTEFVWQ KISDPRSIGE VLKQVYADHS TKVDKVFSRI
361  LETTQHPAAA ASFASIMFAP QGQLSFSEAL SRCQMSNVPI CLMYGKEDPW VRPVWGLQVK
421  RQLLEAPYYE ISPAGHCPHD EVPEVVNYLL RGWIGNLESK GSVTLPLLDD PENIQYGTTK
481  DLEFVREGSK KSVRVHFYGS RFSLWNRIRS YVKSRFEALE INSR
```

Figure 11

SEQ ID NO:10

```
1   MFSPCPLISS GQTQWLDLGM DILTFNVTTS HRTAHFGSKL VDKTKYSCKS KVSTIIKPQV
61  FCARIDQSCG LLRFSSSNKF LDYPKKIEVS KKHNALKGIK VVNSKVLSGN YNGYVIEADE
121 DMESVSGSGE STPEILIPGL PNESSGECGA PINSCFWEWK PKLYVHYEKA GCENVKSPPV
181 LFLPGFGVGS FHFENQLKDL GRDYRVWAID FLGQGMSLPV ENPTLQLREG DILEGKNSFW
241 GFGDETEPWA NELVYSMDLW RDQVRYFIEE VIGEPVYVVG NSLGGFVAIY FAASNPQLVK
301 GVTLLNATPF WGFLPNPIRS PRLARIIPWS GTFPLPASVR KLTEFFWQKI SDPKSIAQVL
361 KQVYADHSTN VDQVFSRILK ITQHPAAAAS FASIMFAPQG QLSFRECLMR CKMNNLPICL
421 LYGREDPWVK PIWGLQVKRQ VPEASYYEIS PAGHCPHDEV PEVCSLSLFL VGIPLLFLVI
481 L
```

Figure 12

SEQ ID NO:11

```
1   MEVVSSSHSC LAFNRTPSSA WRFPGNGLGP GHAKLTRPRS AILCVRSGTA SNPADSGKVH
61  ASHGFYVSDV DAALQGIPKK VGEIEKMIIP SLPEGPESSL ISTGFWEWKP KLSVYYEKSG
121 IDNSKAPSVL FLPGFGVGTF HFEKQLKDLG RDYKVWTMDF LGQGMSLPCE DPAPKSTSGE
181 LDEDTYWGFG QELQPWAEEL VYSIDLWRDQ VQHFIEEVIG EPVYIVGNSL GGFVSLYLAA
241 SCPHLVKGVT LLNATPFWGF LPNPATSPRL SKIFPWAGTF PLPSFVRKLT ETVWQKISDP
301 RSIQGILKQV YADHSTNVDM VFSRIIETTQ HPAAAASFAS IMCAPKGQIS FEEALSRCQR
361 QGIPISLMYG REDPWVRPIW GIKVKQQVPE SPYYEISPAG HCPHDEVPEV INYLLRGWLK
421 NVESEGSVAV PFLEEPSYAE NGVSRELEFV RGGSKKSVHV RLFGSKISLW SQLRSLLKSN
481 TWVISR
```

Figure 13

SEQ ID NO:12

```
1   MEVVSCSHSC SALHQTPAST WRLRGSALGL GLGHARPSRT RRYTVACVGT TSGASNPGGS
61  GKVHAAQGFH VSDVDAALQG IPSMKAGEAE RVMIQGLPEG PDSSPISTGF WEWKPKLTVH
121 YERSGMKNSK APAVLFLPGF GVGTFHFEKQ LRDLGRDHRV WTMDFLGQGM SLPGEDPAPS
181 SIASEDAFWG FGQDSQPWAE ELVYSVDLWQ NQVQHFIEEV IREPVYIVGN SLGGFVALYF
241 AASSPHLVKG VTLLNATPFW GFFPNPATSP RLSKIFPWAG TFPLPSFVRK ITEAVWQKIS
301 DPKSIQDILK QVYADHSTNV DKVFSRIVEI TQHPAAAASF ASIMFAPRGQ ISFQEAISRC
361 QDQGIPISLM YGREDPWIRP IWGLKVKQQV PEAPYYEISP AGHCPHDEVP EVINYLLRGW
421 LKNLESEGSV DLPFLEERSY AERGVSRELE FVREGSRKSV SVRLYGTKIS LWSQLSSFLN
481 TRVPKSRIVL R
```

Figure 14

SEQ ID NO:13

```
1   MEVHSCYSTT YYCIVNVSKC LISNQAKFPI VKERRLYSGL DVYSIKKKRT QRLTITALKG
61  FDSVDSSLLS ESYNSDIIDG KVGTQDVIGS AKSVPKVIVP SLPDETKADS VAVVDSCLWE
121 WKPKLKVHYE KSGCQNVNSA PILFLPGFGV GSFHYEKQLK DLGCDHRIWA LDFLGQGKSL
181 PCEDPTLQSK RLDESERDGN NAVWGFGDEA EPWAKELVYS VDLWRDQVRY FIEEVIKEPV
241 YIVGNSLGGY VALYLAAYYP QLVKGVTLLN ATPFWGFLPN PVRSPRLSRL FPWAGTFPLP
301 DTIRKLTELV WQKISAPESI AEVLKQVYAD HTTKVDKVFS SILEVTEHPA AAASLASILF
361 APRGQLSFKE ALTGCRMNNV PVCLMYGKED PWVMPFWALQ VKRQLPEAPY YQISPAGHCP
421 HDEVPEIVNF LLRGWIKNIE SHSSVALPLL DSPESIEYDI VRDLEFVRQG MKKSVRVQFY
481 GSMTSQWEKL GMFLKSRFQY GVYSP
```

Figure 15

SEQ ID NO:14

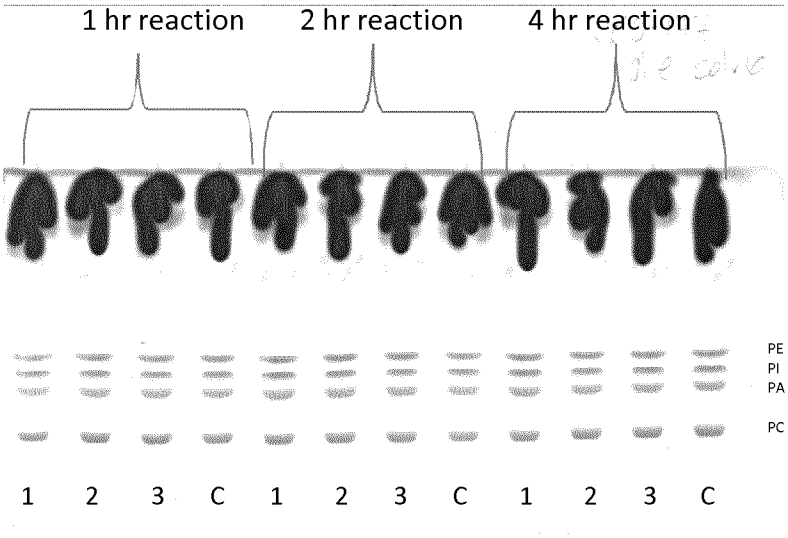
```
1   MEVVSSSHSC LAFNRTPSSA WRFPGNGLGP GHAKLTRPRS AILCVRSGTA SNPADSGKVH
61  ASHGFYVSDV DAALQGIPKK VGEIEKMIIP SLPEGPESSL ISTGFWEWKP KLSVYYEKSG
121 IDNSKAPSVL FLPGFGVGTF HFEKQLKDLG RDYKVWTMDF LGQGMSLPCE DPAPKSTSGE
181 LDEDTYWGFG QELQPWAEEL VYSIDLWRDQ VQHFIEEVIG EPVYIVGNSL GGFVSLYLAA
241 SCPHLVKGVT LLNATPFWGF LPNPATSPRL SKIFPWAGTF PLPSFVRKLT ETVWQKISDP
301 RSIQGILKQV YADHSTNVDM VFSRIIETTQ HPAAAASFAS IMCAPKGQIS FEEALSRCQR
361 QGIPISLMYG REDPWVRPIW GIKVKQQVPE SPYYEISPAG HCPHDEVPEV PGKSLAWWIT
421 GRLQAS
```

Figure 16

SEQ ID NO:15

```
1   IASHIWEWRH RWNIHYECAG TSLNTNAPAM LLLPGFGVGS FHYHQQLRDL GQEYRVWAID
61  FLGQGKSWPS HDPAPEEAEE VVEEIRHWSL GKNPEPWAEG LVYSVDTWRD QVHAFIEKVI
121 GGPVYIVGNS LGGYVGSYFA ATNPELVKGV TLLNATPFWA FTPNSRRYPL LSKLTPWGGL
181 LPVPIFAKAI IRFWWDLLRN PSTIRNMLGA VYANRSAINK KLITQIIEAT DHPAAFAAFA
241 SIVFAPRAHT DFGENLISLK ERRMPMCMIY GKEDPWVVPF WGQRAKQRNP DAIYYELSPA
301 GHCPHHEAPE VLFPAQIVLL ACMVQNIIGK ARPLFKG
```

Figure 17



1: LysoMax Oil®. Test no. 3
2: Lipomod 5µl. Test no 4
3: Purifine 5µl. Test no 5
C: control 5µl Test no 1