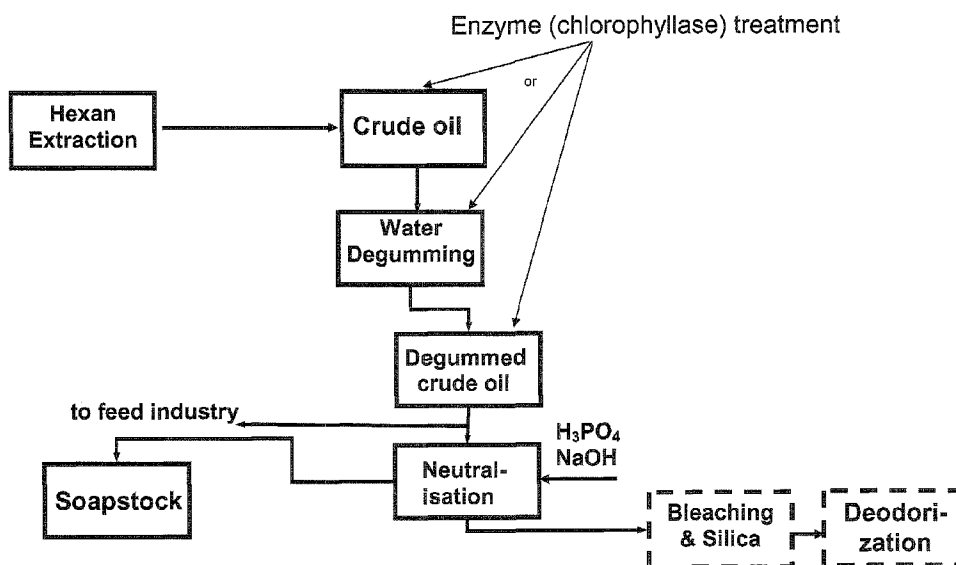




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(57) **Abrégé/Abstract:**

In one aspect, there is provided a process for treating a plant oil, comprising a step of contacting the oil with an enzyme, wherein the enzyme is capable of hydrolysing an a' or b' stereoisomer of chlorophyll or a chlorophyll derivative.

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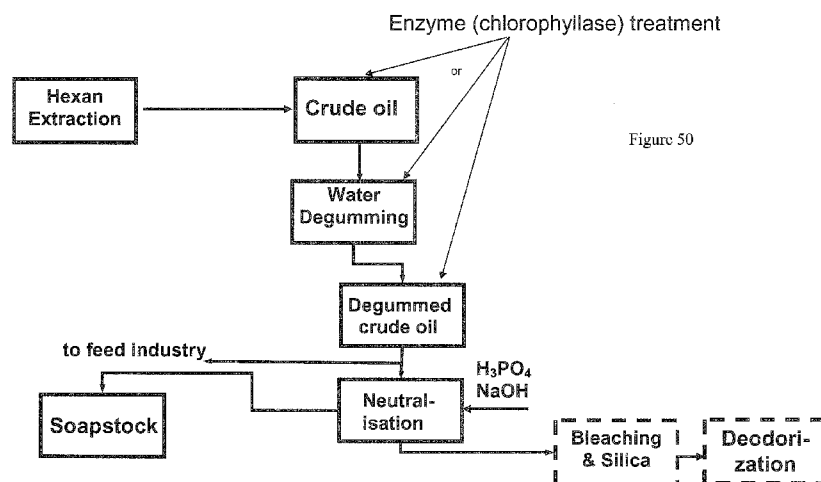


Figure 50

(57) Abstract: In one aspect, there is provided a process for treating a plant oil, comprising a step of contacting the oil with an enzyme, wherein the enzyme is capable of hydrolysing an *a'* or *b'* stereoisomer of chlorophyll or a chlorophyll derivative.

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PROCESS FOR TREATING PLANT OIL

FIELD

The present invention relates to the industrial processing of plant-derived food and feed products, especially vegetable oils. The invention may be employed to reduce or eliminate contamination by chlorophyll and chlorophyll derivatives.

BACKGROUND

Chlorophyll is a green-coloured pigment widely found throughout the plant kingdom. Chlorophyll is essential for photosynthesis and is one of the most abundant organic metal compounds found on earth. Thus many products derived from plants, including foods and feeds, contain significant amounts of chlorophyll.

For example, vegetable oils derived from oilseeds such as soybean, palm or rape seed (canola), cotton seed and peanut oil typically contain some chlorophyll. However the presence of high levels of chlorophyll pigments in vegetable oils is generally undesirable. This is because chlorophyll imparts an undesirable green colour and can induce oxidation 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. 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 pheophorbide is reportedly capable of catalyzing an analogous reaction on pheophorbide, to produce the phytol-free derivative of pyropheophytin known as pyropheophorbide (see Figure 1). 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.

Each of the four chlorophyll derivatives, chlorophyll a and b and pheophytin a and b, exist as a pair of epimers determined by the stereochemistry of H and COOCH₃ around the carbon number 13² (numbering according to the IUPAC system, marked with asterisk in Figure 2). 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. The prime (') forms have S-stereochemistry and non-prime forms have R-stereochemistry about the carbon 13² atom. Epimerization of, for example, the *a* form to *a'* form and vice versa can take place under certain conditions via a common enol, as described in "Epimerization in the pheophytin *a/a'* system", Chemistry letters (1984), 1411-1414. In solution there is typically an equilibrium which dictates the distribution of prime and non-prime chlorophyll compounds and this is often determined by physical parameters such as temperature, pH, solvent and so on.

In general enzymes typically act as stereospecific catalysts by having activity on only one stereoisomer. Previous analyses suggested that chlorophyllases possess a high degree of stereospecificity only catalyzing the hydrolysis of non-prime forms of chlorophyll compounds (see "The stereospecific interaction between chlorophylls and chlorophyllase" J. Biol. Chem. 267(31):22043-22047 (1992)).

In methods for the removal of chlorophyll and chlorophyll derivatives from plant oil which employ chlorophyllases or related enzymes, the stereospecificity of the enzyme may be problematic. In particular, depending on the distribution and equilibrium of the chlorophyll stereoisomers in the oil, a complete degradation of chlorophyll components can be very difficult. For instance, if a significant proportion of the chlorophyll or chlorophyll derivative exists in the prime form, this fraction of the chlorophyll derivatives present in the oil may be resistant to enzymatic degradation. Moreover, a number of enzymes show much lower activity on pyropheophytin than on, for example, pheophytin.

This problem with existing methods is illustrated in Figure 3. Figure 3 shows the epimerization of pheophytin *a* and the conversion to pyropheophytin. The pH of a water/crude plant oil mixture (e.g. comprising about 1-2% water) is typically around 5.0 at about 60°C. Under such conditions, in crude soy bean or rape seed oil the pheophytin *a* epimer distribution is typically around 70% pheophytin *a* (R-stereoisomer) and 30% pheophytin *a'* (S-stereoisomer) and isomerization between the two epimers is slow. Moreover, variable amounts of pyropheophytin may be formed depending on reaction conditions. If the enzyme used in the reaction is predominantly active only on pheophytin *a*, a significant proportion of chlorophyll derivatives present in the oil cannot be hydrolyzed directly by the enzyme at unmodified pH.

There is therefore 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 which enhances the removal of various forms of chlorophyll and chlorophyll derivatives from the oil.

SUMMARY

In one aspect, the present invention provides a process for treating a plant oil, comprising a step of contacting the oil with an enzyme, wherein the enzyme is capable of hydrolysing an *a'* or *b'* stereoisomer of chlorophyll or a chlorophyll derivative.

In one embodiment, the *a'* or *b'* stereoisomer comprises chlorophyll *a'*, pheophytin *a'*, chlorophyll *b'* or pheophytin *b'*. Preferably the stereoisomer is an *a'* stereoisomer of chlorophyll or the chlorophyll derivative, e.g. chlorophyll *a'* or pheophytin *a'*.

In one embodiment, the enzyme has an activity ratio on an *a* stereoisomer of chlorophyll or a chlorophyll derivative, compared to an *a'* stereoisomer of chlorophyll or the chlorophyll derivative, of less than 10, less than 5, or less than 2. In an alternative embodiment, the enzyme has an activity ratio on a *b* stereoisomer of chlorophyll or a chlorophyll derivative, compared to an *b'* stereoisomer of chlorophyll or the chlorophyll derivative, of less than 10, less than 5, or less than 2.

In one embodiment, following treatment with the enzyme the oil comprises at least 50% *a* stereoisomers of chlorophyll or the chlorophyll derivative, based on the total amount of *a* and *a'* stereoisomers of chlorophyll or the chlorophyll derivative in the oil. In an alternative embodiment, following treatment with the enzyme the oil comprises at least 50% *b* stereoisomers of chlorophyll or the chlorophyll derivative, based on the total amount of *b* and *b'* stereoisomers of chlorophyll or the chlorophyll derivative in the oil.

In further embodiments, the enzyme has an activity ratio on pheophytin compared to pyropheophytin of less than 10, less than 8 or less than 5.

In further embodiments, the enzyme comprises chlorophyllase, pheophytinase and/or pyropheophytinase activity, i.e. hydrolytic activity on chlorophyll, pheophytin and/or pyropheophytin.

In further embodiments, the enzyme is derived from *Arabidopsis thaliana* or *Ricinus communis*. For instance, the enzyme may comprise a polypeptide sequence as defined in SEQ ID NO:2 or SEQ ID NO:13, or a functional fragment or variant thereof.

Preferably the enzyme comprises a polypeptide sequence having at least 75% sequence identity to SEQ ID NO:2 or SEQ ID NO:13 over at least 50 amino acid residues. In one embodiment, the enzyme comprises a polypeptide having at least 90% sequence identity to SEQ ID NO:2. In another embodiment, the enzyme comprises a polypeptide having at least 90% sequence identity to SEQ ID NO:13.

In a further aspect, the present invention provides a plant oil obtainable by a method according to any preceding claim.

In a further aspect, the present invention provides use of an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative, for removing an *a'* or *b'* stereoisomer of chlorophyll or the chlorophyll derivative from a plant oil.

As described herein, enzymes have been identified which surprisingly show hydrolytic activity on prime as well as non-prime forms of chlorophyll derivatives. Moreover such enzymes may also show increased hydrolytic activity on pyropheophytin compared to enzymes used in known methods. Such enzymes can be advantageously used to enhance the removal of various forms of chlorophyll derivatives from plant oils.

BRIEF DESCRIPTION OF DRAWINGS

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

Figure 2 shows pheophytin a, where the C-13² according to the IUPAC numbering system is marked with an asterisk.

Figure 3 shows epimerization of pheophytin a molecules and conversion to pyropheophytin a.

Figure 4 shows amino acid and nucleotide sequences showing the fusion of a chlorophyllase gene to a His tag and thrombin site.

Figure 5 shows a schematic presentation of an *E. coli*. expression vector pET28-TRI_CHL containing the TRI_CHL gene encoding a chlorophyllase from *Triticum aestivum* (database acc. no. BT009214).

Figure 6 shows amino acid and nucleotide sequences showing the fusion of a chlorophyllase gene to an AprE signal sequence and an AGK sequence.

Figure 7 shows a schematic presentation of a *B. subtilis* expression vector pBN-TRI_CHL containing the TRI_CHL gene encoding a chlorophyllase from *Triticum aestivum* (database acc. no. BT009214).

Figure 8 shows amino acid and nucleotide sequences showing the fusion of a chlorophyllase gene directly to an AprE promoter.

Figure 9 shows a schematic presentation of a *B. subtilis* expression vector pBN-Spe-TRI_CHL containing the TRI_CHL gene encoding a chlorophyllase from *Triticum aestivum* (database acc. no. BT009214).

Figure 10 shows amino acid and nucleotide sequence showing the fusion of a chlorophyllase gene to the Cel A signal sequence.

Figure 11 shows a schematic presentation of an *S. lividans* expression vector pKB-TRI_CHL containing the TRI_CHL gene encoding a chlorophyllase from *Triticum aestivum* (database acc. no. BT009214).

Figure 12 shows the amino acid sequence of an *Arabidopsis thaliana* chlorophyllase (SEQ ID NO:1).

Figure 13 shows the amino acid sequence of an *Arabidopsis thaliana* chlorophyllase (SEQ ID NO:2).

Figure 14 shows the amino acid sequence of *Citrus sinensis* chlorophyllase (SEQ ID NO:3).

Figure 15 shows the amino acid sequence of a *Triticum aestivum* chlorophyllase (SEQ ID NO:4).

Figure 16 shows the amino acid sequence of a *Triticum aestivum* chlorophyllase (SEQ ID NO:5).

Figure 17 shows the amino acid sequence of a *Brassica oleracea* chlorophyllase (SEQ ID NO:6).

Figure 18 shows the amino acid sequence of a *Brassica oleracea* chlorophyllase (SEQ ID NO:7).

Figure 19 shows the amino acid sequence of a *Brassica oleracea* chlorophyllase (SEQ ID NO:8).

Figure 20 shows the amino acid sequence of a *Zea Mays* chlorophyllase (SEQ ID NO:9).

Figure 21 shows the amino acid sequence of a *Zea Mays* chlorophyllase (SEQ ID NO:10).

Figure 22 shows the amino acid sequence of a *Phyllostachys edulis* chlorophyllase (SEQ ID NO:11).

Figure 23 shows the amino acid sequence of a *Chenopodium album* chlorophyllase (SEQ ID NO:12).

Figure 24 shows the amino acid sequence of a *Ricinus communis* chlorophyllase (SEQ ID NO:13).

Figure 25 shows the amino acid sequence of a *Glycine max* chlorophyllase (SEQ ID NO:14).

Figure 26 shows the amino acid sequence of a *Ginkgo biloba* chlorophyllase (SEQ ID NO:15).

Figure 27 shows the amino acid sequence of a *Pachira macrocarpa* chlorophyllase (SEQ ID NO:16).

Figure 28 shows the amino acid sequence of a *Populus trichocarpa* chlorophyllase (SEQ ID NO:17).

Figure 29 shows the amino acid sequence of a *Sorghum bicolor* chlorophyllase (SEQ ID NO:18).

Figure 30 shows the amino acid sequence of a *Sorghum bicolor* chlorophyllase (SEQ ID NO:19).

Figure 31 shows the amino acid sequence of a *Vitis vinifera* chlorophyllase (SEQ ID NO:20).

Figure 32 shows the amino acid sequence of a *Physcomitrella patens* chlorophyllase (SEQ ID NO:21).

Figure 33 shows the amino acid sequence of a *Aquilegia* chlorophyllase (SEQ ID NO:22).

Figure 34 shows the amino acid sequence of a *Brachypodium distachyon* chlorophyllase (SEQ ID NO:23).

Figure 35 shows the amino acid sequence of a *Medicago truncatula* chlorophyllase (SEQ ID NO:24).

Figure 36 shows the amino acid sequence of a *Piper betle* chlorophyllase (SEQ ID NO:25).

Figure 37 shows the amino acid sequence of a *Lotus japonicus* chlorophyllase (SEQ ID NO:26).

Figure 38 shows the amino acid sequence of a *Oryza sativa Indica* chlorophyllase (SEQ ID NO:27).

Figure 39 shows the amino acid sequence of a *Oryza sativa Japonica* chlorophyllase (SEQ ID NO:28).

Figure 40 shows the amino acid sequence of a *Oryza sativa Japonica* chlorophyllase (SEQ ID NO:29).

Figure 41 shows the amino acid sequence of a *Picea sitchensis* chlorophyllase (SEQ ID NO:30).

Figure 42 shows the amino acid sequence of a *Chlamydomonas* chlorophyllase (SEQ ID NO:31).

Figure 43 shows a phylogenetic tree of the plant chlorophyllases and the *Chlamydomonas* chlorophyllase (CHL_CHL) described herein.

Figure 44 shows a Western blot analysis of *E. coli* extracts containing recombinant chlorophyllases derived from various species. Lane 1: BAM_CHL CoRe 112. Lane 2: CIT_CHL CoRe 113A. Lane 3: ARA_CHL CoRe 114A. Lane 4: CB_CHL CoRe 127. Lane 5: GlyMax_CHL CoRe 133. Lane 6: Sor_CHL CoRe 134. Lane 7: ARA_CHL2 CoRe 135. Lane 8: BRA_CHL1 CoRe 136. See Tables 1 and 2 below for definitions of source species of the enzymes corresponding to the above abbreviations.

Figure 45 shows a Western blot analysis of *E. coli* extracts containing recombinant chlorophyllases derived from various species. Lane 1: SORG_CHL CoRe 137A. Lane 2: TRI_CHL2 CoRe 138A. Lane 3: ZEA_CHL2 CoRe 139. Lane 4: TRI_CHL CoRe 20. Lane 5: BRACH_CHL CoRe 156. Lane 6: PIP_CHL CoRe 158. Lane 7: PICEA_CHL CoRe 163. Lane 8: Vector control. See Tables 1 and 2 below for definitions of source species of the enzymes corresponding to the above abbreviations.

Figure 46 shows activity of recombinant enzymes derived from various species on pheophytin a, as demonstrated by total pheophytin a (pheophytin *a* + *a'*) levels in ppm remaining at various times after treatment with each enzyme.

Figure 47 shows activity of recombinant enzymes derived from various species on pheophytin a, as demonstrated by pyropheophytin a levels in ppm remaining at various times after treatment with each enzyme.

Figure 48 shows the percentage of *a* stereoisomers of pheophytin in an oil sample after treatment with recombinant enzymes derived from various species, based on the total amount of pheophytin *a* and pheophytin *a'* stereoisomers in the oil sample after treatment.

Figure 49 shows an HPLC chromatogram using absorbance detection (430 nm) indicating numbered peaks associated with: 1 = chlorophyllide *b*; 2 = chlorophyllide *a*; 3 = neoxanthin; 3' = neoxanthin isomer; 4 = neochrome; 5 = violaxanthin; 6 = luteoxanthin; 7 = auroxanthin; 8 = anteraxanthin; 8' = anteraxanthin isomer; 9 = mutatoxanthin; 10 = lutein; 10' = lutein isomer; 10'' = lutein isomer; 11 = pheophorbide *b*; 12 = pheophorbide *a*; 13 = chlorophyll *b*; 13' = chlorophyll *b*'; 14 = chlorophyll *a*; 14' = chlorophyll *a*'; 15 = pheophytin *b*; 15' = pheophytin *b*'; 16 = β -carotene; 17 = pheophytin *a*; 17' = pheophytin *a*'; 18 = pyropheophytin *b*; 19 = pyropheophytin *a*.

Figure 50 shows a diagrammatic representation of an oil refining process according to an embodiment of the present invention.

Figure 51 shows the logarithm of substrate concentration for pheophytin *a* and *a*' after ½ hr as a function of ARA_CHL2 (*Arabidopsis* chlorophyllase) dosage.

DETAILED DESCRIPTION

In one aspect the present invention relates to a process for treating a plant oil. Typically the process is used to remove chlorophyll and/or chlorophyll derivatives from the 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.

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 in 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 the oil. 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 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. The process may involve bleaching or de-colorizing to remove the green and/or red/brown colouring of the 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.

As described herein, chlorophyll and chlorophyll derivatives may exist as a pair of epimers determined by the stereochemistry around the carbon number 13² (numbering according to the IUPAC system, marked with asterisk in Figure 2). Thus chlorophyll a exists as the pair of epimers chlorophyll *a* and chlorophyll *a'*, and chlorophyll b comprises *b* and *b'* forms. Pheophytin a comprises the epimers *a* and *a'* and pheophytin b comprises *b* and *b'* forms. The prime (') forms have S-stereochemistry and non-prime forms have R-stereochemistry about the carbon 13² atom. When used generally herein, the term “chlorophyll and chlorophyll derivatives” includes both prime and non-prime forms.

Plant oils

Any plant oil may be treated according to the present process, in order to remove undesirable contamination by chlorophyll and/or chlorophyll derivatives. The oil may be derived from any type of plant, and from any part of a plant, including whole plants, leaves, stems, flowers, roots, plant protoplasts, seeds and plant cells and progeny of

same. The class of plants from which products can be treated in the method of the invention 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.

In preferred embodiments, the oil may comprise a vegetable oil, including oils processed from oil seeds or oil fruits (e.g. seed oils such as canola (rapeseed) oil and fruit oils such as palm). Examples of suitable oils include rice bran, soy, canola (rape seed), palm, olive, cottonseed, corn, palm kernel, coconut, peanut, sesame, Moringa or sunflower. The process of the invention can be used in conjunction with methods for 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 processing high phosphorus oils (e.g. a soy bean oil). Preferably the oil is a crude plant oil.

Chlorophyll and chlorophyll derivatives in oil

The chlorophyll and/or chlorophyll derivatives (e.g. chlorophyll, pheophytin and/or pyropheophytin) may be present in the oil 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 oil. Typically chlorophyll, pheophytin and/or pyropheophytin may be present as a natural contaminant in the oil 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 oil. In further embodiments, the chlorophyll and/or chlorophyll derivatives may be present in the oil 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 oil.

Phytol-free chlorophyll derivatives may also be present in the oil. For instance, chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present at any level in the oil. Typically chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present in the oil, 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 oil. In further

embodiments, the chlorophyllide, pyropheophorbide and/or pyropheophorbide may be present in the composition 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 composition.

Enzymes hydrolysing chlorophyll or a chlorophyll derivative

The process of the present invention comprises a step of contacting the oil with an enzyme which is capable of hydrolysing chlorophyll or a chlorophyll derivative, particularly prime stereoisomers (e.g. *a'* or *b'*) thereof. 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 an oil phase, and optionally also in 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, and in particular prime stereoisomers thereof, 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 prime stereoisomers (e.g. *a'* or *b'*) of 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.

In embodiments of the present invention, the enzyme is capable of hydrolysing an *a'* or *b'* stereoisomer of chlorophyll or a chlorophyll derivative. By this it is meant that the

enzyme has hydrolytic activity on a prime (') form of chlorophyll or a chlorophyll derivative. The prime (') designation refers to the stereochemistry around the carbon number 13² in chlorophyll or the chlorophyll derivative.

Thus prime forms of chlorophyll or chlorophyll derivatives which may be hydrolysed in embodiments of the present invention include chlorophyll *a'*, chlorophyll *b'*, pheophytin *a'* and pheophytin *b'*. Preferably the enzyme is capable of hydrolyzing at least a prime form of an a type chlorophyll derivative, i.e. chlorophyll *a'* or pheophytin *a'*.

Typically the enzyme also has hydrolytic activity on non-prime forms of chlorophyll or chlorophyll derivatives. The enzymes used in the present invention may have reduced stereospecificity, i.e. the enzymes used herein are less specific for non-prime forms of chlorophyll or chlorophyll derivatives than other known chlorophyllases (such as *Triticum aestivum* chlorophyllase, see SEQ ID NO:4).

In one embodiment, the enzyme has an activity ratio on a non-prime (e.g. *a* or *b*) stereoisomer of chlorophyll or a chlorophyll derivative, compared to a prime (e.g. *a'* or *b'*) stereoisomer of chlorophyll or the chlorophyll derivative, of less than 100. Preferably the activity ratio is less than 50, less than 10, less than 5, less than 3, less than 2, less than 1.5, less than 1 or less than 0.5. By "activity ratio" it is meant to refer to the relative activity of the enzyme on the prime form compared to the non-prime form under the same conditions. Thus the activity ratio may be determined by determining (a) hydrolytic activity of the enzyme on a non-prime stereoisomer, and (b) hydrolytic activity of the enzyme on a corresponding prime stereoisomer, and dividing (a) by (b). A low activity ratio is thus indicative of a relatively high activity on prime forms.

Hydrolytic activity may be determined, for example, using methods described below. Typically the activity ratio may be determined under conditions which do not favour epimerization (i.e. transition between prime and non-prime isomers). For instance, the activity ratio may be determined by measuring hydrolytic activity on prime and non-prime isomers in a crude oil with greater than 0.5 ppm pheophytin, about 2% water and at pH 5.0 to 5.5. In one embodiment, the hydrolytic activity of the enzyme on

pheophytin *a* or pheophytin *a'* is calculated at half of the original substrate concentration (see e.g. Example 4)

In another embodiment, the enzyme has an activity ratio on pheophytin compared to pyropheophytin of less than 80, less than 50, less than 10, less than 8, less than 7 or less than 5. For example, the enzyme may have a pheophytinase to pyropheophytinase activity ratio of 0.1 to 10, 1 to 10 or 1 to 5. The pheophytinase to pyropheophytinase activity ratio may be calculated by determining pheophytinase activity and pyropheophytinase activity under the same conditions using methods described below, and dividing the pheophytinase activity by the pyropheophytinase activity. Activity ratios within the above ratios may be determined in respect of corresponding species of pheophytin and pyropheophytin, e.g. pheophytin *a* (comprising both *a* and *a'* forms) to pyropheophytin *a*.

Enzyme (chlorophyllase, pheophytinase or pyropheophytinase) activity assay

Hydrolytic activity on chlorophyll or a chlorophyll derivative, including on prime and non-prime forms thereof, 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, including *a* and *b* and prime and non-prime forms thereof. 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 μ l mM HEPES, pH 7.0 is added 20 μ l 0.3 mM chlorophyll, pheophytin or pyropheophytin dissolved in acetone. The enzyme is dissolved in 50 mM HEPES, pH 7.0. 10 μ l enzyme solution is added to 190 μ l substrate solution to initiate the reaction and incubated at 40°C for various time periods. The reaction was stopped by addition of 350 μ l 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. Prime and non-prime forms of chlorophyll and chlorophyll derivatives may be distinguished by HPLC analysis, as shown in Fig. 49.

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 method 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. Preferably the enzyme has a hydrolytic 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, on a prime (e.g. *a'* or *b'*) stereoisomer of chlorophyll or a chlorophyll derivative (e.g. chlorophyll *a'*, chlorophyll *b'*, pheophytin *a'* or pheophytin *b'*).

In a further embodiment, hydrolytic activity on chlorophyll or a chlorophyll derivative may be determined using a method as described in EP10159327.5.

Chlorophyllases

In one embodiment, the enzyme is capable of hydrolyzing at least a prime (e.g. *a'* or *b'*) stereoisomer of chlorophyll. A polypeptide that catalyses the hydrolysis of a chlorophyll *a'* or *b'* ester bond to yield chlorophyllide *a'* or *b'* and phytol 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). An 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 embodiment, the enzyme may be derived from *Arabidopsis thaliana*. For instance, the enzyme may be a polypeptide comprising the sequence of SEQ ID NO:2 (see Figure 13).

In another embodiment, the chlorophyllase is derived from castor bean, e.g. from *Ricinus communis*. For example, the chlorophyllase may be polypeptide comprising the sequence of SEQ ID NO:13 (see Figure 24).

Further provided herein are enzymes comprising a polypeptide sequence as defined in any one of SEQ ID NO:s 1 to 31, as well as functional fragments and variants thereof, as described below.

Variants and fragments

Functional variants and fragments of known sequences which hydrolyse prime forms of 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 a prime (e.g. *a'* or *b'*) stereoisomer of chlorophyll or a chlorophyll derivative. Typically such variants and fragments show homology to a source 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 source chlorophyllase, pheophytinase or pyropheophytinase amino acid sequence, e.g. to SEQ ID NO:2 or SEQ ID NO: 13, 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 activity on prime forms of chlorophyll or a chlorophyll derivative 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, the motif GHSRG (SEQ ID NO: 32) containing the Ser active site is highly conserved in chlorophyllase sequences. In some embodiments, an enzyme for use in the present invention may comprise such a sequence. 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 Beaucage 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 *ex vivo* 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 prime forms of 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 an enzyme 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 11 and 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 enzyme 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.

Enzyme formulation and dosage

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, 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 enzymatic reactions of the methods of the invention, e.g. the step of contacting the oil with an enzyme which hydrolyses a prime (e.g. *a'* or *b'*) stereoisomer of chlorophyll or a chlorophyll derivative, can be done in one reaction vessel or multiple vessels. In one aspect, the enzymatic reactions of the methods of the invention are done in a vegetable oil refining unit or plant.

The method of the invention can be practiced with immobilized enzymes, e.g. an immobilized chlorophyllase, pheophytinase and/or pyropheophytinase. The enzyme can be immobilized on any organic or inorganic support. Exemplary inorganic supports include alumina, celite, Dowex-1-chloride, glass beads and silica gel. Exemplary organic supports include DEAE-cellulose, alginate hydrogels or alginate beads or equivalents. In various aspects of the invention, immobilization of the enzyme can be optimized by physical adsorption on to the inorganic support. Enzymes used to practice the invention can be immobilized in different media, including water, Tris-HCl buffer

solution and a ternary micellar system containing Tris-HCl buffer solution, hexane and surfactant. The enzyme can be immobilized to any type of substrate, e.g. filters, fibers, columns, beads, colloids, gels, hydrogels, meshes and the like.

The enzyme may be dosed into the oil in any suitable amount. For example, the enzyme may be dosed in a range of about 0.001 to 10U/g of the composition, preferably 0.01 to 1 U/g, e.g. 0.01 to 0.1 U/g of the oil. One unit is defined as the amount of enzyme which hydrolyses 1 μ mol of substrate (e.g. chlorophyll a or b, pheophytin a or b and/or pyropheophytin a or b, or a prime (e.g. *a'* or *b'*) stereoisomer thereof) per minute at 40 °C, e.g. under assay conditions as described in J. Biol. Chem. (1961) 236: 2544-2547.

Enzyme reaction conditions

In general the oil may be incubated (or admixed) with the enzyme between about 5°C to and about 100°C, more preferably between 10°C to about 90°C, more preferably between about 15°C to about 80°C, more preferably between about 20°C to about 75°C.

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 oil is incubated with the enzyme 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 oil above 50 °C during incubation with the enzyme. Accordingly preferred temperature ranges for the incubation of the enzyme with the oil include about 50°C to below about 70°C, about 50°C to about 65°C and about 55°C to about 65°C.

Preferably the temperature of the oil may be at the desired reaction temperature when the enzyme is admixed therewith. The oil may be heated and/or cooled to the desired temperature before and/or during enzyme addition. Therefore in one embodiment it is envisaged that a further step of the process according to the present invention may be the cooling and/or heating of the oil.

Suitably the reaction time (i.e. the time period in which the enzyme is incubated with the oil), preferably with agitation, is for a sufficient period of time to allow hydrolysis of chlorophyll and chlorophyll derivatives, especially prime (e.g. *a'* or *b'*) stereoisomers thereof, to form e.g. phytol and chlorophyllide, pheophorbide and/or pyropheophorbide. For example, the reaction 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 reaction time may be between about 15 minutes to about 6 hours, preferably between about 15 minutes to about 60 minutes, preferably about 30 to about 120 minutes. In some embodiments, the reaction time may up to 6 hours.

Preferably the process is carried out between about pH 4.0 and about pH 10.0, more preferably between about pH 5.0 and about pH 10.0, more preferably between about pH 6.0 and about pH 10.0, more preferably between about pH 5.0 and about pH 7.0, more preferably between about pH 5.0 and about pH 7.0, more preferably between about pH 6.5 and about pH 7.0, e.g. at about pH 7.0 (i.e. neutral pH). In one embodiment preferably the process is carried out between about pH 5.5 and pH 6.0.

Suitably the water content of the oil when incubated (or admixed) with the enzyme is between about 0.5 to about 5% water, more preferably between about 1 to about 3% and more preferably between about 1.5 and about 2%.

When an immobilised enzyme is used, suitably the water activity of the immobilised enzyme may be in the range of about 0.2 to about 0.98, preferably between about 0.4 to about 0.9, more preferably between about 0.6 to about 0.8.

Oil separation

Following an enzymatic treatment step using an enzyme according to the present invention, in one embodiment the treated liquid (e.g. oil) is separated with an

appropriate means such as a centrifugal separator and the processed oil is obtained. Upon completion of the enzyme treatment, if necessary, the processed oil can be additionally washed with water or organic or inorganic acid such as, e.g., acetic acid, citric acid, phosphoric acid, succinic acid, and the like, or with salt solutions.

Chlorophyll and/or chlorophyll derivative removal

The process of the present invention involving an enzyme treatment typically reduces the level of chlorophyll and/or chlorophyll derivatives in the oil, especially prime (e.g. *a'* or *b'*) stereoisomers thereof. For example, the process may reduce the concentration of chlorophyll a or b, pheophytin a or b and/or pyropheophytin a or b, or prime (e.g. *a'* or *b'*) stereoisomers thereof 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 before treatment. Thus in particular embodiments, the concentration of chlorophyll and/or chlorophyll derivatives, or prime (e.g. *a'* or *b'*) stereoisomers thereof, in the 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.

If an enzyme which is stereospecific for non-prime forms of chlorophyll or chlorophyll derivatives is used, following treatment with the enzyme the oil typically comprises a reduced proportion of non-prime stereoisomers, compared to the total amount of non-prime and prime stereoisomers remaining in the oil (see Example 3 and Figure 48 below). In contrast, in embodiments of the present invention, the enzymes used in the present method typically have reduced stereospecificity for non-prime forms of chlorophyll and chlorophyll derivatives, i.e. the enzymes are typically capable of hydrolyzing both prime and non-prime forms. Consequently following a treatment step of the present invention, the proportion of non-prime stereoisomers remaining in the oil typically falls less than when a stereospecific enzyme is used.

It has been found that under typical conditions, crude oils (e.g. crude soy bean oil or rape seed oil) may comprise around 70% non-prime (e.g. pheophytin *a*) and 30% prime (e.g. pheophytin *a'*) stereoisomers. In one embodiment, following treatment with the

enzyme the oil comprises at least 50% non-prime (e.g. *a* and/or *b*) stereoisomers of chlorophyll or the chlorophyll derivative, based on the total amount of non-prime (e.g. *a* and/or *b*) and prime (e.g. *a'* and/or *a''*) stereoisomers of chlorophyll or the chlorophyll derivative in the oil. More preferably, the oil comprises at least 55%, at least 60% or at least 65% non-prime stereoisomers of chlorophyll or the chlorophyll derivative after treatment.

In one embodiment, following treatment with the enzyme the oil comprises at least 50%, at least 60% or at least 65% pheophytin *a*, based on the total amount of pheophytin *a* and pheophytin *a'* in the oil. In one embodiment, following treatment with the enzyme the oil comprises at least 50%, at least 60% or at least 65% pheophytin *b*, based on the total amount of pheophytin *b* and pheophytin *b'* in the oil.

In these embodiments, typical conditions may be, for example, about 20°C to about 70°C (e.g. about 40°C or about 60°C), pH 5 to 8 (e.g. about pH 6.0 or about pH 7.0) and water content of 1 to 3% (e.g. about 2%). The treatment time may comprise, for example, at least 1 hour, preferably 2 hours or more, more preferably 4 hours or more.

Further processing steps

In a typical plant oil processing method, oil is extracted in hexane, 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 50). 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.

In embodiments of the present invention, an enzymatic reaction involving application of the enzyme capable of hydrolyzing chlorophyll or a chlorophyll derivative may be

performed at various stages in this process, are shown in Figure 50. In particular embodiments, the enzyme is contacted with the oil before the degumming step. In another embodiment, the enzyme may be contacted with the oil during a water degumming step. In another embodiment, the enzyme is contacted with water degummed oil, but before degumming is complete (e.g. before a total degumming or caustic neutralization step).

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, particularly in embodiments where the chlorophyll- or chlorophyll derivative-hydrolyzing enzyme is contacted with the oil before the degumming step. Thus suitable degumming methods include water degumming, ALCON oil degumming (e.g., for soybeans), safinco degumming, "super degumming," UF degumming, TOP degumming, uni-degumming, dry degumming and ENZYMAXTM 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. In one embodiment the process comprises contacting the chlorophyll- or chlorophyll derivative-hydrolyzing enzyme with the oil and subsequently performing an enzymatic degumming step using an acyltransferase as described in WO 2006/008508. Acyltransferases suitable for use in 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.

In another embodiment, the process comprises a degumming step using 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 contacting the chlorophyll- or chlorophyll derivative-hydrolyzing enzyme with the oil before 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.

In another embodiment, the degumming step may be a water degumming step. In a further embodiment, an enzymatic degumming step using an enzyme such as phospholipase C (IUB 3.1.4.1) may be used. Polypeptides having phospholipase C activity which are may be used in a degumming step are disclosed, for example, in WO2008143679, WO2007092314, WO2007055735, WO2006009676 and WO03089620. A suitable phospholipase C for use in the present invention is Purifine®, available from Verenium Corporation, Cambridge, MA.

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, including prime and non-prime forms thereof. 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.

For example, the process may reduce the concentration of chlorophyllide, pheophorbide and/or pyropheophorbide, including prime and non-prime forms thereof, 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 chlorophyllide, pheophorbide and/or pyropheophorbide (by weight) present in the oil before the chlorophyllide, pheophorbide and/or pyropheophorbide removal step, i.e. before or after enzyme treatment. Thus in particular embodiments, the chlorophyllide, pheophorbide and/or pyropheophorbide concentration in the oil after the removal step 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 composition (e.g a vegetable oil).

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 (as indicated by the dashed boxes shown in Fig. 50).

Clay treatment

It is particularly preferred that the process does not comprise a clay treatment step. Avoiding the use of clay is advantageous for the reasons described earlier, in particular the reduction in cost, the reduced losses of oil through adherence to the clay and the increased 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, preferably subsequent to the enzyme 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 RTM 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 220 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 deodorization 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.

Further enzyme treatments

In further aspects, the processes of the invention further comprise use of lipid acyltransferases, phospholipases, proteases, phosphatases, phytases, xylanases, amylases (e.g. α -amylases), glucanases, polygalacturonases, galactolipases, cellulases, hemicellulases, pectinases and other plant cell wall degrading enzymes, as well as mixed enzyme preparations and cell lysates. In alternative aspects, the processes of the invention can be practiced in conjunction with other processes, e.g., enzymatic treatments, e.g., with carbohydrases, including cellulase, hemicellulase and other side degrading activities, or, chemical processes, e.g., hexane extraction of soybean oil. In one embodiment the method of the present invention can be practiced in combination with a method as defined in WO 2006031699.

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

EXAMPLE 1

Identification and cloning of chlorophyllases

Using different approaches (including BLAST) to search the NCBI databases, several sequences were identified as chlorophyllases or sequences having homology to chlorophyllases. The names of the sequences, their origin and NCBI database accession numbers are listed in Table 1.

Table 1. Chlorophyllases with accession numbers and names used herein.

Organism	Database acc. no.	CHL name
<i>Arabidopsis thaliana</i>	AAG12547	ARA_CHL
<i>Arabidopsis thaliana</i>	NP_199199	ARA_CHL2
<i>Citrus sinensis</i>	AAF59834	CIT_CHL
<i>Triticum aestivum</i>	BT009214	TRI_CHL
<i>Triticum aestivum</i>	BT008923	TRI_CHL2
<i>Brassica oleracea</i>	AAN51935	BRA_CHL
<i>Brassica oleracea</i>	AAN51933	BRA_CHL1
<i>Brassica oleracea</i>	AAN51934	Brass_CHL2
<i>Zea Mays</i>	ACN32030	ZEA_CHL
<i>Zea Mays</i>	ACG44273	ZEA_CHL2
<i>Phyllostachys edulis</i>	FP092915	BAM_CHL
<i>Chenopodium album</i>	Q9LE89	CHE_CHL
<i>Ricinus communis</i>	XP_002517075	CB_CHL
<i>Glycine max</i>	BAF43704	GlyMax_CHL
<i>Ginkgo biloba</i>	AAP44978	Gin_CHL
<i>Pachira macrocarpa</i>	ACO50429	PAC_CHL2
<i>Populus trichocarpa</i>	XP_002315752	POP_CHL
<i>Sorghum bicolor</i>	XP_002459848	Sor_CHL
<i>Sorghum bicolor</i>	XP_002445588	SORG_CHL
<i>Vitis vinifera</i>	XP_002273926	Vitis_CHL
<i>Physcomitrella patens</i>	EDQ81786	PHYS_CHL
<i>Aquilegia</i>		AQU_CHL
<i>Brachypodium distachyon</i>	ADDN01001446	BRACH_CHL
<i>Medicago truncatula</i>	ACJ85964	MED_CHL
<i>Piper betle</i>	ABI96085	PIP_CHL
<i>Lotus japonicus</i>	AK338339	LOTUS_CHL
<i>Oryza sativa Indica</i>	EEC66959	ORYI_CHL
<i>Oryza sativa Japonica</i>	NP_001064620	ORYJ1_CHL
<i>Oryza sativa Japonica</i>	EEE50970	ORYJ2_CHL

Picea sitchensis	ACN40275	PICEA_CHL
Chlamydomonas	XP_001695577	CHL_CHL

Chlorophyllase sequences

The chlorophyllase sequences identified from the search in NCBI databases are listed in Table 1 and the amino acid sequences are shown in Figures 12 to 42 (SEQ ID NO:s 1 to 31). Multiple sequence alignment of the selected chlorophyllase amino acid sequences showed several conserved residues distributed throughout the sequences. The motif GHSRG (SEQ ID NO: 32) containing the Ser active site is highly conserved. The alignment resulted in a phylogenetic tree as shown in Figure 43.

Cloning in *E. coli*

Synthetic genes encoding the chlorophyllases shown in Table 1 were prepared. Each gene was codon optimized for expression in *E. coli*. For cloning purposes the genes were extended in the 5'-end to contain a restriction site for NheI and in the 3'-end to contain a restriction site for XhoI.

Following digestion with NheI and XhoI restriction enzymes the synthetic DNA was ligated into the *E. coli* expression vector pET-28a(+) (Novagen) digested with the same restriction enzymes. This vector includes a T7 promoter with a Lac operator for controlling expression of inserted genes. The chlorophyllase genes were fused in frame to a His tag and a thrombin cleavage site for purification (example shown in Figure 4). The resulting constructs (an example pET28-TRI_CHL is shown in Figure 5), were transformed into competent *E. coli* TOP10 cells (Invitrogen), and plasmids were isolated from transformed colonies and subjected to nucleotide sequencing to verify the correct sequence and that all fusions were as expected.

Expression in *E. coli*

For expression the plasmids were transformed into the expression host *E. coli* BL21(DE3) (Novagen). The cells were cultured at 37°C in LB containing carbenicillin (50mg/ml) until OD₆₀₀ 0.6-0.8. For induction the culture was added 1 mM IPTG and incubated at 25°C for another 20-24 h before harvesting the cells by centrifugation. The

recombinant chlorophyllases were released from the cell pellet by sonication and cellular debris removed by centrifugation.

Cloning in *B. subtilis*

For cloning and expression in *B. subtilis* the synthetic genes encoding the chlorophyllases (Table 1) were codon optimized for *B. subtilis*. The genes were cloned in two different plasmids, one for intracellular expression and one for secretion into the culture medium (extracellular expression).

Extracellular expression

The genes were extended in the 5'-end to contain a restriction site for BssHII and part of an AprE signal sequence for in frame fusion to the AprE signal sequence as well as a sequence encoding the amino acids A G K to facilitate signal sequence cleavage. In the 3' end the genes were extended with a restriction site for PacI. The BssHII and PacI digested genes were ligated into *B. subtilis* expression vector pBN digested with the same restriction enzymes. The pBN vector contains an AprE promoter and an AprE signal sequence. An example of the resulting fusion of the chlorophyllase genes with the AprE signal sequence is shown in Figure 6. The final constructs (an example pBN-TRI_CHL is shown in Figure 7), were transformed into competent *E. coli* TOP10 cells (Invitrogen), and plasmids were isolated from transformed colonies and subjected to nucleotide sequencing to verify the correct sequence and that all fusions were as expected.

For expression the plasmids were transformed into the expression host *B. subtilis* BG6002. The cells were cultured at 33°C in Grant's II medium for 68 h. The recombinant chlorophyllases were isolated from the culture medium after precipitation of the cells by centrifugation.

Intracellular expression

The genes were extended in the 5'-end to contain a restriction site for SpeI to allow fusion of the genes directly to the AprE promoter in a *B. subtilis* expression vector pBN without the AprE signal sequence. In the 3' end the genes were extended with a

restriction site for HindIII. The fusion of a chlorophyllase gene to the AprE promoter is shown in Figure 8. The resulting constructs (an example pBN-Spe-TRI_CHL is shown in Figure 9), were transformed into competent *E. coli* TOP10 cells (Invitrogen), and plasmids were isolated from transformed colonies and subjected to nucleotide sequencing to verify the correct sequence and that all fusions were as expected.

For expression the plasmids were transformed into the expression host *B. subtilis* BG6002. The cells were cultured at 33°C in Grant's II medium for 68 h. The recombinant chlorophyllases were released from the cultures by treatment with 1 mg/ml Lysozyme for 1 h at 30°C. Cellular debris was removed by centrifugation and the chlorophyllases were recovered from the supernatant.

Cloning in *S. lividans*

For cloning and expression in *S. lividans* the synthetic genes encoding the chlorophyllases (Table 1) were codon optimized for *S. lividans*. For cloning purposes the genes were extended in the 5'-end to contain a restriction site for NheI and part of a Cel A signal sequence for in frame fusion to the Cel A signal sequence. The 3'-end was extended to contain a restriction site for BamHI. The fusion of a chlorophyllase gene (TRI_CHL) to the Cel A signal sequence is shown in Figure 10. The resulting constructs (an example pKB-TRI_CHL is shown in Figure 11), were transformed into competent *E. coli* TOP10 cells (Invitrogen), and plasmids were isolated from transformed colonies and subjected to nucleotide sequencing to verify the correct sequence and that all fusions were as expected.

Expression in *S. lividans*

For expression plasmids were transformed into protoplasts of the expression host *S. lividans* strain g3s3. The cells were pre-cultured for 48h at 30°C in TSG medium supplemented with thiostrepton. The pre-cultures were diluted 10x in Strept Pdxn2 modified medium and incubated at 30°C for 96h. The recombinant chlorophyllases were isolated from the culture medium after precipitation of the cells by centrifugation.

EXAMPLE 2**Activity of chlorophyllases**

A number of chlorophyllases were identified by genome mining as described above and expressed in *E. coli*. The extracts from *E. coli* harboring plasmids containing the chlorophyllase gene were analyzed for pheophytinase activity. The assay may be performed as described in EP10159327.5. Alternatively pheophytinase activity may be determined by a method as described above, e.g. HPLC-based methods. The results are shown in Table 2.

Pheophytinase activity may be determined based on hydrolysis of pheophytin a in a reaction buffer followed by fluorescent measurement of the generated pheophorbide a. The assay can also be adapted to employ pyropheophytin as a substrate. 1U of enzyme activity is defined as hydrolysis of 1 μ mole of pheophytin a or pyropheophytin per minute at 40°C.

Table 2: Pheophytinase activity of enzymes

Enzyme	Ferment	Activity U/ml
BAM_CHL	CoRe 112	0,32
CIT_CHL	CoRe 113-A	0,25
ARA_CHL	CoRe 114-A	5,19
CB_CHL	CoRe 127	0,10
GlyMax_CHL	Core133	0,010
Sor_CHL	Core134	6,14
ARA_CHL2	Core135	0,94
BRA_CHL1	Core136	1,21
SORG_CHL	CoRe 137-A	0,78
TRI_CHL2	Core138 -A	0,19
ZEA_CHL2	Core139	0,03
TRI_CHL	CoRe 20	0,18
BRACH_CHL	CoRe 156	1,50
PIP_CHL	CoRe 158	0,01
PICEA_CHL	CoRe 163	0,05
Control	Empty vector	0,000

The enzymes described in Table 2 were analyzed by western blot analysis using a primary antibody raised in rabbit against purified TRI_CHL. Figures 44 and 45 show that all enzymes from Table 2 react with the raised antibody.

EXAMPLE 3**Hydrolysis of chlorophyll derivatives in plant oils**

Some of the enzymes were tested for the ability to degrade chlorophyll components in an oil system. The recipe is shown in Table 3. Crude rapeseed oil is scaled in a Wheaton glass and heated with magnetic stirring to 60°C. Water and enzyme are added. The sample is treated with high shear mixing for 20 seconds and incubated at 60°C with magnetic stirring. Samples are taken out after 0.5, 2 and 4 hours reaction time. The samples are centrifuged and analysed by HPLC-MS.

Table 3: Recipe for testing chlorophyllases in oil system

	Units/ml		1	2	3	4	5	6
Crude rape seed AKK extracted no 11			10	10	10	10	10	10
water		ml	0,200	0,152	0,159	0,011	0,191	0,162
ARA_CHL2	0,62	ml		0,0484				
BRA_CHL1	3,62	ml			0,0414			
CB_CHL	2,11	ml				0,189		
TRI_CHL	54,19	ml					0,009	
SORG_CHL	0,78	ml						0,0385
Units/ g oil			0,000	0,0030	0,0150	0,0400	0,0500	0,003
% Water			2,000	2,000	2,000	2,000	2,000	2,000
Temperature		°C	60	60	60	60	60	60

The total levels of pheophytin a (pheophytin $a + a'$) as determined by HPLC-MS are shown in Fig. 46. The degradation of pyropheophytin a is shown in Fig. 47. All 5 enzyme candidates can degrade pheophytin and pheophytin but especially the activity on pyropheophytin varies significantly among the 5 tested enzymes.

In the oil samples treated with chlorophyllase we also analyzed the distribution of pheophytin stereoisomers a and a' . Surprisingly we found large differences in the distribution depending on the enzyme applied (see Figure 48). For the BRA_CHL1 and TRI_CHL, the percentage of pheophytin a drops to around half of the initial level whereas ARA_CHL2 and CB_CHL show a distribution which is comparable to the control and initial level. Retaining the initial distribution of stereoisomers throughout the reaction is a clear advantage as this means that the overall reaction rate is not dependent on the epimerization of pheophytin a' to a . These findings also indicate that ARA_CHL2 and CB_CHL are not very sensitive to the groups at C-13². These two

enzymes also show much better activity on pyropheophytin, which has 2 hydrogen atoms at C-13² (see Fig. 47).

Substrate specificity in an *in vitro* assay

The relative activities of the above enzymes on pheophytin and pyropheophytin in an *in vitro* assay system, e.g. as described in EP10159327.5, were measured. Table 4 gives the ratio of pheophytin to pyropheophytin activity.

Table 4. Ratio of pheophytin to pyropheophytin activity

Enzyme	Ratio of activity on pheophytin to pyropheophytin
SORG_CHL	173
ARA_CHL2	4
CB_CHL	4
TRI_CHL	45

It is clear that SORG_CHL has a relatively lower activity on pyropheophytin compared to TRI_CHL. The CB_CHL and ARA2_CHL show a different substrate specificity which is much improved towards pyropheophytin. These findings correlate with what is shown in Figures 46 to 48.

EXAMPLE 4

Relative activity of chlorophyllases on pheophytin *a* and *a'*

Dosage response of chlorophyllases in crude rapeseed oil was tested according to the recipe in Table 5

Table 5

Units/g		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Crude rape seed no 11, AAK		10	10	10	10	10	10	10	10	10	10	10	10	10	10
water	ml	0,200	0,176	0,153	0,106	0,011	0,184	0,173	0,147	0,047	0,006	0,091	0,187	0,162	0,072
CB_CHL CoRe 127-A . 2.12 U/ml	2,12 ml		0,0236	0,0472	0,0943	0,1887					0,0943				
ARA_CHL CoRe 135	0,94 ml						0,0160	0,0266	0,0532	0,0532					
CoRe 137-A SORG_CHL	0,78												0,0128	0,0385	0,1282
Units/g oil		0,000	0,005	0,010	0,020	0,040	0,0015	0,003	0,005	0,005	0,020	0,050	0,001	0,003	0,010
Water	%	2,000	2,000	2,000	2,000	2,000	2,000	2,000	2,000	1,000	1,000	1,000	2,000	2,000	2,000
Temperature	°C	60	60	60	60	60	60	60	60	60	60	60	60	60	60
pH		4,85	5,52	5,29	5,56	5,55	5,40	5,36	5,28	4,99	5,06	4,82	5,01	5,49	5,55

Samples were taken out after ½, 2 and 4 hours reaction time and analysed by HPLC-MS. In order to compare the activity of different chlorophyllases on the two isomers, the enzyme activity on both isomers was calculated at a substrate concentration which is half of the original concentration. The natural logarithm of the substrate concentration is plotted as a function of enzyme dosage (Units/g), as shown in Figure 51 for *Arabidopsis* chlorophyllase (ARA_CHL2).

Based on the graph in Fig. 51, the activity of the enzyme on pheophytin *a* and *a'* is calculated for the substrate concentration which is half the original concentration, as shown in Table 6.

Table 6. Calculation of ARA-CHL2 activity on pheophytin *a* and pheophytin *a'*

	Pheophytin <i>a'</i> Units/g oil	Pheophytin <i>a'</i> Ln (substrate µg/g)		Pheophytin <i>a</i> Units/g oil	Pheophytin <i>a</i> Ln (substrate µg/g)
	0	-0,203		0	0,766
	0,0015	-0,541		0,0015	0,409
	0,0025	-0,685		0,0025	0,259
Conc ½		-0,896	Conc ½		0,073
Slope		-195,3	Slope		-205,5
Intercept		-0,216	Intercept		0,752
Units for conc½		0,003	Units for conc½		0,003
Reciproc µg/u		287,1	Reciproc µg/u		302,5
Reciproc µg/u/hr		574,2	Reciproc µg/u/hr		605,0

Based on the enzyme activity at half the original substrate concentration, it is possible to compare different enzymes under the same conditions. In Table 7 the results from two different chlorophyllases are compared.

Table 7. Chlorophyllase activity on pheophytin *a* and *a'* isomers in crude rape seed oil

Enzyme	Pheophytin <i>a'</i> µg/Unit/hr	Pheophytin <i>a</i> µg/Unit/hr	Relative activity <i>a/a'</i>
ARA_CHL2, CoRe135	574	605	0,95
CB_CHL, CoRe 127-A	141	343	0,41

The results in Table 7 indicate that ARA_CHL2 has almost the same activity on pheophytin *a* and *a'* isomers. This is in agreement with the observations that the ratio between the two isomers does not change during enzymatic degradation with ARA_CHL2 (see Fig. 48). In contrast CB_CHL also shows significant hydrolytic activity on pheophytin *a'*. Expressed in terms of an activity ratios on pheophytin *a* compared to pheophytin *a'* (i.e. the inverse of that shown in Table 7), ARA_CHL2 has an activity ratio of 1.05 and CB_CHL has an activity ratio of 2.44.

Conclusion

We have identified 31 chlorophyllase sequences and furthermore cloned and expressed these in *E. coli*, *B. subtilis* or *S. lividans*. Based on expression in *E. coli* we have detected pheophytinase activity (Table 2) in nearly half of the identified chlorophyllases and all of these reacted with antibody raised against TRI_CHL (Figs 44 and 45). In the protein extracts without detectable pheophytinase activity we could not detect any expressed chlorophyllase enzyme based on western blots with antibody raised against TRI_CHL.

When testing the chlorophyllase candidates in oil applications we found major differences in specificity for the pheophytin and pyropheophytin substrates. The ARA_CHL2 and CB_CHL show much better activity on pyropheophytin compared to the other candidates tested (Fig. 47). For these two candidates we also observed that the ratio of pheophytin *a* to *a'* did not change significantly during incubation in oil trials. For the other candidates tested we saw a clear decrease in this ratio during incubation. The improved activity towards pyropheophytin for ARA_CHL2 and CB_CHL in oil assay was also measured in the *in vitro* assay using pheophytin and pyropheophytin as substrates (Table 4).

HPLC analysis

In the examples herein, chlorophyll derivatives may in general be quantified by HPLC analysis according to the following method. HPLC analysis is performed using a method in general terms as described in "Determination of chlorophylls and carotenoids by high-performance liquid chromatography during olive lactic fermentation", Journal of Chromatography, 585, 1991, 259-266.

The determination of pheophytin, pheophorbide, pyropheophytin and pyropheophorbide is performed by HPLC coupled to a diode array detector. The column employed in the method is packed with C18 material and the chlorophylls were separated by gradient elution. Peaks are assigned using standards of chlorophyll A and B from SigmaAldrich, e.g. based on the representative HPLC chromatogram from Journal of Chromatography, 585, 1991, 259-266 shown in Figure 49.

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 treating a plant oil, comprising a step of contacting the oil with an enzyme, wherein the enzyme hydrolyses an *a'* or *b'* stereoisomer of chlorophyll or a chlorophyll derivative and wherein the enzyme comprises a polypeptide having at least 90% sequence identity to SEQ ID NO:13.
2. A process according to claim 1, wherein the *a'* or *b'* stereoisomer comprises chlorophyll *a'*, pheophytin *a'*, chlorophyll *b'* or pheophytin *b'*.
3. A process according to claim 1 or claim 2, wherein the enzyme hydrolyses an *a'* stereoisomer of chlorophyll or the chlorophyll derivative.
4. A process according to any one of claims 1 to 3, wherein the enzyme has an activity ratio on (a) an *a* stereoisomer of chlorophyll or a chlorophyll derivative compared to an *a'* stereoisomer of chlorophyll or the chlorophyll derivative; or (b) a *b* stereoisomer of chlorophyll or a chlorophyll derivative compared to an *b'* stereoisomer of chlorophyll or the chlorophyll derivative; of less than 10.
5. A process according to any one of claims 1 to 4, wherein following treatment with the enzyme the oil comprises (a) at least 50% *a* stereoisomers of chlorophyll or the chlorophyll derivative, based on the total amount of *a* and *a'* stereoisomers of chlorophyll or the chlorophyll derivative in the oil; or (b) at least 50% *b* stereoisomers of chlorophyll or the chlorophyll derivative, based on the total amount of *b* and *b'* stereoisomers of chlorophyll or the chlorophyll derivative in the oil.
6. A process according to any one of claims 1 to 5, wherein the enzyme has an activity ratio on pheophytin compared to pyropheophytin of less than 10.

7. A process according to any one of claims 1 to 6, wherein the enzyme comprises a chlorophyllase, pheophytinase and/or pyropheophytinase activity.
8. A process according to any one of claims 1 to 7, wherein the enzyme comprises the amino acid sequence GHSRG (SEQ ID NO: 32).
9. A process according to any one of claims 1 to 8, wherein the enzyme is derived from *Ricinus communis*.
10. Use of an enzyme which hydrolyses chlorophyll or a chlorophyll derivative, for removing an *a'* or *b'* stereoisomer of chlorophyll or the chlorophyll derivative from a plant oil, wherein the enzyme has a hydrolytic activity of at least 1000U/g, based on the units of activity per gram of the purified enzyme, on a prime stereoisomer of chlorophyll or a chlorophyll derivative, and wherein one unit of enzyme activity is defined as the amount of enzyme which hydrolyses one micromole of substrate per minute at 40°C, wherein the assay for measuring hydrolytic activity is as follows:
 - a) 170µl 50mM HEPES, pH7 is added to 20µl 0.3mM chlorophyll, pheophytin or pyropheophytin dissolved in acetone;
 - b) the enzyme is dissolved in 50mM HEPES, pH7;
 - c) 10µl enzyme solution is added to 190µl substrate solution to initiate the reaction and incubated at 40°C for various time periods;
 - d) the reaction is stopped by addition of 350µl acetone;
 - e) following centrifugation for 2 min at 18,000g the supernatant is analysed by HPLC and the amounts of (i) chlorophyll and chlorophyllide (ii) pheophytin and pheophorbide or (iii) pyropheophytin and pyropheophorbide determined; wherein the enzyme comprises a polypeptide having at least 90% sequence identity to SEQ ID NO:2 or SEQ ID NO:13.
11. Use according to claim 10, wherein the enzyme has an activity ratio on (a) an *a* stereoisomer of chlorophyll or a chlorophyll derivative compared to an *a'* stereoisomer of

chlorophyll or the chlorophyll derivative; or (b) a *b* stereoisomer of chlorophyll or a chlorophyll derivative compared to a *b'* stereoisomer of chlorophyll or the chlorophyll derivative; of less than 10.

12. Use according to claim 10 or claim 11, wherein the enzyme has an activity ratio on pheophytin compared to pyropheophytin of less than 10.

13. Use according to any one of claims 10 to 12, wherein the *a'* or *b'* stereoisomer comprises chlorophyll *a'*, pheophytin *a'*, chlorophyll *b'* or pheophytin *b'*.

Figure 1

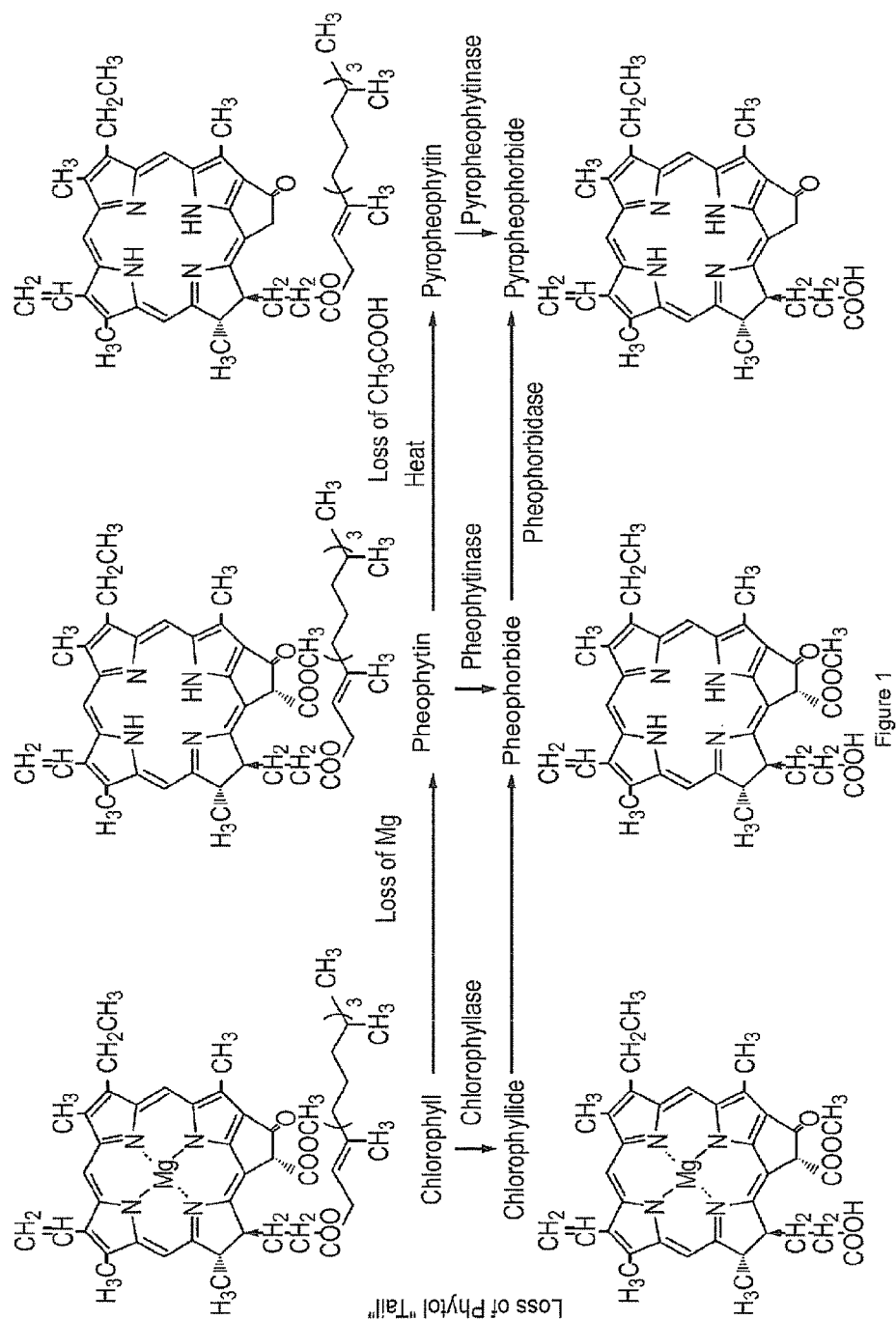


Figure 2

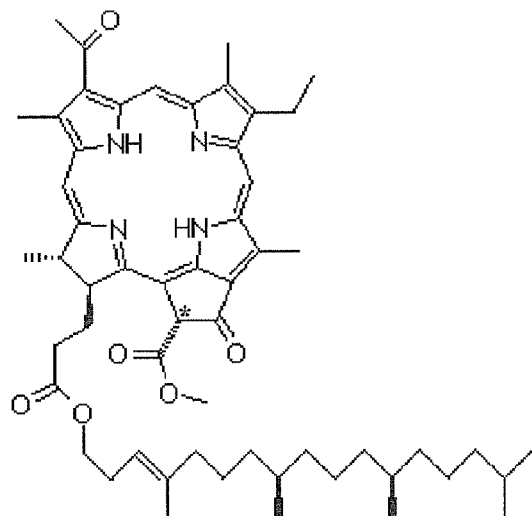


Figure 3

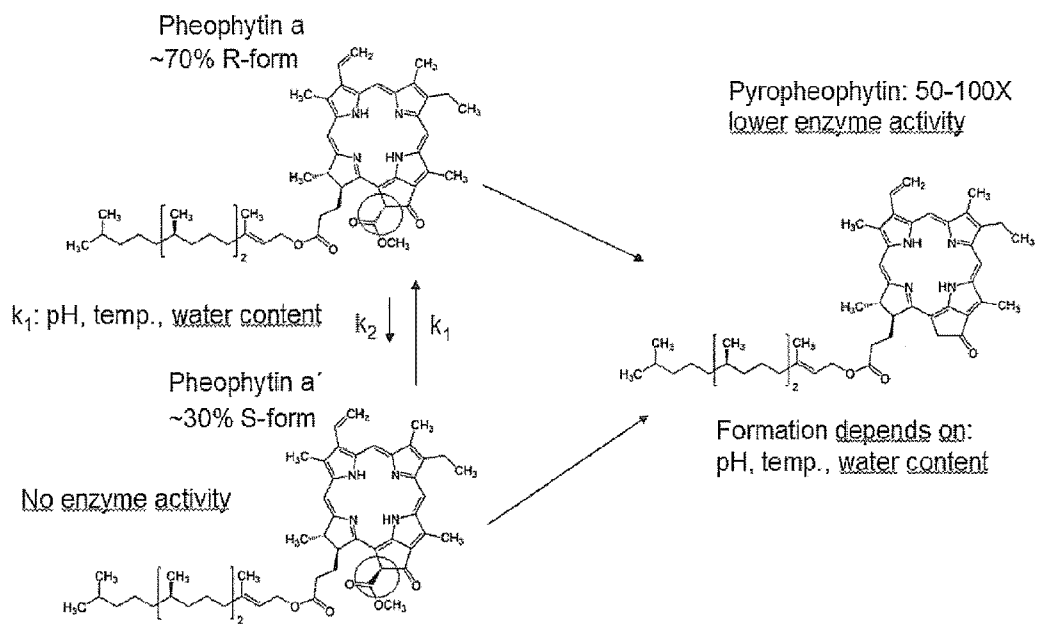


Figure 4

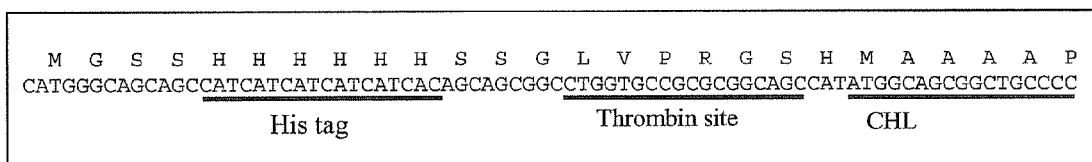


Figure 5

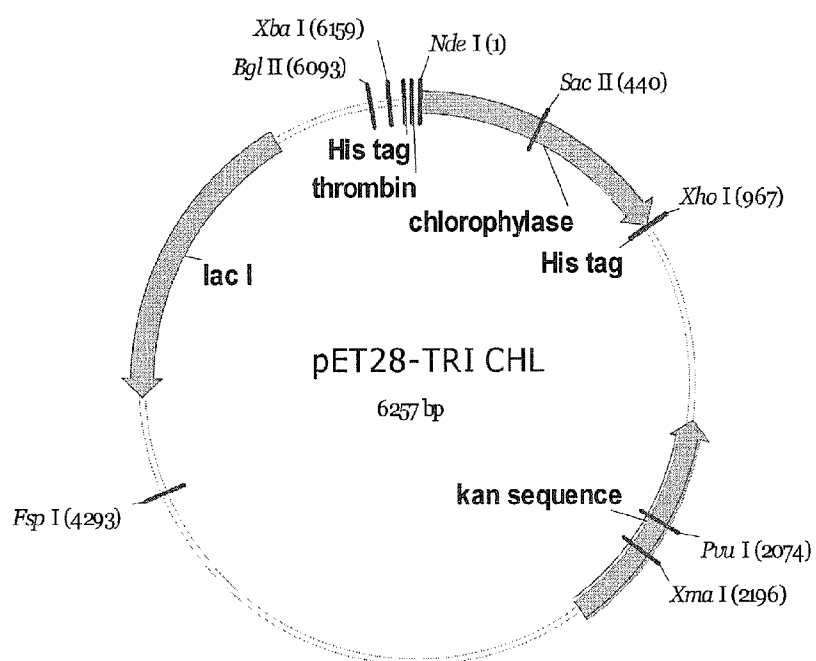


Figure 6

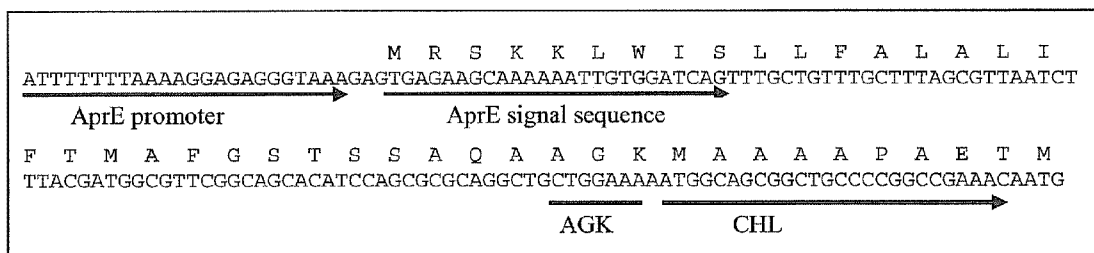
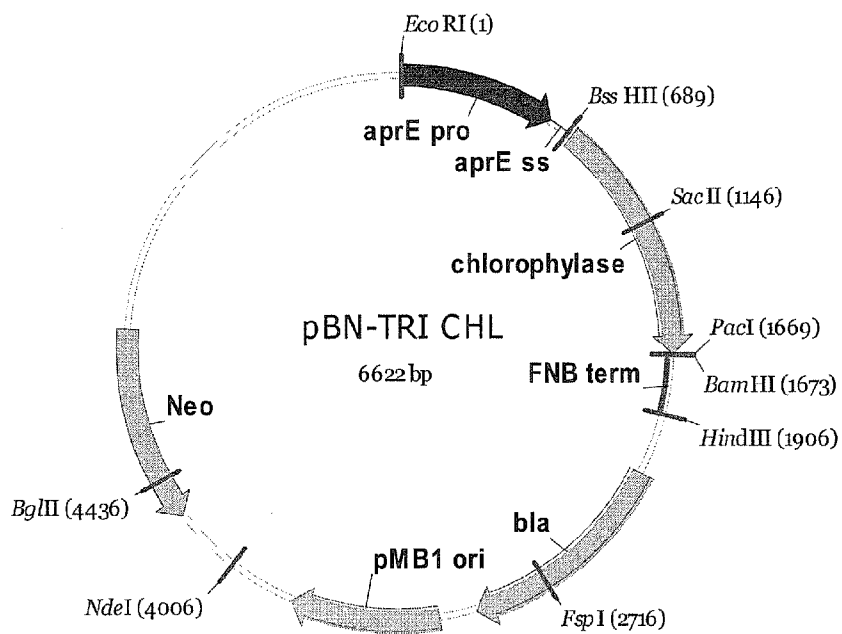


Figure 7



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Figure 8

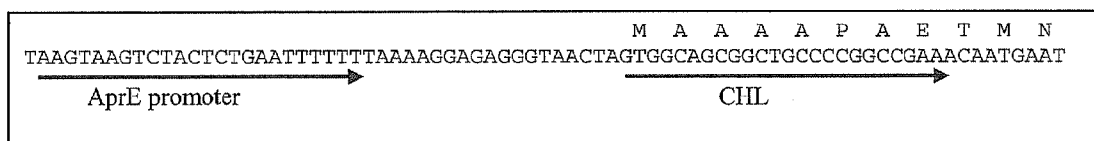
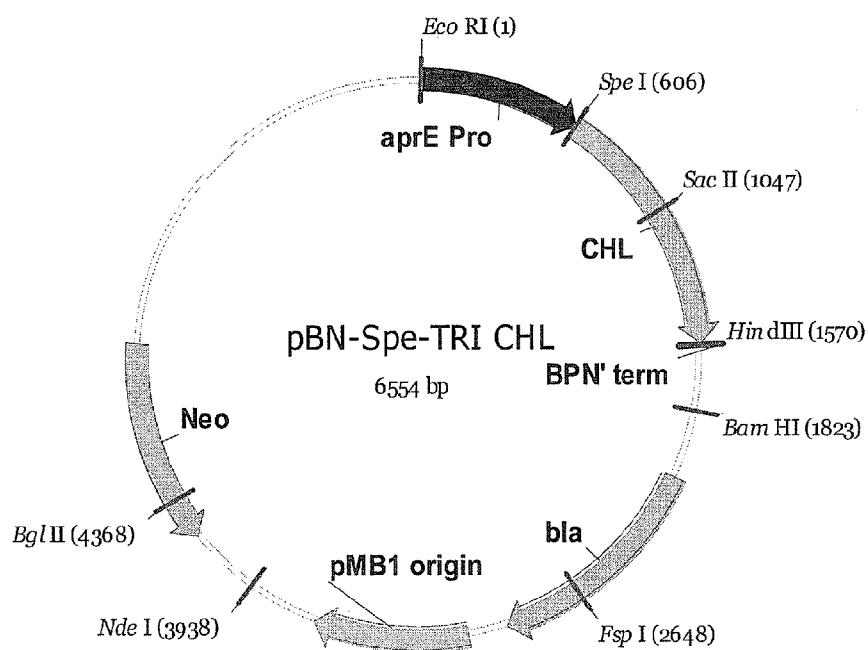


Figure 9



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Figure 10

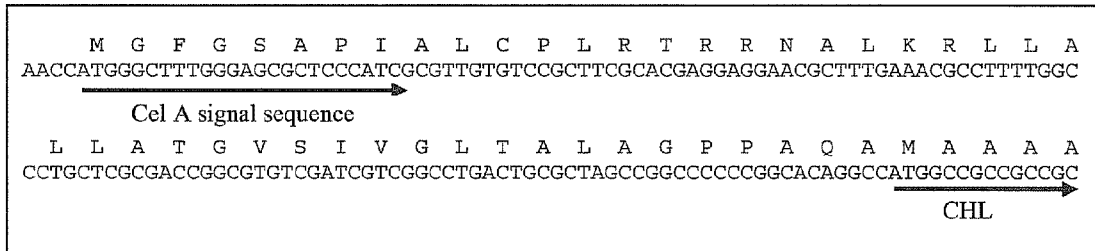


Figure 11

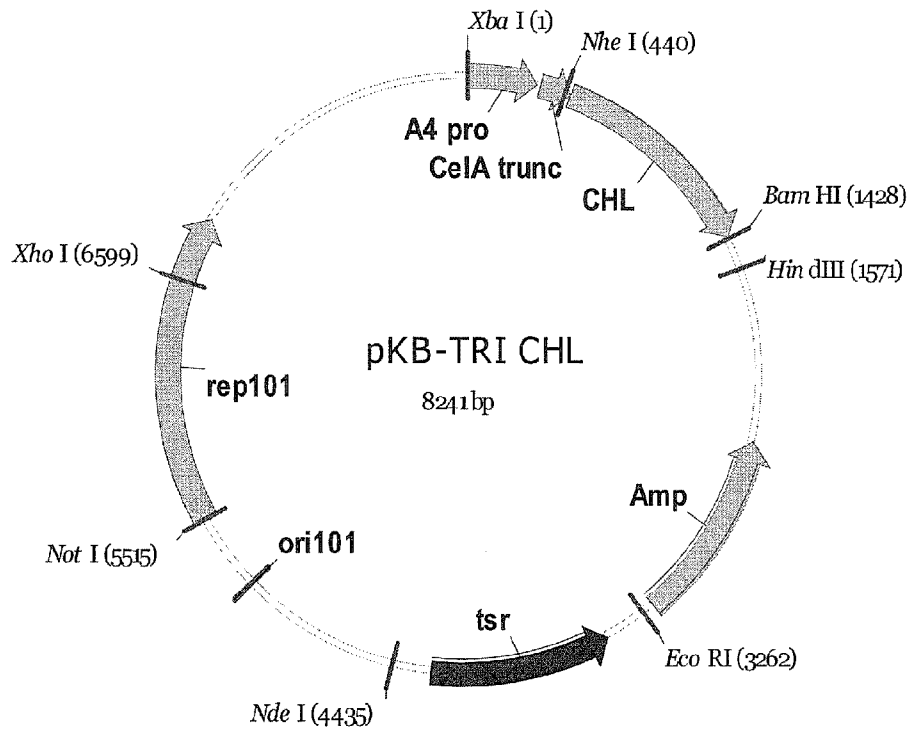


Figure 12 (SEQ ID NO:1)

```

ARA_CHL
  1  MAAIEDSPTF SSVVTPAAFE IGSLPTEIP VDPVENDSTA PPKPVRLTCP
  51  TVAGTYPVVL FFHGFYLRNY FYSDVLNHIA SHGYILVAPQ LCKLLPPGGQ
 101  VEVD DAGSVI NWASENLKAH LPTSVNANGK YTSLVGHSRG GKTAFAVALG
 151  HAATLDPSIT FSALIGIDPV AGTNKYIRTD PHILTYKPES FELDIPVAVV
 201  GTGLGPKWNN VMPPCAPTDL NHEEFYKECK ATKAHFVAAD YGHMDMLDDD
 251  LPGFVGFMAG CMCKNGQRKK SEMRSFVGGI VVAFLKYSIW GEKAEIRLIV
 301  KDPSVSPAKL DPSPELEEAS GIFV

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Figure 13 (SEQ ID NO:2)

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ARA_CHL2
  1  MSSSSSRNAF EDGKYSNLL TLDSSSRCK ITPSSRASPS PPKQLLVATP
  51  VEEGDYPVVM LLHGYLLYNS FYSQMLHVS SHGFILIAPO LYSIAGPDIM
 101  DEIKSTAEIM DWLSVGLNHF LPAQVTPNLS KFALSGHSRG GKTAFAVALK
 151  KFGYSSNLKI STLIGIDPVD GTGKGKQTPP PVLAYLPNSF DLDKTPILVI
 201  GSGLGETARN PLFPPCAPPG VNHREFFREC QGPAWHFVAK DYGHLDMLDD
 251  DTKGIRGKSS YCLCKNGEER RPMRRFVGGL VVSFLKAYLE GDDRELVKIK
 301  DGCHEDVPVE IQEFEVIM

```

Figure 14 (SEQ ID NO:3)

```

CIT_CHL
  1  MAAMVDAKPA ASVQGTPLLA TATLPVFTRG IYSTKRITLE TSSPSSPPPP
  51  KPLIIVTPAG KGTFNVILFL HGTSLSNKSX SKIFDHIASH GFIVVAPOLY
 101  TSIPPPSATN ELNSAAEVAE WLPQGLQQNL PENTEANVSL VAVMGHSRGG
 151  QTAFALSLRY GFGAVIGLDP VAGTSKTTGL DPSILSFDSF DFSIPVTVIG
 201  TGLGGVARI TACAPEGANH EEFNRCKNS SRAHFVATDY GHMDILDDNP
 251  SDVKSWALSK YFCKNGNESR DPMRRCVSGI VVAFLKDFFY GDAEDFRQIL
 301  KDPSFAPIKL DSVEYIDASS MLTTHVKV

```

Figure 15 (SEQ ID NO:4)

```

TRI_CHL
  1  MAAAAPAETM NKSAAGAEVP EAFTSVFQPG KLAVEAIQVD ENAAPTPIPI
  51  VLIVAPKDAG TYPVAMLLHG FFLHNHFYEH LLRHVASHGF IIVAPQFSIS
 101  IIPSGDAEDI AAAAKVADWL PDGLPSVLPK GVEPELSKLA LAGHSRGGHT
 151  AFSLALGHAK TQLTFSALIG LDPVAGTGKS SQLQPKILTY EPSSEGMAMP
 201  VLIGTGIGE EKKNIFFPPC APKDVNHAEF YRECRPPCYF FVTKDYGHLD
 251  MLDDDAKPII TCVCKDGNGC KGKMRRCVAG IMVAFLNAAL GEKDADLEAI
 301  LRDPVAPTIT LDPVEHRVA

```

Figure 16 (SEQ ID NO:5)

```

TRI_CHL2
  1   MAAMATTVFQ AGPMEVDVKH VDKSMIPNLA RPLMVVAPKE TGAYPVIVFL
  51   HGWNMLNSWY EQLLTHVASH GFIAVAPQLY WMVSEPDADD IDATKRITNW
 101   LADHDKGLAH VLKDVLEKLEH VEPDLSEKLAL AGHSRGGQTA FAVALGLGDA
 151   KTKLELKFSF LIGVDPVAGV SRAQQLEPKV LTFEPDCLDV GMPVLVMGTG
 201   LGPKHIGGFP CAPVGVNHAE FYKECAPPRY HLVVKDYGHL DMLDDNVFYI
 251   INNCMCMRNQ HDTKDLARRT MGGAMVAFRL AKLRIDVRDL IAIYHNPEIA
 301   PAVLDQVDEF LPCFVGRPNP SSV

```

Figure 17 (SEQ ID NO:6)

```

BRA_CHL
  1   MSPSFLFFTL FLIKEMSSSS SANSFEDGKY KTDLLTVGLS SCCWKKPSSS
  51   PTPQSPPKRL LVATPVEEGE YPVVMLLHGY LLYNSFYSQL MLHVSSHGFI
 101   VIAPQLYSIA GPDMDDEIKS TAEIIDWLSV GLNHFLPPQV TPNLSKFALS
 151   GHSRGGKTAF ALALKKFGYS SDLKISALIG IDVGTVFWTN GYGQYSGEFF
 201   EQFDCRNDRI VES

```

Figure 18 (SEQ ID NO:7)

```

BRA_CHL1
  1   MAGKEDSETF FSAATPLAFE LGSLPTTVIP ADPSATDLTA PPKPVIITSP
  51   TVAGTYPVVL FFHGFYLRNY FYSDVINHVA SHGYIVVAPQ LCKILPPGGQ
 101   VEVDAGKVI NWTSKNLKAH LPSSVNANGN YTALVGHSRG GKTAFAVALG
 151   HAATLDPSIK FSALVGIDPV AGISKCIIRD PEILTYKPES FDLDMPPAVI
 201   GTGLGPKSNM LMPPCAPAEV NHEEFYIECK ATKGHFVAAD YGHMDMLDDN
 251   LPGFVGFMAG CMCKNGKRKK SEMRSFVGGI VVAFLKYSIW GEMSEIRQIL
 301   KDPSVSPARL DPSPELEEAS GYL

```

Figure 19 (SEQ ID NO:8)

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Brass_CHL2
  1   MSSSSSRNAF VDGKYKPDLL TVDLASRCRC YKTPSSSLT PPKPKSLIV
  51   ATPVEEGEYP VVMLLHGYLL YNSFYSQLML HVSSYGFIVI APQLYNIAGP
 101   DTIDEIKSTA EIIDWLSVGL NHFLPPQVTP NLSKFALTGH SRGGKTAFV
 151   ALKKFGYSSE LKISAIIGVD PVDGTGKGKQ TPPPVLTYEP NSFNLEKMPV
 201   LVIGSGLGEL ARNPLFPPCA PTGVNHREFF QECQGPWWHF VAKDYGHLDL
 251   LDDDTKGLRG KSSYCLCKNG EERKPMRRFI GGIVVSFLMA YLEDDDCLEL
 301   KIKAGCHEGV PVEIQEFVVK K

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Figure 20 (SEQ ID NO:9)

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ZEA_CHL
  1   MAASPVAIGT AVFQRGPLRV EARHVDYSQV PSVPKPLMVV APTDAGVYPV
  51   AVFLHGCNTV NSWYESLLSH VASHGFIAVA PQLYCVTLNM NDLKDIDATR
 101   QVTAWLADKQ QGLAHVLANI LQLHGVRPDL SRLALAGHSR GGDTAFAVAL
 151   GLGPAASDDD DNNADAGTSP AALPLKFSAL IGVDPVAGLS KQAQVEPKVL
 201   TFRPRSLDPG MPALVVG TGL GPKHVGGPPC APAGVNHA EF YDECAPP RYH
 251   VVLRDYG HMD MLDDDGV PYV INN CM MRNT KDTKDLARRA IGGAVVAFLR
 301   ATLEDDDEDL KVVLENRPGL SPAVLDPVGH DLA

```

Figure 21 (SEQ ID NO:10)

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ZEA_CHL
  1   MNLASAVRVF LSYCLLLHRW MGSEQAGGVF DQGGHSVSLT RLDEARAPPR
  51   CAVQSSLSSA ASLPPKPLL V AAPRETGEYP VILFLHG YLA VNSFYSQLFE
 101   HVASHGFIVV GPQLYTISGA DTTEEINSAA AVIDWLATGL PSTLPLGVRA
 151   DLTKVSISGH SRGGKVAFAL ALGHAKAKLA VPLAAVVA VD PVDGMGVGKQ
 201   TPPPILTGRH GSLHVGAP TM VIGTGLGELP RGSLLPPCAP RGVSHAAFYD
 251   ELDGAAPACH LVARDYGHTD MMDDDTPGAR GMLTRTICRS GGARAPMRRF
 301   VAGATVAFLK KWVAGDAAAM DSITARPDQA PIALSVVEFG DEKAIA

```

Figure 22 (SEQ ID NO:11)

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BAM_CHL
  1   MAATAEIKIP STEALEAVTS VFRPGKLAVE LVPVDHNAV P TPPIPI LIVA
  51   PKDAGTYPVA MLLHGFFLQN HFYEHL LKHV ASHGFI MVAP QFHAICTGET
 101   EDIAAAAKVT DWLPEGLPSV LLKGVEADLS KLALAGHSRG GHTAFSLALG
 151   HGKTNLNFAA LIGLDPVAGT GKSSQLPPKI LTYKPSSF DV AMPVLVIGTG
 201   LGEEKKNVLF PPCAPKDVNH REFYYECKPP CY YFVTKDYG HLDMLDDDAP
 251   KFITCLCKDG DNCKDKMRR A VAGIMIAFLR AVLDEKD GDI KVILKDPGLA
 301   PVTLDPVECR LP

```

Figure 23 (SEQ ID NO:12)

```

CHE_CHL
  1   MAKLLLLIFG VFIFVNSQAQ TFPTILEKHN SEKITDV FHK GNFQVTMNPI
  51   RVKRYEFSAP EPLIIISPKE AGVYPVLLFI HGTMLS NEDY SLFFNYIASH
 101   GFIVVAPKLF RLFPPKLPSQ QDEIDMAASV ANWMPYLQV VLQRYVTGVE
 151   GDLEKLAISG HSRGGKS AFA LALGFSNIKL DVTFSALIGV DPVAGRSVDD
 201   RTLPHVLTYK PNSFNLSIPV TVIGSGLGNH TISCAPNHVS HQQFYDECKE
 251   NSSHFVITKY GHMDMLNEFR LSPIAVTMSL MCAQSFRPKA TMRRTLGGIM
 301   VAFLNAYFRD DGRQYYAIIA NRSLAPT NLF AEKKGFNF GF ATTYAQL*

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Figure 24 (SEQ ID NO:13)

CB_CHL

1	MSSSCATVTN	VYENGKYTTV	VAKIESGSCA	RSSLPLPLPP	KPLLIAMPSE
51	AGEFPVLIFL	HGYLLYNSFY	SLLIQHVASH	GFIVIAQPLY	TVAGADSADE
101	IKCTAAITNW	LSKGLHHVLP	PHVQPKLSKL	GLAGHSRGGK	AAFALALQKA
151	GISTALKFSA	LIGVDPVDGM	DKGKQTPPPV	LTYTPHSFDL	DMAAMVIGSG
201	LGEVKRNPMP	PPCAPKGVNH	EDFFKECKKP	AYYFVVKDYG	HLDMLDDDTN
251	GIRGKATYCL	CVNGKSREPM	RRFVGGVLVA	FLKAYLGGDS	SDLMTITDGQ
301	TGPVELQAAE	CYV			

Figure 25 (SEQ ID NO:14)

GlyMax_CHL

1	MAQRAQPALA	TTDVFQKGD	IHWKQFNVETS	TASSSPPKPL	LIFTPTVPGL
51	YPVILFCHGF	CIRTSYYSKL	LAHIVSHGFI	LVAPQLFSIG	VPMFGPEEVK
101	CEGRVVDWLD	NGLOPLLPES	VEAKLEKLVL	VGHSKGGKTA	FAVALGYCKT
151	KLKFSALIGI	DPVAGVSKCK	PCRSLEPDILT	GVPRSFNLNI	PVAVIGTGLG
201	PEKANSLFPP	CAPNGVNHKE	FFSECKPPSA	YFVATDYGHM	DMLDDETPGV
251	IGTMMSKCMC	KNGKKGPRDL	MRRTVGGLVV	AFLRAQLNEQ	WKDFDAILAS
301	PNLAPAKLDD	VRYLPT			

Figure 26 (SEQ ID NO:15)

Gin_CHL

1	MVLVKDVFSE	GPLPVQILAI	PQANSSPCSK	LADKNGTATT	PSPCRPPKPL
51	LIALPSQHGD	YPLILFFHGY	VLLNSFYSQL	LRHVASHGYI	AIAPQMYSVI
101	GPNTTPEIAD	AAAITDWLRD	GLSDNLPQAL	NNHVRPNFEK	FVLAGHSRGG
151	KVAFALALGR	VSQPSLKYSA	LVGLDPVDGM	GKDQQTSHPI	LSYREHSFDL
201	GMPTLVVVGSG	LGPCCKRNPLF	PPCAPQGVNH	HDDFFYECVAP	AYHFVASDYG
251	HLDLFLDDDTK	GIRGKATYCL	CKNGEAREPM	RKFSGGIVVA	FLQAFGLGDN
301	GALNDIMVYP	SHAPVKIEPP	ESLVTEDVKS	PEVELLRRAV	CR

Figure 27 (SEQ ID NO:16)

PAC_CHL

1	MAQLLETKHD	LSTVVPVFVT	GKYHPTSVSV	DPSNSSPSSP	PKPLLIPTPS
51	EQGYTPVILF	FHGfYLRNNF	YTGLLLHISS	HGFIIIVAPQL	SNIIPPSGTE
101	EVEHAAKVAD	WLPSGLPSVL	PGNVEANLAK	LALVGHSRGG	KTAFALALGR
151	AKTAQNFSAL	VGIDPVAGNR	FGETSPKILT	YTPGSFDLSI	PVAVVGTGLG
201	PESKGCMPCP	CAPTQYNHEE	FFNECKPPRV	HFDAKNYGHM	DTLDDNPSGF
251	IGKLSDTICV	NGEGPRDPMR	RCVGGIVVAF	LNYFFFEAEKE	DFMTIMNEPY
301	VAPVTLDQVQ	FNV			

Figure 28 (SEQ ID NO:17)

```

POP_CHL
  1  MSSSSAIATV TTTVFEAGKY TTVLQKVESR TTCCTAKTSP PLPVPPPKPL
 51  LIVMPCEAGE FPLLVLHG YLYNSFYSQL LQHIASHGFI VIAPQLYLVA
101  GQDSSDEIKS VAATTNWLSE GLHHLLPPHV KPNLSKLGLA GHSRGGKTAF
151  ALALEKAAAT LKFSALIGVD PVDGMDKGKQ TPPPVLTYVP HSFDLDMAIM
201  VIGSGLGELK KNPLFPFCAP EGVNHKDFEK ECKGPASYFV VKDYGHLDML
251  DDDTEGIRGK TTYCLCKNGK SREPMRKFIG GVVVAFMKAY LGGDSSDLMA
301  IKGQGTGPVE LQTV EYIL

```

Figure 29 (SEQ ID NO:18)

```

Sor_CHL
  1  MATTPKVL EE PPSAVITSVF QPGKLAVEVI SVEHDARPTP PPIPIIIAAP
 51  KDAGTYPVAI LLHGFFLQNR YYEQLLKHVA SFGFIMVAPQ FHTSLISNSD
101  ADDIAAAAKV TDWLPEGLPT VLPTGVEADL SKLALAGHSR GGHTAFSLAL
151  GYAKTNTSSL LKFSALIGLD PVAGTGKNSQ LPPAILTYEP SSFDIAVPVL
201  VIGTGLGDER ENALFPFCAP VEVNHAEFYR ECRAPCYHLV TKDYGHLDML
251  DDDAPKLVTC LCKEGNTCKD VMRRTVAGIM VAFLKAVMGE DEDGDLKAIL
301  QHPGLAPTIL DPVEYRLA

```

Figure 30 (SEQ ID NO:19)

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SORG_CHL
  1  MASPVAI STT AVFKRGRHPV DTKHVDHSQV PGVPKPLMVV TPTDAGVYPV
 51  AVFLHGCSMY NSWYQTLLSH VASHGFIAVA PQLGGILPPL DMKDLKDIDA
101  TRKVTAWLAD NLAHVLTN IL HLHGVTDL S RLALAGHSRG GDTAFAVALG
151  LGSSSSSSDT TPLKFSALIG VDPVAGLSKE LQLEPKVLTF EPRSIDPGMP
201  ALVVGTGLGP KGLLPFCAPAG VSHGEFYDEC APPRYHVVR DYGHLDMLDD
251  DGVPYVISNC MCKRNTNTTK DLARRAIGGA MVAFLRAKLE DDEDLRAVL
301  QNSPGLSPAV LDPVEYDDDE AMDGPGCAGN NGVAGASG

```

Figure 31 (SEQ ID NO:20)

```

Vitis_CHL
  1  MALLGPNPST QGIKLDLKT TSVFEPGNLS VTCIRVETSN IASPPKPLLI
 51  VTPTIQGTYP VLLFLHGFEL RNTFYTQLLQ LISSHGYIVV APQLYGLLPP
101  SGIQEIKSAA AVTNWLSSGL QSVLPENVKP DLLKLALSGH SRGGKTAFAL
151  ALGYADTSLN FSALLGLDPV GGLSKCSQTV PKILTYVPHS FNLAI PVCVI
201  GTGLGDEPRN CLTCCAPDG VNHVEFFSEC KPCCSHFVTT EYGHLDMLDD
251  HLSGCIGAIS GYICKSGKGP RDPMRRCVGG LFVAFLKAYL EGQTGDFKAI
301  VDEPD LAPVK LDPVEFIEA

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Figure 32 (SEQ ID NO:21)

PHYS_CHL

1	MEDPIPNVHG	GIYEDGPFKI	EIVHVDDASS	SSTCLKKSRA	AVDRENLSFK
51	PLVVALPKEE	GVYPVIQFHH	GFTLQNMFY	QIISHIASYG	FIVVAPQMYK
101	ISGSDATTEI	EDAVQILNWM	PTGLVAALPE	TLKSHRPDFS	KVALVGHSRG
151	AKVVFGLALG	VRNSILQYSA	VVGLDPVDGM	GIGQQTNPPI	LQFSEGLNL
201	GVPTLIIGTG	LGPLRKNFLF	PACAPAGVSH	EAFYYDSAAP	AFHFVASKQG
251	HMDFLNDDCS	GPTGMFSYCL	CKNGPTRKPM	RRFSGGMVVA	FLRAAFFGET
301	APLVAALATP	ELAPIPLDRP	EFKGLGDAF	NKMLAPALT	P

Figure 33 (SEQ ID NO:22)

AQU_CHL

1	MTTSLPPPKP	LLIATPSEEG	QFPVLIFLHG	FLLFNKFYSQ	LIQHIASHGF
51	IVIAPOLYKV	AGPDTTDEIK	SAALVIDWLS	NGLHSVLPPL	VQPNLSKLG
101	GGHSRGGKVA	FALALGHIKT	SLKYSVLLGI	DPVDGMGQGN	QTPPPVLTYT
151	PRSFDFNMPV	LVIGSGLGET	KKNSLFPPCA	PKGVDNENFY	SECCSPACYF
201	VVKDYGHMDM	LDDDTGGVRG	KATYCTCSNG	KAREPMRTFV	GGIMVAFMKA
251	YMENDSRDLM	AIKETQGMAL	IELQSVFRL		

Figure 34 (SEQ ID NO:23)

BRACH_CHL

1	MAATAAAEEL	KKNSGADVLE	AVITSVFQPG	KLAVEVIQVD	HNAVPTPIPI
51	VLIVAPKDAG	TYPVAMLLHG	FFLQNHYYKQ	LLRHVASHGF	IMVAPQFHL
101	MIPTGDTKDI	EAAAKVSDWL	PEGLPSVLPK	GVEPELSKLA	LAGHSRGGHT
151	AFSLALGHAK	SNLSFSALIG	IDPVAGTGKS	SQLAPKILTY	EPSSFNMSSA
201	MPVLVIGTGL	GEEKKNIFTP	PCAPKDVNHR	EFYLECKPPC	YYFVTKDYGH
251	LDMLDDDAPM	VITCLCKDGG	SCKDKMRRCV	AGIMVAFVLS	ALGGKDAAH
301	DLEVIVKDPA	LAPTTLDPVE	CRLE		

Figure 35 (SEQ ID NO:24)

MED_CHL

1	MCSSVSNVFE	TGNYTTKLLR	VDSCSHAQNV	PPPKSLLIAT	PIEGGEFPLL
51	LFLHGYLLLN	SFYSQLIQHV	ASHGFIVIA	QLYTVAGPDI	TEEIYSVAAI
101	TNWLKGLSK	ILPLNIKPNF	HKLALGGHSR	GGKTSFAVAL	RKLNMTTDLK
151	FSAIGVDPV	DGMDKGKQTS	PPIFTYVPHS	FDYDMATLVI	GFGLGDVKKK
201	PLFPPCAPKG	VNHEDFFSEC	EKPSWYFVAK	DYGHVDMMLD	DTKGVRGKVS
251	YCLCKNGESR	KPMRMFVGGV	MVAFLKAYLH	GDNVDLLAIR	DKNLSVPIEM
301	KFDYFV				

Figure 36 (SEQ ID NO:25)

```

PIP_CHL
  1   MAASSVFEMG KLEVHVKSVN QSNSSSPPKS LLISYPSQKG DYGVVFLHGH
 51   FLISNSFYKE LISHISSHGY IVVAPRIIYP CLQDEINSAA QVANWLPEGL
101   QAALPPNVQP NTSKLTLAGH SRGGKAAFCM LLGLAGSPLT VQFSGLIGVD
151   PVAGFQIPGI NYKMEIPPKI ITNNSKPFDI NVPTLIIGTE LGEEAKGCLA
201   PFYAPAGLNY EQFYEKSKEP SYQFVAKGYG HVDMLDDISK NDLMGKLTYC
251   VCKNGKEREP MRRTAGGLMV AFLKAFSDGQ RDDLDAILND PELAPIQLDA
301   GAKLSS

```

Figure 37 (SEQ ID NO:26)

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LOTUS_CHL
  1   MSLSISSVTH PSSVMGSDAS TALTNVFDSDG KYTTKFQRIE SNSCNGTHPD
 51   PPPPKSLLIA TPLEGGEFFV LLFLHGYLLY NSFYSQLIQH IASHGFIVIA
101   PQLYAVAGPD VSGEIHSTAA IKNLWSEGLS KFLPPNVTPN SSKLALAGHS
151   RGGKTAFAVA LRKLNITTDL KFSALVGVDV VDGLDRGKQT PPPVLTYPVP
201   SFDMPAMV IGSGLGDKVR NPLFPPCAPK TVNHEDFFNE CNKPAWYFVA
251   KDYGHVMDLD DDTNGIIGKA TYCLCKNGES RKPMRTFVGG LVVAPLKAYL
301   QGDNRDSLAI KDKHLSAPVE LKFDYFV

```

Figure 38 (SEQ ID NO:27)

```

ORYI_CHL
  1   MIAFAAQILA FCLLLLLLLL LQLQTTMAGD SSFSGVFDHG SHGVTLVKVD
 51   EAPRKCSSAA AAKKTDDDTA PAGGAPPKPL LVAAPCDAGV YPVVFLHGY
101   LAYNSFYSQL FEHVASHGFV VVGPNQNSI LIYYFSYIRC LDRIPPTRST
151   RRAAVINWLA AGGLTSKLPP NVRADATKIS ISGHSRGGKV AFALALGHAN
201   VSLRGGAGGA TIAALVAVDP VDGATGKQT PPPILTYGGA NSLRVPAPVM
251   VIGTGLGGLA RAAPLLPACA PPGVSHGEFY GECAAPACHL VARDYGHTDM
301   VVDVTPGSA SLRVPCAGAS APGRPCVGSS SAPWSRS

```

Figure 39 (SEQ ID NO:28)

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ORYJ1_CHL
  1   MIAFAAQILA FCLLLLLLLL LQLQTTMAGD SSFSGVFDHG SHGVTLVKVD
 51   EAPRKCSSAA AAKKTDDDTA PAGGAPPKPL LVAAPCDAGV YPVVFLHGY
101   LAYNSFYSQL FEHVASHGFV VVGPNQNSI LIYYFSYIRC LDRIPPTRST
151   GGLTSKLPPN VRADATKISI SGHSRGGKVA FALALGHANV SLRGGAGGAT
201   IALVAVDPV DGFAAGKQTP PPILTYGGAN SLRVAPAPVMV IGTGLGGLAR
251   AAPLLPACAP PGVSHGEFYG ECAAPACHLV ARDYGHTDMM DDVTPGARGL
301   ATRAVCRSGG ARAPMRRFFG GAMVAFVKRW VEGEPELLDC VRARPETAPV
351   VLSAVEFRDE AIANHSY

```

Figure 40 (SEQ ID NO:29)

ORYJ2_CHL

1	MIAFAAQILA	FCLLLLLLLL	LQLQTTMAGD	SSFSGVFDHG	SHGVTLVKVD
51	EAPRKCSSAA	AAKKTDDDTA	PAGGAPPKPL	LVAAPCDAGV	YPVVVFLHGY
101	LAYNSFYSQL	FEHVASHGFV	VVGPQLFLGC	ELILSNNFDA	KMLYTMSPGD
151	TTDEINSAAA	VINWLAAGGL	TSKLPPNVRA	DATKISISGH	SRGGKVAFAL
201	ALGHANQTPR	PILTYGGANS	LRLPAPVMVI	GTGLGGLARA	APLLPACAPP
251	GVSHGEFYGE	CAAPACHLVA	RDYGHTDMMD	DVTPGARGLA	TRAVCRSGGA
301	RAPMRRFFGG	AMVAFVKRWV	EGEPELLDCV	RARPETAPVV	LSAVEFRDEA
351	IANHSY				

Figure 41 (SEQ ID NO:30)

PICEA_CHL

1	MGQQGEEPWE	DVFKPGRFPV	RILKIPQRTT	HGSTTAAAPK	PLLLALPAQP
51	GEYPVLLFFH	GYLLLSFYT	QLLOHIASHG	YIAIAPQMYC	VTGADATPEI
101	ADAAAICNWL	LQGLSSYLPD	DVRPDFQNV	MAGHSRGGKV	AFGLALDRTS
151	QTTELKFSAL	VGVDPVDGMA	RGRQTQPRIL	TYKPHSFDSV	IPTLIVGSGL
201	GAVKRNPFLP	PCAPEGVSHR	EFFSECSAPA	YHFVASDYGH	MDFLDDETGG
251	VKGQSSYCLC	KNGVAREPMR	RFCGGLIVAF	LVNCLQNDG	AFNDLLVHPS
301	HAPVKLEPPE	SFVSEVEHQA	VESLLPQTV		

Figure 42 (SEQ ID NO:31)

CHL_CHL

1	MPSTQFLGAS	TLLLFGLRAV	MSSDDYIKRG	DLPTSKWSGR	VTLRVDSAMA
51	VPLDVVITYP	SSGAAAYPVL	VMYNGFQAKA	PWYRGIVDHV	SSWGYTVVQY
101	TNGGLFPIVV	DRVELTYLEP	LLTWLETQSA	DAKSPLYGRA	DVSRLGTMGH
151	SRGGKLAALQ	FAGRTDVSGC	VLFDVPDVGSP	MTPESADYPS	ATKALAAAGR
201	SAGLVGAAIT	GSCNPVGQNY	PKFWGALAPG	SWQMVLSQAG	HMQFARTGNP
251	FLDWSLDRLC	GRGTMMSSDV	ITYSAAFTVA	WFEGIFRPAQ	SQMGISNFKT
301	WANTQVAARS	ITFDIKPMQS	PQ		

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Figure 43

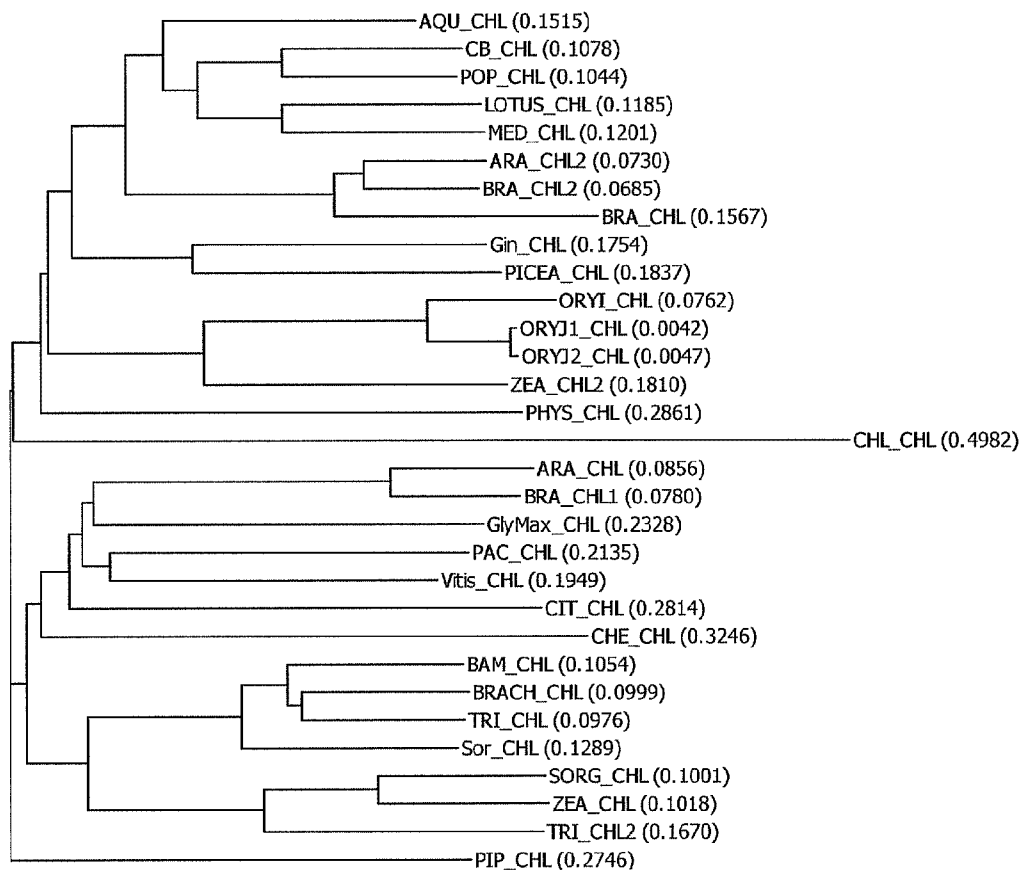


Figure 44

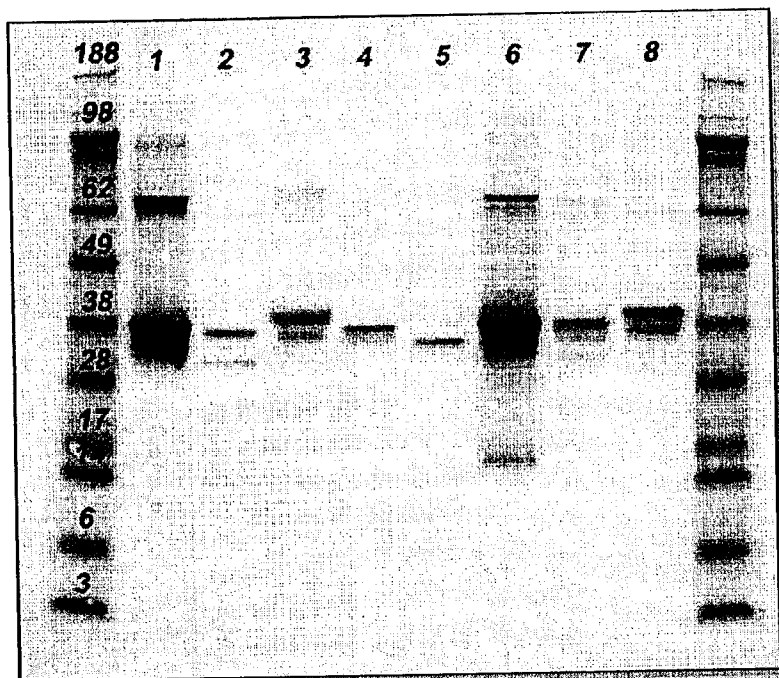
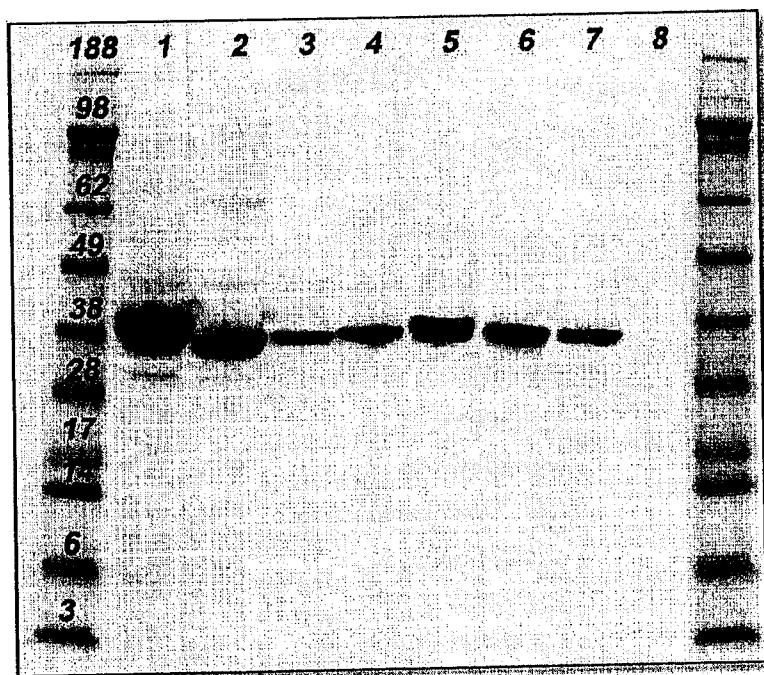


Figure 45



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Figure 46

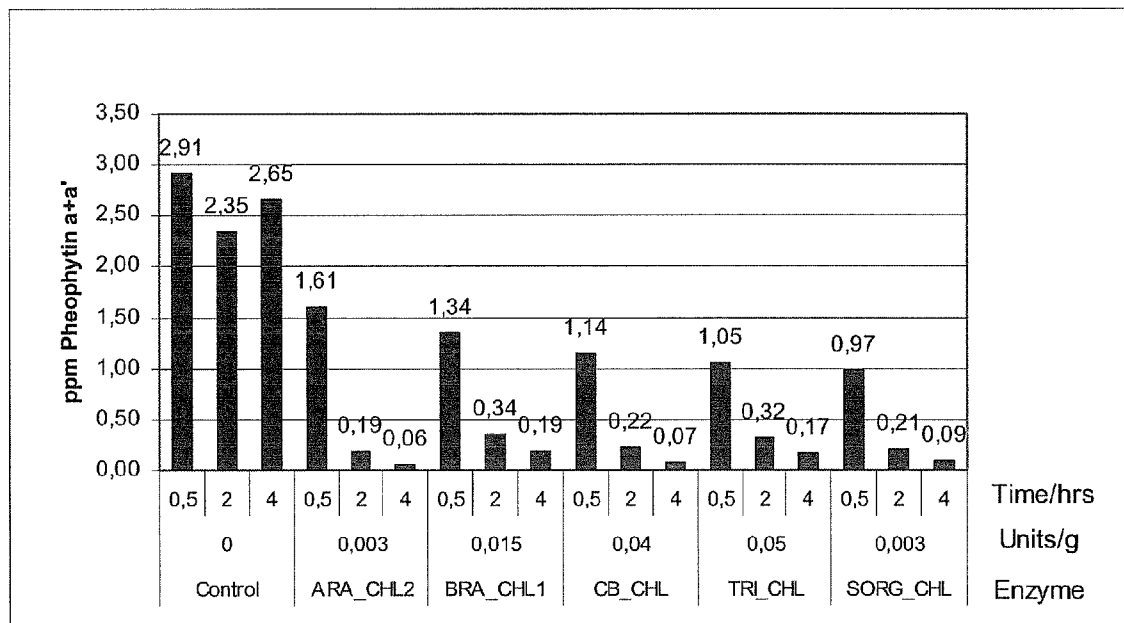


Figure 47

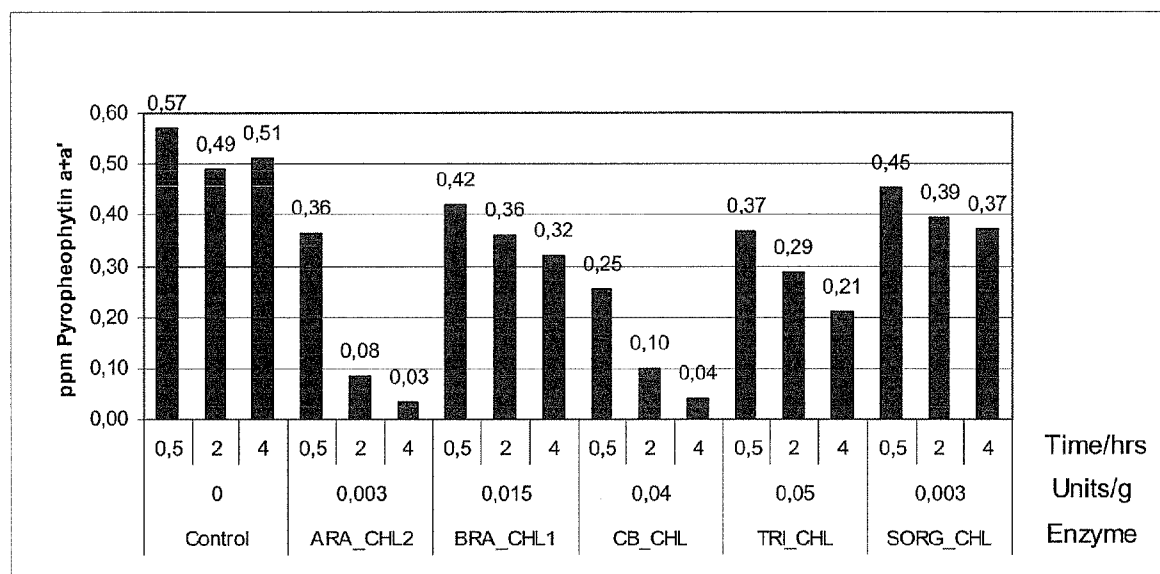


Figure 48

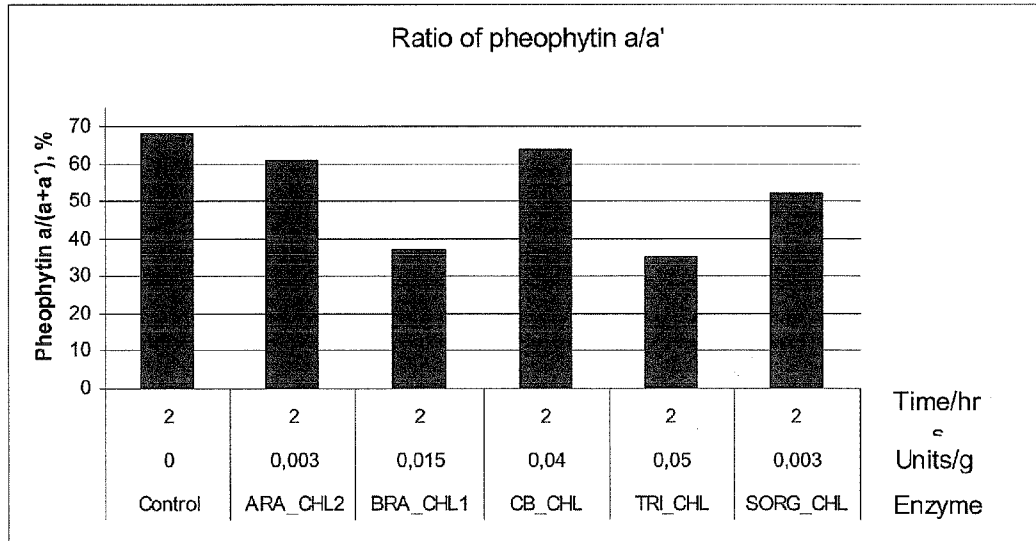
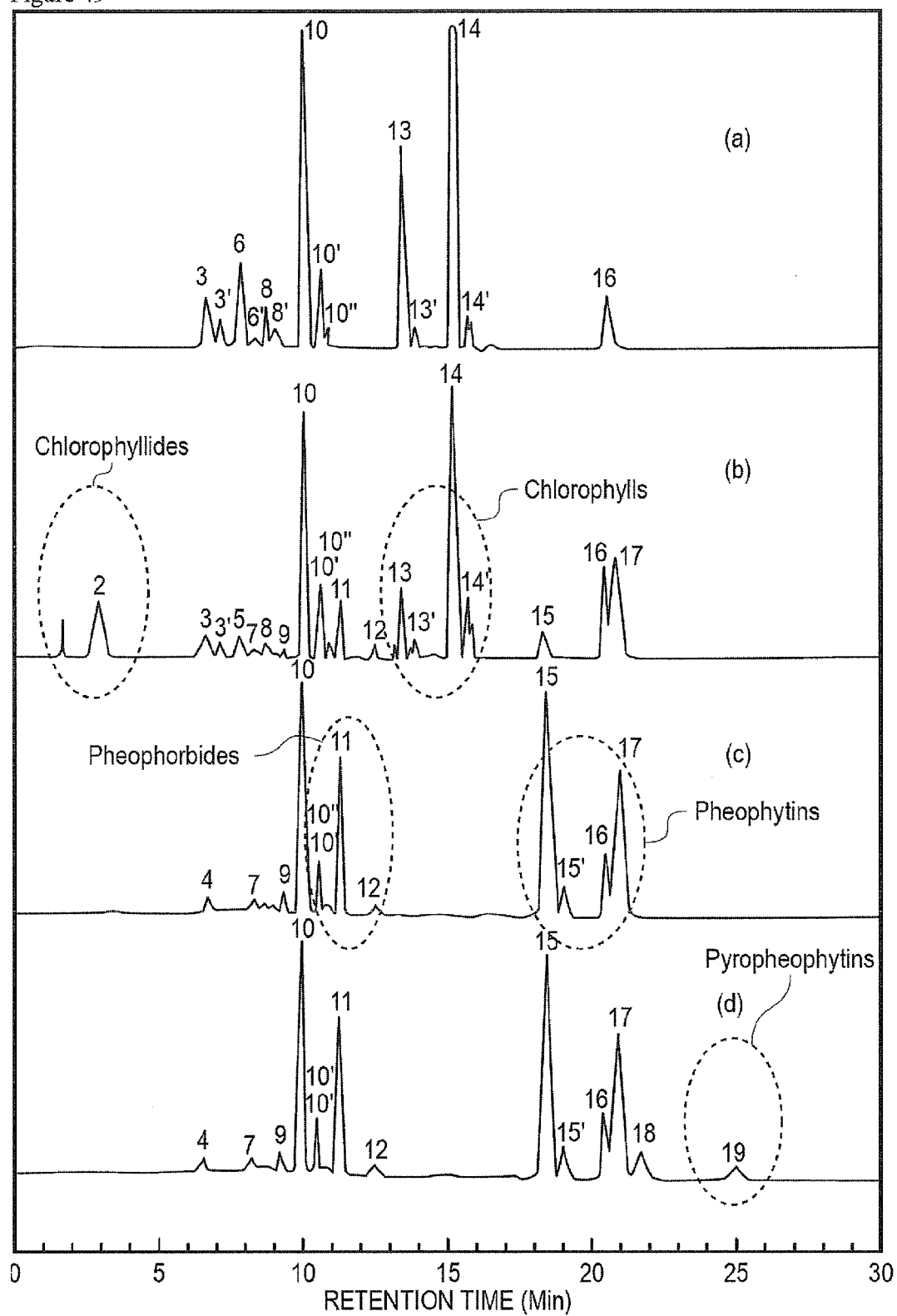


Figure 49



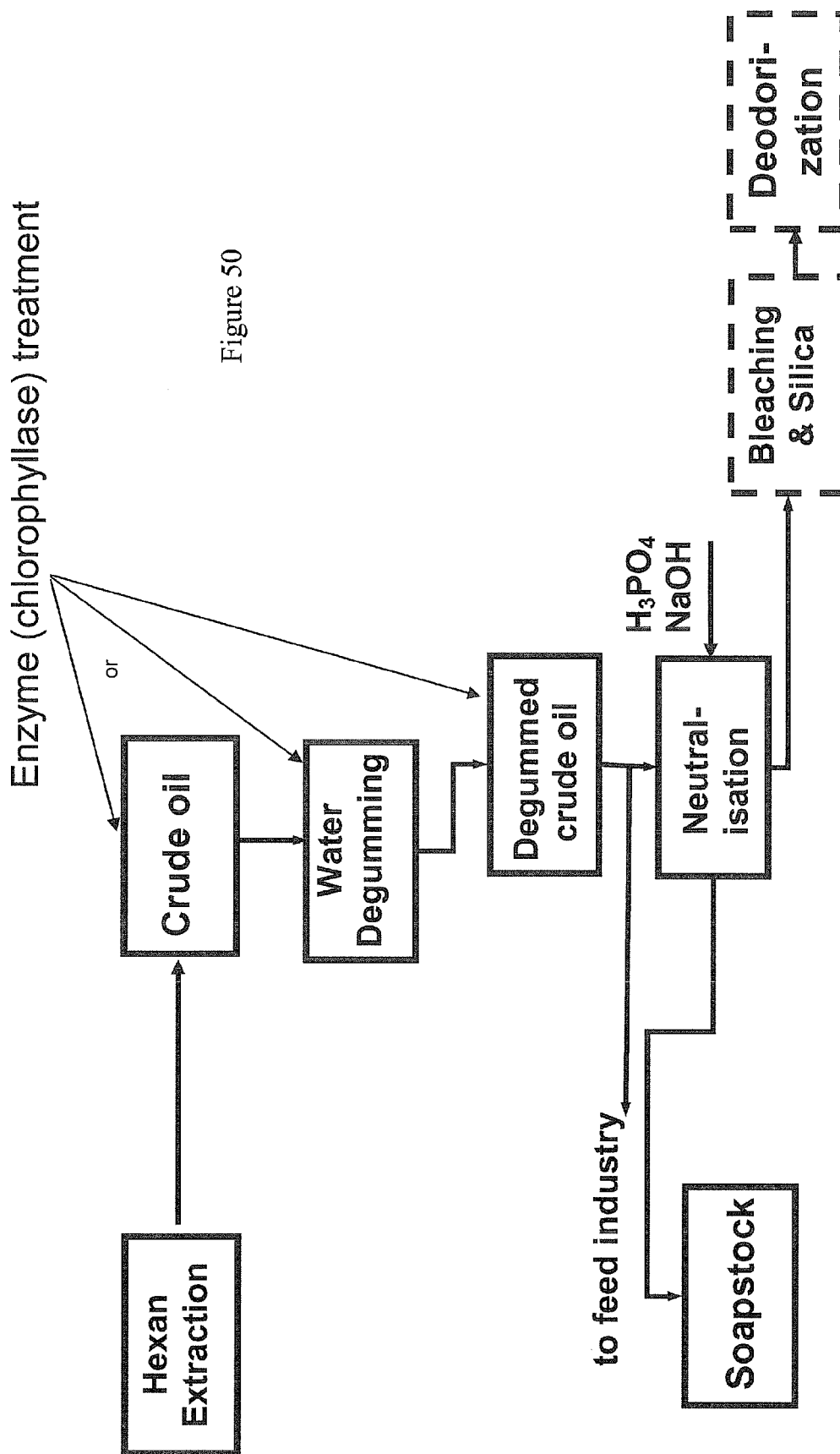


Figure 50

Figure 51

