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(71) Applicant(s)
Novozymes Biotech, Inc.; Novozymes Japan, Ltd.

(72) Inventor(s)
Randy M. Berka; Michael W. Rey; Tony Byun; Ryoko Itami; Noriko Tsutsumi; Alan Klotz

(74) Agent/Attorney
SPRUSON and FERGUSON,GPO Box 3898,SYDNEY NSW 2001

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NOVOZYMES BIOTECH, INC.

(71) Applicants: ~~NOVO NORDISK BIOTECH, INC.~~ [US/US]; 1445 Drew Avenue, Davis, CA 95616 (US). ~~NOVO NORDISK BIOINDUSTRY, LTD.~~ [JP/JP]; Makuharui Techno Garden CB-6, 3, Nakasa, 1-chome, Chiba-shi 261-01 (JP).

(72) Inventors: BERKA, Randy, M.; 3609 Modoc, Davis, CA 95616 (US). REY, Michael, W.; 605 Robin Place, Davis, CA 95616 (US). BYUN, Tony; Apartment B-1, 2680 Sycamore Lane, Davis, CA 95616 (US). ITAMI, Ryoko; 6-1, Wadamichi, Sukagawa-shi, Fukushima-ken 962-0818 (JP). TSUTSUMI, Noriko; 3-2-16-2, Higashi Sugano, Ichikawa-shi, Chiba-ken 272-0823 (JP). KLOTZ, Alan; 725 Collier Drive, Dixon, CA 95620 (US).

(74) Agents: ZELSON, Steve, T. et al.; Novo Nordisk of North America, Inc., 405 Lexington Avenue, Suite 6400, New York, NY 10174 (US).

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(54) Title: POLYPEPTIDES HAVING LYSOPHOSPHOLIPASE ACTIVITY AND NUCLEIC ACIDS ENCODING SAME

(57) Abstract

The present invention relates to isolated polypeptides having lysophospholipase activity and isolated nucleic acid sequences encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing and using the polypeptides.

POLYPEPTIDES HAVING LYSOPHOSPHOLIPASE ACTIVITY AND NUCLEIC ACIDS ENCODING SAME

Background of the Invention

Field of the Invention

The present invention relates to isolated polypeptides having lysophospholipase activity and isolated nucleic acid sequences encoding the polypeptides. The invention also relates to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing and using the polypeptides.

Description of the Related Art

Phospholipases are enzymes that participate in the hydrolysis of phospholipids which consist of a glycerol backbone 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 may, in turn, be esterified to an amino alcohol.

Several types of phospholipase activity can be distinguished which hydrolyze the fatty acyl moieties. Phospholipase A1 and A2 catalyze the deacylation of one fatty acyl group in the sn-1 and sn-2 positions, respectively, from a diacylglycerophospholipid to produce lysophospholipid. Lysophospholipase (also called phospholipase B by the Nomenclature Committee of the International Union of Biochemistry on the Nomenclature and Classification of Enzymes {Enzyme Nomenclature, Academic Press, New York, 1992}) catalyzes the hydrolysis of the remaining fatty acyl group in a lysophospholipid. A phospholipase B has been reported from *Penicillium notatum* (Saito *et al.*, 1991, *Methods in Enzymology* 197:446-456) which catalyzes the deacylation of both fatty acids from a diacylglycerophospholipid and intrinsicly possesses lysophospholipase activity.

Fungal enzymes with phospholipase activity have been reported from various sources, including *Cryptococcus neoformans* (Chen *et al.*, *Infection and Immunity* 65: 405-411), *Fusobacterium necrophorum* (Fifis *et al.*, 1996, *Veterinary Microbiology* 49: 219-233), *Penicillium notatum* (also known as *Penicillium chrysogenum*; Kawasaki,

1975, *Journal of Biochemistry* 77: 1233-1244; Masuda *et al.*, 1991, *European Journal of Biochemistry* 202: 783-787), *Penicillium cyclopium* (Mustranta *et al.*, 1995, *Process Biochemistry* 30: 393-401), *Saccharomyces cerevisia* (Ichimasa *et al.*, 1985, *Agric. Biol. Chem.* 49: 1083-1089; Paultauf *et al.*, 1994, *Journal of Biological Chemistry* 269: 19725-19730), *Torulaspora delbrueckii* (old name *Saccharomyces rosei*, Kuwabara, 1988, *Agric. Biol. Chem.* 52: 2451-2458; Watanabe *et al.*, 1994, *FEMS Microbiological Letters* 124: 29-34), *Schizosaccharomyces pombe* (Oishi *et al.*, 1996, *Biosci. Biotech. Biochem.* 60: 1087-1092), *Neurospora crassa* (Chakravarti *et al.*, 1981, *Archives of Biochemistry and Biophysics* 206: 393-402), *Aspergillus niger* (Technical Bulletin, G-zyme™ G6999, Enzyme Bio-Systems Ltd.; Mustranta *et al.*, 1995, *supra*), *Corticium centrifugum* (Uehara *et al.*, 1979, *Agric. Biol. Chem.* 43: 517-525), *Fusarium oxysporum* (WO 98/26057), and *Fusarium solani* (Tsung-Che *et al.*, 1968, *Phytopathological Notes* 58:1437-38).

Fungal phospholipase genes have been cloned from several sources including *Penicillum notatum* (Masuda *et al.*, 1991, *supra*), *Torulaspora delbrueckii* (Watanabe *et al.*, 1994, *FEMS Microbiology Letters* 124: 29-34), *Saccharomyces cerevisiae* (Lee *at al.*, 1994, *Journal of Biological Chemistry* 269: 19725-19730), *Aspergillus* (JP 10155493), *Neurospora crassa* (EMBL O42791), and *Schizosaccharomyces pombe* (EMBL O13857).

It is an object of the present invention to provide improved polypeptides having lysophospholipase activity and nucleic acid encoding the polypeptides.

Summary of the Invention

According to a first embodiment of the invention, there is provided isolated polypeptides having lysophospholipase activity selected from the group consisting of:

(a) a polypeptide having an amino acid sequence which has at least 65% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16;

(b) a polypeptide encoded by a nucleic acid sequence which hybridizes under high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15; (ii) the genomic DNA sequence containing

nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii) of at least 100 nucleotides, or (iv) a complementary strand of (i), (ii) or (iii);

(c) an allelic variant of (a) or (b); and

(d) a functional fragment of (a), (b) or (c), wherein the fragment has lysophospholipase activity.

According to a second embodiment of the invention, there is provided a polypeptide having the same lysophospholipase activity as the polypeptide in accordance with the first embodiment of the present invention.

According to a third embodiment of the invention, there is provided an isolated nucleic acid sequence comprising a nucleic acid sequence which encodes the polypeptide in accordance with the first or second embodiment of the present invention.

According to a fourth embodiment of the invention, there is provided an isolated nucleic acid sequence comprising a nucleic acid sequence having at least one mutation in the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, in which the mutant nucleic acid sequence encodes a polypeptide consisting of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, respectively.

According to a fifth embodiment of the invention, there is provided an isolated nucleic acid sequence produced by (a) hybridizing under high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii) of at least 100 nucleotides, or (iv) a complementary strand of (i), (ii) or (iii); and (b) isolating the nucleic acid sequence.

According to a sixth embodiment of the invention, there is provided a nucleic acid construct comprising the nucleic acid sequence in accordance with the third embodiment of the present invention operably linked to one or more control sequences that direct the production of the polypeptide in a suitable expression host.

According to a seventh embodiment of the invention, there is provided a recombinant expression vector comprising the nucleic acid construct in accordance with the sixth embodiment of the present invention.

According to an eighth embodiment of the invention, there is provided a recombinant host cell comprising the nucleic acid construct in accordance with the sixth embodiment of the present invention.

According to a ninth embodiment of the invention, there is provided a method for producing a mutant nucleic acid sequence, comprising (a) introducing at least one mutation into the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, wherein the mutant nucleic acid sequence encodes a polypeptide consisting of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, respectively; and (b) recovering the mutant nucleic acid sequence.

According to a tenth embodiment of the invention, there is provided a method for producing a polypeptide comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide, wherein the host cell comprises a mutant nucleic acid sequence having at least one mutation in the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, wherein the mutant nucleic acid sequence encodes a polypeptide consisting of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, respectively, and (b) recovering the polypeptide.

According to an eleventh embodiment of the invention, there is provided a method for producing the polypeptide in accordance with the first or second embodiment of the present invention comprising (a) cultivating a homologously recombinant cell, having incorporated therein a new transcription unit comprising a regulatory sequence, an exon, and/or a splice donor site operably linked to a second exon of an endogenous nucleic acid sequence encoding the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

According to a twelfth embodiment of the invention, there is provided a method for producing a mutant of a parent cell, which comprises disrupting or deleting a nucleic acid sequence encoding the polypeptide in accordance with the first or second embodiment or a portion thereof, or the promoter of said nucleic acid or a portion thereof, which results in the mutant cell producing less of the polypeptide than the parent cell.

According to a thirteenth embodiment of the invention, there is provided a nucleic acid construct comprising a gene encoding a protein operably linked to one or both of a first nucleic acid sequence encoding a signal peptide consisting of nucleotides 100 to 150 of SEQ ID NO. 1 or nucleotides 1 to 48 of SEQ ID NO. 15, or a second nucleic acid

sequence encoding a propeptide consisting of nucleotides 151 to 213 of SEQ ID NO. 1, wherein the gene is foreign to the first and second nucleic acid sequences.

According to a fourteenth embodiment of the invention, there is provided a recombinant expression vector comprising the nucleic acid construct in accordance with the thirteenth embodiment of the present invention.

According to a fifteenth embodiment of the invention, there is provided a recombinant host cell comprising the nucleic acid construct in accordance with the thirteenth embodiment of the present invention.

According to a sixteenth embodiment of the invention, there is provided a method for reducing the phospholipid content in an edible oil, comprising treating the oil with the polypeptide in accordance with the first or second embodiment of the present invention to hydrolyze the phospholipid and separating the hydrolyzed phospholipid from the oil.

According to a seventeenth embodiment of the invention, there is provided a method for reducing the phospholipid content of an aqueous carbohydrate solution or slurry, comprising treating the aqueous carbohydrate solution or slurry with the polypeptide in accordance with the first or second embodiment of the present invention to hydrolyze the phospholipid and separating the hydrolyzed phospholipid from the aqueous carbohydrate solution or slurry.

According to an eighteenth embodiment of the invention, there is provided a method for preparing a dough, comprising incorporating into the dough an effective amount of the polypeptide in accordance with the first or second embodiment of the present invention to improve one or more properties of the dough and/or a baked product obtained from the dough.

According to a nineteenth embodiment of the invention, there is provided a method for preparing a baked product, comprising baking a dough produced by the method in accordance with the eighteenth embodiment of the present invention to produce a baked product.

According to a twentieth embodiment of the invention, there is provided a dough prepared by the method in accordance with the eighteenth embodiment of the present invention.

The disclosure herein also relates to isolated nucleic acid sequences encoding the polypeptides and to nucleic acid constructs, vectors, and host cells comprising the nucleic acid sequences as well as methods for producing and using the polypeptides.

Brief Description of the Figures

Figures 1A, 1B and 1C show the cDNA sequence and the deduced amino acid sequence of a *Fusarium venenatum* lysophospholipase (SEQ ID NOS. 1 and 2, respectively).

Figure 2 shows a restriction map of pDM181.

Figure 3 shows a restriction map of pSheB1.

Figure 4 shows a restriction map of pRaMB54.

Figure 5 shows the pH activity profile of a *Fusarium venenatum* lysophospholipase.

Figures 6A, 6B and 6C show the cDNA sequence and the deduced amino acid sequence of a *Fusarium verticillioides* lysophospholipase (SEQ ID NOS. 15 and 16, respectively).

Detailed Description of the Invention

Polypeptides Having Lysophospholipase Activity

The term "lysophospholipase activity" is defined herein as a carboxylic ester hydrolysis activity which catalyzes the deacylation of one or both of the fatty acyl groups in the sn-1 and sn-2 positions of a diacylglycerophospholipid. For purposes of the present invention, lysophospholipase activity is determined by incubating the lysophospholipase with lysolecithin (or L-α-lysophosphatidylcholine) in the presence of

calcium chloride at 37°C, pH 5 or 7 for 10 minutes and measuring the release of fatty acid using any method known in the art such as the NEFA C assay kit (Wako Chemicals, Richmond, VA) according to the manufacturer's instructions. One unit of lysophospholipase activity is defined as 1.0 μmole of free fatty acid produced per minute at 37°C, ph 5.0 or pH 7.0.

In a first embodiment, the present invention relates to isolated polypeptides having an amino acid sequence which has a degree of identity to amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, of at least about 65%, preferably at least about 70%, more preferably at least about 80%, even more preferably at least about 90%, most preferably at least about 95%, and even most preferably at least about 97%, which have lysophospholipase activity (hereinafter "homologous polypeptides"). In a preferred embodiment, the homologous polypeptides have an amino acid sequence which differs by five amino acids, preferably by four amino acids, more preferably by three amino acids, even more preferably by two amino acids, and most preferably by one amino acid from amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16. For purposes of the present invention, the degree of identity between two amino acid sequences is determined by the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters were Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

Preferably, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO. 2 or an allelic variant thereof; or a fragment thereof that has lysophospholipase activity. In a more preferred embodiment, the polypeptide of the present invention comprise the amino acid sequence of SEQ ID NO. 2. In another preferred embodiment, the polypeptide of the present invention comprises amino acids 38 to 654 of SEQ ID NO. 2, which is the mature polypeptide of SEQ ID NO. 2, or an allelic variant thereof; or a fragment thereof, wherein the fragment has lysophospholipase activity. In another preferred embodiment, the polypeptide of the present invention comprises amino acids 38 to 654 of SEQ ID NO. 2. In another preferred embodiment, the polypeptide of the present invention consists of the amino acid sequence of SEQ ID NO. 2 or an allelic variant thereof; or a fragment thereof, wherein the fragment has

lysophospholipase activity. In another preferred embodiment, the polypeptide of the present invention consists of the amino acid sequence of SEQ ID NO. 2. In another preferred embodiment, the polypeptide consists of amino acids 38 to 654 of SEQ ID NO. 2 or an allelic variant thereof; or a fragment thereof, wherein the fragment has lysophospholipase activity. In another preferred embodiment, the polypeptide consists of amino acids 38 to 654 of SEQ ID NO. 2.

A fragment of SEQ ID NO. 2 is a polypeptide having one or more amino acids deleted from the amino and/or carboxyl terminus of this amino acid sequence. Preferably, a fragment contains at least 500 amino acid residues, more preferably at least 550 amino acid residues, and most preferably at least 600 amino acid residues.

Preferably, the polypeptides of the present invention comprise the amino acid sequence of SEQ ID NO. 16 or an allelic variant thereof; or a fragment thereof that has lysophospholipase activity. In a more preferred embodiment, the polypeptide of the present invention comprise the amino acid sequence of SEQ ID NO. 16. In another preferred embodiment, the polypeptide of the present invention comprises amino acids 17 to 648 of SEQ ID NO. 16, which is the mature polypeptide of SEQ ID NO. 16, or an allelic variant thereof; or a fragment thereof, wherein the fragment has lysophospholipase activity. In another preferred embodiment, the polypeptide of the present invention comprises amino acids 17 to 648 of SEQ ID NO. 16. In another preferred embodiment, the polypeptide of the present invention consists of the amino acid sequence of SEQ ID NO. 16 or an allelic variant thereof; or a fragment thereof, wherein the fragment has lysophospholipase activity. In another preferred embodiment, the polypeptide of the present invention consists of the amino acid sequence of SEQ ID NO. 16. In another preferred embodiment, the polypeptide consists of amino acids 17 to 648 of SEQ ID NO. 16 or an allelic variant thereof; or a fragment thereof, wherein the fragment has lysophospholipase activity. In another preferred embodiment, the polypeptide consists of amino acids 17 to 648 of SEQ ID NO. 16.

A fragment of SEQ ID NO. 16 is a polypeptide having one or more amino acids deleted from the amino and/or carboxyl terminus of this amino acid sequence. Preferably, a fragment contains at least 500 amino acid residues, more preferably at least 550 amino acid residues, and most preferably at least 600 amino acid residues.

An allelic variant denotes any of two or more alternative forms of a gene

occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences. An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

The amino acid sequences of the homologous polypeptides may differ from the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16, or the mature polypeptide thereof, by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In*, *The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

In a second embodiment, the present invention relates to isolated polypeptides having lysophospholipase activity which are encoded by nucleic acid sequences which hybridize under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very

high stringency conditions with a nucleic acid probe which hybridizes under the same conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii) (J. Sambrook, E.F. Fritsch, and T. Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York). The subsequence of SEQ ID NO. 1 or SEQ ID NO. 15 may be at least 100 nucleotides or preferably at least 200 nucleotides. Moreover, the subsequence may encode a polypeptide fragment which has lysophospholipase activity. The polypeptides may also be allelic variants or fragments of the polypeptides that have lysophospholipase activity.

The nucleic acid sequence of SEQ ID NO. 1 or SEQ ID NO. 15 or a subsequence thereof, as well as the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16, or a fragment thereof, may be used to design a nucleic acid probe to identify and clone DNA encoding polypeptides having lysophospholipase activity from strains of different genera or species according to methods well known in the art. In particular, such probes can be used for hybridization with the genomic or cDNA of the genus or species of interest, following standard Southern blotting procedures, in order to identify and isolate the corresponding gene therein. Such probes can be considerably shorter than the entire sequence, but should be at least 15, preferably at least 25, and more preferably at least 35 nucleotides in length. Longer probes can also be used. Both DNA and RNA probes can be used. The probes are typically labeled for detecting the corresponding gene (for example, with ^{32}P, ^{3}H, ^{35}S, biotin, or avidin). Such probes are encompassed by the present invention.

Thus, a genomic DNA or cDNA library prepared from such other organisms may be screened for DNA which hybridizes with the probes described above and which encodes a polypeptide having lysophospholipase activity. Genomic or other DNA from such other organisms may be separated by agarose or polyacrylamide gel electrophoresis, or other separation techniques. DNA from the libraries or the separated DNA may be transferred to and immobilized on nitrocellulose or other suitable carrier material. In order to identify a clone or DNA which is homologous with SEQ ID NO. 1 or SEQ ID NO. 15, or a subsequence thereof, the carrier material is used in a Southern blot. For

purposes of the present invention, hybridization indicates that the nucleic acid sequence hybridizes to a nucleic acid probe corresponding to the nucleic acid sequence shown in SEQ ID NO. 1 or SEQ ID NO. 15, its complementary strand, or a subsequence thereof, under low to very high stringency conditions. Molecules to which the nucleic acid probe hybridizes under these conditions are detected using X-ray film.

In a preferred embodiment, the nucleic acid probe is a nucleic acid sequence which encodes the polypeptide of SEQ ID NO. 2, or a subsequence thereof. In another preferred embodiment, the nucleic acid probe is SEQ ID NO. 1. In another preferred embodiment, the nucleic acid probe is nucleotides 214 to 2061 of SEQ ID NO. 1, which encodes a mature polypeptide having lysophospholipase activity. In another preferred embodiment, the nucleic acid probe is the nucleic acid sequence contained in plasmid pFB0346 which is contained in *Escherichia coli* NRRL B-30073, wherein the nucleic acid sequence encodes a polypeptide having lysophospholipase activity. In another preferred embodiment, the nucleic acid probe is the nucleic acid sequence encoding the mature polypeptide coding region contained in plasmid pFB0346 which is contained in *Escherichia coli* NRRL B-30073.

In another preferred embodiment, the nucleic acid probe is a nucleic acid sequence which encodes the polypeptide of SEQ ID NO. 16, or a subsequence thereof. In another preferred embodiment, the nucleic acid probe is SEQ ID NO. 15. In another preferred embodiment, the nucleic acid probe is nucleotides 49 to 1944 of SEQ ID NO. 15, which encodes a mature polypeptide having lysophospholipase activity. In another preferred embodiment, the nucleic acid probe is the nucleic acid sequence contained in *Fusarium verticillioides* CBS 650.96, wherein the nucleic acid sequence encodes a polypeptide having lysophospholipase activity. In another preferred embodiment, the nucleic acid probe is the nucleic acid sequence encoding the mature polypeptide coding region contained in *Fusarium verticillioides* CBS 650.96.

For long probes of at least 100 nucleotides in length, very low to very high stringency conditions are defined as prehybridization and hybridization at 42°C in 5X SSPE, 0.3% SDS, 200 µg/ml sheared and denatured salmon sperm DNA, and either 25% formamide for very low and low stringencies, 35% formamide for medium and medium-high stringencies, or 50% formamide for high and very high stringencies, following standard Southern blotting procedures.

For long probes of at least 100 nucleotides in length, the carrier material is finally washed three times each for 15 minutes using 2 x SSC, 0.2% SDS preferably at least at 45°C (very low stringency), more preferably at least at 50°C (low stringency), more preferably at least at 55°C (medium stringency), more preferably at least at 60°C (medium-high stringency), even more preferably at least at 65°C (high stringency), and most preferably at least at 70°C (very high stringency).

For short probes which are about 15 nucleotides to about 70 nucleotides in length, stringency conditions are defined as prehybridization, hybridization, and washing post-hybridization at 5°C to 10°C below the calculated T_m using the calculation according to Bolton and McCarthy (1962, *Proceedings of the National Academy of Sciences USA* 48:1390) in 0.9 M NaCl, 0.09 M Tris-HCl pH 7.6, 6 mM EDTA, 0.5% NP-40, 1X Denhardt's solution, 1 mM sodium pyrophosphate, 1 mM sodium monobasic phosphate, 0.1 mM ATP, and 0.2 mg of yeast RNA per ml following standard Southern blotting procedures.

For short probes which are about 15 nucleotides to about 70 nucleotides in length, the carrier material is washed once in 6X SCC plus 0.1% SDS for 15 minutes and twice each for 15 minutes using 6X SSC at 5°C to 10°C below the calculated T_m.

In a third embodiment, the present invention relates to variants of the polypeptide having an amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16, comprising a substitution, deletion, and/or insertion of one or more amino acids.

The amino acid sequences of the variant polypeptides may differ from the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16, or the mature polypeptide thereof, by an insertion or deletion of one or more amino acid residues and/or the substitution of one or more amino acid residues by different amino acid residues. Preferably, amino acid changes are of a minor nature, that is conservative amino acid substitutions that do not significantly affect the folding and/or activity of the protein; small deletions, typically of one to about 30 amino acids; small amino- or carboxyl-terminal extensions, such as an amino-terminal methionine residue; a small linker peptide of up to about 20-25 residues; or a small extension that facilitates purification by changing net charge or another function, such as a poly-histidine tract, an antigenic epitope or a binding domain.

Examples of conservative substitutions are within the group of basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid),

polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). Amino acid substitutions which do not generally alter the specific activity are known in the art and are described, for example, by H. Neurath and R.L. Hill, 1979, *In, The Proteins*, Academic Press, New York. The most commonly occurring exchanges are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly as well as these in reverse.

In a fourth embodiment, the present invention relates to isolated polypeptides having immunochemical identity or partial immunochemical identity to the polypeptide having the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16, or the mature polypeptide thereof. The immunochemical properties are determined by immunological cross-reaction identity tests by the well-known Ouchterlony double immunodiffusion procedure. Specifically, an antiserum containing polyclonal antibodies which are immunoreactive or bind to epitopes of the polypeptide having the amino acid sequence of SEQ ID NO. 2 or the mature polypeptide thereof are prepared by immunizing rabbits (or other rodents) according to the procedure described by Harboe and Ingild, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 23, or Johnstone and Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, 1982 (more specifically pages 27-31). A polypeptide having immunochemical identity is a polypeptide which reacts with the antiserum in an identical fashion such as total fusion of precipitates, identical precipitate morphology, and/or identical electrophoretic mobility using a specific immunochemical technique. A further explanation of immunochemical identity is described by Axelsen, Bock, and Krøll, *In* N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 10. A polypeptide having partial immunochemical identity is a polypeptide which reacts with the antiserum in a partially identical fashion such as partial fusion of precipitates, partially identical precipitate morphology, and/or partially identical electrophoretic mobility using a specific immunochemical technique. A further explanation of partial immunochemical identity is described by Bock and Axelsen, *In*

N.H. Axelsen, J. Krøll, and B. Weeks, editors, *A Manual of Quantitative Immunoelectrophoresis*, Blackwell Scientific Publications, 1973, Chapter 11.

The antibody may also be a monoclonal antibody. Monoclonal antibodies may be prepared and used, *e.g.*, according to the methods of E. Harlow and D. Lane, editors, 1988, *Antibodies, A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor, New York.

The polypeptides of the present invention have at least 20%, preferably at least 40%, more preferably at least 60%, even more preferably at least 80%, even more preferably at least 90%, and most preferably at least 100% of the lysophospholipase activity of the mature polypeptide of SEQ ID NO. 2 or SEQ ID NO. 16.

A polypeptide of the present invention may be obtained from microorganisms of any genus. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide encoded by the nucleic acid sequence is produced by the source or by a cell in which the nucleic acid sequence from the source has been inserted. In a preferred embodiment, the polypeptide is secreted extracellularly.

A polypeptide of the present invention may be a bacterial polypeptide. For example, the polypeptide may be a gram positive bacterial polypeptide such as a *Bacillus* polypeptide, *e.g.*, a *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, or *Bacillus thuringiensis* polypeptide; or a *Streptomyces* polypeptide, *e.g.*, a *Streptomyces lividans* or *Streptomyces murinus* polypeptide; or a gram negative bacterial polypeptide, *e.g.*, an *E. coli* or a *Pseudomonas* sp. polypeptide.

A polypeptide of the present invention may be a fungal polypeptide, and more preferably a yeast polypeptide such as a *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* polypeptide; or more preferably a filamentous fungal polypeptide such as an *Acremonium*, *Aspergillus*, *Aureobasidium*, *Cryptococcus*, *Filibasidium*, *Fusarium*, *Humicola*, *Magnaporthe*, *Mucor*, *Myceliophthora*, *Neocallimastix*, *Neurospora*, *Paecilomyces*, *Penicillium*, *Piromyces*, *Schizophyllum*, *Talaromyces*, *Thermoascus*, *Thielavia*, *Tolypocladium*, or *Trichoderma* polypeptide.

In a preferred embodiment, the polypeptide is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* polypeptide.

In another preferred embodiment, the polypeptide is an *Aspergillus aculeatus*, *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, *Aspergillus oryzae*, *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* polypeptide.

In another preferred embodiment, the polypeptide is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sporotrichioides*, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, or *Fusarium verticillioides* polypeptide.

In a more preferred embodiment, the *Fusarium venenatum* cell is *Fusarium venenatum* A3/5, which was originally deposited as *Fusarium graminearum* ATCC 20334 and recently reclassified as *Fusarium venenatum* by Yoder and Christianson, 1998, *Fungal Genetics and Biology* 23: 62-80 and O'Donnell *et al.*, 1998, *Fungal Genetics and Biology* 23: 57-67; as well as taxonomic equivalents of *Fusarium venenatum* regardless of the species name by which they are currently known. In another preferred embodiment, the *Fusarium venenatum* cell is a morphological mutant of *Fusarium venenatum* A3/5 or *Fusarium venenatum* ATCC 20334, as disclosed in WO 97/26330.

In a more preferred embodiment, the *Fusarium verticillioides* cell is *Fusarium verticillioides* CBS 650.96.

It will be understood that for the aforementioned species, the invention encompasses both the perfect and imperfect states, and other taxonomic equivalents, *e.g.*, anamorphs, regardless of the species name by which they are known. Those skilled in the art will readily recognize the identity of appropriate equivalents. For example,

taxonomic equivalents of *Fusarium* are defined by D.L. Hawksworth, P.M. Kirk, B.C. Sutton, and D.N. Pegler (editors), 1995, *In* Ainsworth & Bisby's *Dictionary of the Fungi*, Eighth Edition, CAB International, University Press, Cambridge, England, pp. 173-174.

Strains of these species are readily accessible to the public in a number of culture collections, such as the American Type Culture Collection (ATCC), Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSM), Centraalbureau Voor Schimmelcultures (CBS), and Agricultural Research Service Patent Culture Collection, Northern Regional Research Center (NRRL).

Furthermore, such polypeptides may be identified and obtained from other sources including microorganisms isolated from nature (*e.g.*, soil, composts, water, etc.) using the above-mentioned probes. Techniques for isolating microorganisms from natural habitats are well known in the art. The nucleic acid sequence may then be derived by similarly screening a genomic or cDNA library of another microorganism. Once a nucleic acid sequence encoding a polypeptide has been detected with the probe(s), the sequence may be isolated or cloned by utilizing techniques which are known to those of ordinary skill in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

As defined herein, an "isolated" polypeptide is a polypeptide which is essentially free of other non-lysophospholipase polypeptides, *e.g.*, at least about 20% pure, preferably at least about 40% pure, more preferably about 60% pure, even more preferably about 80% pure, most preferably about 90% pure, and even most preferably about 95% pure, as determined by SDS-PAGE.

Polypeptides encoded by nucleic acid sequences of the present invention also include fused polypeptides or cleavable fusion polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by fusing a nucleic acid sequence (or a portion thereof) encoding another polypeptide to a nucleic acid sequence (or a portion thereof) of the present invention. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and that expression of the fused polypeptide is under control of the same promoter(s) and terminator.

Nucleic Acid Sequences

The present invention also relates to isolated nucleic acid sequences which encode a polypeptide of the present invention. In a preferred embodiment, the nucleic acid sequence is set forth in SEQ ID NO. 1. In another preferred embodiment, the nucleic acid sequence is set forth in SEQ ID NO. 15. In a more preferred embodiment, the nucleic acid sequence is the sequence contained in plasmid pFB0346 that is contained in *Escherichia coli* NRRL B-30073. In another more preferred embodiment, the nucleic acid sequence is the sequence contained in *Fusarium verticilliodes* CBS 650.96. In another preferred embodiment, the nucleic acid sequence is the mature polypeptide coding region of SEQ ID NO. 1. In another preferred embodiment, the nucleic acid sequence is the mature polypeptide coding region of SEQ ID NO. 15. In another more preferred embodiment, the nucleic acid sequence is the mature polypeptide coding region contained in plasmid pFB0346 that is contained in *Escherichia coli* NRRL B-30073. In another more preferred embodiment, the nucleic acid sequence is the mature polypeptide coding region contained in *Fusarium verticilliodes* CBS 650.96. The present invention also encompasses nucleic acid sequences which encode a polypeptide having the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 15, or the mature polypeptide thereof, which differ from SEQ ID NO. 1 or SEQ ID NO. 15, respectively, by virtue of the degeneracy of the genetic code. The present invention also relates to subsequences of SEQ ID NO. 1 or SEQ ID NO. 15, which encode fragments of SEQ ID NO. 2 or SEQ ID NO. 17, respectively, that have lysophospholipase activity.

A subsequence of SEQ ID NO. 1 is a nucleic acid sequence encompassed by SEQ ID NO. 1 except that one or more nucleotides from the 5' and/or 3' end have been deleted. Preferably, a subsequence contains at least 1500 nucleotides, more preferably at least 1650 nucleotides, and most preferably at least 1800 nucleotides.

A subsequence of SEQ ID NO. 15 is a nucleic acid sequence encompassed by SEQ ID NO. 15 except that one or more nucleotides from the 5' and/or 3' end have been deleted. Preferably, a subsequence contains at least 1500 nucleotides, more preferably at least 1650 nucleotides, and most preferably at least 1800 nucleotides.

The present invention also relates to mutant nucleic acid sequences comprising at least one mutation in the mature polypeptide coding sequence of SEQ ID NO. 1, in which the mutant nucleic acid sequence encodes a polypeptide which consists of amino acids 38 to 654 of SEQ ID NO. 2.

The present invention also relates to mutant nucleic acid sequences comprising at least one mutation in the mature polypeptide coding sequence of SEQ ID NO. 15, in which the mutant nucleic acid sequence encodes a polypeptide which consists of amino acids 17 to 648 of SEQ ID NO. 16.

The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are known in the art and include isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequences of the present invention from such genomic DNA can be effected, *e.g.*, by using the well known polymerase chain reaction (PCR) or antibody screening of expression libraries to detect cloned DNA fragments with shared structural features. See, *e.g.*, Innis *et al.*, 1990, *PCR: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT) and nucleic acid sequence-based amplification (NASBA) may be used. The nucleic acid sequence may be cloned from a strain of *Fusarium*, or another or related organism and thus, for example, may be an allelic or species variant of the polypeptide encoding region of the nucleic acid sequence.

The term "isolated nucleic acid sequence" as used herein refers to a nucleic acid sequence which is essentially free of other nucleic acid sequences, *e.g.*, at least about 20% pure, preferably at least about 40% pure, more preferably at least about 60% pure, even more preferably at least about 80% pure, and most preferably at least about 90% pure as determined by agarose electrophoresis. For example, an isolated nucleic acid sequence can be obtained by standard cloning procedures used in genetic engineering to relocate the nucleic acid sequence from its natural location to a different site where it will be reproduced. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a host cell where multiple copies or clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The present invention also relates to nucleic acid sequences which have a degree of homology to the mature polypeptide coding sequence of SEQ ID NO. 1 (*i.e.*, nucleotides 214 to 2061) or SEQ ID NO. 15 (*i.e.*, nucleotides 49 to 1944) of at least

about 65%, preferably about 70%, preferably about 80%, more preferably about 90%, even more preferably about 95%, and most preferably about 97% homology, which encode an active polypeptide. For purposes of the present invention, the degree of homology between two nucleic acid sequences is determined by the Wilbur-Lipman method (Wilbur and Lipman, 1983, *Proceedings of the National Academy of Science USA* 80: 726-730) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10 and gap length penalty of 10. Pairwise alignment parameters were Ktuple=3, gap penalty=3, and windows=20.

Modification of a nucleic acid sequence encoding a polypeptide of the present invention may be necessary for the synthesis of polypeptides substantially similar to the polypeptide. The term "substantially similar" to the polypeptide refers to non-naturally occurring forms of the polypeptide. These polypeptides may differ in some engineered way from the polypeptide isolated from its native source, *e.g.*, variants that differ in specific activity, thermostability, pH optimum, or the like. The variant sequence may be constructed on the basis of the nucleic acid sequence presented as the polypeptide encoding part of SEQ ID NO. 1 or SEQ ID NO. 16, *e.g.*, a subsequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the polypeptide encoded by the nucleic acid sequence, but which correspond to the codon usage of the host organism intended for production of the enzyme, or by introduction of nucleotide substitutions which may give rise to a different amino acid sequence. For a general description of nucleotide substitution, see, *e.g.*, Ford *et al.*, 1991, *Protein Expression and Purification* 2: 95-107.

It will be apparent to those skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active polypeptide. Amino acid residues essential to the activity of the polypeptide encoded by the isolated nucleic acid sequence of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis (see, *e.g.*, Cunningham and Wells, 1989, *Science* 244: 1081-1085). In the latter technique, mutations are introduced at every positively charged residue in the molecule, and the resultant mutant molecules are tested for lysophospholipase activity to identify amino acid residues that are critical to the

activity of the molecule. Sites of substrate-enzyme interaction can also be determined by analysis of the three-dimensional structure as determined by such techniques as nuclear magnetic resonance analysis, crystallography or photoaffinity labelling (see, *e.g.*, de Vos *et al.*, 1992, *Science* 255: 306-312; Smith *et al.*, 1992, *Journal of Molecular Biology* 224: 899-904; Wlodaver *et al.*, 1992, *FEBS Letters* 309: 59-64).

The present invention also relates to isolated nucleic acid sequences encoding a polypeptide of the present invention, which hybridize under very low stringency conditions, preferably low stringency conditions, more preferably medium stringency conditions, more preferably medium-high stringency conditions, even more preferably high stringency conditions, and most preferably very high stringency conditions with a nucleic acid probe which hybridizes under the same conditions with the nucleic acid sequence of SEQ ID NO. 1 or SEQ ID NO. 16, or its complementary strand; or allelic variants and subsequences thereof (Sambrook *et al.*, 1989, *supra*), as defined herein.

The present invention also relates to isolated nucleic acid sequences produced by (a) hybridizing a DNA under very low, low, medium, medium-high, high, or very high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii), or (iv) a complementary strand of (i), (ii), or (iii); and (b) isolating the nucleic acid sequence. The subsequence is preferably a sequence of at least 100 nucleotides such as a sequence which encodes a polypeptide fragment which has lysophospholipase activity.

Methods for Producing Mutant Nucleic Acid Sequences

The present invention further relates to methods for producing a mutant nucleic acid sequence, comprising introducing at least one mutation into the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, or a subsequence thereof, wherein the mutant nucleic acid sequence encodes a polypeptide which consists of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, or a fragment thereof which has lysophospholipase activity.

The introduction of a mutation into the nucleic acid sequence to exchange one nucleotide for another nucleotide may be accomplished by site-directed mutagenesis

using any of the methods known in the art. Particularly useful is the procedure which utilizes a supercoiled, double stranded DNA vector with an insert of interest and two synthetic primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, extend during temperature cycling by means of *Pfu* DNA polymerase. On incorporation of the primers, a mutated plasmid containing staggered nicks is generated. Following temperature cycling, the product is treated with *Dpn*I which is specific for methylated and hemimethylated DNA to digest the parental DNA template and to select for mutation-containing synthesized DNA. Other procedures known in the art may also be used.

Nucleic Acid Constructs

The present invention also relates to nucleic acid constructs comprising a nucleic acid sequence of the present invention operably linked to one or more control sequences which direct the expression of the coding sequence in a suitable host cell under conditions compatible with the control sequences. Expression will be understood to include any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion.

"Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct is synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence of the present invention. The term "coding sequence" is defined herein as a portion of a nucleic acid sequence which directly specifies the amino acid sequence of its protein product. The boundaries of the coding sequence are generally determined by a ribosome binding site (prokaryotes) or by the ATG start codon (eukaryotes) located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

An isolated nucleic acid sequence encoding a polypeptide of the present invention may be manipulated in a variety of ways to provide for expression of the polypeptide. Manipulation of the nucleic acid sequence prior to its insertion into a vector may be desirable or necessary depending on the expression vector. The techniques for modifying nucleic acid sequences utilizing recombinant DNA methods are well known in the art.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of a polypeptide of the present invention. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader, polyadenylation sequence, propeptide sequence, promoter, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter, and transcriptional and translational stop signals. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide. The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the coding sequence of the DNA sequence such that the control sequence directs the expression of a polypeptide.

The control sequence may be an appropriate promoter sequence, a nucleic acid sequence which is recognized by a host cell for expression of the nucleic acid sequence. The promoter sequence contains transcriptional control sequences which mediate the expression of the polypeptide. The promoter may be any nucleic acid sequence which shows transcriptional activity in the host cell of choice including mutant, truncated, and hybrid promoters, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the host cell.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention, especially in a bacterial host cell, are the promoters obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis xylA* and *xylB* genes, and prokaryotic beta-lactamase gene

(Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80: 21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242: 74-94; and in Sambrook *et al.*, 1989, *supra*.

Examples of suitable promoters for directing the transcription of the nucleic acid constructs of the present invention in a filamentous fungal host cell are promoters obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Rhizomucor miehei* aspartic proteinase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* or *Aspergillus awamori* glucoamylase (*glaA*), *Rhizomucor miehei* lipase, *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* triose phosphate isomerase, *Aspergillus nidulans* acetamidase, and *Fusarium oxysporum* trypsin-like protease (WO 96/00787), as well as the NA2-tpi promoter (a hybrid of the promoters from the genes for *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), and mutant, truncated, and hybrid promoters thereof.

In a yeast host, useful promoters are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* galactokinase (GAL1), *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP), and *Saccharomyces cerevisiae* 3-phosphoglycerate kinase. Other useful promoters for yeast host cells are described by Romanos *et al.*, 1992, *Yeast* 8: 423-488.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a host cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide. Any terminator which is functional in the host cell of choice may be used in the present invention.

Preferred terminators for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Aspergillus niger* alpha-glucosidase, and *Fusarium oxysporum* trypsin-like protease.

Preferred terminators for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase, *Saccharomyces cerevisiae* cytochrome C (CYC1),

and *Saccharomyces cerevisiae* glyceraldehyde-3-phosphate dehydrogenase. Other useful terminators for yeast host cells are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA which is important for translation by the host cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence that is functional in the host cell of choice may be used in the present invention.

Preferred leaders for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase and *Aspergillus nidulans* triose phosphate isomerase.

Suitable leaders for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* enolase (ENO-1), *Saccharomyces cerevisiae* 3-phosphoglycerate kinase, *Saccharomyces cerevisiae* alpha-factor, and *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase (ADH2/GAP).

The control sequence may also be a polyadenylation sequence, a sequence operably linked to the 3' terminus of the nucleic acid sequence and which, when transcribed, is recognized by the host cell as a signal to add polyadenosine residues to transcribed mRNA. Any polyadenylation sequence which is functional in the host cell of choice may be used in the present invention.

Preferred polyadenylation sequences for filamentous fungal host cells are obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* glucoamylase, *Aspergillus nidulans* anthranilate synthase, *Fusarium oxysporum* trypsin-like protease, and *Aspergillus niger* alpha-glucosidase.

Useful polyadenylation sequences for yeast host cells are described by Guo and Sherman, 1995, *Molecular Cellular Biology* 15: 5983-5990.

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of a polypeptide and directs the encoded polypeptide into the cell's secretory pathway. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to the coding sequence. The

foreign signal peptide coding region may be required where the coding sequence does not naturally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to enhance secretion of the polypeptide. However, any signal peptide coding region which directs the expressed polypeptide into the secretory pathway of a host cell of choice may be used in the present invention.

Effective signal peptide coding regions for bacterial host cells are the signal peptide coding regions obtained from the genes for *Bacillus* NCIB 11837 maltogenic amylase, *Bacillus stearothermophilus* alpha-amylase, *Bacillus licheniformis* subtilisin, *Bacillus licheniformis* beta-lactamase, *Bacillus stearothermophilus* neutral proteases (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis prsA*. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

Effective signal peptide coding regions for filamentous fungal host cells are the signal peptide coding regions obtained from the genes for *Aspergillus oryzae* TAKA amylase, *Aspergillus niger* neutral amylase, *Aspergillus niger* glucoamylase, *Rhizomucor miehei* aspartic proteinase, *Humicola insolens* cellulase, and *Humicola lanuginosa* lipase.

In a preferred embodiment, the signal peptide coding region is nucleotides 100 to 150 of SEQ ID NO. 1 which encodes amino acids 1 to 17 of SEQ ID NO. 2.

In a preferred embodiment, the signal peptide coding region is nucleotides 1 to 48 of SEQ ID NO. 15 which encodes amino acids 1 to 16 of SEQ ID NO. 16.

Useful signal peptides for yeast host cells are obtained from the genes for *Saccharomyces cerevisiae* alpha-factor and *Saccharomyces cerevisiae* invertase. Other useful signal peptide coding regions are described by Romanos *et al.*, 1992, *supra*.

The control sequence may also be a propeptide coding region that codes for an amino acid sequence positioned at the amino terminus of a polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to a mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide. The propeptide coding region may be obtained from the genes for *Bacillus subtilis* alkaline protease (*aprE*), *Bacillus subtilis* neutral protease (*nprT*), *Saccharomyces cerevisiae* alpha-factor, *Rhizomucor miehei* aspartic proteinase, and *Myceliophthora thermophila* laccase (WO 95/33836).

In a preferred embodiment, the propeptide coding region is nucleotides 151 to 213 of SEQ ID NO. 1 which encodes amino acids 18 to 37 of SEQ ID NO. 2.

Where both signal peptide and propeptide regions are present at the amino terminus of a polypeptide, the propeptide region is positioned next to the amino terminus of a polypeptide and the signal peptide region is positioned next to the amino terminus of the propeptide region.

It may also be desirable to add regulatory sequences which allow the regulation of the expression of the polypeptide relative to the growth of the host cell. Examples of regulatory systems are those which cause the expression of the gene to be turned on or off in response to a chemical or physical stimulus, including the presence of a regulatory compound. Regulatory systems in prokaryotic systems include the *lac*, *tac*, and *trp* operator systems. In yeast, the ADH2 system or GAL1 system may be used. In filamentous fungi, the TAKA alpha-amylase promoter, *Aspergillus niger* glucoamylase promoter, and *Aspergillus oryzae* glucoamylase promoter may be used as regulatory sequences. Other examples of regulatory sequences are those which allow for gene amplification. In eukaryotic systems, these include the dihydrofolate reductase gene which is amplified in the presence of methotrexate, and the metallothionein genes which are amplified with heavy metals. In these cases, the nucleic acid sequence encoding the polypeptide would be operably linked with the regulatory sequence.

The present invention also relates to nucleic acid constructs for altering the expression of an endogenous gene encoding a polypeptide of the present invention. The constructs may contain the minimal number of components necessary for altering expression of the endogenous gene. In one embodiment, the nucleic acid constructs preferably contain (a) a targeting sequence, (b) a regulatory sequence, (c) an exon, and (d) a splice-donor site. Upon introduction of the nucleic acid construct into a cell, the construct inserts by homologous recombination into the cellular genome at the endogenous gene site. The targeting sequence directs the integration of elements (a)-(d) into the endogenous gene such that elements (b)-(d) are operably linked to the endogenous gene. In another embodiment, the nucleic acid constructs contain (a) a targeting sequence, (b) a regulatory sequence, (c) an exon, (d) a splice-donor site, (e) an intron, and (f) a splice-acceptor site, wherein the targeting sequence directs the integration of elements (a)-(f) such that elements (b)-(f) are operably linked to the

endogenous gene. However, the constructs may contain additional components such as a selectable marker.

In both embodiments, the introduction of these components results in production of a new transcription unit in which expression of the endogenous gene is altered. In essence, the new transcription unit is a fusion product of the sequences introduced by the targeting constructs and the endogenous gene. In one embodiment in which the endogenous gene is altered, the gene is activated. In this embodiment, homologous recombination is used to replace, disrupt, or disable the regulatory region normally associated with the endogenous gene of a parent cell through the insertion of a regulatory sequence which causes the gene to be expressed at higher levels than evident in the corresponding parent cell. The activated gene can be further amplified by the inclusion of an amplifiable selectable marker gene in the construct using methods well known in the art (see, for example, U.S. Patent No. 5,641,670). In another embodiment in which the endogenous gene is altered, expression of the gene is reduced.

The targeting sequence can be within the endogenous gene, immediately adjacent to the gene, within an upstream gene, or upstream of and at a distance from the endogenous gene. One or more targeting sequences can be used. For example, a circular plasmid or DNA fragment preferably employs a single targeting sequence, while a linear plasmid or DNA fragment preferably employs two targeting sequences.

The regulatory sequence of the construct can be comprised of one or more promoters, enhancers, scaffold-attachment regions or matrix attachment sites, negative regulatory elements, transcription binding sites, or combinations of these sequences.

The constructs further contain one or more exons of the endogenous gene. An exon is defined as a DNA sequence which is copied into RNA and is present in a mature mRNA molecule such that the exon sequence is in-frame with the coding region of the endogenous gene. The exons can, optionally, contain DNA which encodes one or more amino acids and/or partially encodes an amino acid. Alternatively, the exon contains DNA which corresponds to a 5' non-encoding region. Where the exogenous exon or exons encode one or more amino acids and/or a portion of an amino acid, the nucleic acid construct is designed such that, upon transcription and splicing, the reading frame is in-frame with the coding region of the endogenous gene so that the appropriate reading frame of the portion of the mRNA derived from the second exon is unchanged.

The splice-donor site of the constructs directs the splicing of one exon to another exon. Typically, the first exon lies 5' of the second exon, and the splice-donor site overlapping and flanking the first exon on its 3' side recognizes a splice-acceptor site flanking the second exon on the 5' side of the second exon. A splice-acceptor site, like a splice-donor site, is a sequence which directs the splicing of one exon to another exon. Acting in conjunction with a splice-donor site, the splicing apparatus uses a splice-acceptor site to effect the removal of an intron.

Expression Vectors

The present invention also relates to recombinant expression vectors comprising a nucleic acid sequence of the present invention, a promoter, and transcriptional and translational stop signals. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence of the present invention may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with the appropriate control sequences for expression.

The recombinant expression vector may be any vector (*e.g.*, a plasmid or virus) which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence. The choice of the vector will typically depend on the compatibility of the vector with the host cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids.

The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the host cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. Furthermore, a single vector or plasmid or two or more vectors or

plasmids which together contain the total DNA to be introduced into the genome of the host cell, or a transposon may be used.

The vectors of the present invention preferably contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such as ampicillin, kanamycin, chloramphenicol or tetracycline resistance. Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3. Selectable markers for use in a filamentous fungal host cell include, but are not limited to, *amdS* (acetamidase), *argB* (ornithine carbamoyltransferase), *bar* (phosphinothricin acetyltransferase), *hph* (hygromycin phosphotransferase), *niaD* (nitrate reductase), *pyrG* (orotidine-5'-phosphate decarboxylase), *sC* (sulfate adenyltransferase), and *trpC* (anthranilate synthase), as well as equivalents thereof. Preferred for use in an *Aspergillus* cell are the *amdS* and *pyrG* genes of *Aspergillus nidulans* or *Aspergillus oryzae* and the *bar* gene of *Streptomyces hygroscopicus*.

The vectors of the present invention preferably contain an element(s) that permits stable integration of the vector into the host cell genome or autonomous replication of the vector in the cell independent of the genome of the cell.

For integration into the host cell genome, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous or nonhomologous recombination. Alternatively, the vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the host cell. The additional nucleic acid sequences enable the vector to be integrated into the host cell genome at a precise location(s) in the chromosome(s). To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 10,000 base pairs, preferably 400 to 10,000 base pairs, and most preferably 800 to 10,000 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the host cell. Furthermore, the

integrational elements may be non-encoding or encoding nucleic acid sequences. On the other hand, the vector may be integrated into the genome of the host cell by non-homologous recombination.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the host cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAMß1 permitting replication in *Bacillus*. Examples of origins of replication for use in a yeast host cell are the 2 micron origin of replication, ARS1, ARS4, the combination of ARS1 and CEN3, and the combination of ARS4 and CEN6. The origin of replication may be one having a mutation which makes its functioning temperature-sensitive in the host cell (see, *e.g.*, Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1433).

More than one copy of a nucleic acid sequence of the present invention may be inserted into the host cell to increase production of the gene product. An increase in the copy number of the nucleic acid sequence can be obtained by integrating at least one additional copy of the sequence into the host cell genome or by including an amplifiable selectable marker gene with the nucleic acid sequence where cells containing amplified copies of the selectable marker gene, and thereby additional copies of the nucleic acid sequence, can be selected for by cultivating the cells in the presence of the appropriate selectable agent.

The procedures used to ligate the elements described above to construct the recombinant expression vectors of the present invention are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

Host Cells

The present invention also relates to recombinant host cells, comprising a nucleic acid sequence of the invention, which are advantageously used in the recombinant production of the polypeptides. A vector comprising a nucleic acid sequence of the present invention is introduced into a host cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extra-chromosomal vector as described earlier. The term "host cell" encompasses any progeny of a parent cell that is not

identical to the parent cell due to mutations that occur during replication. The choice of a host cell will to a large extent depend upon the gene encoding the polypeptide and its source.

The host cell may be a unicellular microorganism, *e.g.*, a prokaryote, or a non-unicellular microorganism, *e.g.*, a eukaryote.

Useful unicellular cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus clausii*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* and *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial host cell is a *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, or *Bacillus subtilis* cell. In another preferred embodiment, the *Bacillus* cell is an alkalophilic *Bacillus*.

The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278).

The host cell may be a eukaryote, such as a mammalian, insect, plant, or fungal cell.

In a preferred embodiment, the host cell is a fungal cell. "Fungi" as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, *In, Ainsworth and Bisby's Dictionary of The Fungi*, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*).

In a more preferred embodiment, the fungal host cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast,

and yeast belonging to the Fungi Imperfecti (Blastomycetes). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in *Biology and Activities of Yeast* (Skinner, F.A., Passmore, S.M., and Davenport, R.R., eds, *Soc. App. Bacteriol. Symposium Series* No. 9, 1980).

In an even more preferred embodiment, the yeast host cell is a *Candida*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia* cell.

In a most preferred embodiment, the yeast host cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis* or *Saccharomyces oviformis* cell. In another most preferred embodiment, the yeast host cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast host cell is a *Yarrowia lipolytica* cell.

In another more preferred embodiment, the fungal host cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are generally characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative.

In an even more preferred embodiment, the filamentous fungal host cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Thielavia*, *Tolypocladium*, or *Trichoderma*.

In a most preferred embodiment, the filamentous fungal host cell is an *Aspergillus awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger* or *Aspergillus oryzae* cell. In another most preferred embodiment, the filamentous fungal host cell is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium*

sporotrichioides, *Fusarium sulphureum*, *Fusarium torulosum*, *Fusarium trichothecioides*, *Fusarium venenatum*, or *Fusarium verticillioides* cell. In an even most preferred embodiment, the filamentous fungal parent cell is a *Fusarium venenatum* (Nirenberg sp. nov.) cell. In another most preferred embodiment, the filamentous fungal host cell is a *Humicola insolens*, *Humicola lanuginosa*, *Mucor miehei*, *Myceliophthora thermophila*, *Neurospora crassa*, *Penicillium purpurogenum*, *Thielavia terrestris*, *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Fungal cells may be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known *per se*. Suitable procedures for transformation of *Aspergillus* host cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474. Suitable methods for transforming *Fusarium* species are described by Malardier *et al.*, 1989, *Gene* 78: 147-156, and WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, *In* Abelson, J.N. and Simon, M.I., editors, *Guide to Yeast Genetics and Molecular Biology*, *Methods in Enzymology*, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

Methods of Production

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a strain, which in its wild-type form is capable of producing the polypeptide, to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide. Preferably, the strain is of the genus *Fusarium*, and more preferably *Fusarium venenatum* or *Fusarium verticillioides*.

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide, wherein the host cell comprises a mutant nucleic acid

sequence having at least one mutation in the mature polypeptide coding region of SEQ ID NO. 1 or SEQ ID NO. 15, wherein the mutant nucleic acid sequence encodes a polypeptide which consists of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, respectively, and (b) recovering the polypeptide.

The present invention further relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a homologously recombinant cell, having incorporated therein a new transcription unit comprising a regulatory sequence, an exon, and/or a splice donor site operably linked to a second exon of an endogenous nucleic acid sequence encoding the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. The methods are based on the use of gene activation technology, for example, as described in U.S. Patent No. 5,641,670.

In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, and small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, or disappearance of an enzyme substrate. For example, an enzyme assay may be used to determine the activity of the polypeptide as described herein.

The resulting polypeptide may be recovered by methods known in the art. For example, the polypeptide may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-

drying, evaporation, or precipitation.

The polypeptides of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), SDS-PAGE, or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

Plants

The present invention also relates to a transgenic plant, plant part, or plant cell which has been transformed with a nucleic acid sequence encoding a polypeptide having lysophospholipase activity of the present invention so as to express and produce the polypeptide in recoverable quantities. The polypeptide may be recovered from the plant or plant part. Alternatively, the plant or plant part containing the recombinant polypeptide may be used as such for improving the quality of a food or feed, *e.g.*, improving nutritional value, palatability, and rheological properties, or to destroy an antinutritive factor.

The transgenic plant can be dicotyledonous (a dicot) or monocotyledonous (a monocot). Examples of monocot plants are grasses, such as meadow grass (blue grass, Poa), forage grass such as festuca, lolium, temperate grass, such as Agrostis, and cereals, *e.g.*, wheat, oats, rye, barley, rice, sorghum, and maize (corn).

Examples of dicot plants are tobacco, legumes, such as lupins, potato, sugar beet, pea, bean and soybean, and cruciferous plants (family Brassicaceae), such as cauliflower, rape seed, and the closely related model organism *Arabidopsis thaliana*.

Examples of plant parts are stem, callus, leaves, root, fruits, seeds, and tubers. Also specific plant tissues, such as chloroplast, apoplast, mitochondria, vacuole, peroxisomes, and cytoplasm are considered to be a plant part. Furthermore, any plant cell, whatever the tissue origin, is considered to be a plant part.

Also included within the scope of the present invention are the progeny of such plants, plant parts and plant cells.

The transgenic plant or plant cell expressing a polypeptide of the present invention may be constructed in accordance with methods known in the art. Briefly, the

plant or plant cell is constructed by incorporating one or more expression constructs encoding a polypeptide of the present invention into the plant host genome and propagating the resulting modified plant or plant cell into a transgenic plant or plant cell.

Conveniently, the expression construct is a nucleic acid construct which comprises a nucleic acid sequence encoding a polypeptide of the present invention operably linked with appropriate regulatory sequences required for expression of the nucleic acid sequence in the plant or plant part of choice. Furthermore, the expression construct may comprise a selectable marker useful for identifying host cells into which the expression construct has been integrated and DNA sequences necessary for introduction of the construct into the plant in question (the latter depends on the DNA introduction method to be used).

The choice of regulatory sequences, such as promoter and terminator sequences and optionally signal or transit sequences is determined, for example, on the basis of when, where, and how the polypeptide is desired to be expressed. For instance, the expression of the gene encoding a polypeptide of the present invention may be constitutive or inducible, or may be developmental, stage or tissue specific, and the gene product may be targeted to a specific tissue or plant part such as seeds or leaves. Regulatory sequences are, for example, described by Tague *et al.*, 1988, *Plant Physiology* 86: 506.

For constitutive expression, the 35S-CaMV promoter may be used (Franck *et al.*, 1980, *Cell* 21: 285-294). Organ-specific promoters may be, for example, a promoter from storage sink tissues such as seeds, potato tubers, and fruits (Edwards & Coruzzi, 1990, *Ann. Rev. Genet.* 24: 275-303), or from metabolic sink tissues such as meristems (Ito *et al.*, 1994, *Plant Mol. Biol.* 24: 863-878), a seed specific promoter such as the glutelin, prolamin, globulin, or albumin promoter from rice (Wu *et al.*, 1998, *Plant and Cell Physiology* 39: 885-889), a *Vicia faba* promoter from the legumin B4 and the unknown seed protein gene from *Vicia faba* (Conrad *et al.*, 1998, *Journal of Plant Physiology* 152: 708-711), a promoter from a seed oil body protein (Chen *et al.*, 1998, Plant and Cell Physiology 39: 935-941), the storage protein napA promoter from *Brassica napus*, or any other seed specific promoter known in the art, *e.g.*, as described in WO 91/14772. Furthermore, the promoter may be a leaf specific promoter such as the *rbcs* promoter from rice or tomato (Kyozuka *et al.*, 1993, *Plant Physiology* 102: 991-

1000, the chlorella virus adenine methyltransferase gene promoter (Mitra and Higgins, 1994, *Plant Molecular Biology* 26: 85-93), or the *aldP* gene promoter from rice (Kagaya *et al.*, 1995, *Molecular and General Genetics* 248: 668-674), or a wound inducible promoter such as the potato pin2 promoter (Xu *et al.*, 1993, *Plant Molecular Biology* 22: 573-588).

A promoter enhancer element may also be used to achieve higher expression of the enzyme in the plant. For instance, the promoter enhancer element may be an intron which is placed between the promoter and the nucleotide sequence encoding a polypeptide of the present invention. For instance, Xu *et al.*, 1993, *supra* disclose the use of the first intron of the rice actin 1 gene to enhance expression.

The selectable marker gene and any other parts of the expression construct may be chosen from those available in the art.

The nucleic acid construct is incorporated into the plant genome according to conventional techniques known in the art, including *Agrobacterium*-mediated transformation, virus-mediated transformation, microinjection, particle bombardment, biolistic transformation, and electroporation (Gasser *et al.*, 1990, *Science* 244: 1293; Potrykus, 1990, *Bio/Technology* 8: 535; Shimamoto *et al.*, 1989, *Nature* 338: 274).

Presently, *Agrobacterium tumefaciens*-mediated gene transfer is the method of choice for generating transgenic dicots (for a review, see Hooykas and Schilperoort, 1992, *Plant Molecular Biology* 19: 15-38). However it can also be used for transforming monocots, although other transformation methods are generally preferred for these plants. Presently, the method of choice for generating transgenic monocots is particle bombardment (microscopic gold or tungsten particles coated with the transforming DNA) of embryonic calli or developing embryos (Christou, 1992, *Plant Journal* 2: 275-281; Shimamoto, 1994, *Current Opinion Biotechnology* 5: 158-162; Vasil *et al.*, 1992, *Bio/Technology* 10: 667-674). An alternative method for transformation of monocots is based on protoplast transformation as described by Omirulleh *et al.*, 1993, *Plant Molecular Biology* 21: 415-428.

Following transformation, the transformants having incorporated therein the expression construct are selected and regenerated into whole plants according to methods well-known in the art.

The present invention also relates to methods for producing a polypeptide of the present invention comprising (a) cultivating a transgenic plant or a plant cell comprising a nucleic acid sequence encoding a polypeptide having lysophospholipase activity of the present invention under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

Removal or Reduction of Lysophospholipase Activity

The present invention also relates to methods for producing a mutant cell of a parent cell, which comprises disrupting or deleting a nucleic acid sequence encoding the polypeptide or a control sequence thereof, which results in the mutant cell producing less of the polypeptide than the parent cell when cultivated under the same conditions.

The construction of strains which have reduced lysophospholipase activity may be conveniently accomplished by modification or inactivation of a nucleic acid sequence necessary for expression of the polypeptide having lysophospholipase activity in the cell. The nucleic acid sequence to be modified or inactivated may be, for example, a nucleic acid sequence encoding the polypeptide or a part thereof essential for exhibiting lysophospholipase activity, or the nucleic acid sequence may have a regulatory function required for the expression of the polypeptide from the coding sequence of the nucleic acid sequence. An example of such a regulatory or control sequence may be a promoter sequence or a functional part thereof, *i.e.*, a part which is sufficient for affecting expression of the polypeptide. Other control sequences for possible modification are described above.

Modification or inactivation of the nucleic acid sequence may be performed by subjecting the cell to mutagenesis and selecting or screening for cells in which the lysophospholipase producing capability has been reduced. The mutagenesis, which may be specific or random, may be performed, for example, by use of a suitable physical or chemical mutagenizing agent, by use of a suitable oligonucleotide, or by subjecting the DNA sequence to PCR generated mutagenesis. Furthermore, the mutagenesis may be performed by use of any combination of these mutagenizing agents.

Examples of a physical or chemical mutagenizing agent suitable for the present purpose include ultraviolet (UV) irradiation, hydroxylamine, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), O-methyl hydroxylamine, nitrous acid, ethyl methane

sulphonate (EMS), sodium bisulphite, formic acid, and nucleotide analogues.

When such agents are used, the mutagenesis is typically performed by incubating the cell to be mutagenized in the presence of the mutagenizing agent of choice under suitable conditions, and selecting for cells exhibiting reduced lysophospholipase activity or production.

Modification or inactivation of production of a polypeptide of the present invention may be accomplished by introduction, substitution, or removal of one or more nucleotides in the nucleic acid sequence encoding the polypeptide or a regulatory element required for the transcription or translation thereof. For example, nucleotides may be inserted or removed so as to result in the introduction of a stop codon, the removal of the start codon, or a change of the open reading frame. Such modification or inactivation may be accomplished by site-directed mutagenesis or PCR generated mutagenesis in accordance with methods known in the art. Although, in principle, the modification may be performed *in vivo*, *i.e.*, directly on the cell expressing the nucleic acid sequence to be modified, it is preferred that the modification be performed *in vitro* as exemplified below.

An example of a convenient way to eliminate or reduce production by a host cell of choice is by gene replacement or gene interruption. In the gene interruption method, a nucleic acid sequence corresponding to the endogenous gene or gene fragment of interest is mutagenized *in vitro* to produce a defective nucleic acid sequence which is then transformed into the host cell to produce a defective gene. By homologous recombination, the defective nucleic acid sequence replaces the endogenous gene or gene fragment. It may be desirable that the defective gene or gene fragment also encodes a marker which may be used for selection of transformants in which the gene encoding the polypeptide has been modified or destroyed.

Alternatively, modification or inactivation of the nucleic acid sequence may be performed by established anti-sense techniques using a nucleotide sequence complementary to the polypeptide encoding sequence. More specifically, production of the polypeptide by a cell may be reduced or eliminated by introducing a nucleotide sequence complementary to the nucleic acid sequence encoding the polypeptide which may be transcribed in the cell and is capable of hybridizing to the polypeptide mRNA produced in the cell. Under conditions allowing the complementary anti-sense nucleotide sequence to hybridize to the polypeptide mRNA, the amount of polypeptide

translated is thus reduced or eliminated.

It is preferred that the cell to be modified in accordance with the methods of the present invention is of microbial origin, for example, a fungal strain which is suitable for the production of desired protein products, either homologous or heterologous to the cell.

The present invention further relates to a mutant cell of a parent cell which comprises a disruption or deletion of a nucleic acid sequence encoding the polypeptide or a control sequence thereof, which results in the mutant cell producing less of the polypeptide than the parent cell.

The polypeptide-deficient mutant cells so created are particularly useful as host cells for the expression of homologous and/or heterologous polypeptides. Therefore, the present invention further relates to methods for producing a homologous or heterologous polypeptide comprising (a) cultivating the mutant cell under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide. The term "heterologous polypeptides" is defined herein as polypeptides which are not native to the host cell, a native protein in which modifications have been made to alter the native sequence, or a native protein whose expression is quantitatively altered as a result of a manipulation of the host cell by recombinant DNA techniques.

In a further aspect, the present invention relates to a method for producing a protein product essentially free of lysophospholipase activity by fermentation of a cell which produces both a polypeptide of the present invention as well as the protein product of interest by adding an effective amount of an agent capable of inhibiting lysophospholipase activity to the fermentation broth before, during, or after the fermentation has been completed, recovering the product of interest from the fermentation broth, and optionally subjecting the recovered product to further purification.

In a further aspect, the present invention relates to a method for producing a protein product essentially free of lysophospholipase activity by cultivating the cell under conditions permitting the expression of the product, subjecting the resultant culture broth to a combined pH and temperature treatment so as to reduce the lysophospholipase activity substantially, and recovering the product from the culture broth. Alternatively, the combined pH and temperature treatment may be performed on an enzyme preparation recovered from the culture broth. The combined pH and temperature treatment may

optionally be used in combination with a treatment with a lysophospholipase inhibitor.

In accordance with this aspect of the invention, it is possible to remove at least 60%, preferably at least 75%, more preferably at least 85%, still more preferably at least 95%, and most preferably at least 99% of the lysophospholipase activity. Complete removal of lysophospholipase activity may be obtained by use of this method.

The combined pH and temperature treatment is preferably carried out at a pH in the range of 6.5-8.0 and a temperature in the range of 45-70°C for a sufficient period of time to attain the desired effect, where typically, 30 to 60 minutes is sufficient.

The methods used for cultivation and purification of the product of interest may be performed by methods known in the art.

The methods of the present invention for producing an essentially lysophospholipase-free product is of particular interest in the production of eukaryotic polypeptides, in particular fungal proteins such as enzymes. The enzyme may be selected from, *e.g.*, an amylolytic enzyme, lipolytic enzyme, proteolytic enzyme, cellulytic enzyme, oxidoreductase, or plant cell-wall degrading enzyme. Examples of such enzymes include an aminopeptidase, amylase, amyloglucosidase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, galactosidase, beta-galactosidase, glucoamylase, glucose oxidase, glucosidase, haloperoxidase, hemicellulase, invertase, isomerase, laccase, ligase, lipase, lyase, mannosidase, oxidase, pectinolytic enzyme, peroxidase, phytase, phenoloxidase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transferase, transglutaminase, or xylanase. The lysophospholipase-deficient cells may also be used to express heterologous proteins of pharmaceutical interest such as hormones, growth factors, receptors, and the like.

It will be understood that the term "eukaryotic polypeptides" includes not only native polypeptides, but also those polypeptides, *e.g.*, enzymes, which have been modified by amino acid substitutions, deletions or additions, or other such modifications to enhance activity, thermostability, pH tolerance and the like.

In a further aspect, the present invention relates to a protein product essentially free from lysophospholipase activity which is produced by a method of the present invention.

Uses

The present invention is also directed to methods for using the polypeptides having lysophospholipase activity.

The polypeptides of the present invention may be used in any application where it is desired to hydrolyze the fatty acyl group(s) of a phospholipid or lysophospholipid, such as lecithin or lysolecithin. The polypeptides of the present invention are preferably used at a pH optimal for activity.

A polypeptide having lysophospholipase activity of the present invention may be used for degumming an aqueous carbohydrate solution or slurry to improve its filterability, particularly, a starch hydrolysate, especially a wheat starch hydrolysate which is difficult to filter and yields cloudy filtrates. The treatment may be performed using methods well known in the art. See, for example, EP 219,269 and EP 808,903.

A polypeptide having lysophospholipase activity of the present invention may be used in a process to reduce the phospholipid content in an edible oil by treating the oil with the polypeptide to hydrolyze a major portion of the phospholipid and separating an aqueous phase containing the hydrolyzed phospholipid from the oil. Such a process is applicable to the purification of any edible oil which contains phospholipid, *e.g.*, vegetable oil such as soybean oil, rape seed oil, and sunflower oil.

Prior to the lysophospholipase treatment, the 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 beginning of the treatment with the lysophospholipase, and the treatment may reduce the phosphorus value to below 5-10 ppm.

The lysophospholipase treatment is conducted by dispersing an aqueous solution of the lysophospholipase, preferably as droplets with an average diameter below 10 μm. 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 lysophospholipase treatment can be conducted at a pH in the range of about 1.5 to about 5.0. The process pH may be in the range of about 3.5 to about 5 to maximize the enzyme's performance, or a pH in the range of about 1.5 to about 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

hydrochloric acid.

A suitable temperature is generally 30-70°C (particularly 30-45°C, *e.g.*, 35-40°C). The reaction time will typically be 1-12 hours (*e.g.*, 2-6 hours). A suitable enzyme dosage will usually be 0.1-10 mg per liter (*e.g.*, 0.5-5 mg per liter).

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

The treatment may be performed using any of the methods known in the art. See, for example, U.S. Patent No. 5,264,367, EP 654,527, JP-A 2-153997.

A polypeptide having lysophospholipase activity of the present invention may be used in the preparation of dough, bread, or cakes. The lysophospholipase is added to the ingredients of a dough which is kneaded and baked to make the bread using methods well known in the art. See, for example, U.S. Patent No. 4,567,046, EP 426,211, JP-A 60-78529, JP-A 62-111629, and JP-A 63-258528.

The present invention also relates to methods for preparing a dough or a baked product comprising incorporating into the dough an effective amount of a polypeptide of the present invention which improves one or more properties of the dough or the baked product obtained from the dough relative to a dough or a baked product in which the polypeptide is not incorporated.

The phrase "incorporating into the dough" is defined herein as adding the polypeptide having lysophospholipase activity to the dough, any ingredient from which the dough is to be made, and/or any mixture of dough ingredients from which the dough is to be made. In other words, the lysophospholipase may be added in any step of the dough preparation and may be added in one, two, or more steps. The lysophospholipase is added to the ingredients of a dough which is kneaded and baked to make the baked product using methods well known in the art. See, for example, U.S. Patent No. 4,567,046, EP 426,211, JP-A 60-78529, JP-A 62-111629, and JP-A 63-258528.

The term "effective amount" is defined herein as an amount of the polypeptide having lysophospholipase activity that is sufficient for providing a measurable effect on

at least one property of interest of the dough and/or baked product.

The term "improved property" is defined herein as any property of a dough and/or a product obtained from the dough, particularly a baked product, which is improved by the action of the lysophospholipase activity relative to a dough or product in which the polypeptide having lysophospholipase activity is not incorporated. The improved property may include, but is not limited to, increased strength of the dough, increased elasticity of the dough, increased stability of the dough, reduced stickiness of the dough, improved extensibility of the dough, improved machinability of the dough, increased volume of the baked product, improved crumb structure of the baked product, improved softness of the baked product, improved flavor of the baked product, and/or improved antistaling of the baked product.

The improved property may be determined by comparison of a dough and/or a baked product prepared with and without addition of a polypeptide having lysophospholipase activity of the present invention in accordance with the methods of the present invention. Techniques which can be used to determine improvements achieved by use of the methods of present invention are described below in the Examples. Organoleptic qualities may be evaluated using procedures well established in the baking industry, and may include, for example, the use of a panel of trained taste-testers.

The term "increased strength of the dough" is defined herein as the property of a dough that has generally more elastic properties and/or requires more work input to mould and shape.

The term "increased elasticity of the dough" is defined herein as the property of a dough which has a higher tendency to regain its original shape after being subjected to a certain physical strain.

The term "increased stability of the dough" is defined herein as the property of a dough that is less susceptible to mechanical abuse thus better maintaining its shape and volume.

The term "reduced stickiness of the dough" is defined herein as the property of a dough that has less tendency to adhere to surfaces, *e.g.*, in the dough production machinery, and is either evaluated empirically by the skilled test baker or measured by the use of a texture analyzer (*e.g.*, TAXT2) as known in the art.

The term "improved extensibility of the dough" is defined herein as the property

of a dough that can be subjected to increased strain or stretching without rupture.

The term "improved machinability of the dough" is defined herein as the property of a dough that is generally less sticky and/or more firm and/or more elastic.

The term "increased volume of the baked product" is measured as the specific volume of a given loaf of bread (volume/weight) determined typically by the traditional rape seed displacement method.

The term "improved crumb structure of the baked product" is defined herein as the property of a baked product with finer and/or thinner cell walls in the crumb and/or more uniform/homogenous distribution of cells in the crumb and is usually evaluated empirically by the skilled test baker.

The term "improved softness of the baked product" is the opposite of "firmness" and is defined herein as the property of a baked product that is more easily compressed and is evaluated either empirically by the skilled test baker or measured by the use of a texture analyzer (*e.g.*, TAXT2) as known in the art.

The term "improved flavor of the baked product" is evaluated by a trained test panel.

The term "improved antistaling of the baked product" is defined herein as the properties of a baked product that have a reduced rate of deterioration of quality parameters, *e.g.*, softness and/or elasticity, during storage.

The term "dough" is defined herein as a mixture of flour and other ingredients firm enough to knead or roll. The dough may be fresh, frozen, pre-bared, or pre-baked. The preparation of frozen dough is described by Kulp and Lorenz in *Frozen and Refrigerated Doughs and Batters.*

The term "baked product" is defined herein as any product prepared from a dough, either of a soft or a crisp character. Examples of baked products, whether of a white, light or dark type, which may be advantageously produced by the present invention are bread (in particular white, whole-meal or rye bread), typically in the form of loaves or rolls, French baguette-type bread, pasta, pita bread, tortillas, tacos, cakes, pancakes, biscuits, cookies, pie crusts, steamed bread, and crisp bread, and the like.

The polypeptide having lysophospholipase activity and/or additional enzymes to be used in the methods of the present invention may be in any form suitable for the use in question, *e.g.*, in the form of a dry powder, agglomerated powder, or granulate, in

particular a non-dusting granulate, liquid, in particular a stabilized liquid, or protected enzyme. Granulates and agglomerated powders may be prepared by conventional methods, *e.g.*, by spraying the lysophospholipase onto a carrier in a fluid-bed granulator. The carrier may consist of particulate cores having a suitable particle size. The carrier may be soluble or insoluble, *e.g.*, a salt (such as NaCl or sodium sulfate), sugar (such as sucrose or lactose), sugar alcohol (such as sorbitol), starch, rice, corn grits, or soy. The lysophospholipase and/or additional enzymes may be contained in slow-release formulations. Methods for preparing slow-release formulations are well known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding nutritionally acceptable stabilizers such as a sugar, sugar alcohol, or another polyol, and/or lactic acid or another organic acid according to established methods.

For inclusion in pre-mixes or flour it is advantageous that the polypeptide having lysophospholipase activity is in the form of a dry product, *e.g.*, a non-dusting granulate, whereas for inclusion together with a liquid it is advantageously in a liquid form.

One or more additional enzymes may also be incorporated into the dough. The additional enzyme may be of any origin, including mammalian and plant, and preferably of microbial (bacterial, yeast or fungal) origin and may be obtained by techniques conventionally used in the art.

In a preferred embodiment, the additional enzyme may be an amylase, such as an alpha-amylase (useful for providing sugars fermentable by yeast and retarding staling) or beta-amylase, cyclodextrin glucanotransferase, peptidase, in particular, an exopeptidase (useful in flavour enhancement), transglutaminase, lipase (useful for the modification of lipids present in the dough or dough constituents so as to soften the dough), phospholipase (useful for the modification of lipids present in the dough or dough constituents so as to soften the dough and improve gas retention in the dough), cellulase, hemicellulase, in particular a pentosanase such as xylanase (useful for the partial hydrolysis of pentosans which increases the extensibility of the dough), protease (useful for gluten weakening in particular when using hard wheat flour), protein disulfide isomerase, *e.g.*, a protein disulfide isomerase as disclosed in WO 95/00636, glycosyltransferase, peroxidase (useful for improving the dough consistency), laccase, or oxidase, *e.g.*, an aldose oxidase, glucose oxidase, pyranose oxidase, lipoxygenase, or L-amino acid oxidase (useful in improving dough consistency).

When one or more additional enzyme activities are to be added in accordance with the methods of the present invention, these activities may be added separately or together with the polypeptide having lysophospholipase activity, optionally as constituent(s) of the bread-improving and/or dough-improving composition. The other enzyme activities may be any of the enzymes described above and may be dosed in accordance with established baking practices.

The present invention also relates to methods for preparing a baked product, comprising baking a dough obtained by a method of the present invention to produce a baked product. The baking of the dough to produce a baked product may be performed using methods well known in the art.

The present invention also relates to doughs and baked products, respectively, produced by the methods of the present invention.

The present invention further relates to a pre-mix, *e.g.*, in the form of a flour composition, for dough and/or baked products made from dough, in which the pre-mix comprises a polypeptide having lysophospholipase activity of the present invention. The term "pre-mix" is defined herein to be understood in its conventional meaning, *i.e.*, as a mix of baking agents, generally including flour, which may be used not only in industrial bread-baking plants/facilities, but also in retail bakeries. The pre-mix may be prepared by mixing the polypeptide or a bread-improving and/or dough-improving composition of the invention comprising the polypeptide with a suitable carrier such as flour, starch, a sugar, or a salt. The pre-mix may contain other dough-improving and/or bread-improving additives, *e.g.*, any of the additives, including enzymes, mentioned above.

The present invention further relates to baking additives in the form of a granulate or agglomerated powder, which comprise a polypeptide having lysophospholipase activity of the present invention. The baking additive preferably has a narrow particle size distribution with more than 95% (by weight) of the particles in the range from 25 to 500 μm.

Signal Peptide and Propeptide

The present invention also relates to nucleic acid constructs comprising a gene encoding a protein operably linked to one or both of a first nucleic acid sequence consisting of nucleotides 100 to 150 of SEQ ID NO. 1 encoding a signal peptide

consisting of amino acids 1 to 17 of SEQ ID NO. 2 or nucleotides 1 to 48 of SEQ ID NO. 15 encoding a signal peptide consisting of amino acids 1 to 16 of SEQ ID NO. 16, and a second nucleic acid sequence consisting of nucleotides 151 to 213 of SEQ ID NO. 1 encoding a propeptide consisting of amino acids 18 to 37 of SEQ ID NO. 2, wherein the gene is foreign to the first and second nucleic acid sequences.

The present invention also relates to recombinant expression vectors and recombinant host cells comprising such nucleic acid constructs.

The present invention also relates to methods for producing a protein comprising (a) cultivating such a recombinant host cell under conditions suitable for production of the protein; and (b) recovering the protein.

The first and second nucleic acid sequences may be operably linked to foreign genes individually with other control sequences or in combination with other control sequences. Such other control sequences are described *supra*. As noted earlier, where both signal peptide and propeptide regions are present at the amino terminus of a protein, the propeptide region is positioned next to the amino terminus of a protein and the signal peptide region is positioned next to the amino terminus of the propeptide region.

The protein may be native or heterologous to a host cell. The term "protein" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The term "protein" also encompasses two or more polypeptides combined to form the encoded product. The proteins also include hybrid polypeptides which comprise a combination of partial or complete polypeptide sequences obtained from at least two different proteins wherein one or more may be heterologous or native to the host cell. Proteins further include naturally occurring allelic and engineered variations of the above mentioned proteins and hybrid proteins.

Preferably, the protein is a hormone or variant thereof, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. In a more preferred embodiment, the protein is an oxidoreductase, transferase, hydrolase, lyase, isomerase, or ligase. In an even more preferred embodiment, the protein is an aminopeptidase, amylase, carbohydrase, carboxypeptidase, catalase, cellulase, chitinase, cutinase, cyclodextrin glycosyltransferase, deoxyribonuclease, esterase, alpha-galactosidase, beta-galactosidase, glucoamylase, alpha-glucosidase, beta-glucosidase, invertase, laccase,

lipase, mannosidase, mutanase, oxidase, pectinolytic enzyme, peroxidase, phytase, polyphenoloxidase, proteolytic enzyme, ribonuclease, transglutaminase or xylanase.

The gene may be obtained from any prokaryotic, eukaryotic, or other source.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Examples

Chemicals used as buffers and substrates were commercial products of at least reagent grade.

Media and Solutions

COVE trace metals solution was composed per liter of 0.04 g of $NaB_4O_7 \cdot 10H_2O$, 0.4 g of $CuSO_4 \cdot 5H_2O$, 1.2 g of $FeSO_4 \cdot 7H_2O$, 0.7 g of $MnSO_4 \cdot H_2O$, 0.8 g of $Na_2MoO_2 \cdot 2H_20$, and 10 g of $ZnSO_4 \cdot 7H_2O$.

50X COVE salts solution was composed per liter of 26 g of KCl, 26 g of $MgSO_4 \cdot 7H_2O$, 76 g of KH_2PO_4, and 50 ml of COVE trace metals.

COVE medium was composed per liter of 342.3 g of sucrose, 20 ml of 50X COVE salt solution, 10 ml of 1 M acetamide, 10 ml of 1.5 M $CsCl_2$, and 25 g of Noble agar.

50X Vogels medium was composed per liter of 150 g of sodium citrate, 250 g of KH_2PO_4, 10 g of $MgSO_4 \cdot 7H_2O$, 10 g of $CaCl_2 \cdot 2H_2O$, 2.5 ml of biotin stock solution, and 5.0 ml of trace metals solution.

Trace metals solution was composed per liter of 14.3 g of $ZnSO_4 \cdot 7H_2O$, 2.5 g of $CuSO_4 \cdot 5H_2O$, 0.5 g of $NiCl_2$, 13.8 g of $FeSO_4$, 8.5 g of $MnSO_4$, and 3.0 g of citric acid.

COVE top agarose was composed per liter of 20 ml of 50X COVE salts, 0.8 M sucrose, 1.5 M cesium chloride, 1.0 M acetamide, and 10 g of low melt agarose, pH adjusted to 6.0.

RA sporulation medium was composed per liter of 50 g of succinic acid, 12.1 g of $NaNO_3$, 1 g of glucose, 20 ml of 50X Vogels, and 0.5 ml of a 10 mg/ml $NaMoO_4$ stock solution, pH to 6.0.

YEPG medium was composed per liter of 10 g of yeast extract, 20 g of peptone, and 20 g of glucose.

STC was composed of 0.8 M sorbitol, 25 mM Tris pH 8, 25 mM $CaCl_2$.

SPTC was composed of 40% PEG 4000, 0.8 M sorbitol, 25 mM Tris pH 8, 25 mM $CaCl_2$.

M400Da medium was composed per liter of 50 g of maltodextrin, 2 g of $MgSO_4 \cdot 7H_2O$, 2 g of KH_2PO_4, 4 g of citric acid, 8 g of yeast extract, 2 g of urea, and 1 ml of COVE trace metals solution.

10X basal salts w/o amino acid was composed per liter of 66.8 g of yeast nitrogen base w/o amino acids (Diffco), 100 g of succinic acid, and 60 g of NaOH.

SC Ura-glc medium was composed per liter of 100 ml of 20% glucose, 100 ml of 10X basal salts w/o amino acid, 25 ml of 20% (w/v) casamino acid, 4 ml of 5% of threonine, 10 ml of 1% tryptophan, and 20 g of Agar Noble.

SC Ura-gal medium was composed per liter of 100 ml of 20% galactose, 100 ml of 10X basal salts w/o amino acid , 25 ml of 20% (w/v) casamino acid, 4 ml of 5% of threonine, 10 ml of 1% tryptophan and 20 g of Noble agar.

YPD medium was composed per liter of 10 g of yeast extract, 20 g of bactopeptone, and 100 ml of 20% glucose.

1X TE/LiAc was composed per 10 ml of 1ml of 10X TE (100 mM of Tris and 10 mM of EDTA at pH), 1 ml of 1 M lithium acetate, and 8 ml of milli Q water.

PEG/LiAc solution was composed of 50 ml of 40% of PEG and 1 ml of 5 M lithium acetate.

Assay plates for detecting lysophospholipase activity were composed per liter of 5 g of L-alpha phosphatidylcholine 95%, 2.5 g of cholic acid, 50 ml of 1 M Tris-HCl pH 8.0 buffer, 100 ml of 100 mM $CaCl_2$, 15 ml of 2% Brilliant green, and 20 g of Agar Noble. The solution was poured into Falcon 1058 plates in 50 ml aliquots.

STET solution was composed of 8% of sucrose, 50 mM Tris-HCl pH 8.0, 50mM EDTA, and 5% of Triton X-100.

Example 1: Fermentation and Mycelial Tissue

Fusarium venenatum CC1-3, a morphological mutant of *Fusarium* strain ATCC 20334 (Wiebe *et al.*, 1991, *Mycol. Research* 95: 1284-1288), was grown in a two-liter

lab-scale fermentor using a fed-batch fermentation scheme with NUTRIOSE™ (Roquette Freres, S.A., Beinheim, France) as the carbon source and yeast extract. Ammonium phosphate was provided in the feed. The pH was maintained at 6 to 6.5, and the temperature was kept at 30°C with positive dissolved oxygen.

Mycelial samples were harvested at 2, 4, 6, and 8 days post-inoculum and quick-frozen in liquid nitrogen. The samples were stored at -80°C until they were disrupted for RNA extraction.

Example 2: cDNA Library Construction

Total cellular RNA was extracted from the mycelial samples described in Example 1 according to the method of Timberlake and Barnard (1981, *Cell* 26: 29-37), and the RNA samples were analyzed by Northern hybridization after blotting from 1% formaldehyde-agarose gels (Davis *et al.*, 1986, *Basic Methods in Molecular Biology*, Elsevier Science Publishing Co., Inc., New York). Polyadenylated mRNA fractions were isolated from total RNA with an mRNA Separator Kit™ (Clontech Laboratories, Inc., Palo Alto, CA) according to the manufacturer's instructions. Double-stranded cDNA was synthesized using approximately 5 µg of poly(A)+ mRNA according to the method of Gubler and Hoffman (1983, *Gene* 25: 263-269) except a *Not*I-(dT)18 primer (Pharmacia Biotech, Inc., Piscataway, NJ) was used to initiate first strand synthesis. The cDNA was treated with mung bean nuclease (Boehringer Mannheim Corporation, Indianapolis, IN) and the ends were made blunt with T4 DNA polymerase (New England Biolabs, Beverly, MA).

The cDNA was digested with *Not*I, size selected by agarose gel electrophoresis (ca. 0.7-4.5 kb), and ligated with pZErO-2.1 (Invitrogen Corporation, Carlsbad, CA) which had been cleaved with *Not*I plus *Eco*RV and dephosphorylated with calf-intestine alkaline phosphatase (Boehringer Mannheim Corporation, Indianapolis, IN). The ligation mixture was used to transform competent *E. coli* TOP10 cells (Invitrogen Corporation, Carlsbad, CA). Transformants were selected on 2YT agar plates (Miller, 1992, *A Short Course in Bacterial Genetics. A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*, Cold Spring Harbor Press, Cold Spring Harbor, New York) which contained kanamycin at a final concentration of 50 µg/ml.

Two independent directional cDNA libraries were constructed using the plasmid

cloning vector pZErO-2.1. Library A was made using mRNA from mycelia harvested at four days, and Library B was constructed with mRNA from the six day time point. Neither cDNA library was amplified in order to examine a representative "snapshot" of the gene expression profile in the cells. Instead the libraries were plated, titered, and independent clones from each was analyzed by DNA sequencing.

Library A (4 day cells) consisted about 7.5 x 10^4 independent clones and Library B (6 day cells) consisted of roughly 1.2 x 10^5 clones. Miniprep DNA was isolated from forty colonies in each library and checked for the presence and size of cDNA inserts. In this analysis 39 of 40 colonies (97.5%) from Library A contained inserts with sizes ranging from 600 bp to 2200 bp (avg. = 1050 bp). Similarly, 39 of 40 colonies (97.5%) picked from Library B had inserts with sizes ranging from 800 bp to 3600 bp (avg. = 1380 bp).

Example 3: Template Preparation and Nucleotide Sequencing

From each cDNA library described in Example 2, 1192 transformant colonies were picked directly from the transformation plates into 96-well microtiter dishes which contained 200 µl of 2YT broth (Miller, 1992, *supra)* with 50 µg/ml kanamycin. The plates were incubated overnight at 37°C without shaking. After incubation 100 µl of sterile 50% glycerol was added to each well. The transformants were replicated into secondary, deep-dish 96-well microculture plates (Advanced Genetic Technologies Corporation, Gaithersburg, MD) containing 1 ml of Magnificent Broth™ (MacConnell Research, San Diego, CA) supplemented with 50 µg of kanamycin per ml in each well. The primary microtiter plates were stored frozen at -80°C. The secondary deep-dish plates were incubated at 37°C overnight with vigorous agitation (300 rpm) on rotary shaker. To prevent spilling and cross-contamination, and to allow sufficient aeration, each secondary culture plate was covered with a polypropylene pad (Advanced Genetic Technologies Corporation, Gaithersburg, MD) and a plastic microtiter dish cover.

DNA was isolated from each well using the 96-well Miniprep Kit protocol of Advanced Genetic Technologies Corporation (Gaithersburg, MD) as modified by Utterback *et al.* (1995, *Genome Sci. Technol.* 1: 1-8). Single-pass DNA sequencing was done with a Perkin-Elmer Applied Biosystems Model 377 Sequencer XL (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) using dye-terminator chemistry (Giesecke *et*

al., 1992, *Journal of Virology Methods* 38: 47-60) and the reverse lac sequencing primer.

Example 4: Analysis of DNA Sequence Data

Nucleotide sequence data were scrutinized for quality, and samples giving improper spacing or ambiguity levels exceeding 2% were discarded or re-run. Vector sequences were trimmed manually with assistance of FACTURA™ software (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA). In addition, sequences were truncated at the end of each sample when the number of ambiguous base calls increased. All sequences were compared to each other to determine multiplicity using AutoAssembler™ software (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA). Lastly, all sequences were translated in three frames and searched against a non-redundant database (NRDB) using GeneAssist™ software (Perkin-Elmer Applied Biosystems, Inc., Foster City, CA) with a modified Smith-Waterman algorithm using the BLOSUM 62 matrix with a threshold score of 70. The NRDB was assembled from Genpept, Swiss-Prot, and PIR databases.

Example 5: Identification of lysophospholipase cDNA Clone

Putative lysophospholipase clones were identified by partial sequencing of random cDNA clones using an Applied Biosystems Model 377 XL Automated DNA Sequencer according to the manufacturer's instructions and comparison of the deduced amino acid sequence to the amino acid sequence of *Penicillium notatum* lysophospholipase (Swissprot accession number P39457) as described in Example 4. Among several clones discovered in this manner, one was presumed to be full-length on the basis of its alignment to the *Penicillium notatum* lysophospholipase amino acid sequence and the presence of a possible signal peptide, detected using the Signal-P computer program (Nielsen, *et al.*, 1997, *Protein Engineering* 10: 1-6). This clone designated *E. coli* FB0346 containing pFB0346 was selected for nucleotide sequence analysis and expression studies.

Example 6: Nucleotide sequencing and characterization of the *Fusarium venenatum* lysophospholipase cDNA

DNA sequencing was performed with an Applied Biosystems Model 377 XL

Automated DNA Sequencer using dye-terminator chemistry. Contiguous sequences were generated using a transposon insertion strategy (Primer Island Transposition Kit, Perkin-Elmer/Applied Biosystems, Inc., Foster City, CA). The lysophospholipase clone from *E. coli* FB0346 was sequenced to an average redundancy of 6.9.

The lysophospholipase clone encoded an open reading frame of 1962 bp encoding a polypeptide of 654 amino acids. The nucleotide sequence (SEQ ID NO. 1) and deduced amino acid sequence (SEQ ID NO. 2) are shown in Figures 1A, 1B, and 1C. Using the SignalP program (Nielsen *et al.*, 1997, *Protein Engineering* 10: 1-6), a signal peptide of 17 residues was predicted and N-terminal analysis of the secreted protein indicated the presence of a pro region of 20 amino acids (see Example 11), hence indicating a predicted molecular weight of approximately 67 kDa for the secreted lysophospholipase. Thus, the mature lysophospholipase is composed of 617 amino acids.

A comparative alignment of lysophospholipase sequences was undertaken using the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10, and gap length penalty of 10. Pairwise alignment parameters were Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

The comparative alignment showed that the *Fusarium venenatum* lysophospholipase shares regions of identity with lysophospholipase proteins from *Neurospora crassa* of 59% (TREMBL O42791), *Penicillium notatum* of 52% (Swissprot P39457), *Saccharomyces cerevisiae* of 44% (Swissprot P39105), and *Schizosaccharomyces pombe* of 39% (TREMBL O13857). The identities are highest between regions that are likely to be important for catalytic and/or structural roles of the enzyme. There are 19 potential N-linked glycosylation sites (Asn-X-Ser/Thr) within *Fusarium venenatum* lysophospholipase, and 11 of these are conserved in *Neurospora crassa* lysophospholipase, whereas 10 are conserved in *Penicillium notatum* lysophospholipase. The alignment also indicates the presence of eight Cys residues whose positions are strictly conserved among *Fusarium venenatum, Neurospora crassa, Penicillium notatum, Saccharomyces pombe,* and *Saccharomyces cerevisiae* lysophospholipases.

Example 7: Construction of pDM181

Plasmid pDM181 was constructed using the technique of splice overlap extension to fuse the 1.2 kb *Fusarium oxysporum* trypsin promoter (SP387) to the 1.1 kb *Fusarium oxysporum* trypsin terminator (SP387). A polylinker containing *Swa*I, *Kpn*I and *Pac*I restriction sites was inserted between the promoter and terminator as part of the overlapping PCR strategy. At the 5' end of the promoter a *Xho*I site was added and the native *EcoR*I site was preserved. At the 3' end of the terminator *EcoR*I, *Hind*III and *Nsi*I sites were incorporated by the PCR reaction.

A PCR fragment containing -1208 to -1 of the *Fusarium oxysporum* trypsin promoter plus a 25 base pair polylinker was generated from plasmid pJRoy20 (Royer *et al.*, 1995, *Biotechnology* 13: 1479-1483) using the following primers:

Primer 1 (sense):

5'-GAGCTCGAGGAATTCTTACAAACCTTCAAC-3' (SEQ ID NO. 3)

*Xho*I *Eco*RI

Primer 2 (antisense):

5'-TTAATTAAGGTACCTGAATTTAAATGGTGAAGAGATAGATATCCAAG-3' (SEQ ID NO. 4)

*Pac*I *Kpn*I *Swa*I

The 100 µl PCR reaction contained 1X *Pwo* buffer (Boehringer Mannheim, Indianapolis, IN), 200 µM each of dATP, dCTP, dGTP, and dTTP, 10 ng of pJRoy20, and 5 units of *Pwo* DNA polymerase (Boehringer Mannheim, Indianapolis, IN). PCR conditions used were 95°C for 3 minutes followed by 25 cycles each at 95°C for 30 seconds, 50°C for 1 minute, and 72°C for 1 minute. The final extension cycle was at 72°C for 5 minutes.

Using the same PCR conditions, a second PCR fragment containing bp -5 to -1 of the *Fusarium oxysporum* trypsin promoter, a 25 base pair polylinker, and 1060 base pairs of the 3' untranslated region of the *Fusarium oxysporum* trypsin gene (terminator region) was generated from plasmid pJRoy20 using the following primers:

Primer 3 (sense):

5'-TCACCATTTAAATTCAGGTACCTTAATTAAATTCCTTGTTGGAAGCGTCGA-3'

*Swa*I *Kpn*I *Pac*I (SEQ ID NO.

5)

Primer 4 (antisense):

5'-TGGTATGCATAAGCTTGAATTCAGGTAAACAAGATATAATTT-3' (SEQ ID NO. 6)

NsiI *HindIII* *EcoRI*

The final 2.3 kb overlapping PCR fragment which contained -1208 to -1 of the *Fusarium oxysporum* trypsin promoter, the 25 base pair polylinker and 1060 base pairs of the *Fusarium oxysporum* trypsin terminator was obtained using 0.2 µl of the first PCR (promoter) reaction and 3 µl of the second (terminator) reaction as templated and primers 1 and 4. The PCR conditions used were 95°C for 3 minutes followed by 30 cycles each at 95°C for 30 seconds, 62°C for 1 minute, and 72°C for 3 minutes. The final extension cycle was at 72°C for 5 minutes. *Pwo* DNA polymerase was also used for this reaction.

The resulting 2.3 kb fragment containing the trypsin promoter, the polylinker, and the trypsin terminator was digested with *EcoRI* and ligated into the *EcoRI* digested vector pMT1612 containing the *bar* gene (WO 97/26330) to create pDM181 (Figure 2).

Example 8: Construction of plasmid pSheB1

The *Fusarium venenatum* expression vector pSheB1 (Figure 3) was generated by modification of pDM181. The modifications included (a) removal of two *NcoI* sites within the pDM181 sequence, and (b) restoration of the natural translation start of the Fusarium oxysporum trypsin promoter (reconstruction of an *NcoI* site at the ATG start codon).

Removal of two *NcoI* sites within the pDM181 sequence was accomplished using the QuikChange™ site-directed mutagenesis kit (Stratagene Cloning Systems, La Jolla, CA) according to the manufacturer's instruction with the following pairs of mutagenesis primers:

5'-dCAGTGAATTGGCCTCGATGGCCGCGGCCGCGAATT-3' plus (SEQ ID NO. 7)

5'-dAATTCGCGGCCGCGGCCATCGAGGCCAATTCACTG-3' (SEQ ID NO. 8)

5'-dCACGAAGGAAAGACGATGGCTTTCACGGTGTCTG-3' plus (SEQ ID NO. 9)

5'-dCAGACACCGTGAAAGCCATCGTCTTTCCTTCGTG-3' (SEQ ID NO. 10)

Restoration of the natural translation start of the *Fusarium oxysporum* trypsin promoter was also accomplished using the Stratagene QuikChange™ site directed

mutagenesis kit in conjunction with the following pair of mutagenesis primers: 5'-dCTATCTCTTCACCATGGTACCTTAATTAAATACCTTGTTGGAAGCG-3' plus (SEQ ID NO. 11)

5'-dCGCTTCCAACAAGGTATTTAATTAAGGTACCATGGTGAAGAGATAG-3' (SEQ ID NO. 12)

All site-directed changes were confirmed by DNA sequence analysis of the appropriate vector regions.

Example 9: Construction of expression vector pRaMB54

The lysophospholipase-expression vector, pRaMB54, was constructed as shown in Figure 4. The lysophospholipase coding region was amplified from clone FB0346 using the following pair of primers: 5'-dGGCACATGTTGGGCCCTCTCGTCTTTACT-3' (forward) (SEQ ID NO. 13) and 5'-dGACTTAATTAATTTACGAAATAGCCAAGAATAAAGC-3' (reverse) (SEQ ID NO. 14). The forward primer introduces a *Bsp*LU11I restriction site at the start codon, and the reverse primer introduces a *Pac*I site after the stop codon.

The amplification reaction (100 µl) contained the following components: 0.8 µg of clone FB0346 cDNA, 40 pmol of the forward primer, 40 pmol of the reverse primer, 200 µM each of dATP, dCTP, dGTP, and dTTP, 1X *Pwo* DNA polymerase buffer, and 2.5 units of *Pwo* DNA polymerase. The reactions were incubated in a Perkin-Elmer Model 480 Thermal Cycler programmed for 30 cycles each at 95°C for 3 minutes, 58°C for 2 minutes, and 72°C for 2 minutes. The reaction products were isolated on a 1.5% agarose gel (Eastman Kodak, Rochester, NY) where a 2 kb product band was excised from the gel and purified using Qiaex II (Qiagen, Chatsworth, CA) according to the manufacturer's instructions.

The amplified lysophospholipase segment was digested with *Bsp*LU11I and *Pac*I, purified by agarose gel electrophoresis using standard methods (Sambrook *et al.*, 1989, *supra*), and ligated to the vector pSheB1 which had been previously cleaved with *Nco*I and *Pac*I (note: *Bsp*LU11I and *Nco*I generate compatible cohesive ends). The resulting expression plasmid was designated as pRaMB54.

Example 10: Expression of lysophospholipase cDNA in *Fusarium venenatum*

Spores of *Fusarium venenatum* CC1-3 (MLY-3) were generated by inoculating a flask containing 500 ml of RA sporulation medium with 10 plugs from a 1X Vogels medium plate (2.5% Noble agar) supplemented with 2.5% glucose and 2.5 mM sodium nitrate and incubating at 28°C, 150 rpm for 2 to 3 days. Spores were harvested through Miracloth (Calbiochem, San Diego, CA) and centrifuged 20 minutes at 7000 rpm in a Sorvall RC-5B centrifuge (E. I. DuPont De Nemours and Co., Wilmington, DE). Pelleted spores were washed twice with sterile distilled water, resuspended in a small volume of water, and then counted using a hemocytometer.

Protoplasts were prepared by inoculating 100 ml of YEPG medium with 4 X 10^7 spores of *Fusarium venenatum* CC1-3 and incubating for 16 hours at 24°C and 150 rpm. The culture was centrifuged for 7 minutes at 3500 rpm in a Sorvall RT 6000D (E. I. DuPont De Nemours and Co., Wilmington, DE). Pellets were washed twice with 30 ml of 1 M $MgSO_4$ and resuspended in 15 ml of 5 mg/ml of NOVOZYME 234™ (batch PPM 4356, Novo Nordisk A/S, Bagsværd, Denmark) in 1 M $MgSO_4$. Cultures were incubated at 24°C and 150 rpm until protoplasts formed. A volume of 35 ml of 2 M sorbitol was added to the protoplast digest and the mixture was centrifuged at 2500 rpm for 10 minutes. The pellet was resuspended, washed twice with STC, and centrifuged at 2000 rpm for 10 minutes to pellet the protoplasts. Protoplasts were counted with a hemocytometer and resuspended in an 8:2:0.1 solution of STC:SPTC:DMSO to a final concentration of 1.25 x 10^7 protoplasts/ml. The protoplasts were stored at -80°C, after controlled-rate freezing in a Nalgene Cryo 1°C Freezing Container (VWR Scientific, Inc., San Francisco, CA).

Frozen protoplasts of *Fusarium venenatum* CC1-3 were thawed on ice. Five µg of pRaMB54 described in Example 9 and 5 µl of heparin (5 mg per ml of STC) was added to a 50 ml sterile polypropylene tube. One hundred µl of protoplasts was added, mixed gently, and incubated on ice for 30 minutes. One ml of SPTC was added and incubated 20 minutes at room temperature. After the addition of 25 ml of 40°C COVE top agarose, the mixture was poured onto an empty 150 mm diameter plate and incubated overnight at room temperature. Then an additional 25 ml of 40°C COVE top agarose containing 10 mg of BASTA™ per ml was poured on top of the plate and incubated at room temperature for up to 14 days. The active ingredient in the herbicide BASTA™ is phosphinothricin. BASTA™ was obtained from AgrEvo (Hoechst Schering, Rodovre,

Denmark) and was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1), and once with chloroform:isoamyl alcohol (24:1) before use.

Fifteen transformants were picked directly from the selection plates (COVE underlay with COVE-BASTA™ overlay) into 125 ml shake flasks containing 25 ml of M400Da medium supplemented with 1 mM $CaCl_2$ and 100 µg/ml ampicillin (to prevent bacterial contamination) and incubated at 28°C, 200 rpm on a platform shaker for 7 days. The untransformed recipient strain was also included as a negative control.

Flasks were sampled at 5 days. Cells were removed by centrifugation, and 10 µl of each supernatant sample was heated to 95°C for 5 minutes with an equal volume of SDS-PAGE sample buffer (Novex Experimental Technology, San Diego, CA). The denatured supernatant proteins were separated on a 10-20% gradient gel (Novex Experimental Technology, San Diego, CA) and stained with Coomassie blue.

Lysophospholipase activity in culture supernatants was also measured using egg yolk lysolecithin as the substrate with a NEFA C assay kit (Wako Chemicals, Richmond, VA). Specifically, a 10 µl sample of the supernatant was added to 160 µl of 20 mM MOPS pH 7.0 buffer and 30 µl of a stock solution of 5 mg of lysolecithin per ml of 100 mM NaCl and incubated at 37°C for 20 minutes. Then 20 µl of the reaction was added to 200 µl of Reagent A (Wako Chemicals, Richmond, VA) and incubated at 37°C for 10 minutes. Finally, 400 µl of Reagent B (Wako Chemicals, Richmond, VA) was added and the solution incubated at 37°C for an additional 10 minutes. The absorbance at 550 nm was measured by transferring 200 µl of the final solution to a 96 well plate and measuring the absorbance with a SpectraMax Model 340 (Molecular Dynamics, Sunnyvale, CA) relative to a standard curve constructed with oleic acid as the standard.

The results shown in Table 1 clearly demonstrate the presence of increased lysophospholipase activity in these samples. SDS-PAGE analysis showed that the lysophospholipase-producing transformants secrete a prominent polypeptide with an apparent molecular weight of approximately 116 kDa. The discrepancy between the predicted molecular weight of mature lysophospholipase (ca. 69 kDa) versus that which was observed by SDS-PAGE suggests that the protein is heavily glycosylated. As noted previously, there are 19 potential sites for N-linked glycosylation within the deduced amino acid sequence of *Fusarium venenatum* lysophospholipase.

Table 1. Lysophospholipase activity present in culture supernatants from *Fusarium venenatum*/pRaMB54 transformants.

Transformant	Relative Lysophospholipase Activity[†]
RaMB54.01	1.00
RaMB54.02	0.86
RaMB54.03	0.88
RaMB54.04	0.88
RaMB54.05	0.96
RaMB54.06	0.08
RaMB54.10	0.85
RaMB54.11	0.88
RaMB54.12	0.88
RaMB54.14	0.35
RaMB54.15	1.00
RaMB54.16	0.88
RaMB54.17	0.88
RaMB54.18	0.81
RaMB54.19	0.85
MLY-3 control	N.D.

[†]Activity reflects the rate of hydrolysis of egg yolk lysolecithin at pH 7 and 37°C measured in micromoles of product per minute per ml relative to the activity of the highest producer RamB54.01 which is normalized to 1.00. N.D., not detected.

Example 11: Purification of recombinant *Fusarium venenatum* lysophospholipase

Fusarium venenatum/pRaMB54 was cultivated as described in Example 10 for 4 days in two 500 ml shake flasks containing 100 ml of M400Da medium. The 4 day whole culture broths were filtered through Miracloth followed by a 0.45 µm syringe filter (Whatman, Inc., Fairfield, NJ) to yield a sample volume of approximately 150 ml. Then 25 mM PMSF in 75% ethanol/25% methanol was added to the filtered broth to a final concentration of 0.5 mM. The sample was then diluted with water and 20 mM sodium phosphate pH 7 to achieve a pH of 7.45 and a conductivity of 2.3 mS.

Q-Sepharose Big Beads (Pharmacia Biotech, Inc., Piscataway, NJ) were prepared in a XK-26 column with a volume of approximately 80 ml of resin. The column had been pre-equilibrated with 500 ml of 20 mM sodium phosphate buffer pH 7.0. The sample was then loaded, followed by washing with 20 mM sodium phosphate pH 7.0 until baseline was achieved. A 600 ml gradient was run from 0 to 0.35 M NaCl at a flow rate of 5 ml/min for 120 minutes. Fractions of 12.5 ml were collected and assayed using lauroyl lysophosphatidylcholine (Sigma Chemical Co., St. Louis, MO) as substrate at a concentration of 1.2 mg/ml using the same method described in Example 10. The release of lauric acid was measured using a NEFA C kit. Active fractions were pooled, $(NH_4)_2SO_4$ was added to the pooled fractions to achieve a concentration of 2.2 M, and the pH was adjusted to 7.0 using 1 M NaOH.

The pooled fractions were then loaded onto a Phenyl Superose 16/10 column (Pharmacia Biotech, Inc., Piscataway, NJ) washed with 100 ml of 50 mM sodium phosphate pH 7.0 and pre-equilibrated with 200 ml of 2.2 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0. The column was then washed with 2.2 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0 buffer until baseline was achieved. Then a 200 ml gradient was run from 2.2 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate pH 7.0 to 50 mM sodium phosphate pH 7.0 containing no $(NH_4)_2SO_4$ at a flow rate of 2 ml/min for 100 minutes. Fractions of 4 ml were collected and assayed using the same method described above. Active fractions were also analyzed by SDS PAGE which demonstrated that lysophospholipase was 90-95% pure.

Example 12: Protein sequencing and amino acid analysis of recombinant *Fusarium venenatum* lysophopholipase

N-terminal sequencing of a semi-purified lysophospholipase obtained as described in Example 11 and an unpurified lysophospholipase isolated from a broth (5 days) prepared as described in Example 10 was performed on an Applied Biosystems 476A Protein Sequencer (Perkin Elmer/Applied Biosystems Division, Foster City, CA) with on-line HPLC and liquid phase trifluoroacetic acid (TFA) delivery. The lysophospholipase preparations were submitted to SDS-PAGE using Novex 8-16% Tris-glycine SDS-PAGE gels under reducing conditions in the presence of 1 mM PMSF. The gels were transblotted to PVDF membranes (Novex, San Diego, CA) for 2 hours at 25

volts in 10 mM CAPS pH 11.0 buffer. The PVDF membranes were stained in 0.1% Commassie Blue R250 in 40% methanol/1% acetic acid and the observed bands excised. The excised bands were sequenced from a blot cartridge using sequencing reagents (Perkin Elmer/Applied Biosystems Division, Foster City, CA). Detection of phenylthiohydantoin-amino acids was accomplished by on-line HPLC using Buffer A containing 3.5% tetrahydrofuran in water with 18 ml of the Premix concentrate (Perkin Elmer/Applied Biosystems Division, Foster City, CA) containing acetic acid, sodium acetate, and sodium hexanesulfonate and Buffer B containing acetonitrile. Data was collected and analyzed on a Macintosh IIsi using Applied Biosystems 610 Data Analysis software.

SDS-PAGE of the purified lysophospholipase revealed major bands at approximately 130 kDa and 90 kDa, and minor bands at approximately 45, 40, and 35 kDa. Molecular weights were based on pre-stained Multi-Mark SDS page markers which do not reflect accurate MW determinations. N-terminal sequencing of the excised bands produced the following sequences.

Run# AB0909 - 130 kDa band sequence: ALPDSPSGGY (SEQ ID NO. 2).

Run# AB0910 - 90 kDa band sequence: NTAKYWDDIKDTVDEKADGW (SEQ ID NO. 2) (Internal peptide following leucine).

Run# AB0912 - 45 kDa band sequence: ALPDSPSGGYA (SEQ ID NO. 2)

Run# AB0913 - 40 kDa band sequence: ALPDSPSGGYAPKV (SEQ ID NO. 2)

Run# AB0915 - 35 kDa band sequence: ALPD(S)P?GGYAP (SEQ ID NO. 2).

SDS-PAGE of the unpurified lysophospholipase revealed a major band at 116 kDa based on Novex Mark 12 SDS-page markers. N-terminal sequencing of the excised 116 kDa band produced the following sequence:

Run# AB0917 - 116 kDa band sequence: ALPDSPSGGYAPKVVD?P (SEQ ID NO. 2) (?=Cys which is undetected without modification in Edman chemistry). The N-terminus appeared to be processed with a 20 amino acid pro-peptide. Cleavage occurred following an arginine.

The overall results are summarized as follows:

Signal Sequence: MLGPLVFTLWLTSSAIA (SEQ ID NO. 2)

Pro-peptide: APDDAGLVAAPAIGKSLSIR (SEQ ID NO. 2)
N-terminus: ALPDSPSGGYAPKVVDCP (SEQ ID NO. 2)

Example 13: Characterization of recombinant *Fusarium venenatum* lysophospholipase

The lysophospholipase purified as described in Example 11 was assayed using lysolecithin, dilauroyl L-phosphotidyl choline, and lecithin as substrates. The assay was performed as follows: Lysophospholipase activity in culture supernatants was also measured using egg yolk lysolecithin as the substrate with a NEFA C assay kit (Wako Chemicals, Richmond, VA). Specifically, a 10 µl sample of the supernatant was added to 160 µl of 20 mM MOPS pH 7.0 buffer and 30 µl of a stock solution of 4 mg of lysolecithin, 5 mg of dilauroyl L-phosphotidyl choline (dispersed by sonication), or 5 mg of lecithin (dispersed by sonication) per ml of 100 mM NaCl and incubated at 37°C for 20 minutes. Then 20 µl of the reaction was added to 200 µl of Wako Chemicals Reagent A and incubated at 37°C for 10 minutes. Finally, 400 µl of Wako Chemicals Reagent B was added and the solution incubated at 37°C for an additional 10 minutes. The absorbance at 550 nm was measured by transferring 200 µl of the final solution to a 96 well plate and measuring the absorbance with a SpectraMax Model 340 relative to a standard curve constructed with oleic acid as the standard. No significant activity was observed with L-phosphatidylcholine and lecithin as substrates.

The pH optimum of the lysophospholipase was determined as follows. Solutions of 0.125 M glycine-acetate-MES-phosphate were prepared at pHs 2,3,4,5,6,7, and 8. A 50 µl volume of a 16 mg/ml solution of lysophosphatidyl choline, lauroyl in 100 mM NaCl was added to 445 µl of the buffer solutions at the different pHs followed by 5 µl of the enzyme solution. After a 5 minute incubation, 1 ml of 5% TCA was added to stop the reaction, followed by1.5 ml of hexane and 0.5 ml of ethanol. The solution was vortexed and 500 µl of the hexane layer was removed and added to a conical vial. The hexane was evaporated under nitrogen and the fatty acids were esterified using the Methyl 8 reagent (Pierce, Rockville, IL). The resulting samples were the analyzed by gas chromatography using a DB WAX column (length 30 m, I.D. 0.32 mm, film 0.5 µm) (J&W Scientific, Folsom, CA) and a temperature gradient from 100 to 200°C at 4°C per minute followed by 200 to 270°C at 35°C per minute. Detection was by flame ionization.

A Grain Fatty Acid Methyl Ester Mix (Supelco, Inc., Bellefonte, PA) was used to determine the retention time for lauroyl fatty acid methyl ester and also for quantitation of the fatty acid peaks.

The results are shown in Figure 5. No hydrolysis of the substrate was detected at pH 2 and 8. The pH optimum was determined to be ~ 4-6.

Example 14: Construction of *Fusarium verticillioides* lysophospholipase cDNA library

Mycelia of *Fusarium verticillioides* were prepared similarly as described in Example 1. Poly A^+ mRNA was isolated from mycelia of *Fusarium verticillioides* and cDNA was synthesized from the Poly A^+ mRNA with *Bst*XI and *Not*I linkers similarly as described in Example 2. The cDNA cloned into the yeast expression vector pYES 2.0 (Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions.

Example 15: Transformation of *Saccharomyces cerevisiae* with *Fusarium verticillioides* lysophospholipase cDNA library

Competent cells of *Saccharomyces cerevisiae* YNG318 were prepared using the following procedure. *Saccharomyces cerevisiae* YNG318 was grown in 20 ml of YPD medium overnight at 30°C. A sufficient seed culture was transferred to 300 ml of YPD medium and cultivated at 30°C for 3 hours at 230-250 rpm until the OD_{600} = 0.2-0.3. Then the cells were harvested by centrifugation at 5000 rpm for 5 minutes at 20°C. The pellet was suspended in 50 ml of sterile water and the suspension was centrifuged again. The pellet was then suspended in 1.5 ml of 1X TE/LiAc and glycerol was added to a final concentration of 15%. The competent cells were stored at -80°C until use.

Transformation of *Saccharomyces cerevisiae* YNG318 was performed with the *Fusarium verticillioides* cDNA library described in Example 14 using the following procedure. A 100 µl volume of competent cells was thawed on ice and transferred into a sterile tube containing 10 µl of carrier DNA (yeast marker carrier DNA; Clontech Laboratories, Inc., Palo Alto, CA). One µg of plasmid DNA from Example 14 was added to the tube and the tube was gently mixed. Then 0.6 ml of sterile PEG/LiAc solution was added to the tube. The tube was incubated at 30°C with shaking at 200 rpm for 30 minutes and then heat-shocked at 42°C for 15 minutes. After heat-shock the tube was

placed on ice and then centrifuged for 5 seconds. The supernatant was removed and the pellet was dissolved in 1.5 ml of YPD. The cells were incubated at 30°C with shaking at 200 rpm for 45 minutes. The transformants were spread onto sterilized cellulose acetate filters, placed on SC Ura-glc plates, and incubated for 1 day at 30°C. The cellulose acetate filters were transferred onto sterilized Hybond N+ filter, placed onto SC Ura-gal plates, and incubated for 3 days at 30°C.

A total of 89,000 yeast transformants were obtained and screened using the following procedure for expression of lysophospholipase activity. Hybond N+ filters were placed on assay plates and incubated overnight at 30°C. One transformant containing p87YES showed lysophospholipase activity on the assay plate as a green halo.

p87YES was recovered from the yeast transformant using the following procedure. The yeast transformant was grown at 30°C in 1.5 ml of YPD medium overnight. The cells were harvested by centrifugation and the pellet was re-suspended in 100 µl of STET. A 0.2 g quantity of 0.45 mn glass beads were added and they were mixed with vortex for 5 minutes. Another 100 µl of STET was added and the tube was boiled for 3 minutes. A 100 µl volume of supernatant was transferred to a fresh tube and plasmid DNA was precipitated with ethanol. *E. coli* HB101 was transformed with the precipitated plasmid DNA using standard methods. p87YES was isolated from one of the *E. coli* transformants and sequenced to determine the nucleotide sequence of the *Fusarium verticillioides* lysophospholipase gene.

Example 16: Nucleotide sequencing and characterization of the *Fusarium verticillioides* lysophospholipase cDNA

DNA sequencing of p87YES was performed with an ABI PRISM 310 Genetic Analyzer Automated DNA Sequencer using a Dye Terminator Cycle Sequencing FS Ready Reaction Kit according to manufacturer's instructions.

The lysophospholipase clone encoded an open reading frame of 1944 bp encoding a polypeptide of 648 amino acids. The nucleotide sequence (SEQ ID NO. 15) and deduced amino acid sequence (SEQ ID NO. 16) are shown in Figures 6A, 6B, and 6C. Using the SignalP program (Nielsen *et al.*, 1997, *supra*), a signal peptide of 16 residues was predicted. Thus, the mature lysophospholipase is composed of 632 amino acids.

A comparative alignment of lysophospholipase sequences was undertaken using

the Clustal method (Higgins, 1989, *CABIOS* 5: 151-153) using the LASERGENE™ MEGALIGN™ software (DNASTAR, Inc., Madison, WI) with an identity table and the following multiple alignment parameters: Gap penalty of 10, and gap length penalty of 10. Pairwise alignment parameters were Ktuple=1, gap penalty=3, windows=5, and diagonals=5.

The comparative alignment showed that the *Fusarium verticillioides* lysophospholipase shares regions of identity with phospholipase proteins *Neurospora crassa* of 55.4% (042791), *Penicillium notatum* of 49.3% (Swissprot P39457), *Saccharomyces cerevisiae* of 37.0% (EMBL L23089), and *Torulaspora delbrueckii* of 38.3% (EMBL D32134). The identities are highest between regions that are likely to be important for catalytic and/or structural roles of the enzyme.

Deposit of Biological Materials

The following biological materials have been deposited under the terms of the Budapest Treaty with the Agricultural Research Service Patent Culture Collection, Northern Regional Research Center, 1815 University Street, Peoria, Illinois, 61604, and Centraalbureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, 3740 AG Baarn, The Netherlands, and given the following accession numbers:

Deposit	Accession Number	Date of Deposit
E. coli TOP10 (pFB0346)	NRRL B-30073	October 27, 1998
Fusarium verticillioides	CBS 650.96	June 5, 1996

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims. In the case of conflict, the present disclosure including definitions will control.

Various references are cited herein, the disclosures of which are incorporated by reference in their entireties.

EDITORIAL NOTE

APPLICATION NUMBER - 21484/00

The following Sequence Listing pages 1 to 7 are part of the description. The claims pages follow on pages "64" to "70".

SEQUENCE LISTING

<110> Randy M. Berka
Michael W. Rey
Tony Byun
Ryoko Itami
Noriko Tsutsumi
Alan Klotz

<120> Polypeptides Having Lysophospholipase Activity And Nucleic Acids Enocoding Same

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        35                  40                  45
Asp Cys Pro Ser Thr Arg Pro Lys Ala Arg Leu Ala Asp Gly Leu Ser
    50                  55                  60
Ser Glu Glu Glu Ser Trp Val Arg Arg Arg Arg Asn Asn Thr Ile Asp
65                  70                  75                  80
Asp Leu Lys Thr Phe Leu Ser Arg Ala Asn Ile Ser Gly Phe Asp Ala
                85                  90                  95
Glu Ser Phe Val Glu Lys His Lys Asn Asn Ala Thr Gly Leu Pro Asn
            100                 105                 110
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        115                 120                 125
Ala Gly Phe Leu Ser Ala Ala Asp Ser Arg Asn Asn Lys Thr Gly Pro
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Ile Ser Gly Leu Leu Gln Ser Ala Thr Tyr Leu Ala Gly Leu Ser Gly
145                 150                 155                 160
Gly Gly Trp Leu Val Gly Ser Ile Phe Ala Asn Asn Phe Ser Thr Val
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Pro Asp Leu Gln Ser Gly Asp Lys Val Trp Arg Phe Asp Arg Ser Ile
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Phe Lys Gly Pro Lys Ser Ser Gly Ile Ser Leu Leu Asn Thr Ala Glu
        195                 200                 205
Tyr Trp Asp Glu Met Lys Asp Ala Val Asp Asp Lys Asp Lys Gly Trp
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225                 230                 235                 240
Val Asn Ala Pro Glu Gly Gly Pro Ser Tyr Thr Phe Ser Ser Ile Ala
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625                 630                 635                 640
Ala Ala Ala Leu Phe Ala Leu Met
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```

The claims defining the invention are as follows:

1. An isolated polypeptide having lysophospholipase activity, selected from the group consisting of:

(a) a polypeptide having an amino acid sequence which has at least 65% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16;

(b) a polypeptide encoded by a nucleic acid sequence which hybridizes under high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii) of at least 100 nucleotides, or (iv) a complementary strand of (i), (ii) or (iii);

(c) an allelic variant of (a) or (b); and

(d) a functional fragment of (a), (b) or (c), wherein the fragment has lysophospholipase activity.

2. The polypeptide of claim 1, having an amino acid sequence which has at least 65% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16.

3. The polypeptide of claim 2, having an amino acid sequence which has at least 70% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16.

4. The polypeptide of claim 3, having an amino acid sequence which has at least 80% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16.

5. The polypeptide of claim 4, having an amino acid sequence which has at least

90% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16.

6. The polypeptide of claim 5, having an amino acid sequence which has at least 95% identity with amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16.

7. The polypeptide of any one of claims 1-6, comprising the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16.

8. The polypeptide of any one of claims 1-7, consisting of the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16, or a functional fragment thereof.

9. The polypeptide of claim 8, consisting of the amino acid sequence of SEQ ID NO. 2 or SEQ ID NO. 16.

10. The polypeptide of claim 9, which consists of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16.

11. The polypeptide of claim 1, which is encoded by a nucleic acid sequence which hybridizes under high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii) of at least 100 nucleotides, or (iv) a complementary strand of (i), (ii) or (iii).

12. The polypeptide of claim 11, which is encoded by a nucleic acid sequence which hybridizes under high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, or (iii) a complementary strand of (i) or (ii).

13. The polypeptide of any of claims 1-12, which is encoded by the nucleic acid sequence contained in plasmid pFB0345 contained in *E. coli* NRRL B-30073.

14. The polypeptide of any of claims 1-12, which is encoded by the nucleic acid sequence contained in *Fusarium verticillioides* CBS 650.96.

15. The polypeptide of any of claims 1-14 which has at least 20% of the lysophospholipase activity of SEQ ID NO. 2 or SEQ ID NO. 16.

16. A polypeptide having the same lysophospholipase activity as the polypeptide of any of claims 1-15.

17. An isolated nucleic acid sequence comprising a nucleic acid sequence which encodes the polypeptide of any of claims 1-16.

18. An isolated nucleic acid sequence comprising a nucleic acid sequence having at least one mutation in the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, in which the mutant nucleic acid sequence encodes a polypeptide consisting of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, respectively.

19. An isolated nucleic acid sequence produced by (a) hybridizing under high stringency conditions with (i) nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (ii) the genomic DNA sequence containing nucleotides 214 to 2061 of SEQ ID NO. 1 or nucleotides 49 to 1944 of SEQ ID NO. 15, (iii) a subsequence of (i) or (ii) of at least 100 nucleotides, or (iv) a complementary strand of (i), (ii), or (iii); and (b) isolating the nucleic acid sequence.

20. A nucleic acid construct comprising the nucleic acid sequence of claim 17 operably linked to one or more control sequences that direct the production of the polypeptide in a suitable expression host.

21. A recombinant expression vector comprising the nucleic acid construct of claim 20.

22. A recombinant host cell comprising the nucleic acid construct of claim 20.

23. A method for producing a mutant nucleic acid sequence, comprising (a) introducing at least one mutation into the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, wherein the mutant nucleic acid sequence encodes a polypeptide consisting of amino acids 38 to 654 of SEQ ID NO. 2 or amino acids 17 to 648 of SEQ ID NO. 16, respectively; and (b) recovering the mutant nucleic acid sequence.

24. A mutant nucleic acid sequence produced by the method of claim 23.

25. A method for producing a polypeptide, comprising (a) cultivating a strain comprising the mutant nucleic acid sequence of claim 24 encoding the polypeptide to produce a supernatant comprising the polypeptide; and (b) recovering the polypeptide.

26. A method for producing the polypeptide of any of claims 1-16 comprising (a) cultivating a strain under conditions suitable for production of the polypeptide; and (b) recovering the polypeptide.

27. A method for producing the polypeptide of any of claims 1-16 comprising (a) cultivating a host cell comprising a nucleic acid construct comprising a nucleic acid sequence encoding the polypeptide under conditions suitable for production of the polypeptide; and (b) recovering the polypeptide.

28. A method for producing a polypeptide comprising (a) cultivating a host cell under conditions conducive for production of the polypeptide, wherein the host cell comprises a mutant nucleic acid sequence having at least one mutation in the mature polypeptide coding sequence of SEQ ID NO. 1 or SEQ ID NO. 15, wherein the mutant nucleic acid sequence encodes a polypeptide consisting of amino acids 38 to 654 of SEQ ID NO. 2 or

amino acids 17 to 648 of SEQ ID NO. 16, respectively, and (b) recovering the polypeptide.

29. A method for producing the polypeptide of any one of claims 1-16 comprising (a) cultivating a homologously recombinant cell, having incorporated therein a new transcription unit comprising a regulatory sequence, an exon, and/or a splice donor site operably linked to a second exon of an endogenous nucleic acid sequence encoding the polypeptide, under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

30. A method for producing a mutant of a parent cell, which comprises disrupting or deleting a nucleic acid sequence encoding the polypeptide of any one of claims 1-16 or a portion thereof, or the promoter of said nucleic acid or a portion thereof, which results in the mutant cell producing less of the polypeptide than the parent cell.

31. A mutant cell produced by the method of claim 30.

32. The mutant cell of claim 31, which further comprises a nucleic acid sequence encoding a heterologous protein.

33. A method for producing a heterologous polypeptide comprising (a) cultivating the mutant cell of claim 32 under conditions conducive for production of the polypeptide; and (b) recovering the polypeptide.

34. A nucleic acid construct comprising a gene encoding a protein operably linked to one or both of a first nucleic acid sequence encoding a signal peptide consisting of nucleotides 100 to 150 of SEQ ID NO. 1 or nucleotides 1 to 48 of SEQ ID NO. 15, or a second nucleic acid sequence encoding a propeptide consisting of nucleotides 151 to 213 of SEQ ID NO. 1, wherein the gene is foreign to the first and second nucleic acid sequences.

35. A recombinant expression vector comprising the nucleic acid construct of claim

34.

36. A recombinant host cell comprising the nucleic acid construct of claim 34.

37. A method for producing a protein comprising (a) cultivating the recombinant host cell of claim 36 under conditions suitable for production of the protein; and (b) recovering the protein.

38. A method for reducing the phospholipid content in an edible oil, comprising treating the oil with the polypeptide of any one of claims 1-16 to hydrolyze the phospholipid and separating the hydrolyzed phospholipid from the oil.

39. A method for reducing the phospholipid content of an aqueous carbohydrate solution or slurry, comprising treating the aqueous carbohydrate solution or slurry with the polypeptide of any one of claims 1-16 to hydrolyze the phospholipid and separating the hydrolyzed phospholipid from the aqueous carbohydrate solution or slurry.

40. A method for preparing a dough, comprising incorporating into the dough an effective amount of the polypeptide of any one of claims 1-16 to improve one or more properties of the dough and/or a baked product obtained from the dough.

41. The method of claim 40, wherein the one or more improved properties are selected from the group consisting of increased strength of the dough, increased stability of the dough, reduced stickiness of the dough, improved machinability of the dough, increased volume of the baked product, improved crumb structure of the baked product, improved softness of the baked product, improved flavor of the baked product, and improved antistaling of the baked product.

42. A method for preparing a baked product, comprising baking a dough produced by the method of claim 40 or 41 to produce a baked product.

43. A dough prepared by the method of claim 40 or 41.

44. A baked product produced by the method of claim 42.

45. An isolated polypeptide having lysophospholipase activity, substantially as hereinbefore described with reference to any one of the examples.

46. An isolated nucleic acid sequence comprising a nucleic acid sequence which encodes the polypeptide of claim 45.

47. A method for producing a mutant nucleic acid sequence, substantially as hereinbefore described with reference to any one of the examples.

48. A method for producing a polypeptide, substantially as hereinbefore described with reference to any one of the examples.

49. A method for producing a mutant of a parent cell, substantially as hereinbefore described with reference to any one of the examples.

50. A method for producing a protein, substantially as hereinbefore described with reference to any one of the examples.

51. A method for reducing the phospholipid content in an edible oil, substantially as hereinbefore described with reference to any one of the examples.

52. A method for reducing the phospholipid content of an aqueous carbohydrate solution or slurry, substantially as hereinbefore described with reference to any one of the examples.

53. A method for preparing a dough, substantially as hereinbefore described with reference to any one of the examples.

54. A method for preparing a baked product, comprising baking a dough produced by the method of claim 53 to produce a baked product.

55. A dough prepared by the method of claim 53.

56. A baked product prepared by the method of claim 54.

Dated 17 October, 2003
Novozymes Biotech, Inc.
Novozymes Japan, Ltd.

Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

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  S  S  T  Y  L  A  G  L  S  G  G  G  W  L  V  G  S  I  F  A  N  N  F  T  T  I

CCGACCTACAAAAGGGAGACAAGGGTTCAGATATCTGGGCTTTTGACCGTTCAATTTTCAAAGGACCCGAAAAGTCAGGC 720
P  D  L  Q  K  G  D  K  G  S  D  I  W  A  F  D  R  S  I  F  K  G  P  E  K  S  G

TTGAACGTTTTGAACACGGCTAAATACTGGGATGACATAAAAGACACCGTTGACGAAAAGGCCGATGGGTGGAATACTAC 800
 L  N  V  L  N  T  A  K  Y  W  D  D  I  K  D  T  V  D  E  K  A  D  G  W  N  T  T
```

Fig. 1A

```
ACTCACTGACTGGTGGGGTCGTGCTCTGTCTTACCAGCTGATCGATGCCTCTGAGGGTGGTCCTGCGTATACTTTCTCCT 880
  L  T  D  W  W  G  R  A  L  S  Y  Q  L  I  D  A  S  E  G  G  P  A  Y  T  F  S

CCATCGCCGATACTTCCAACTTCAAGGACGCCGATACCCCATTTCCTATCCTCGTTGCCGATGGTCGTGCTCCTGGTCAA 960
S  I  A  D  T  S  N  F  K  D  A  D  T  P  F  P  I  L  V  A  D  G  R  A  P  G  Q

CGCATCGTTTCACTCAACGCAACTGTGTACGAGTTCAACCCGTTCGAGTTCGGAACATGGGATCCCACTAGTTACGGGTT 1040
  R  I  V  S  L  N  A  T  V  Y  E  F  N  P  F  E  F  G  T  W  D  P  T  S  Y  G  F

TGCTCCCGTCGAGTACATCGGCTCTAACTTCACGAATGGAACTATCGAAAAGGGCGGCGGCGAATGTGTACGTGGCTTTGATC 1120
  A  P  V  E  Y  I  G  S  N  F  T  N  G  T  I  E  K  G  G  E  C  V  R  G  F  D

AGTTCGGCTTTGTTATGGGCACATCCTCCTCGTTATTCAACCAGTTCCTGCTCAACAACATCACCAAGATTGGAGAAGAA 1200
Q  F  G  F  V  M  G  T  S  S  S  L  F  N  Q  F  L  L  N  N  I  T  K  I  G  E  E

AATGACATTCCTTCACTTGTCGTGAAGGCTATCCAGGGATTCCTGGTAGCTTTGGATACCAATGATGAGGACATTGCGGA 1280
N  D  I  P  S  L  V  V  K  A  I  Q  G  F  L  V  A  L  D  T  N  D  E  D  I  A  D

TTATTCTCCCAACCCATTCTACCAGTGGAACGTGACAGGAAAAAGCTACAACGCCAAGGACCATCAATTGACTCTTGTCG 1360
  Y  S  P  N  P  F  Y  Q  W  N  V  T  G  K  S  Y  N  A  K  D  H  Q  L  T  L  V

ACGGAGGGGAGGATCTGCAGAATATCCCACTCCATCCCTTGATCCAGCCTGTTCGTGGTGTCGACATCATCTTTGCCATC 1440
D  G  G  E  D  L  Q  N  I  P  L  H  P  L  I  Q  P  V  R  G  V  D  I  I  F  A  I

GATTCTTCAGCGGATACGGACAACAATTGGCCCAATGGTACCGCTCTTCGTGCGACATACGATCGTGTCGATTCCAGCTT 1520
D  S  S  A  D  T  D  N  N  W  P  N  G  T  A  L  R  A  T  Y  D  R  V  D  S  S  L

AGGAAACGGAACTCAGTTTCCCTCTATTCCATCAGCTGAGACTTTCATTAATGAGAAGTTGAACCAACGCCCAACACTCT 1600
  G  N  G  T  Q  F  P  S  I  P  S  A  E  T  F  I  N  E  K  L  N  Q  R  P  T  L
```

Fig. 1B

```
TTGGCTGTGATGCAGACAACTTCACGCTTTCAGACGGCAAAGCTCCTCCTCCTCTTGTCTTCTACATTCCCAACGCGCCC 1680
F  G  C  D  A  D  N  F  T  L  S  D  G  K  A  P  P  P  L  V  F  Y  I  P  N  A  P

TACACATTCTTGAGCAATGTCTCTACCTTCGATCTCTCATACAGCATCCCTGAGCGTGACAGTATCATTCTCAATGCTCT 1760
 Y  T  F  L  S  N  V  S  T  F  D  L  S  Y  S  I  P  E  R  D  S  I  I  L  N  A  L

GAACGGTGCCACTCAGGGCAATGGTACTATTGATAAGGAATGGCCCACGTGTGTCGTTTGTGCCATCATGAGCCGAAGTT 1840
  N  G  A  T  Q  G  N  G  T  I  D  K  E  W  P  T  C  V  V  C  A  I  M  S  R  S

GGTGGAAGTCCAATGAGACTGTTCCCAAAGAGTGCAGTACGTGTTTTGACAGATACTGCTGGGACGGAAAGTCGAATAAC 1920
W  W  K  S  N  E  T  V  P  K  E  C  S  T  C  F  D  R  Y  C  W  D  G  K  S  N  N

ACAGCTGTTAAGACTTACGAGCCTGGATTTATCATTGCTAACGAGACTGCGGCGGCGGAAGACAATGCAGCCGTTGCCGG 2000
 T  A  V  K  T  Y  E  P  G  F  I  I  A  N  E  T  A  A  A  E  D  N  A  A  V  A  G

TTTCACACCGAGTTGGTATATGTCTGTGTTTGTTGGAGTGGCTTTATTCTTGGCTATTTCGTAATTAGCATGTAGTTTCT 2080
  F  T  P  S  W  Y  M  S  V  F  V  G  V  A  L  F  L  A  I  S  .

GAGTTTGAATATATCTACATCATGCCAATAATATAATTAAAAAAAAAAAAAAAAAAAAAAA 2141
```

Fig. 1C

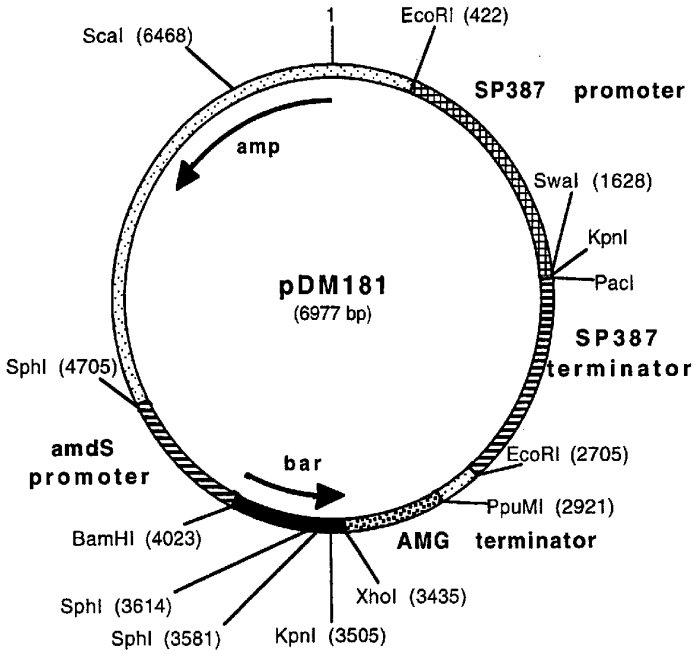

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EcoRI (422)
ScaI (6468)
SP387 promoter
amp
SwaI (1628)
KpnI
PacI
pDM181
(6977 bp)
SP387
terminator
SphI (4705)
amdS
promoter
bar
EcoRI (2705)
PpuMI (2921)
BamHI (4023)
AMG terminator
XhoI (3435)
SphI (3614)
SphI (3581)
KpnI (3505)

Fig. 2

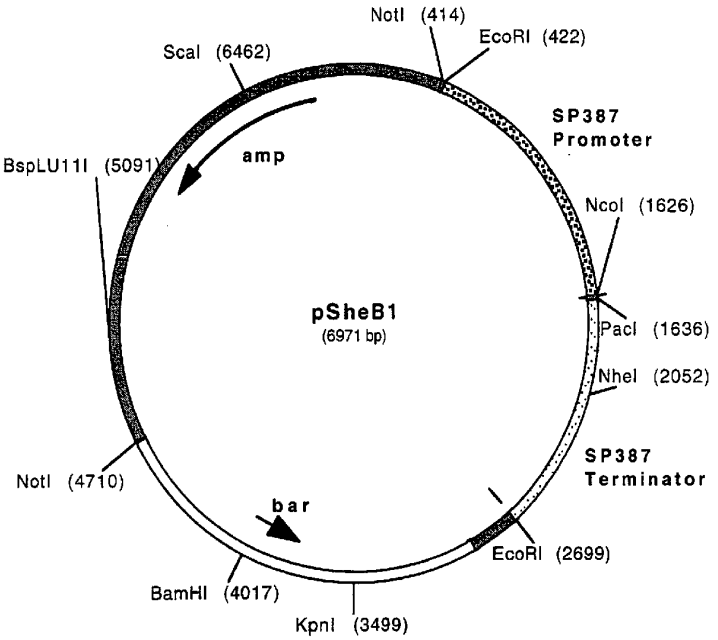

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EcoRI (422)
ScaI (6462)
SP387
Promoter
amp
BspLU11I (5091)
NcoI (1626)
pSheB1
(6971 bp)
PacI (1636)
NheI (2052)
SP387
Terminator
NotI (4710)
bar
EcoRI (2699)
BamHI (4017)
KpnI (3499)

Fig. 3

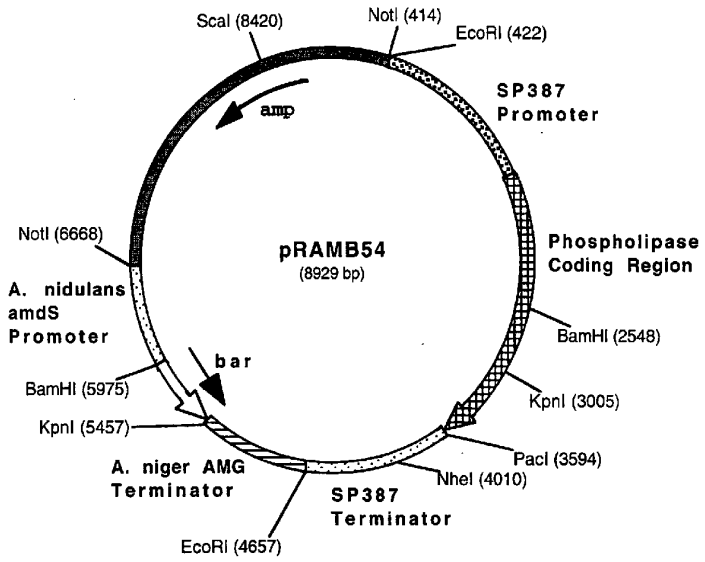

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NotI (414)
EcoRI (422)
SP387
Promoter
amp
NotI (6668)
pRAMB54
(8929 bp)
Phospholipase
Coding Region
A. nidulans
amdS
Promoter
BamHI (2548)
bar
BamHI (5975)
KpnI (3005)
KpnI (5457)
PacI (3594)
A. niger AMG
Terminator
NheI (4010)
SP387
Terminator
EcoRI (4657)

Fig. 4

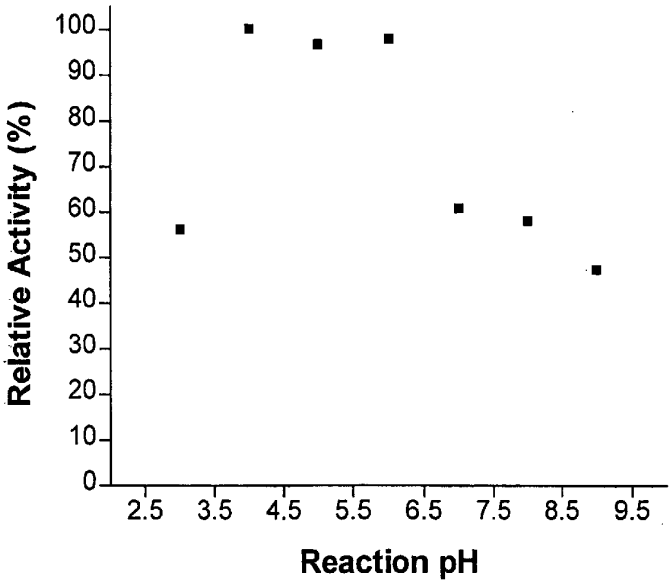

Relative Activity (%)
100
90
80
70
60
50
40
30
20
10
0
2.5
3.5
4.5
5.5
6.5
7.5
8.5
9.5
Reaction pH

Fig. 5

```
                M  L  G  F  V  A  L  T  L  W  L  S  T  A  I  A  A  P  E  D
    1   ATGCTCGGTT TTGTGGCCTT GACGCTCTGG CTCAGCACAG CCATTGCCGC TCCGGAAGAC
                T  A  L  I  P  R  V  N  S  V  E  I  R  A  L  P  N  S  P  S
   61   ACGGCTTTGA TCCCCAGGGT CAATTCAGTC GAGATCCGAG CCCTACCAAA TTCTCCGAGT
                G  G  Y  A  P  K  V  V  D  C  P  S  T  R  P  K  A  R  L  A
  121   GGCGGATATG CTCCGAAAGT AGTCGATTGC CCTTCAACAC GTCCAAAAGC TCGTCTTGCC
                D  G  L  S  S  E  E  E  S  W  V  R  R  R  N  N  T  I  D
  181   GATGGACTGT CTAGCGAGGA AGAATCCTGG GTTCGTCGTC GCAGAAACAA CACAATAGAT
                D  L  K  T  F  L  S  R  A  N  I  S  G  F  D  A  E  S  F  V
  241   GACCTAAAGA CATTCTTGTC GCGAGCCAAC ATTTCCGGCT TCGATGCCGA GAGCTTCGTT
                E  K  H  K  N  N  A  T  G  L  P  N  I  A  I  A  A  S  G  G
  301   GAGAAGCATA AAAACAATGC GACTGGTCTA CCCAACATCG CCATCGCCGC GTCCGGCGGT
                G  Y  R  A  L  M  N  G  A  G  F  L  S  A  A  D  S  R  N  N
  361   GGTTATCGCG CTTTGATGAA CGGTGCAGGC TTTCTCTCAG CTGCTGATTC ACGCAACAAC
                K  T  G  P  I  S  G  L  L  Q  S  A  T  Y  L  A  G  L  S  G
  421   AAGACCGGCC CCATCAGCGG CCTTCTCCAG TCAGCAACGT ATCTAGCTGG TCTTTCTGGC
                G  G  W  L  V  G  S  I  F  A  N  N  F  S  T  V  P  D  L  Q
  481   GGTGGCTGGC TCGTTGGTTC TATTTTCGCC AACAATTTCT CAACAGTGCC AGACTTGCAA
                S  G  D  K  V  W  R  F  D  R  S  I  F  K  G  P  K  S  S  G
  541   TCTGGTGACA AAGTCTGGCG ATTTGATCGC TCCATCTTCA AGGGCCCCAA GAGCTCTGGA
                I  S  L  L  N  T  A  E  Y  W  D  E  M  K  D  A  V  D  D  K
  601   ATCAGTCTCC TCAACACTGC CGAGTACTGG GATGAGATGA AAGATGCCGT TGACGACAAG
                D  K  G  W  N  T  T  L  T  D  W  W  G  R  A  L  S  Y  Q  L
  661   GACAAGGGCT GGAATACTAC TCTCACTGAT TGGTGGGCCC GCGCATTGTC GTATCAGCTC
                V  N  A  P  E  G  G  P  S  Y  T  F  S  S  I  A  D  T  S  N
  721   GTCAATGCGC CTGAGGGTGG ACCTTCATAC ACTTTCTCTT CCATTGCCGA TACCTCCAAC
                F  K  D  A  D  T  P  F  P  I  L  V  A  D  G  R  A  P  G  E
  781   TTCAAGGACG CAGACACACC CTTCCCCATC CTCGTTGCTG ATGGTCGTGC TCCAGGTGAG
```

Fig. 6A

```
      R V I   S L N A   T V Y   E F N   P Y E F   G T W
841   CGCGTCATCT CCCTGAATGC TACTGTATAC GAGTTCAACC CATACGAGTT CGGTACATGG
        D P T   T F G F   V P T   E Y L   A S N F   T N G
901   GATCCTACAA CCTTTGGCTT CGTACCCACT GAGTATCTCG CTTCCAACTT CACCAACGGC
        S I S   S K G E   C V R   G F D   Q I G F   V M G
961   AGCATCAGCT CCAAGGGCGA ATGCGTTCGT GGCTTTGATC AGATCGGCTT TGTTATGGGA
        T S S   S L F N   Q F L   L N N   I T K V   G K E
1021  ACATCCTCTT CACTATTCAA CCAGTTCCTT CTCAACAACA TCACCAAGGT CGGCAAAGAG
        N D I   P D I V   V K A   I E G   V L V G   L D E
1081  AATGATATTC CCGACATTGT TGTCAAAGCC ATTGAAGGCG TCTTGGTTGG CTTGGACGAG
        D D E   D I A Q   Y A P   N P F   F G W N   P T D
1141  GATGATGAGG ATATCGCCCA GTATGCACCC AACCCCTTCT TCGGATGGAA CCCTACCGAT
        K S V   N S K D   R Q L   T L V   D G G E   D L Q
1201  AAGAGCGTCA ATTCCAAGGA CCGCCAGTTG ACTCTTGTCG ATGGTGGAGA GGATCTGCAG
        N I P   L H P L   I Q P   V R G   V D I I   F A I
1261  AACATTCCTC TTCACCCATT GATCCAGCCC GTTCGTGGCG TTGACATCAT TTTTGCCATT
        D S S   A D T D   N N W   P N G   T A L R   A T Y
1321  GATTCGTCTG CTGATACGGA CAACAACTGG CCCAATGGTA CTGCTCTTCG TGCTACATAT
        D R V   G S S I   G N G   T L F   P S V P   S A E
1381  GACCGTGTTG GTTCCAGCAT TGGCAACGGC ACACTATTTC CCTCAGTCCC ATCAGCTGAG
        T F I   N E K L   N Q R   P T L   F G C N   A N N
1441  ACCTTTATCA ACGAGAAGTT GAACCAGCGC CCTACGCTCT TTGGCTGTAA CGCGAACAAC
        F T L   S D G E   V P P   P L I   L Y I P   N A P
1501  TTCACTCTTT CAGACGGCGA AGTTCCCCCT CCGCTGATCT TGTACATCCC CAACGCTCCC
        Y T Y   H S N V   S T F   D M S   Y T T T   E R D
1561  TACACCTACC ACAGTAACGT CTCCACCTTC GACATGTCGT ATACCACTAC CGAACGCGAC
```

Fig. 6B

```
      N  I  I  L  N  A  L  N  G  A  T  Q  G  N  A  T  I  D  K  E
1621  AACATCATTC TTAATGCTCT CAATGGAGCT ACTCAGGGTA ACGCTACCAT TGACAAGGAG
        W  P  T  C  V  A  C  A  V  M  S  R  S  W  W  K  A  N  E  A
1681  TGGCCTACTT GCGTGGCGTG TGCTGTCATG AGCAGAAGCT GGTGGAAGGC GAATGAGGCT
        V  P  D  A  C  K  T  C  F  D  R  Y  C  W  D  G  K  S  N  N
1741  GTTCCTGACG CATGCAAGAC CTGCTTCGAT CGCTACTGCT GGGATGGAAA GTCTAACAAC
        T  A  V  K  S  Y  E  P  E  Y  I  I  G  G  N  A  T  A  E  A
1801  ACTGCTGTCA AGAGCTATGA GCCCGAGTAC ATCATTGGAG GAAATGCGAC CGCGGAAGCC
        A  D  N  A  A  G  A  R  L  G  P  S  W  F  V  S  A  G  V  G
1861  GCAGACAACG CTGCTGGAGC CAGGCTTGGT CCAAGCTGGT TTGTATCGGC TGGTGTCGGG
        A  A  A  L  F  A  L  M  *
1921  GCGGCTGCTT TGTTTGCTCT CATGTGA
```

Fig. 6C