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<td>(57) Abstract: An acidic phospholipase is obtained from a strain of the genus <em>Hyphozyma</em>. It is able to hydrolyze both fatty acyl groups in intact phospholipid. Advantageously, it has no lipase activity and is active at very low pH; these properties make it very suitable for use in oil degumming, as enzymatic and alkaline hydrolysis (saponification) of the oil can both be suppressed. The phospholipase is not membrane bound, making it suitable for commercial production and purification.</td>
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NOVEL PHOSPHOLIPASE, PRODUCTION AND USE THEREOF

TECHNICAL FIELD

This invention relates to a novel phospholipase, DNA encoding it and to its production and use.

5 BACKGROUND ART

Phospholipids, such as lecithin or phosphatidylcholine, consist of glycerol esterified with two fatty acids in an outer (sn-1) and the middle (sn-2) positions and esterified with phosphoric acid in the third position; the phosphoric acid, in turn, may be esterified to an amino-alcohol. Phospholipases are enzymes which participate in the hydrolysis of phospholipids. Several types of phospholipase activity can be distinguished, including phospholipase A1 and A2 which hydrolyze one fatty acyl group (in the sn-1 and sn-2 position, respectively) to form lysophospholipid; and lysophospholipase (or phospholipase B) which can hydrolyze the remaining fatty acyl group in lysophospholipid. This invention relates to a phospholipase that has the ability to hydrolyze both fatty acyl groups in a phospholipid. Enzymes with this activity are also sometimes called phospholipase B.


It is known to use phospholipase in, e.g., enzymatic oil degumming (US 5,264,367, Metallgesellschaft, Röhm), treatment of starch hydrolysate (particularly from wheat starch) to improve the filterability (EP 219,269, CPC International) and as an additive to bread dough to improve the elasticity of the bread (US 4,567,046, Kyowa Hakko).

It is the object of this invention to provide an improved phospholipase for use in such processes.
STATEMENT OF THE INVENTION

The present inventors have found that an acidic phospholipase can be obtained from a strain of the genus *Hyphozyma*. It is able to hydrolyze both fatty acyl groups in intact phospholipid. Advantageously, it has no lipase activity and is active at very low pH; these properties make it very suitable for use in oil degumming, as enzymatic and alkaline hydrolysis (saponification) of the oil can both be suppressed. The phospholipase is not membrane bound, making it suitable for commercial production and purification.

WO 93/24619 (Novo Nordisk) discloses a lipase from *Hyphozyma sp.* LF-132 (CBS 648.91), but the production of phospholipase by this genus has never been reported. We have found that the phospholipase of this invention can be obtained from the same strain as the known lipase, and that the two enzymes can be separated.

Accordingly, a first aspect of the invention provides an isolated phospholipase which is able to hydrolyze both fatty acyl groups in a phospholipid, is derivable from a strain of *Hyphozyma*, and has optimum phospholipase activity at about 50°C and pH 3 measured at the conditions described in Example 3.

The invention also provides an isolated phospholipase which is able to hydrolyze both fatty acyl groups in a phospholipid, and is a polypeptide comprising at its N-terminal a partial amino acid sequence which is the sequence shown in positions 1-497 of SEQ ID NO: 11, or is at least 50 % identical therewith.

In another aspect, the invention provides an isolated phospholipase which is able to hydrolyze both fatty acyl groups in a phospholipid, and is a polypeptide containing amino acid sequences which are at least 50 % identical with the amino acid sequences shown in SEQ ID NO: 1-8, disregarding Xaa.

The invention further provides an isolated DNA sequence which encodes said phospholipase.

Yet another aspect of the invention provides a method of producing a phospholipase, comprising cultivation of a phospholipase-producing strain of *Hyphozyma* in a suitable nutrient medium, followed by recovery of the phospholipase.

A further aspect of the invention provides a method for producing a phospholipase, comprising isolating a DNA sequence encoding the phospholipase from a phospholipase-producing strain of *Hyphozyma*, combining the DNA fragment with appropriate expression signal(s) in an appropriate vector, transforming a suitable heterologous host organism with the vector, cultivating the transformed host organism under conditions leading to expression of the phospholipase, and recovering the phospholipase from the culture medium.
The invention also provides use of said phospholipase in a process comprising treatment of a phospholipid or lysophospholipid with the phospholipase so as to hydrolyze fatty acyl groups.

Finally, the invention provides a process for reducing the content of phospholipid in a vegetable oil, comprising treating the oil with an aqueous dispersion of an acidic phospholipase at pH 1.5-3 so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

BRIEF DESCRIPTION OF DRAWINGS

Figs. 1, 2 and 3 show the temperature profile, pH profile and thermostability, respectively, of phospholipase from *Hyphozyma* sp. CBS 648.91. Further details are given in Example 3.

Fig. 4a-d gives a comparison of SEQ ID NO: 11 with 3 prior-art sequences.

DETAILED DISCLOSURE OF THE INVENTION

**Phospholipase**

The phospholipase of the invention is able to hydrolyze both acyl groups in a phospholipid molecule (such as phosphatidyl choline or lecithin) without intermediate accumulation of lysophospholipid and is also able to hydrolyze the fatty acyl group of a lysophospholipid (such as lysophosphatidyl choline or lyso-lecithin). Advantageously, the phospholipase of the invention is not membrane bound.

A preferred enzyme is derived from *Hyphozyma* sp. strain CBS 648.91. Its molecular weight is about 94 kDa by SDS, about 87 kDa by gel filtration, and 92 kDa by mass spectrometry. It is believed to be glycosylated. It has an iso-electric point of about 5.6. It has no lipase activity, i.e. it does not hydrolyze triglycerides.

The influence of pH and temperature on the activity of this phospholipase is shown in Fig. 1 and 2. As shown in these figures, the enzyme has optimum activity at about pH 3 and 50°C.

Fig. 3 shows the thermostability of this enzyme, expressed as the residual activity after 10 minutes at pH 7 at various temperatures. It is seen that the enzyme retains more than 90% activity at temperatures up to 50°C, more than 75% up to 60°C and more than 50% up to 70°C.

**Phospholipase Activity Assay**

Two different units are used in this specification:
1 unit (phospholipase activity unit) is the amount of phospholipase that releases one \( \mu \) (micro)-mole of fatty acid per minute from DPPC (dipalmitoyl phosphatidylcholine) at 40°C and pH 4. The amount of released fatty acid is determined by NEFA-C test Wako.

1 International Unit (IU) is the amount of phospholipase that releases one \( \mu \) (micro)-equivalent of free fatty acid per minute from egg yolk in the presence of calcium and deoxycholate at pH 8.0 and 40°C in a pH-stat. The released fatty acids are titrated with 0.1 N sodium hydroxide and the base volume is monitored as a function of time.

**Assay for action pattern of phospholipase**

The following test is used to identify if a given enzyme has the ability to hydrolyze both fatty acyl groups of a phospholipid without the accumulation of lysophospholipid.

A substrate solution is prepared containing 2% L-\( \alpha \)(alpha)-phosphatidylcholine, dipalmitoyl (product of Wako Pure Chemical Industries Ltd.) and 2% Triton X-100. A buffer solution is prepared containing 0.4 M citrate buffer (pH 5). Enzyme solutions are prepared containing various amounts of the sample to be analyzed.

0.5 ml of the substrate solution, 0.25 ml of the buffer solution and 0.05 ml of 0.1 N CaCl\(_2\) are mixed and incubated at 40°C. 0.1 ml of the enzyme solution is added and incubated for 1 hour. The reaction is terminated by adding 0.1 ml of 1 N HCl.

2 ml of CHCl\(_3\)-methanol (1:1) is added to the reaction mixture and mixed vigorously. Approx. 1 \( \mu \) (micro)l of the CHCl\(_3\)-methanol is taken and applied to a TLC rod (in triplicate or quadruplicate). The TLC rods are dried and developed for 45 minutes with CHCl\(_3\) : methanol : NH\(_3\) (25% solution) = 65:25:5. After the development, the rods are scanned by TLC-FID (latroscan), and the chromatograms are integrated.

The amounts of palmitate, the substrate, lysophosphatidyl choline (LPC) and glycerophosphatidyl choline (GPC) are calculated from the areas of peaks appearing in that order.

The result of the test is considered positive if GPC is formed without any LPC formation.

**Amino acid sequence**

Partial sequences SEQ ID NO: 1-8 were determined by sequencing of phospholipase from *Hyphozyma sp.* CBS 648.91 after enzymatic hydrolysis. In these sequences, Xaa represents an amino acid that could not be determined. SEQ ID NO:
1 is an N-terminal sequence, and the others are internal sequences. Xaa in SEQ ID NO: 1 is believed to be a Pro residue. Xaa in SEQ ID NO: 3, 7 and 8 and both Xaa in
SEQ ID NO: 5 are believed to be glycosylated Asn residues.

A nearly complete DNA sequence (SEQ ID NO: 9) was determined for the
gene encoding the phospholipase from *Hyphozyma* sp. CBS 648.91. This sequence
was determined from the genomic locus and includes an open reading frame of 552
amino acids and 213 base pairs of sequence upstream of the putative translation
initiation codon. The methods used for sequence isolation and determination are well
known in the art. Details are given in the examples.

The long, uninterrupted open reading frame identified in this sequence was
translated and compared to the partial peptide sequences SEQ ID NO: 1-8. The
translated sequence was identical to seven of the partial peptide sequences at all
positions, SEQ ID NO:1-7, and overlapped the most distal partial peptide sequence,
SEQ ID NO: 8 by 10 amino acids. By combining the translation with partial peptide
NO: 8, a sequence of 573 amino acid residues (shown as SEQ ID NO: 11) has been
determined. The amino terminus of the mature peptide is determined by comparison
with SEQ ID NO: 1. The sequenced open reading frame extends upstream an
additional 115 amino acids. There is only one Met codon in this region, 76 amino acids
from the start of the mature peptide (position -76). The 14 amino acids immediately
following this methionine residue appear to constitute a secretion signal sequence (G.
von Heijne, Nucleic Acids Res, 14, 4683-4690, 1986), indicating both that this is the
translation initiation codon and that the encoded protein is secreted. The intervening
61 amino acids must constitute a propeptide.

The peptide sequence from *Hyphozyma* was aligned with the phospholipase B
sequences from three other fungi, *Penicillium notatum* (Genbank X60348),
*Saccharomyces cerevisiae* (Genbank L23089) and *Torulaspora delbrueckii* (Genbank
D32134), as shown in Fig. 4a-d. In this alignment a dash (-) indicates an inserted gap,
a circle (o) above the alignment marks a position at which the same amino acid is
found in all proteins, and a vertical line (|) above the alignment indicates similar
residues in all proteins. The portion of the *Hyphozyma* phospholipase sequence we
have determined is 38% identical to the phospholipase from *Penicillium notatum*, 37%
identical to the phospholipase from *Saccharomyces cerevisiae*, and 38% identical to
the phospholipase from *Torulaspora delbrueckii*. The full length *Penicillium*,
*Saccharomyces*, and *Torulaspora* sequences extend from 112 to 145 residues further
than the partial *Hyphozyma* sequence, suggesting that the full length for the translated
*Hyphozyma* peptide is approximately 700 amino acid residues.
Thus, the phospholipase of the invention may contain an N-terminal sequence as shown at positions 1-497 of SEQ ID NO: 11 or a sequence derived therefrom by substitution, deletion or insertion of one or more amino acids. The derived sequence may be at least 50 % identical, e.g. at least 60%, preferably at least 70%, especially at least 80 or at least 90% identical with said partial sequence. The phospholipase of the invention may contain a further 150-250 (e.g. 180-220) amino acid residues at the C-terminal.

Microorganism

The phospholipase of this invention may be derived from a fungal strain of the genus *Hyphozyma*, a genus of yeast-like *Hyphomycetes* described in de Hoog, G.S & Smith, M.Th., Antonie van Leeuwenhoek, 47, 339-352 (1981).

Preferably, the strain belongs to the species defined by the strain *Hyphozyma sp.* LF132, CBS 648.91, which is described in WO 93/24619. This strain was classified in the genus *Hyphozyma*, but it did not match any of the previously described species of *Hyphozyma*, so it is believed to define a new species. It is particularly preferred to use said strain or a mutant or variant thereof having the ability to produce phospholipase.

The preferred *Hyphozyma sp.* strain (designated LF132 by the inventors) has been deposited on 12 November 1991, for the purpose of patent procedures according to the Budapest Treaty at Centraal Bureau voor Schimmelcultures (CBS), Oosterstraat 1, 3740 AG Baarn, Netherlands, and was given the accession number CBS 648.91.

Production of phospholipase by cultivation of *Hyphozyma*

The phospholipase of the invention may be produced by cultivation of the microorganism described above in a suitable nutrient medium, containing carbon and nitrogen sources and inorganic salts, followed by recovery of the enzyme. The nutrient medium may be formulated according to principles well known in the art.

The phospholipase may be recovered from the culture broth and purified to remove lipase activity, e.g. as described in the examples of this specification.

Production by cultivation of transformant

An alternative method of producing the phospholipase of the invention comprises transforming a suitable host cell with a DNA sequence encoding the phospholipase, cultivating the transformed organism under conditions permitting the production of the enzyme, and recovering the enzyme from the culture.
The host organism is preferably a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, preferably a strain of Aspergillus, Fusarium, Trichoderma or Saccharomyces, most preferably A. niger, A. oryzae, F. graminearum, F. sambucinum, F. cerealis or S. cerevisiae. The production of the phospholipase in such host organisms may be done by the general methods described in EP 238,023 (Novo Nordisk), WO 96/00787 (Novo Nordisk) or EP 244,234 (Alko).

The DNA sequence can be isolated from a phospholipase-producing Hyphozyma strain by extraction of DNA by methods known in the art, e.g. as described by Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY.

The DNA sequence of the invention can also be isolated by any general method involving
- cloning, in suitable vectors, a cDNA library from a phospholipase-producing Hyphozyma strain,
- transforming suitable yeast host cells with said vectors,
- culturing the host cells under suitable conditions to express any enzyme of interest encoded by a clone in the cDNA library,
- screening for positive clones by determining any phospholipase activity of the enzyme produced by such clones, and
- isolating the enzyme encoding DNA from such clones.

A general isolation method has been disclosed in WO 93/11249 or WO 94/14953, the contents of which are hereby incorporated by reference.

Alternatively, the DNA encoding a phospholipase of the invention may, in accordance with well-known procedures, conveniently be isolated from a phospholipase-producing Hyphozyma strain, by use of synthetic oligonucleotide probes prepared on the basis of a peptide sequence disclosed herein.

**Use of phospholipase**

The phospholipase of the invention can be used in any application where it is desired to hydrolyze the fatty acyl group(s) of a phospholipid or lyso-phospholipid, such as lecithin or lyso-lecithin. The phospholipase is preferably used at pH 1.5-5 (e.g. 3-5, particularly 3.5-4.5) and at 30-70°C (particularly 40-60°C). If desired, the phospholipase may be inactivated after the reaction by a heat treatment, e.g. at pH 7, 80°C for 1 hour or 90°C for 10 minutes.

As an example, the phospholipase of the invention can be used in the preparation of dough, bread and cakes, e.g. to improve the elasticity of the bread or
cake. Thus, the phospholipase can be used in a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread. This can be done in analogy with US 4,567,046 (Kyowa Hakko), JP-A 60-78529 (QP Corp.), JP-A 62-111629 (QP Corp.), JP-A 63-258528 (QP Corp.) or EP 426211 (Unilever).

The phospholipase of the invention can also be used to improve the filterability of an aqueous solution or slurry of carbohydrate origin by treating it with the phospholipase. This is particularly applicable to a solution or slurry containing a starch hydrolysate, especially a wheat starch hydrolysate since this tends to be difficult to filter and to give cloudy filtrates. The treatment can be done in analogy with EP 219,269 (CPC International).

Treatment of vegetable oil

The phospholipase of the invention can be used in a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. This process is applicable to the purification of any edible oil which contains phospholipid, e.g., vegetable oil such as soy bean oil, rape seed oil and sunflower oil.

Prior to the enzymatic treatment, the vegetable oil is preferably pretreated to remove slime (mucilage), e.g. by wet refining. Typically, the oil will contain 50-250 ppm of phosphorus as phospholipid at the start of the treatment with phospholipase, and the process of the invention can reduce this value to below 5-10 ppm.

The enzymatic treatment is conducted by dispersing an aqueous solution of the phospholipase, preferably as droplets with an average diameter below 10 \( \mu \text{m} \). The amount of water is preferably 0.5-5\% by weight in relation to the oil. An emulsifier may optionally be added. Mechanical agitation may be applied to maintain the emulsion.

The enzymatic treatment can be conducted at a pH in the range 1.5-5. The process pH may be in the range 3.5-5 in order to maximize the enzyme performance, or a pH in the range 1.5-3 (e.g. 2-3) may be used in order to suppress the alkaline hydrolysis of triglycerides (saponification). The pH may be adjusted by adding citric acid, a citrate buffer or HCl.

A suitable temperature is generally 30-70\(^\circ\)C (particularly 30-45\(^\circ\)C, e.g. 35-40\(^\circ\)C). The reaction time will typically be 1-12 hours (e.g. 2-6 hours), and a suitable enzyme dosage will usually be 100-5000 IU per liter of oil (e.g. 200-2000 IU/l) or 0.1-10 mg/l (e.g. 0.5-5 mg/l).
The enzymatic treatment may be conducted batchwise, e.g. in a tank with stirring, or it may be continuous, e.g. a series of stirred tank reactors.

The enzymatic treatment is followed by separation of an aqueous phase and an oil phase. This separation may be performed by conventional means, e.g. centrifugation. The aqueous phase will contain phospholipase, and the enzyme may be re-used to improve the process economy.

In other respects, the process can be conducted according to principles known in the art, e.g. in analogy with US 5,264,367 (Metallgesellschaft, Röhm); K. Dahike & H. Buchold, INFORM, 6 (12), 1284-91 (1995); H. Buchold, Fat Sci. Technol., 95 (8), 300-304 (1993); JP-A 2-153997 (Showa Sangyo); or EP 654,527 (Metallgesellschaft, Röhm).

EXAMPLES

Example 1
Production of phospholipase by cultivation of *Hyphozyma*

The strain *Hyphozyma* sp. CBS 648.91, was cultivated in a nutrient medium containing the following components:

- Glucose 20 g/l
- Peptone 10 g/l
- MgSO₄, 7 H₂O 1 g/l
- Yeast Extract 10 g/l
- K₂HPO₄ 5 g/l

pH adjusted to 6.5 with NaOH

The strain was cultivated at 27-30°C for 3-4 days. The culture broth was subjected to liquid/solid separation by centrifugation. After centrifugation, a phospholipase activity of 1 unit/g culture broth was obtained (unit defined above). The supernatant was desalted and freeze-dried resulting in a crude powder preparation.

Example 2
Purification of phospholipase

Freeze dried phospholipase powder obtained according to Example 1 (300 units/g) was applied on a Butyl Toyopearl 650M column after adjusting the salt concentration to 3.5 M ammonium acetate. Bound phospholipase activity was eluted with H₂O and separated from lipase activity which was also present in the crude powder preparation.
Fractions containing phospholipase activity were pooled, concentrated and dialyzed. The concentrated preparation was treated by anion exchange column chromatography using DEAE Toyopearl 650M. The adsorption condition was pH 7.5 (50 mM Tris-HCl) and elution was carried out by a linear gradient of 0-0.5M NaCl.

The last step was gel filtration column chromatography using HiLoad 26/60 Superdex 200pg. The condition was 50 mM Tris-HCl pH 7.5 including 0.5M NaCl. The resulting purified phospholipase was used in the following examples.

**Example 3**

**Characterization of phospholipase**

The molecular weight (MW) of the phospholipase was found to be about 94 kDa on SDS PAGE and 87 kDa by gel filtration column chromatography. The polypeptide is believed to be glycosylated. The pI is around 5.6 on IEF PAGE.

The temperature profile was determined at pH 3.0 and 4.0 in a range of 40 to 70°C. The phospholipase was incubated for 10 minutes, and the activity was determined by the method described above. The temperature profile is presented in Fig. 1 as relative activity (taking the maximum activity as 100%). It is seen from this figure that both at pH 3 and 4, the phospholipase has high activity (more than 50% of optimum) at temperatures of 40 to 60°C with a temperature optimum around 50°C.

The pH profile was determined at 40°C using glycine-HCl buffer at pH 2, 2.5 and 3, and citrate buffer at pH 3, 4, 5 and 6. The results are presented in Fig. 2 as relative activity (taking the maximum activity as 100%). Due to a change of buffer system (glycine-HCl, citrate), the figure is made up of two curves, one representing the interval of pH 2.0 to 3.0 and the other representing the interval of pH 3.0 to 6.0. From the figure it appears that the phospholipase is active at pH values of 2 to 5, and the pH optimum is around 3.

The thermostability was determined by incubating in 0.1 M phosphate buffer (pH 7) for 10 minutes at temperatures of 40-80°C and determining the residual activity after the incubation. The results were 100% at 40°C, 95% at 50°C, 82% at 60°C, 55% at 70°C and 9% at 80°C. These results are also shown in Fig. 3.

**Example 4**

**Hydrolysis of phospholipid**

A substrate solution was prepared by dissolving 2% of crude soy bean lecithin (phosphatidyl choline) in water. An enzyme solution was prepared by 50 times dilution of the purified enzyme from Example 2. 0.5 ml of the substrate solution, 0.25 ml of 0.4 M citrate buffer (pH 4) and 0.05 ml of 0.1 N CaCl₂ were mixed and incubated at 60°C.
0.1 ml of the enzyme solution was added and incubated for 1 hour at 60°C. The reaction was terminated by adding 0.1 ml of 1 N HCl. The mixture after the reaction was analyzed by TLC-latroscan as described above in the assay for reaction pattern. The results showed that fatty acid was formed and that no lecithin remained after the reaction. A solid precipitate was observed at the bottom of the reaction vessel. This was believed to be a mixture of phospholipid and fatty acid.

Example 5
Hydrolysis of lyso-phospholipid

Lyso-phosphatidylcholine (LPC) was treated for 10 minutes at 40°C, other conditions being the same as described in Example 4. The chromatogram showed that about two thirds of the LPC was hydrolyzed, and that fatty acid was formed together with a small amount of phosphatidylcholine.

Example 6
Enzymatic degumming of edible oil

Vegetable oil was degummed by treating it with phospholipase from *Hyphozyma* as follows. The enzyme dosage, the reaction pH and temperature were varied, and the resulting content of phospholipid was measured.

The equipment consisted of a 1 l jacketed steel reactor fitted with a steel lid, a propeller (600 rpm), baffles, a temperature sensor, an inlet tube at the top, a reflux condenser (4 °C) at the top, and an outlet tube at the bottom. The reactor jacket was connected to a thermostat bath. The outlet tube was connected via silicone tubing to an in-line mixer head equipped with a high shear screen (8500 rpm, flow ca. 1.1 l/minute). The mixer head was fitted with a cooling coil (5-10 °C) and an outlet tube, which was connected to the inlet tube of the reactor via silicone tubing. A temperature sensor was inserted in the silicone tubing just after the mixer head. The only connection from the reactor/mixer head system to the atmosphere was through the reflux condenser.

In each experiment, 0.6 l (ca. 560 g) of water-degummed rape seed oil with a P content of 186-252 ppm was loaded into the reactor with the thermostat and lab mixer running and pre-treated for 30 minutes with 0.6 g (2.86 mmol) of citric acid monohydrate in 27 g of water (added water vs. oil equals 4.8% w/w; [citric acid] in water phase = 106 mM, in water/oil emulsion = 4.6 mM) at time= 0. After the pre-treatment, the pH was adjusted by adding a NaOH solution followed by the enzyme solution. The mixture was then incubated for 6 hours, and samples for P-analysis and pH determination were drawn at intervals throughout the experiment.
The determination of phosphorous content in the oil was done according to procedure 2.421 in "Standard Methods for the Analysis of Oils, Fats, and Derivatives, 7th ed. (1987)" after separating the emulsion by heating and centrifugation.

The initial performance was calculated from the initial rate of phosphorus removal from the oil, taking the optimum as 100%.

**Degumming at various pH**

The oil was treated at 40°C with an enzyme dosage of 1.3 mg/kg oil (as pure enzyme protein). The results at various pH were as follows:

<table>
<thead>
<tr>
<th>pH</th>
<th>Initial performance (relative to optimum)</th>
<th>P content after 6 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>40</td>
<td>74 ppm</td>
</tr>
<tr>
<td>3.7</td>
<td>90</td>
<td>&lt;10 ppm</td>
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<td>4.4</td>
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<td>&lt;10 ppm</td>
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<tr>
<td>4.8</td>
<td>80</td>
<td>&lt;10 ppm</td>
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</table>

**Degumming at various temperatures**

The oil was treated at pH 4.5 with an enzyme dosage of 1.3 mg/kg oil (as pure enzyme protein). The results at various temperatures were as follows:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Initial performance (relative to optimum)</th>
<th>P content after 6 hours</th>
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<tbody>
<tr>
<td>35°C</td>
<td>90</td>
<td>&lt;10 ppm</td>
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<tr>
<td>40°C</td>
<td>100</td>
<td>&lt;10 ppm</td>
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</tbody>
</table>

**Degumming with various enzyme dosages**

The oil was treated at pH 4.5, 40°C. The results at various enzyme dosages (given as pure enzyme protein) were as follows:

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<th>Enzyme dosage (mg/kg oil)</th>
<th>Initial performance (relative to optimum)</th>
<th>P content after 6 hours</th>
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<td>0.65</td>
<td>70</td>
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<td>1.3</td>
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<td>2.6</td>
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<td>&lt;10 ppm</td>
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The results show good degumming performance at pH 3.5-5, 35-40°C. Good degumming to a phosphorus content below 10 ppm was obtained in 6 hours with a dosage of 1.3 mg/kg oil, and in 3 hours at a dosage of 2.6 mg/kg.

Measurement of the free fatty acids generated during degumming showed a low level of free fatty acids, corresponding very well to the amount of phospholipid in the substrate oil.
For reference, similar experiments were done with prior-art phospholipase from porcine pancreas. It was found that degumming to below 10 ppm of phosphorus could be obtained at 60°C, pH 5.5, but the performance of the prior-art enzyme dropped sharply at lower pH, and satisfactory degumming could not be achieved at pH lower than 5.5.

Example 7

Partial determination of the DNA sequence encoding the phospholipase

DNA encoding the phospholipase of *Hyphozyma* was isolated by two different methods. The 5' end of the gene was isolated by cloning. A genomic library of *Hyphozyma* DNA partially digested with Sau3A was screened at high stringency using standard methods (Sambrook et al., (1989), Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Lab.; Cold Spring Harbor, NY) with a probe specific to the phospholipase sequence. This probe was amplified from total *Hyphozyma* DNA with degenerate primers designed using the previously determined partial peptide sequences with SEQ ID NO: 1 and 5. Standard PCR conditions were used for amplification (Saiki et al., Science, 239, 487-491, 1988), including 0.5mM MgCl₂, a 45°C annealing temperature, and primers PLMStr1 (SEQ ID NO: 12) and PLMStr6 (SEQ ID NO: 13). The clone pMStr16 hybridized to the probe, and therefore was isolated and a portion of the insert was sequenced.

An additional internal portion of the phospholipase-encoding gene was isolated using PCR with *Hyphozyma* DNA and the primers PLHaW2 (SEQ ID NO: 14) and PLMStr7 (SEQ ID NO: 15). PLHaW2 was designed using the sequence determined from pMStr16, and PLMStr7 was designed from the sequence of the partial peptide with SEQ ID NO: 8. Standard conditions were used for the PCR reactions, with 1.5 mM MgCl₂, and a 46°C annealing temperature. The resulting amplified fragment was isolated and sequenced.
SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
   (A) NAME: Novo Nordisk A/S
   (B) STREET: Novo Alle
   (C) CITY: Bagsvaerd
   (E) COUNTRY: Denmark
   (F) POSTAL CODE (ZIP): DK-2880
   (G) TELEPHONE: +45-4444-8888
   (I) TELEX: 45-4449-3256

(ii) TITLE OF INVENTION: Novel Phospholipase, Production and Use Thereof

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
   (A) MEDIUM TYPE: Floppy disk
   (B) COMPUTER: IBM PC compatible
   (C) OPERATING SYSTEM: PC-DOS/MS-DOS
   (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

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   (B) TYPE: amino acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: N-terminal

(vi) ORIGINAL SOURCE:
   (A) ORGANISM: Hyphozyma sp.
   (B) STRAIN: CBS 648.91

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   (v) FRAGMENT TYPE: internal
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       (B) STRAIN: CBS 648.91

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       (B) STRAIN: CBS 648.91

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(B) STRAIN: CBS 648.91

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(v) FRAGMENT TYPE: internal
(vi) ORIGINAL SOURCE:
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(B) STRAIN: CBS 648.91
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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Hyphozyma sp.
(B) STRAIN: CBS 648.91

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(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(v) FRAGMENT TYPE: internal
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20   25   30

(2) INFORMATION FOR SEQ ID NO: 8:
   (i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 31 amino acids
   (B) TYPE: amino acid
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   (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: peptide
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      (A) ORGANISM: Hyphozyma sp.
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(iii) HYPOTHETICAL: NO
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(A) ORGANISM: Hyphozyma sp.
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CCA A 1870
Pro

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 552 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xii) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

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(2) INFORMATION FOR SEQ ID NO: 11:
   (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 573 amino acids
       (B) TYPE: amino acid
       (C) STRANDEDNESS: single
       (D) TOPOLOGY: linear
   (ii) MOLECULE TYPE: peptide
   (v) FRAGMENT TYPE: N-terminal
   (vi) ORIGINAL SOURCE:
       (A) ORGANISM: Hyphozyma sp.
(B) STRAIN: CBS 648.91

(xi) FEATURE:
(A) NAME/KEY: Protein
(B) LOCATION: 1..497

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

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   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(ix) FEATURE:
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   (B) LOCATION:3..18
   (D) OTHER INFORMATION:/mod_base= OTHER
       /note= "deoxyinosine"

(x) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

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   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: single
   (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

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   (B) LOCATION:6
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GGCATGTACA CGATGAT
A. The indications made below relate to the microorganism referred to in the description on page 6, lines 18-22.

B. IDENTIFICATION OF DEPOSIT

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<td>12 November 1991</td>
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C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet

Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). As far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

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FORM PCT/RO/134 (JULY 1992)
CLAIMS

1. A phospholipase which:
   a) is able to hydrolyze both fatty acyl groups in a phospholipid,
   b) is derivable from a strain of *Hyphozyma*,
   c) has a temperature optimum measured for 10 minutes at pH 3-4 of about 50°C, and
   d) has a pH optimum measured at 40°C for 10 minutes of about pH 3.

2. A phospholipase which:
   a) is able to hydrolyze both fatty acyl groups in a phospholipid, and
   b) is a polypeptide comprising an N-terminal amino acid sequence which is the sequence shown in positions 1-497 of SEQ ID NO: 11, or is at least 50 % identical therewith.

3. A phospholipase which:
   a) is able to hydrolyze both fatty acyl groups in a phospholipid, and
   b) is a polypeptide containing amino acid sequences which are at least 50% identical with the amino acid sequences shown in SEQ ID NO: 1-8.

4. The phospholipase of claim 2 or 3 wherein said identity of sequences is at least 60%, preferably at least 70%, more preferably at least 80%, and most preferably at least 90%.

5. The phospholipase of any preceding claim which is derivable from *Hyphozyma* sp. strain CBS 648.91.

6. The phospholipase of any preceding claim which is essentially free from lipase activity.

7. A DNA sequence which encodes the phospholipase of claim 2.

8. The DNA sequence of the preceding claim which comprises the sequence shown in positions 457-1870 of SEQ ID NO: 9.
9. A method of producing a phospholipase, comprising cultivation of a phospholipase-producing strain of *Hyphozyma* in a suitable nutrient medium, followed by recovery of the phospholipase.

10. The method of the preceding claim wherein the strain is *Hyphozyma* sp. strain CBS 648.91.

11. The method of claim 9 or 10 wherein the recovery comprises separation to remove lipase activity.

12. A method for producing a phospholipase, comprising:
   a) isolating a DNA sequence encoding the phospholipase from a phospholipase-producing strain of *Hyphozyma*,
   b) combining the DNA fragment with appropriate expression signal(s) in an appropriate vector,
   c) transforming a suitable heterologous host organism with the vector,
   d) cultivating the transformed host organism under conditions leading to expression of the phospholipase, and
   e) recovering the phospholipase from the culture medium.

13. The method of the preceding claim, wherein the host organism is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell, preferably a strain of *Aspergillus*, *Fusarium*, *Trichoderma* or *Saccharomyces*, most preferably *A. niger*, *A. oryzae*, *F. graminearum*, *F. sambucinum*, *F. cerealis* or *S. cerevisiae*.

14. The method of claim 12 or 13 wherein the DNA sequence is isolated by a method comprising:
   a) cloning, in suitable vectors, a cDNA library from the phospholipase-producing strain of *Hyphozyma*,
   b) transforming suitable yeast host cells with said vectors,
   c) cultivating the transformed yeast host cells under suitable conditions to express the phospholipase,
   d) screening for positive clones by determining the phospholipase activity expressed in step (c).
15. The method of any of claims 12-14, wherein the *Hyphozyma* strain is *Hyphozyma sp.* strain CBS 648.91.

16. A process for hydrolyzing fatty acyl groups in a phospholipid or lysophospholipid, comprising treating the phospholipid or lysophospholipid with the phospholipase of any of claims 1-6.

17. The process of the preceding claim wherein the phospholipid or lysophospholipid comprises lecithin or lysolecithin.

18. The process of claim 16 or 17 wherein the treatment is conducted at pH 1.5-5 (preferably 2-4) and 30-70°C.

19. The process of any of claims 16-18, which is a process for improving the filterability of an aqueous solution or slurry of carbohydrate origin which contains phospholipid.

20. The process of the preceding claim wherein the solution or slurry contains a starch hydrolysate, particularly a wheat starch hydrolysate.

21. The process of any of claims 16-18 which is a process for making bread, comprising adding the phospholipase to the ingredients of a dough, kneading the dough and baking the dough to make the bread.

22. The process of any of claims 16-18 which is a process for reducing the content of phospholipid in an edible oil, comprising treating the oil with the phospholipase so as to hydrolyze a major part of the phospholipid, and separating an aqueous phase containing the hydrolyzed phospholipid from the oil.

23. A process for removing phospholipid from an edible oil, comprising:
   a) treating the oil at pH 1.5-3 with a dispersion of an aqueous solution of a phospholipase having the ability to hydrolyze the intact phospholipid at said pH, so as to hydrolyze a major part of the phospholipid, and
   b) separating an aqueous phase containing the hydrolyzed phospholipid from the oil.
24. The method of the preceding claim wherein the oil is treated to remove mucilage prior to the treatment with the phospholipase.

25. The method of claim 23 or 24 wherein the oil prior to the treatment with the phospholipase contains the phospholipid in an amount corresponding to 50-250 ppm as phosphorus.

26. The method of any of claims 23-25 wherein the phospholipase is the phospholipase of any of claims 1-6.

27. The process of any of claims 23-26 wherein the treatment with phospholipase is done at 30-45°C for 1-12 hours at a phospholipase dosage of 0.1-10 mg/l in the presence of 0.5-5% of water.
Relative activity (%)

Citrate buffer, 10 min reaction

Temperature profile

FIG. 1
Relative activity (%)

- Glycine-HCl buffer
- Citrate buffer

40°C, 10 min reaction

pH profile

FIG. 2
FIG. 3

Remaining activity (%)

10 min treatment (pH 7, phosphate buf.)
reaction pH : 4 (citrate buffer)
reaction time : 10 min

Thermostability

°C
FIG. 4a
FIG. 4b
FIG. 4c
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**FIG. 4d**
**INTERNATIONAL SEARCH REPORT**

International application No.
PCT/DK 97/00490

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC6: C12N 9/18, C11B 3/00
According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C11B

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

SE, DK, FI, NO classes as above

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

WPI, CA, MEDLINE, BIOSIS, DBA, FSTA, EMBL/PIR/SWISSPROT/GENESEQ

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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

* Special categories of cited documents:
  *A* document defining the general state of the art which is not considered to be of particular relevance
  *E* earlier document published on or after the international filing date
  *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  *O* document referring to an oral disclosure, use, exhibition or other means
  *P* document published prior to the international filing date but later than the priority date claimed

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

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

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

*E* document of the same patent family

Date of the actual completion of the international search

18 February 1998

Date of mailing of the international search report

24 - 02- 1998

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer
Carolina Palmcrantz
Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)
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|                                      |                 | US 5532163 A            | 02/07/96        |
|                                      |                 | CA 2122069 A            | 26/10/94        |