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[Continued on next page]

(54) Title: PHYTASE ENZYMES, NUCLEIC ACID SEQUENCES ENCODING PHYTASE ENZYMES AND VECTORS AND HOST CELLS INCORPORATING SAME

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TGCACTACTGTCGATGGCGGTTACCAATGCAATTCCGAGCTCTCACACAAGTGGGGCCAGTATTCGCCCTATTTCTCTCT
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CTCGATTCCCGTCCGCGAAGAAGAGCAAGGTATATGCCAAGCTCATTGAAAATATCCAAGCGAACGCGACTGCATACAAT
GGCAACACGAAGTTCCTCCGCTCATACAAGTACACCATGGGCGGTGATGATTTGGTACCCTTCGGAGTGAACCAGACGGT
GGACTCGGGGACCAAATTCTACCAGCGCTACGAGGCGTTGGCGAAGAAAGCTGTGCCCTTCATTCGGTCATCTGACTCAG
GGCGGGTTGTGGCTTCAGGCGTGAACTTTATCAAGGGATTCCAGCAGGCAAAGTTGGATGATAAAAATGCCAATCACCGT
CAGCCAAGCCCCAAAACCAACGTCATCATCTCAGAAGAGTCTGGCACCAACAACACTCTGAACCACAGCGAGATCTGTCC
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TCGAGGCCGATCTCCCTGGCGTTAAACTTGAAGACATCGATGTTGTCAGTCTGATGGACATCTGCCCTTTCGAGACAGTG
TCTTCAAGTGACGACGCAGCCGAGCTATCTCCATTCTGCGACCTCTTCACCCCGACCGAATGGAGCCAATATGACTACCT
CCAGTCGTTAAGCAAGTACTATGGTTATGGCGCCGGCAATCCTCTCGGCCCGACCCAGGGTGTCGGTTTCGTAAACGAAC
TGATTGCCCGACTCACTCGCCACCCAGTGAGAGACCACACAAGCACAAACCGTGCGCTCGATGCCCCCGGCGCTGCGACA
TTCCCCCTCAACTACACCATGTATGCCGACTTCACGCATGACAACGGAATGATCCCGTTCTTCTTTGCTTTGGGGCTGTA
CAACGGCACCGCTCCACTCTCGCTCACCCACGTCCAGTCTCCTAGCCAAACAGACGGGTTCTCATCCGCCTGGACAGTCC
CCTTCGGTGCTCGGGCTTATGTTGAGATGATGCAATGTCGTCGGGAACCTGAGCCGCTCGTGCGAGTCCTCGTTAATGAC
CGTGTTATTCCGCTGCACGGTTGCCCGGTGGATAAACTTGGCCGTTGTCGCCGTCGTGATTTCGTGAAAGGGCTTACTTT
CGCACGCTCTGGCGGCGACTGGGCCAGGTGTTATAAA
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(57) **Abstract:** DNA is provided which encodes an enzyme having phytase activity isolated from Penicillium, Fusarium, Humicola and Emericella. Also provided for is a method of isolating DNA encoding an enzyme having phytase activity from organisms which possess such DNA, transformation of the DNA into a suitable host organism, expression of the transformed DNA and the use of the expressed phytase protein in feed as a supplement.

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PHYTASE ENZYMES, NUCLEIC ACID SEQUENCES ENCODING PHYTASE ENZYMES AND VECTORS AND HOST CELLS INCORPORATING SAME

FIELD OF THE INVENTION

The present invention relates to phytase, nucleic acid sequences encoding phytase, as well as the production of phytase and its use.

REFERENCES

al-Batshan et al., Poultry Science 73(10):1590-1596 (1994).

Altschul, S.F., Gish, W., Miller, W., Myers, E.W. & Lipman, D.J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410.

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. (1997) "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs." Nucleic Acids Res. 25:3389-3402.

Aplin and Wriston, Crit. Rev. Biochem., pp. 259-306 (1981).

ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Chapters 2, 3, 6 and 7, Ed. Y.S. Sanghui and P. Dan Cook.

Ausubel et al. (eds.) (1995) Current Protocols In Molecular Biology ,3rd edition, John Wiley & Sons,Inc.

Baker et al., U.S. Patent No.5,571,706 (1996).

Beaucage et al. (1993) Tetrahedron 49(10):1925.

Benner, Steven A., U.S. Patent No. 5,216,141 (1993).

Bennett & Lasure, More Gene Manipulations in Fungi, Academic Press, San Diego, pp. 70-76 (1991).

Benton, W. and Davis, R., 1977, Science 196:180.

Berger and Kimmel, (1987), Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol. 152, Academic Press, San Diego CA.

Birnboim, H.C. and Doly, J. (1979). Nucleic Acids Research 7: 1513-23.

Botstein, D. and Shortle, D. (1985) Science 229:1193-1201.

Bowen et al., U.S. Patent No. 5,736,369 (1998).

Bremel et al., U.S. Patent No. 6,291,740 (2001).

Bremel et al., U.S. Patent No. 6,080,912 (2000).

Brisson et al (1984) Nature 310:511-514.

Briu et al. (1989) J. Am. Chem. Soc. 111:2321.

Broglie et al (1984) Science 224:838-843).

Cadwell, R.C. and Joyce, G.F., 1992, PCR Methods Applic. 2: 28-33.

Canadian Journal of Animal Science 75(3):439-444 (1995).

Committee on Food Chemicals Codex, Institute of Medicine, Food Chemicals Codex, 4th Edition, National Academy Press, Washington, DC, 1996.

Carlsson et al., Nature 380:207 (1996).

Clark, H. Fred, U.S. Patent No. 5,610,049 (1997).

Conklin et al., U.S. Patent No. 5,750,386 (1998).

Cook et al., U.S. Patent No. 5,637,684 (1997).

Coruzzi et al (1984) EMBO J 3:1671-1680.

Creighton, T.E. , Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)

Cromwell , G.L.T., T.S. Stahly, R.D. Coffey, H.J. Monegue, and J.H. Randolph. 1993. Efficacy of phytase in improving bioavailability of phosphorus in soybean and corn-soybean meal diets for pigs. J. Anim. Sci. 71:1831.

Damron et al., Poultry Science 74(5):784-787 (1995).

Dayhoff, M.O., Schwartz, R.M. & Orcutt, B.C. (1978) "A model of evolutionary change in proteins." In "Atlas of Protein Sequence and Structure, vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 345-352, Natl. Biomed. Res. Found., Washington, DC.

Deutscher, Methods in Enzymology, 182 (1990).

DeBoer et al, U.S. Patent No. 6,066,725 (2000).

De Clercq et al., U.S. Patent No. 5,589,615 (1996).

De Mesmaeker et al., U.S. Patent No. 5,602,240 (1997).

De Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994).

Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995).

Devlin, Robert H., U.S. Patent No. 5,998,697 (1999).

Dieffenbach CW and Dveksler GS, 1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY.

Eckert, K.A. and Kunkel, T.A., 1991, PCR Methods Applic. 1: 17-24.

Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press.

Edge et al., Anal. Biochem., 118:131 (1981).

Egholm (1992) J. Am. Chem. Soc. 114:1895.

Ehrlich, K. C., Montalbano, B. G., Mullaney, E. J., Dischinger Jnr., H. C. & Ullah, A. H. J. (1993). Identification and cloning of a second phytase gene (*phy B*) from *Aspergillus niger* (*ficum*). Biochemical and Biophysical Research Communications 195, 53-57.

Elander, R.P., Microbial screening, Selection and Strain Improvement, in Basic Biotechnology, J. Bullock and B. Kristiansen Eds., Academic Press, New York, 1987, 217.

Evan et al., Molecular and Cellular Biology, 5:3610-3616 (1985).

Field et al., Mol. Cell. Biol., 8:2159-2165 (1988).

Finkelstein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi. Technology and Products (eds by Finkelstein & Bill) 113-156.

Fiske, C.H. and SubbaRow, Y. (1925). Journal of Biological Chemistry 66:375-392.

Fungaro et al. (1995) Transformation of Aspergillus nidulans by microprojection bombardment on intact conidia, FEMS Microbiology Letters 125 293-298.

Gelvin *et al.*, J. Bacteriol. 172(3):1600-1608 (1990).

Gish, W. & States, D.J. (1993) "Identification of protein coding regions by database similarity search." Nature Genet. 3:266-272.

Glover, DM and Hames, BD (Eds.), DNA Cloning: A Practical Approach, *Vols 1 and 2,* Second Edition.

Glover, DM and Hames, BD (Eds.), 1995, DNA Cloning 1: A Practical Approach, Oxford University Press, Oxford).

Glover, DM and Hames, BD (Eds.), 1995, DNA Cloning 2: A Practical Approach, Oxford University Press, Oxford).

Groot et al. (1998) Agrobacterium tumefaciens-mediated transformation of filamentous fungi, Nature Biotechnology 16 839-842.

Grunstein, M. and Hogness, D., 1975, Proc. Natl. Acad. Sci. USA 72:3961.

Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987).

Hale & Marham, **THE HARPER COLLINS DICTIONARY OF BIOLOGY,** Harper Perennial, NY (1991).

Henikoff & Henikoff, Proc. Natl. Acad. Sci. USA 89:10915 (1989).

Hershey et al., U.S. Patent No. 5,268,526 (1993).

Higgins D.G., Bleasby A.J., Fuchs R. (1992) CLUSTAL V: improved software for multiple sequence alignment. Comput. Appl. Biosci. 8:189-191.

Hobbs S or Murry LE (1992) in McGraw Hill Yearbook of Science and Technology , McGraw Hill, New York, N.Y., pp 191-196.

Hodges et al., U.S. Patent No. 5,677,175 (1997).

Hopp et al., BioTechnology, 6:1204-1210 (1988).

Houdebine et al., U.S. Patent No. 6,268,545 (2001).

Jaynes et al., U.S. Patent No. 5,597,945 (1997).

Jeffs et al., J. Biomolecular NMR 34:17 (1994).

Jenkins et al., Chem. Soc. Rev. (1995) pp169-176.

Jeroch et al., Bodenkultur Vo. 45(4):361-368 (1994).

Karatzas et al., U.S. Patent No. 5,907,080 (1999).

Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA 90:5873-5787 (1993).

Kerovuo, J., Lauraeus, M., Nurminen, P., Kalkkinen, N., Apajalahti, J. (1988) Isolation, characterization and molecular gene cloning, and sequencing of a novel phytase from *Bacillus subtilis.* Appl. Environ. Micro., 64, 6, 2079-2085.

Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991).

Kornegay, E.T., D.M. Denbow, Z. Yi., and V. Ravindran. 1996. Response of broilers to graded levels of Natuphosâ phytase added to corn-soybean meal-based diets containing three levels of nonphytate phosphorus. Br. J. Nutr.

Lebrun et al., U.S. Patent No. 5,510,471 (1996).

Letsinger, J. Org. Chem. 35:3800 (1970).

Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994).

Letsinger et al. (1988) J. Am. Chem. Soc. 110:4470.

Letsinger et al. (1986) Nucl. Acids Res. 14:3487.

Leung, D.W., Chen, E., and Goeddel, D.V., 1989, Technique 1: 11-15.

Lubon et al., U.S. Patent No. 6,262,336 (2001).

Lundquist et al., U.S. Patent No. 5,780,708 (1998).

Lundquist et al., U.S. Patent No. 5,538,880 (1996);

Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990).

Madden, T.L., Tatusov, R.L. & Zhang, J. (1996) "Applications of network BLAST server" Meth. Enzymol. 266:131-141.

Mag et al. (1991) Nucleic Acids Res. 19:1437.

Martin et al., Science, 255:192-194 (1992).

Meier et al. (1992) Chem. Int. Ed. Engl. 31:1008.

The Merck Veterinary Manual (Seventh Edition, Merck & Co., Inc., Rahway, N.J., USA, 1991, page 1268).

Myers, R.M., Lerman, L.S., and Maniatis, T., 1985, Science 229: 242-247.

Mitchell, D.B., Vogel, K., Weimann, B.J., Pasamontes, L. and van Loon, A.P., Microbiology 143 (Pt 1), 245-252 (1997)).

Moloney et al., U.S. Patent No. 5,750,871(1998).

Mullis, Kary B., U.S. Patent No. 4,683,202 (1990).

Needleman & Wunsch, J. Mol. Biol. 48:443 (1970).

Nielsen (1993) Nature,365:566.

Oakley et al., <u>Gene</u> 61(3): 385-99 (1987).

Paborsky et al., Protein Engineering, 3(6):547-553 (1990).

Pasamontes, L., Haiker, M., Henriquez-Huecas, M., Mitchell, D.B. and van Loon, A.P., Cloning of the phytases from Emericella nidulans and the thermophilic fungus Talaromyces thermophilus, Biochim. Biophys. Acta 1353 (3), 217-223 (1997).

Pasamontes, L., Haiker, M., Wyss, M., Tessier, M. and van Loon, A.P., Gene cloning, purification, and characterization of a heat-stable phytase from the fungus Aspergillus fumigatus, Appl. Environ. Microbiol. 63 (5), 1696-1700 (1997).

Pauwels et al. (1986) Chemica Scripta 26:141.

Pearson & Lipman, Proc. Nat'l. Acad. Sci. USA 85:2444 (1988).

Piedrahita et al., U.S. Patent Nos. 6,271,436 (2001).

Piddington, C. S., Houston, C. S., Paloheimo, M., Cantrell, M., Miettinen-Oinonen, A., Nevalainen, H. & Rambosek, J. (1993). The cloning and sequencing of the genes encoding phytase (*PhyA*) and pH 2.5-optimum acid phosphatase (*aph*) from *Aspergillus niger* var. *awamori.* Gene 133, 55-62.

Powar, V.K. and Jagannathan V., (1982) J. Bacteriology, 151 (3), 1102-1108.

Rawls, C & E News June 2, 1997 page 35.

Roland et al., Poultry Science, 75(1):62-68 (1996).

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloning – A Laboratory Manual, 2nd Ed. Cold Spring Harbour Press.

Sambrook *et al.* (2001). Molecular Cloning, A Laboratory Manual, 3d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sanchez, O. and J. Aguirre. 1996. Efficient transformation of Aspergillus nidulans by electroporation of germinated conidia. Fungal Genetics Newsletter 43: 48-51.

Sanger, F., Nilken, S. and Coulson, A.R. (1977). Proc. Nat'l. Acad. Sci. USA, 74: 5463-5467.

Sanghvi et al. U.S. Patent No. 5,386,023 (1995)

Sawai et al. (1984) Chem. Lett. 805.

Schwartz, R.M. & Dayhoff, M.O. (1978) "Matrices for detecting distant relationships." In "Atlas of Protein Sequence and Structure, vol. 5, suppl. 3." M.O. Dayhoff (ed.), pp. 353-358, Natl. Biomed. Res. Found., Washington, DC.

Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982).

Shimizu, M., (1992) Biosci. Biotech. Biochem., 56 (8), 1266-1269.

Shimizu, M., Japanese Patent Application 6-38745 (1994).

Singleton, *et al.,* DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994).

Skinner et al., J. Biol. Chem., 266:15163-15166 (1991).

Smith & Waterman, Adv. Appl. Math. 2:482 (1981).

Somers et al., U.S. Patent No. 5,773,269 (1998).

Sprinzl et al. (1977) Eur. J. Biochem. 81:579.

Summerton et al., U.S. Patent No. 5,235,033 (1993).

Summerton et al., U.S. Patent No. 5,034,506 (1991).

Takamatsu et al (1987) EMBO J 6:307-311.

Thotakura et al., Meth. Enzymol., 138:350 (1987).

T'so et al., U.S. Patent No. 4,469,863 (1984).

Ullah, H.J. and Gibson, D.M., Preparative Biochemistry, 17 (1) (1987), 63-91.

van Gorcom, Robert Franciscus Maria; van Hartingsveldt, Willem; van Paridon, Peter Andreas; Veenstra, Annemarie Eveline; Luiten, Rudolf Gijsbertus Marie; Selten, Gerardus Cornelis Maria; EP 420 358 (1991).

van Hartingsveldt, W., van Zeijl, C. M. J., Harteveld, G. M., Gouka, R. J., Suykerbuyk, M. E. G., Luiten, R. G. M., van Paridon, P. A., Selten, C. G. M., Veenstra, A. E., van Gorcom, R. F. M. & van den Hondel, C. A. J. J. (1993). Cloning, characterization and over expression of the phytase-encoding gene (*PhyA*) of *Aspergillus niger.* Gene 127:87-94.

Van Loon, A. and Mitchell, D.; EP 684 313 (1995).

Weber, K. L. et al., *Biotechniques* 25(3): 415-9 (1998).

Weidner, G., d'Enfert, C., Koch, A., Mol, P., and Brakhage, A.A. (1998) Development of a homologous transformation system for the human pathogenic fungus Aspergillus fumigatus based on the pyrG gene encoding orotidine monophosphate decarboxylase. Current Genet. 33: 378-385.

Weissbach and Weissbach (1988) Methods for Plant Molecular Biology, Academic Press, New York, N.Y., pp 421-463.

Wheeler, Mathew B., U.S. Patent No. 5,942,435 (1999).

Winter J and Sinibaldi RM (1991) Results Probl Cell Differ 17:85-105.

Yau, Eric K., U.S. Patent No. 5,644,048 (1997).

Yamada et al., Agr. Biol. Chem., 32 (10) (1968), 1275-1282.

BACKGROUND OF THE INVENTION

Phosphorous (P) is an essential element for growth. A substantial amount of the phosphorous found in conventional livestock feed, e.g., cereal grains, oil seed meal, and by products that originate from seeds, is in the form of phosphate which is covalently bound in a molecule know as phytate (myo-inositol hexakisphosphate). The bioavailability of phosphorus in this form is generally quite low for non-ruminants, such as poultry and swine, because they lack digestive enzymes for separating phosphorus from the phytate molecule.

Several important consequences of the inability of non-ruminants to utilize phytate may be noted. For example, expense is incurred when inorganic phosphorus (e.g., dicalcium phosphate, defluorinated phosphate) or animal products (e.g., meat and bone meal, fish meal) are added to meet the animals' nutritional requirements for phosphorus. Additionally, phytate can bind or chelate a number of minerals (e.g., calcium, zinc, iron, magnesium, copper) in the gastrointestinal tract, thereby rendering them unavailable for absorption. Furthermore, most of the phytate present in feed passes through the gastrointestinal tract, elevating the amount of phosphorous in the manure. This leads to an increased ecological phosphorous burden on the environment.

Ruminants, such as cattle, in contrast, readily utilize phytate thanks to an enzyme produced by rumen microorganisms known as phytase. Phytase catalyzes the hydrolysis of phytate to (1) myo-inositol and/or (2) mono-, di-, tri-, tetra- and/or penta-phosphates thereof and (3) inorganic phosphate. Two different types of phytases are known: (1) a so-called 3-phytase (myo-inositol hexaphosphate 3-phosphohydrolase, EC 3.1.3.8) and (2) a so-called 6-phytase (myo- inositol hexaphosphate 6-phosphohydrolase, EC 3.1.3.26). The 3-phytase preferentially hydrolyzes first the ester bond at the 3-position, whereas the 6-phytase preferentially hydrolyzes first the ester bond at the 6-position.

Microbial phytase, as a feed additive, has been found to improve the bioavailability of phytate phosphorous in typical non-ruminant diets (See, e.g., Cromwell, et al, 1993). The result is a decreased need to add inorganic phosphorous to animal feeds, as well as lower phosphorous levels in the excreted manure (See, e.g., Kornegay, et al, 1996).

Despite such advantages, few of the known phytases have gained widespread acceptance in the feed industry. The reasons for this vary from enzyme to enzyme. Typical concerns relate to high manufacture costs and/or poor stability/activity of the enzyme in the environment of the desired application (e.g., the pH/temperature encountered in the processing of feedstuffs, or in the digestive tracts of animals).

It is, thus, generally desirable to discover and develop novel enzymes having good stability and phytase activity for use in connection with animal feed, and to apply advancements in fermentation technology to the production of such enzymes in order to make them commercially viable. It is also desirable to ascertain nucleotide sequences which can be used to produce more efficient genetically engineered organisms capable of expressing such phytases in quantities suitable for industrial production. It is still further desirable to develop a phytase expression system via genetic engineering which will enable the purification and utilization of working quantities of relatively pure enzyme.

SUMMARY OF THE INVENTION

The present invention provides for a purified enzyme having phytase activity which is derived from a microbial source, and preferably from a fungal source, such as, a *Penicillium* species, e.g., *P. chrysogenum* (deposit no. NRRL 1951), a *Fusarium* species, e.g. *F. javanicum* (deposit no. CBS 203.32) or *F. vertisillibodes*, a *Humicola* species, e.g., *H. grisea* (deposit no. ATCC 22081 or CBS 225.63), or an *Emericella* species, e.g., *E. desertorum* (deposit no. CBS 653.73).

The present invention further provides a polynucleotide sequence coding for the enzyme comprising a DNA as shown in Figure 1 or Figures 19A-19C; a polynucleotide which encodes the amino acid sequence shown in Figure 2, 3 or 19A-19C; a polynucleotide which encodes a phytase which comprises an amino acid segment which differs from the sequence in Figure 2 or Figure 3 or Figures 19A-19C, provided that the polynucleotide encodes a derivative of the phytase specifically described herein; and a polynucleotide which encodes a phytase that comprises an amino acid sequence which differs from the sequence in Figure 2 or Figure 3 or Figures 19A-19C, provided that the polynucleotide hybridizes under medium to high stringency conditions with a nucleic acid sequence comprising all or part of the nucleic acid sequence in Figure 1 or Figures 19A-19C.

The present invention also provides a polynucleotide encoding an enzyme having phytate hydrolyzing activity and including a nucleotide sequence as shown in Figure 4, 7, 18A-18C or 21; a polynucleotide which encodes the amino acid sequence shown in Figure 5, 6, 8, 18A-18C or 21; a polynucleotide which encodes a phytase which comprises an amino acid segment which differs from the sequence in Figure 5, 6, 8, 18A-18C or 21, provided that the polynucleotide encodes a derivative of the phytase specifically described herein; and a polynucleotide which encodes a phytase that comprises an amino acid segment which differs from the sequence in Figure 5, 6, 8, 18A-18C or 21, provided that the polynucleotide hybridizes under medium to high stringency conditions with a nucleotide sequence as shown in Figure 4, 7, 18A-18C or 21.

The present invention further provides a polynucleotide encoding an enzyme having phytate hydrolyzing activity and including a nucleotide sequence as shown in Figure 9 or Figures 20A-20C; a polynucleotide which encodes the amino acid sequence shown in Figure 10, 11, or 20A-20B; a polynucleotide which encodes a phytase which comprises an amino acid segment which differs from the sequence in Figure 10, 11, or 20A-20B, provided that the polynucleotide encodes a derivative of the phytase specifically described herein; and a polynucleotide which encodes a phytase that comprises an amino acid segment which differs from the sequence in Figure 10, 11, or 20A-20B, provided that the polynucleotide hybridizes under medium to high stringency conditions with a nucleotide sequence as shown in Figure 9 or Figures 20A-20C.

The present invention further provides a polynucleotide encoding an enzyme having phytate hydrolyzing activity and including a nucleotide sequence as shown in Figure 17A; a polynucleotide which encodes the amino acid sequence shown in Figure 17B; a polynucleotide which encodes a phytase which comprises an amino acid segment which differs from the sequence in Figure 17B, provided that the polynucleotide encodes a derivative of the phytase specifically described herein; and a polynucleotide which encodes a phytase that comprises an amino acid segment which differs from the sequence in Figure 17B, provided that the polynucleotide hybridizes under medium to high stringency conditions with a nucleotide sequence as shown in Figure 17A.

Additionally, the present invention encompasses vectors which include the polynucleotide sequences described above, host cells which have been transformed with such polynucleotides or vectors, fermentation broths comprising such host cells and phytase proteins encoded by such polynucleotides which are expressed by the host cells. Preferably, the polynucleotide of the invention is in purified or isolated form and is used to prepare a transformed host cell capable of producing the encoded protein product thereof. Additionally, polypeptides which are the expression product of the polynucleotide sequences described above are within the scope of the present invention.

In one embodiment, the present invention provides an isolated or purified polynucleotide derived from a fungal source of the genus Penicillium, which polynucleotide comprises a nucleotide sequence encoding an enzyme having phytase activity. The fungal source can be, for example, from Penicillium chrysogenum. In another embodiment, the invention provides an isolated or purified polynucleotide derived from a fungal source of the genus Fusarium, which polynucleotide comprises a nucleotide sequence encoding an enzyme having phytase activity. The fungal source can be selected, for example, from the group consisting of Fusarium javanicum and Fusarium vertisillibodes. In yet another embodiment, the present invention provides an isolated or purified polynucleotide derived from a fungal source of the genus Humicola, which polynucleotide comprises a nucleotide sequence encoding an enzyme having phytase activity. The fungal source can be, for example, from Humicola grisea. In still another embodiment, the present invention provides an isolated or purified polynucleotide derived from a fungal source of the genus Emericella, which polynucleotide comprises a nucleotide sequence encoding an enzyme having phytase activity. The fungal source can be, for example, from Emericella desertorum.

According to one embodiment, the polynucleotide encodes a phytate-hydrolyzing enzyme including an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 2, 3 or 19A-19C.

One embodiment of the present invention provides an isolated polynucleotide comprising a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 1 or 19A-19C, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 1 or 19A-19C under conditions of intermediate to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 1 or 19A-19C.

Another aspect of the present invention provides an isolated polynucleotide encoding an enzyme having phytase activity, wherein the enzyme is derived from a Penicillium source. The source can be, for example, Penicillium chrysogenum.

In one embodiment, the polynucleotide encodes a phytate-hydrolyzing enzyme that includes an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75%

identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21.

In another embodiment, the polynucleotide encoding a phytate-hydrolyzing enzyme has at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 1 or 19A-19C, or (ii) is capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 1 or 19A-19C under conditions of medium to high stringency, or (iii) is complementary to the nucleotide sequence disclosed in Figure 1 or 19A-19C.

Yet a further aspect of the present invention provides an expression construct including a polynucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 1 or 19A-19C, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 1 or 19A-19C under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 1 or 19A-19C. Also provided are a vector (e.g., a plasmid) including such expression construct, and a host cell (such as an Aspergillus, e.g., Aspergillus niger or Aspergillus nidulans) transformed with such a vector.

In another of its aspects, the present invention provides a probe for use in detecting nucleic acid sequences coding for an enzyme having phytase activity derived from a microbial source, comprising: a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 1 or 19A-19C, or (ii) being capable of hybridizing to a polynucleotide including a sequence as disclosed in Figure 1 or 19A-19C under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 1 or 19A-19C.

In one embodiment, the microbial source is a fungal source, e.g., a Penicillium species, such as Penicillium chrysogenum.

The present invention additionally provides a food or animal feed including an enzyme having phytase activity, wherein the enzyme comprises an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 2, 3 or 19A-19C.

The present invention provides food or animal feed including an enzyme having phytase activity, wherein the enzyme is derived from a fungal source such as Penicillium chrysogenum.

Another aspect of the present invention provides a method of producing an enzyme having phytase activity, comprising:

(a) providing a host cell transformed with an expression vector comprising a polynucleotide as described herein;
(b) cultivating the transformed host cell under conditions suitable for the host cell to produce the phytase; and
(c) recovering the phytase.

According to one embodiment, the host cell is an Aspergillus species, such as A. niger or A. nidulans.

In one embodiment, the host cell is a plant cell. In this embodiment, cells or entire transformed plants may be grown and used.

Another aspect of the present invention provides a method of producing an enzyme having phytase activity, comprising:

(a) providing a host cell transformed with an expression vector comprising a polynucleotide as described herein;
(b) cultivating the transformed host cell under conditions suitable for the host cell to produce the phytase. The transformed cells, as well as organisms grown from such cells, may be used without further isolation of the enzyme.

In another aspect, the invention provides a purified enzyme having phytase activity, produced by the methods described above.

In yet another of its aspects, the present invention provides a method of separating phosphorous from phytate, comprising the step of treating the phytate with an enzyme comprising an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as
disclosed in Figure 2, 3 or 19A-19C.

The present invention further provides a method of separating phosphorous from phytate, comprising the step of treating the phytate with an enzyme as defined above.

Another aspect of the present invention provides a phytate-hydrolyzing enzyme that includes an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21. In a different embodiment, the compares in the same way to the sequence as disclosed in Figure 10, 11 or 20A-20B.

A further aspect of the present invention provides an isolated polynucleotide including a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 5, 6, 8, 18A-18C or 21 under conditions of intermediate to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 5, 6, 8, 18A-18C or 21.

In another embodiment, the invention provides an isolated polynucleotide including a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 10, 11 or 20A-20B, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 10, 11 or 20A-20B under conditions of intermediate to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 10, 11 or 20A-20B.

In one embodiment, the isolated polynucleotide encodes a phytate-hydrolyzing enzyme derived from a member of the Fusarium genus, preferably from F. javanicum or F. vertisillibodes. The enzyme includes, according to one embodiment, an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21.

In a different embodiment, the isolated polynucleotide encodes a phytate-hydrolyzing enzyme derived from a member of the Humicola genus, preferably from H. grisea. The enzyme includes, according to one embodiment, an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 10, 11 or 20A-20B.

In still another embodiment, the isolated polynucleotide encodes a phytate-hydrolyzing enzyme derived from a member of the Emericella genus, preferably from E. desertorum. The enzyme includes, according to one embodiment, an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 17B.

In another embodiment, the polynucleotide encoding a phytate-hydrolyzing enzyme includes a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 4, 7, 18A-18C, and 21, or (ii) capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 4, 7, 18A-18C, and 21 under conditions of medium to high stringency, or (iii) complementary to the nucleotide sequence disclosed in Figure 4, 7, 18A-18C, and 21.

In still another embodiment, the polynucleotide encoding a phytate-hydrolyzing enzyme includes a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 9 or 20A-20C, or (ii) capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 9 or 20A-20C under conditions of medium to high stringency, or (iii) complementary to the nucleotide sequence disclosed in Figure 9 or 20A-20C.

In still another embodiment, the polynucleotide encoding a phytate-hydrolyzing enzyme includes a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 17A, or (ii) capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 17A under conditions of medium to high stringency, or (iii) complementary to the nucleotide sequence disclosed in Figure 17A.

Another aspect of the present invention provides an expression construct comprising a polynucleotide including a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 4, 7, 18A-18C, and 21, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 4, 7, 18A-18C, and 21 under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 4, 7, 18A-18C, and 21.

Alternatively, the present invention provides an expression construct comprising a polynucleotide including a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 9 or 20A-20C, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 9 or 20A-20C under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 9 or 20A-20C.

In another embodiment, the present invention provides an expression construct comprising a polynucleotide including a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 17A, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 17A under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 17A.

The present invention further provides a vector (e.g., plasmid) including such an expression construct, as well as a host cell (e.g., Aspergillus niger or Aspergillus nidulans) transformed with a vector as described above.

The present invention additionally provides a probe for use in detecting nucleic acid sequences coding for an enzyme having phytase activity derived from a microbial source, comprising: a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 4, 7, 18A-18C, and 21, or (ii) being capable of hybridizing to a polynucleotide including a sequence as disclosed in Figure 4, 7, 18A-18C, and 21 under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 4, 7, 18A-18C, and 21.

In another aspect, the invention provides a probe for use in detecting nucleic acid sequences coding for an enzyme having phytase activity derived from a microbial source, comprising: a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 9 or 20A-20C, or (ii) being capable of hybridizing to a polynucleotide including a sequence as disclosed in Figure 9 or 20A-20C under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 9 or 20A-20C.

In another aspect, the invention provides a probe for use in detecting nucleic acid sequences coding for an enzyme having phytase activity derived from a microbial source, comprising: a nucleotide sequence (i) having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to a nucleotide sequence as disclosed in Figure 17A, or (ii) being capable of hybridizing to a polynucleotide including a sequence as disclosed in Figure 17A under conditions of medium to high stringency, or (iii) being complementary to the nucleotide sequence disclosed in Figure 17A.

In one embodiment, the microbial source is a fungal source, e.g., a Penicillium species, such as P. chrysogenum, a Fusarium species, such as F. javanicum or F. vertisillibodes, an Emericella species such as E. desertorum or a Humicola species, such as H. grisea.

The present invention further provides a food or animal feed including an enzyme having phytase activity, wherein the enzyme includes an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 10, 11, 17B, 18A-18C, 19A-19C, 20A-20B or 21.

Still further, the present invention provides a method of separating phosphorous from phytate, comprising the step of treating the phytate with an enzyme (i) having phytate hydrolyzing activity and (ii) including an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 5, 6, 8,18A-18C or 21. In another aspect, the invention provides a method of separating phosphorous from phytate, comprising the step of treating the phytate with an enzyme (i) having phytate hydrolyzing activity and (ii) including an amino acid sequence having at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to an amino acid sequence as disclosed in Figure 10, 11 or 20A-20B, or as disclosed in Figure 17B.

As will be appreciated, an advantage of the present invention is that a polynucleotide has been isolated which provides the capability of isolating further polynucleotides which encode proteins having phytase activity.

Another advantage of the present invention is that, by virtue of providing a polynucleotide encoding a protein having phytase activity, it is possible to produce, through recombinant means, a host cell which is capable of producing the protein having phytase activity in relatively large quantities.

Yet another advantage of the present invention is that commercial application of proteins having phytase activity is made practical. For example, the present invention provides animal feed incorporating the phytase described herein.

Other objects and advantages of the present invention will become apparent from the following detailed specification.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a nucleic acid sequence corresponding to 1317 base pairs of a gene encoding a phytase hydrolyzing enzyme derived from *Penicillium chrysogenum.*

Fig. 2 shows an amino acid sequence of a phytase enzyme encoded by the nucleic acid sequence of Figure 1.

Fig. 3 shows an amino acid sequence of a mature chimeric phytase enzyme, produced via the expression of a nucleic acid sequence encoding an Aspergillus signal sequence, linked to a nucleic acid sequence encoding a phytase hydrolyzing enzyme derived from *Penicillium chrysogenum.*

Fig. 4 shows a nucleic acid sequence corresponding to 1299 base pairs of a gene encoding a phytase hydrolyzing enzyme derived from *Fusarium javanicum.*

Fig. 5 shows an amino acid sequence of a phytase enzyme encoded by the nucleic acid sequence of Figure 4.

Fig. 6 shows an amino acid sequence of a mature chimeric phytase enzyme, produced via the expression of a nucleic acid sequence encoding an Aspergillus signal sequence, linked to a nucleic acid sequence encoding a phytase hydrolyzing enzyme derived from *Fusarium javanicum.*

Figure 7 shows a nucleic acid sequence corresponding to 224 base pairs of a gene encoding a phytase hydrolyzing enzyme derived from *Fusarium vertisillibodes.*

Fig. 8 shows an amino acid sequence of a phytase enzyme encoded by the nucleic acid sequence of Figure 7.

Figure 9 shows a nucleic acid sequence corresponding to 224 base pairs of a gene encoding a phytase hydrolyzing enzyme derived from *Humicola grisea*.

Fig. 10 shows an amino acid sequence of a phytase enzyme encoded by the nucleic acid sequence of Figure 9.

Fig. 11 shows an amino acid sequence of a mature chimeric phytase enzyme, produced via the expression of a nucleic acid sequence encoding an Aspergillus signal sequence, linked to a nucleic acid sequence encoding a phytase hydrolyzing enzyme derived from *Humicola grisea*.

Figure 12 shows a nucleic acid sequence corresponding to 192 base pairs of a gene fragment encoding the amino end, including a signal sequence, for a phytase hydrolyzing enzyme derived from *Aspergillus niger*. This sequence includes an ATG start codon at the 5□ end and an intron extending from residues 46 to 147.

Fig. 13 shows an amino acid sequence of the amino end, including a signal sequence, of a phytase enzyme encoded by the nucleic acid sequence of Figure 12.

Figures 14A-14D show alignments of amino acid sequences disclosed herein with published amino acid sequences of known phytase enzymes. Figure 14A shows a GAP alignment, as further described below, of the amino acid sequence of a mature (i.e., lacking the signal sequence corresponding to amino acids 1-23) phytase from A. niger (accession number P34752, 444 amino acids; top row of each pair) and a phytase derived from P. chrysogenum (Figure 3, 446 amino acids; bottom row of each pair). Straight lines between the aligned sequences indicate identical residues, dots between the aligned sequences indicate similar residues. The two sequences show 65% identity, 70% similarity.

Figure 14B shows a BLAST alignment (TBLASTN 2.0.5 program), as further described below, of residues 1-445 of the amino acid sequence from P. chrysogenum (P.c.) disclosed in Figure 3 and the amino acid sequence determined from nucleic acid residues 407 to 1732 of a cDNA sequence encoding an *Aspergillus fumigatus* (A.f.) phytase (accession number U59804). Letters between the aligned sequences indicate identical amino acid residues, pluses indicate similar residues. These portions of the two sequences show 62% identity, 75% similarity.

Figure 14C shows a BLAST alignment of residues 4-445 of the amino acid sequence from *P. chrysogenum* (P.c.) disclosed in Figure 3 and the amino acid sequence determined from nucleic acid residues 411 to 1730 of a cDNA sequence encoding an *Aspergillus terreus* (A.t.) phytase (accession number U60412). These portions of the two sequences show 60% identity, 73% similarity.

Figure 14D shows a BLAST alignment of residues 7-445 of the amino acid sequence from *P. chrysogenum* (P.c.) disclosed in Figure 3 and the amino acid sequence determined from nucleic acid residues 293 to 1594 of a cDNA sequence encoding an *Emericella nidulans* (*Aspergillus nidulans*; A.t.) phytase (accession number U59803). These portions of the two sequences show 60% identity, 75% similarity.

Figures 15A-15C show alignments of amino acid sequences disclosed herein with published amino acid sequences of known phytase enzymes. Figure 15A shows a GAP alignment of the 444 amino acid sequence of a mature phytase from *A. niger* (accession number P34752) and a 440 amino acid phytase sequence derived from *F. javanicum* (disclosed in Figure 6). The two sequences show 50% identity, 56% similarity.

Figure 15B shows a GAP alignment of a 440 amino acid phytase sequence derived from F. javanicum (disclosed in Figure 6) and the 463 amino acid sequence of a phytase from Emericella nidulans (E.n.) (Aspergillus nidulans; accession number U59803).. The two sequences show 52% identity, 60% similarity.

Figure 15C shows a BLAST alignment of residues 7-438 of a phytase amino acid sequence from *F. javanicum* (F.j.) disclosed in Figure 6 and the amino acid sequence determined from nucleic acid residues 2379 to 3719 of a cDNA sequence encoding an *Myceliopthora thermophila* (M.t.) phytase (accession number U59806). These portions of the two sequences show 52% identity, 68% similarity.

Figures 16A-16C show alignments of amino acid sequences disclosed herein with published amino acid sequences of known phytase enzymes. Figure 16A shows a GAP alignment of a 487 amino acid sequence of a phytase from *M. thermophila* (accession number U59806) and a 449 amino acid phytase sequence derived from *H. grisea* (disclosed in Figure 11). The two sequences show 66% identity, 72% similarity.

Figure 16B shows a GAP alignment of a 449 amino acid phytase sequence derived from H. grisea (disclosed in Figure 11) and the 444 amino acid sequence of a mature phytase from A. niger (accession number P34752). The two sequences show 51% identity, 59% similarity.

Figure 16C shows a BLAST alignment of residues 8-448 of a phytase amino acid sequence from H. grisea (H.g) disclosed in Figure 11 and the amino acid sequence determined from nucleic acid residues 2340 to 3722 of a cDNA sequence encoding an *Myceliopthora thermophila* (M.t.) phytase (accession number U59806). These portions of the two sequences show 65% identity, 74% similarity.

Figures 17A and 17B show the DNA encoding and amino acid sequence of a phytase from *E. desertorum*. Figure 17A shows the sequence of genomic DNA encoding the gene for the phytase. Lower case lettering depicts a putative intron. Figure 17B shows the putative amino acid sequence encoded by the *E. desertorum* phytase gene.

Figures 18A-18C show the genomic DNA sequence encoding a phytase from *F. javanicum*. The putative amino acid sequence of the phytase is indicated below the DNA sequence. A putative intron is indicated below the DNA sequence by a horizontal line. Box arrows below the DNA sequence indicate sequences of primers useful for amplifying the gene. Restriction sites are indicated above the sequence in bold.

Figures 19A-19C show the genomic DNA sequence encoding a phytase from *P. chrysogenum.* The putative amino acid sequence of the phytase is indicated below the DNA sequence. A putative intron is indicated below the DNA sequence by a horizontal box. Box arrows below the DNA sequence indicate sequences of primers useful for amplifying the gene. Restriction sites are indicated above the sequence in bold.

Figures 20A-20C show the genomic DNA sequence encoding a phytase from *H. grisea*. The putative amino acid sequence of the phytase is indicated below the DNA sequence. A putative intron is indicated below the DNA sequence by a horizontal line. Restriction sites are indicated above the sequence in bold.

Figure 21 shows a partial genomic DNA sequence encoding a phytase from *F. vertisillibodes*. The putative amino acid sequence of the phytase is indicated below the DNA sequence. A putative intron is indicated below the DNA sequence by a horizontal line. Restriction sites are indicated above the sequence in bold.

Figure 22 shows the DNA sequence of a gene encoding a phytase from *E. desertorum* obtained using the procedure described in Example 1. Below the DNA sequence is indicated the putative amino acid sequence of the phytase. The arrows above the DNA sequence indicate ligation sequences (primers GSP1rev:fyt037 and GSP2rev:fyt036) used to obtain upstream sequences of the gene (*see* Fig. 17A).

Figure 23 shows the DNA sequence of a gene encoding a phytase from *F. javanicum* obtained using the procedure described in Example 1. The putative amino acid sequence of the phytase is indicated below the DNA sequence. The arrows above the DNA sequence indicate ligation sequences (primers GSP1rev:fyt039 and GSP2rev:fyt038) used to obtain upstream sequences of the gene (*see* Figs. 18A-18B).

Figure 24 shows results of expression of recombinantly produced phytase described herein. Figure 24A shows an isoelectric focusing (IEF) gel stained with Comassie blue. This gel sows protein present in the supernatant from cultures of *Aspergillus niger* which had been transformed with a vector encoding chimeric phytase from *P. chrysogenum* (lanes 5-8) and *F. javanicum* (lanes 9-12), as described in Examples 3 and 4. The transformed host cells were grown under conditions designed to facilitate expression of the proteins encoded in the expression vector. Lanes 1-3 (as marked) have nothing in them. Lane 4 has fermentation broth from an *A. niger* transformed with the same vector as used for the F. javanicum and P. chrysogenum phytases, but comprising a nucleic acid sequence encoding the native A. niger phytase enzyme. Lanes 5-8 have fermentation broth from four different clones transformed with vector comprising the P. chrysogenum chymeric phytase, selected for their apparent high (lanes 5 and 8), moderate (lane 7) and low (lane 6) phytase activity, as determined in a preliminary test. Lanes 9-12 have fermentation broth from four different clones transformed with vector comprising the F. javanicum chymeric phytase, selected for their apparent high (lane 11), moderate (lanes 9 and 10) and low (lane 12) phytase activity, as determined in a preliminary test. The Comassie stained gel indicates novel protein bands corresponding to phytase activity, as shown in the zymogram described in Figure 24B, for each of the transformant types and no such novel protein bands for clones in which no activity was found.

Figure 24B shows a zymogram produced as an overlay of the IEF gel described in Fig. 24A, made prior to staining of the gel, showing the phosphatase activity of the proteins in the gel. The zymogram indicates phytase activity associated with the novel Comassie stained bands from the hosts transformed with chimeric phytase.

DETAILED DESCRIPTION OF THE INVENTION

I. Definitions

Unless defined otherwise herein, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Singleton, *et al.*, DICTIONARY OF MICROBIOLOGY AND MOLECULAR BIOLOGY, 2D ED., John Wiley and Sons, New York (1994), and Hale & Marham, THE HARPER COLLINS DICTIONARY OF BIOLOGY, Harper Perennial, NY (1991) provide one of skill with a general dictionary of many of the terms used in this invention. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. Numeric ranges are inclusive of the numbers defining the range. Unless otherwise indicated, nucleic acid sequences are written left to right in 5' to 3' orientation; amino acid sequences are written left to right in amino to carboxy orientation, respectively. The headings provided herein are not limitations of the various aspects or embodiments of the invention which can be had by reference to the specification as a whole. Accordingly, the terms defined immediately below are more fully defined by reference to the specification as a whole.

"Protein", as used herein, includes proteins, polypeptides, and peptides. As will be appreciated by those in the art, the nucleic acid sequences of the invention, as defined below and further described herein, can be used to generate protein sequences.

As used herein, the term "phytase" or "phytase activity" refers to a protein or polypeptide which is capable of catalyzing the hydrolysis of phytate to (1) myo-inositol and/or (2) mono-, di-, tri-, tetra- and/or penta-phosphates thereof and (3) inorganic phosphate. For example, enzymes having catalytic activity as defined in Enzyme Commission EC number 3.1.3.8, or EC number 3.1.3.26.

In the broadest sense, by "nucleic acid sequence" , "polynucleotide" or "oligonucleotide" or grammatical equivalents herein means at least two nucleotides covalently linked together. A nucleic acid sequence of the present invention will generally contain phosphodiester bonds, although in some cases, as outlined below, nucleic acid sequence analogs are included that may have alternate backbones, comprising, for example, phosphoramidate (Beaucage et al., Tetrahedron 49(10):1925 (1993) and references therein; Letsinger, J. Org. Chem. 35:3800 (1970); Sprinzl et al., Eur. J. Biochem. 81:579 (1977); Letsinger et al., Nucl. Acids Res. 14:3487 (1986); Sawai et al, Chem. Lett. 805 (1984), Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); and Pauwels et al., Chemica Scripta 26:141 91986)), phosphorothioate (Mag et al., Nucleic Acids Res. 19:1437 (1991); and U.S. Patent No. 5,644,048), phosphorodithioate (Briu et al., J. Am. Chem. Soc. 111:2321 (1989)), O-methylphophoroamidite linkages (see Eckstein, Oligonucleotides and Analogues: A Practical Approach, Oxford University Press), and peptide nucleic acid backbones and linkages (see Egholm, J. Am. Chem. Soc. 114:1895 (1992); Meier et al., Chem. Int. Ed. Engl. 31:1008 (1992); Nielsen, Nature, 365:566 (1993); Carlsson et al., Nature 380:207 (1996), all of which are incorporated by reference). Other analog nucleic acids include those with positive backbones (Denpcy et al., Proc. Natl. Acad. Sci. USA 92:6097 (1995); non-ionic backbones (U.S. Patent Nos. 5,386,023, 5,637,684, 5,602,240, 5,216,141 and 4,469,863; Kiedrowshi et al., Angew. Chem. Intl. Ed. English 30:423 (1991); Letsinger et al., J. Am. Chem. Soc. 110:4470 (1988); Letsinger et al., Nucleoside & Nucleotide 13:1597 (1994); Chapters 2 and 3, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook; Mesmaeker et al., Bioorganic & Medicinal Chem. Lett. 4:395 (1994); Jeffs et al., J. Biomolecular NMR 34:17 (1994); Tetrahedron Lett. 37:743 (1996)) and non-ribose backbones, including those described in U.S. Patent Nos. 5,235,033 and 5,034,506, and Chapters 6 and 7, ASC Symposium Series 580, "Carbohydrate Modifications in Antisense Research", Ed. Y.S. Sanghui and P. Dan Cook. Nucleic acids containing one or more carbocyclic sugars are also included within one definition of nucleic acids (see Jenkins et al., Chem. Soc. Rev. (1995) pp169-176). Several nucleic acid analogs are described in Rawls, C & E News June 2, 1997 page 35. All of these references are hereby expressly incorporated by reference. These modifications of the ribose-phosphate backbone may be done for a variety of reasons, for example to increase the stability and half-life of such molecules in physiological or food processing environments.

As will be appreciated by those in the art, all of these nucleic acid analogs may find use in the present invention. In addition, mixtures of naturally occurring nucleic acids and analogs can be made; alternatively, mixtures of different nucleic acid analogs, and mixtures of naturally occurring nucleic acids and analogs may be made.

Particularly preferred are peptide nucleic acids (PNA) which includes peptide nucleic acid analogs. These backbones are substantially non-ionic under neutral conditions, in contrast to the highly charged phosphodiester backbone of naturally occurring nucleic acids. This results in two advantages. First, the PNA backbone exhibits improved hybridization kinetics. PNAs have larger changes in the melting temperature (Tm) for mismatched versus perfectly matched basepairs. DNA and RNA typically exhibit a 2-4□C drop in Tm for an internal mismatch. With the non-ionic PNA backbone, the drop is closer to 7-9□C. Similarly, due to their non-ionic nature, hybridization of the bases attached to these backbones is relatively insensitive to salt concentration. In addition, PNAs are not degraded by cellular enzymes, and thus can be more stable.

The nucleic acid sequences may be single stranded or double stranded, as specified, or contain portions of both double stranded or single stranded sequence. As will be appreciated by those in the art, the depiction of a single strand ("Watson") also defines the sequence of the other strand ("Crick"); thus the sequences described herein also includes the complement of the sequence. The nucleic acid sequence may be DNA, both genomic and cDNA, RNA or a hybrid, where the nucleic acid sequence contains any combination of deoxyribo- and ribo-nucleotides, and any combination of bases, including uracil, adenine, thymine, cytosine, guanine, inosine, xanthine hypoxanthine, isocytosine, isoguanine, etc. As used herein, the term "nucleoside" includes nucleotides and nucleoside and nucleotide analogs, and modified nucleosides such as amino modified nucleosides. In addition, "nucleoside" includes non-naturally occurring analog structures. Thus for example the individual units of a peptide nucleic acid sequence, each containing a base, are referred to herein as a nucleoside.

The term "identical" in the context of two nucleic acid sequences or polypeptide sequences refers to the residues in the two sequences that are the same when aligned for maximum correspondence, as measured using one of the following sequence comparison or analysis algorithms.

"Optimal alignment" is defined as an alignment giving the highest percent identity score. Such alignment can be performed using a variety of commercially available sequence analysis programs, such as the local alignment program LALIGN using a ktup of 1, default parameters and the default PAM. A preferred alignment is the pairwise alignment performed using the CLUSTAL-W program in MACVECTOR, operated in "slow" alignment mode using default parameters, including an open gap penalty of 10.0, an extend gap penalty of 0.1, and a BLOSUM30 similarity matrix. If a gap needs to be inserted into a first sequence to optimally align it with a second sequence, the percent identity is calculated using only the residues that are paired with a corresponding amino acid residue (i.e., the calculation does not consider residues in the second sequences that are in the "gap" of the first sequence).

Optimal alignment of sequences for comparison can also be conducted, *e.g.,* by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math. 2*:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol. 48*:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA 85*:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by visual inspection.

"Percent sequence identity", with respect to two amino acid or polynucleotide sequences, refers to the percentage of residues that are identical in the two sequences when the sequences are optimally aligned. Thus, 80% amino acid sequence identity means that 80% of the amino acids in two optimally aligned polypeptide sequences are identical.
Percent identity can be determined, for example, by a direct comparison of the sequence information between two molecules by aligning the sequences, counting the exact number of matches between the two aligned sequences, dividing by the length of the shorter sequence, and multiplying the result by 100. Readily available computer programs can be used to aid in the analysis, such as ALIGN, Dayhoff, M.O. in "Atlas of Protein Sequence and Structure", M.O. Dayhoff ed.,* 5 Suppl. 3:353-358, National Biomedical Research Foundation, Washington, DC, which adapts the local homology algorithm of Smith and Waterman (1981) Advances in Appl. Math. 2:482-489 for peptide analysis. Programs for determining nucleotide sequence identity are available in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, WI) for example, the BESTFIT, FASTA and GAP programs, which also rely on the Smith and Waterman algorithm. These programs are readily utilized with the default parameters recommended by the manufacturer and described in the Wisconsin Sequence Analysis Package referred to above.

An example of an algorithm that is suitable for determining sequence similarity is the BLAST algorithm, which is described in Altschul, *et al., J. Mol. Biol. 215*:403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology

Information (http://www.ncbi.nlm.nih.gov/). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence that either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. These initial neighborhood word hits act as starting points to find longer HSPs containing them. The word hits are expanded in both directions along each of the two sequences being compared for as far as the cumulative alignment score can be increased. Extension of the word hits is stopped when: the cumulative alignment score falls off by the quantity X from a maximum achieved value; the cumulative score goes to zero or below; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a wordlength (W) of 11, the BLOSUM62 scoring matrix (*see* Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA 89*:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M'5, N'-4, and a comparison of both strands.

The BLAST algorithm then performs a statistical analysis of the similarity between two sequences (*see, e.g.,* Karlin & Altschul, *Proc. Nat'l. Acad. Sci. USA 90*:5873-5787 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P(N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. For example, a nucleic acid sequence is considered similar to a phytase nucleic acid sequence of this invention if the smallest sum probability in a comparison of the test nucleic acid sequence to a phytase nucleic acid sequence is less than about 0.1, more preferably less than about 0.01, and most preferably less than about 0.001. Where the test nucleic acid sequence encodes a phytase polypeptide, it is considered similar to a specified phytase nucleic acid sequence if the comparison results in a smallest sum probability of less than about 0.5, and more preferably less than about 0.2.

The phrase "substantially identical" in the context of two nucleic acid sequences or polypeptides thus typically means that a polynucleotide or polypeptide comprises a sequence that has at least 60% sequence identity, preferably at least 80%, more preferably at least 90% and most preferably at least 95%, compared to a reference sequence using the programs described above (e.g., BLAST, ALIGN, CLUSTAL) using standard parameters. One indication that two polypeptides are substantially identical is that the first polypeptide is immunologically cross-reactive with the second polypeptide. Typically, polypeptides that differ by conservative amino acid substitutions are immunologically cross-reactive. Thus, a polypeptide is substantially identical to a second polypeptide, for example, where the two peptides differ only by a conservative substitution. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions (e.g., within a range of medium to high stringency).

"Hybridization" includes any process by which a strand of a nucleic acid sequence joins with a second nucleic acid sequence strand through base-pairing. Thus, strictly speaking, the term refers to the ability of a target sequence to bind to a test sequence, or vice-versa.

"Hybridization conditions" are typically classified by degree of "stringency" of the conditions under which hybridization is measured. The degree of stringency can be based, for example, on the calculated (estimated) melting temperature (Tm) of the nucleic acid sequence binding complex or probe. Calculation of Tm is well known in the art (*see, e.g.* page 9.50-9.51 of Sambrook (1989), below). For example, "maximum stringency" typically occurs at about Tm-5°C (5° below the Tm of the probe); "high stringency" at about 5-10° below the Tm; "intermediate stringency" at about 10-20° below the Tm of the probe; and "low stringency" at about 20-25° below the Tm. In general, hybridization conditions are carried out under high ionic strength conditions, for example, using 6XSSC or 6XSSPE. Under high stringency conditions, hybridization is followed by two washes with low salt solution, for example 0.5XSSC, at the calculated temperature. Under medium stringency conditions, hybridization is followed by two washes with medium salt solution, for example 2XSSC. Under low stringency conditions, hybridization is followed by two washes with high salt solution, for example 6XSSC. Functionally, maximum stringency conditions may be used to identify nucleic acid sequences having strict identity or near-strict identity with the hybridization probe; while high stringency conditions are used to identify nucleic acid sequences having about 80% or more sequence identity with the probe.

For applications requiring high selectivity, one will typically desire to employ relatively stringent conditions to form the hybrids, e.g., one will select relatively high temperature conditions. Hybridization conditions, including moderate stringency and high stringency, are provided in Sambrook et al.,Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press (1989); Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 3d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001) incorporated herein by reference.

The term “complementary”, in the context of a nucleic acid sequence, means a nucleic acid sequence having a sequence relationship to a second nucleic acid sequence such that there is perfect alignment of Watson-Crick base pairs along the entire length of both nucleic acid sequences.

The term "isolated" or "purified" means that a material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, the material is said to be "purified" when it is present in a particular composition in a higher or lower concentration than exists in a naturally occurring or wild type organism or in combination with components not normally present upon expression from a naturally occurring or wild type organism. For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector, and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment. A nucleic acid sequence or protein is said to be purified, for example, if it gives rise to essentially one band in an electrophoretic gel.

The present invention provides for the production of recombinant nucleic acids and proteins. By "recombinant" and grammatical equivalents thereof is meant produced using recombinant technology, whereby novel nucleic acids are made (recombinant nucleic acids) and proteins are produced therefrom (recombinant proteins). Such techniques are well known in the art and many are described in great detail herein. In a broad sense, a recombinant nucleic acid sequence may be any nucleic acid sequence not in its naturally occurring form, whether it be a sequence isolated from its naturally occurring adjoining sequence, or combined with other sequences with which it was not joined in nature to form a new nucleic acid sequence, such as in a vector. Recombinant nucleic acid sequences also include those that are produced from recombinant nucleic acid sequences, for example complementary sequences made through polymerization, additional copies made though replication, or RNA transcribed from recombinant DNA. Recombinant protein is protein produced by translation of recombinant nucleic acid sequences.

As used herein in referring to phytate hydrolyzing enzymes (phytases), the term "derived from" is intended not only to indicate a phytase produced or producible by a strain of the organism in question, but also a phytase encoded by a DNA sequence isolated from such strain and produced in a host organism containing such DNA sequence. Additionally, the term is intended to indicate a phytase which is encoded by a DNA sequence of synthetic and/or cDNA origin and which has the identifying characteristics of the phytase in question. To exemplify, "phytases derived from *Penicillium*" refers to those enzymes having phytase activity which are naturally-produced by *Penicillium*, as well as to phytases like those produced by *Penicillium* sources but which through the use of genetic engineering techniques are produced by non-*Penicillium* organisms transformed with a nucleic acid sequence encoding said phytases. The present invention encompasses phytate hydrolyzing enzymes that are equivalent to those that are derived from the particular microbial strain mentioned. Being "equivalent," in this context, means that the phytate hydrolyzing enzymes are encoded by a polynucleotide capable of hybridizing to the polynucleotide having the sequence as shown in any one of Figures 1, 4, 7 and 9 under conditions of medium to high stringency. Being equivalent means that the phytate hydrolyzing enzyme comprises at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to the phytate hydrolyzing enzyme having the amino acid sequence disclosed in one of Figures 2, 3, 5, 6, 8, 10 and 11. The present invention also encompasses mutants, variants and derivatives of the phytate hydrolyzing enzymes of the present invention as long as the mutant, variant or derivative phytate hydrolyzing enzyme is able to retain at least one characteristic activity of the naturally occurring phytate hydrolyzing enzyme. As used herein, the term "mutants and variants", when referring to phytate hydrolyzing enzymes, refers to phytate hydrolyzing enzymes obtained by alteration of the naturally occurring amino acid sequence and/or structure thereof, such as by alteration of the DNA nucleotide sequence of the structural gene and/or by direct substitution and/or alteration of the amino acid sequence and/or structure of the phytate hydrolyzing enzyme.

The term "derivative" or "functional derivative" as it relates to phytase is used herein to indicate a derivative of phytase which has the functional characteristics of phytase of the present invention. Functional derivatives of phytase encompass naturally occurring, synthetically or recombinantly produced peptides or peptide fragments, mutants or variants which may have one or more amino acid deletions, substitutions or insertions which have the general characteristics of the phytase of the present invention.

The term "functional derivative" as it relates to nucleic acid sequences encoding phytase is used throughout the specification to indicate a derivative of a nucleic acid sequence which has the functional characteristics of a nucleic acid sequence which encodes phytase. Functional derivatives of a nucleic acid sequence which encode phytase of the present invention encompass naturally occurring, synthetically or recombinantly produced nucleic acid sequences or fragments, mutants or variants thereof which may have one or more nucleic acid deletions, substitutions or insertions and encode phytase characteristic of the present invention. Variants of nucleic acid sequences encoding phytase according to the invention include alleles and variants based on the degeneracy of the genetic code known in the art. Mutants of nucleic acid sequences encoding phytase according to the invention include mutants produced via site-directed mutagenesis techniques (see for example, Botstein, D. and Shortle, D., 1985, Science 229:1193-1201 and Myers, R.M., Lerman, L.S., and Maniatis, T., 1985, Science 229: 242-247), error-prone PCR (see for example, Leung, D.W., Chen, E., and Goeddel, D.V., 1989, Technique 1: 11-15; Eckert, K.A. and Kunkel, T.A., 1991, PCR Methods Applic. 1: 17-24; and Cadwell, R.C. and Joyce, G.F., 1992, PCR Methods Applic. 2: 28-33) and/or chemical-induced mutagenesis techniques known in the art (see for example, Elander, R.P., Microbial screening, Selection and Strain Improvement, in Basic Biotechnology, J. Bullock and B. Kristiansen Eds., Academic Press, New York, 1987, 217).

“Expression vector” means a DNA construct comprising a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of the DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable ribosome-binding sites on the mRNA, and sequences which control termination of transcription and translation. Different cell types are preferably used with different expression vectors. A preferred promoter for vectors used in *Bacillus subtilis* is the AprE promoter; a preferred promoter used in *E. coli* is the Lac promoter and a preferred promoter used in *Aspergillus niger* is *glaA*. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, under suitable conditions, integrate into the genome itself.

In the present specification, plasmid and vector are sometimes used interchangeably. However, the invention is intended to include other forms of expression vectors which serve equivalent functions and which are, or become, known in the art. Thus, a wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, may consist of segments of chromosomal, non-chromosomal and synthetic DNA sequences such as various known derivatives of SV40 and known bacterial plasmids, e.g., plasmids from *E. coli* including col E1, pCR1, pBR322, pMb9, pUC 19 and their derivatives, wider host range plasmids, e.g., RP4, phage DNAs e.g., the numerous derivatives of phage l, e.g., NM989, and other DNA phages, e.g., M13 and filamentous single stranded DNA phages, yeast plasmids such as the 2m plasmid or derivatives thereof, vectors useful in eukaryotic cells, such as vectors useful in animal cells and vectors derived from combinations of plasmids and phage DNAs, such as plasmids which have been modified to employ phage DNA or other expression control sequences.

Expression techniques using the expression vectors of the present invention are known in the art and are described generally in, for example, Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Press (1989); Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 3d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001). Often, such expression vectors including the DNA sequences of the invention are transformed into a unicellular host by direct insertion into the genome of a particular species through an integration event (see e.g., Bennett & Lasure, More Gene Manipulations in Fungi, Academic Press, San Diego, pp. 70-76 (1991) and articles cited therein describing targeted genomic insertion in fungal hosts, incorporated herein by reference).

“Host strain” or “host cell” means a suitable host for an expression vector comprising DNA according to the present invention. Host cells useful in the present invention are generally procaryotic or eucaryotic hosts, including any transformable microorganism in which expression can be achieved. For example, host strains can be *Bacillus subtilis, Escherichia coli, Trichoderma longibrachiatum, Saccharomyces cerevisiae*, *Aspergillus niger*, and *Aspergillus nidulans.* Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of both replicating vectors encoding phytase and its variants (mutants) or expressing the desired peptide product.

Examples of appropriate expression hosts include: bacterial cells, such as E. coli, Streptomyces, Salmonella typhimurium; fungal cells, such as Aspergillus and Penicillium; insect cells such as Drosophila and Spodoptera Sf9; animal cells such as CHO, COS, HEK 293 or Bowes melanoma; plant cells, etc. The selection of an appropriate host is deemed to be within the scope of those skilled in the art from the teachings herein. It should be noted that the invention is not limited by the particular host cells employed.

II. Phytase enzymes and nucleic acid sequences encoding phytase enzymes

One aspect of the present invention provides proteins or polypeptides which are capable of catalyzing the hydrolysis of phytate and releasing inorganic phosphate; for example, enzymes having catalytic activity as defined in Enzyme Commission EC number 3.1.3.8, or in EC number 3.1.3.26. In one preferred embodiment, the invention provides a so-called 3-phytase. The present invention additionally encompasses polynucleotides (e.g., DNA) which encode such phytate hydrolyzing proteins or polypeptides.

Preferably, the phytase and/or polynucleotides encoding the phytase according to the present invention is derived from a fungus, preferably from an anaerobic fungus or thermophilic fungus and most preferably from *Penicillium sp.*, e.g., *Penicillium chrysogenum*, *Fusarium sp.*, e.g., *Fusarium javanicum* or *Fusarium vertisillibodes* or *Humicola sp.*, e.g. *Humicola grisea*. Thus, it is contemplated that the phytase or the DNA encoding the phytase according to the invention can be derived from *Absidia sp.; Acremonium sp.; Actinomycetes sp.; Agaricus sp.; Anaeromyces sp.; Aspergillus sp.*, including *A. auculeatus, A. awamori, A. flavus, A. foetidus, A. fumaricus, A. fumigatus, A. nidulans, A. niger, A. oryzae, A. terreus* and *A. versicolor; Aeurobasidium sp.; Cephalosporum sp.; Chaetomium sp.; Coprinus sp.; Dactyllum sp.; Fusarium sp.,* including *F. conglomerans, F. decemcellulare, F. javanicum, F. lini, F. oxysporum* and *F. solani; Gliocladium sp.; Humicola sp.,* including *H. insolens* and *H. lanuginosa; Mucor sp.; Myceliopthora* ssp., including *M. thermophila*; *Neurospora sp.,* including *N. crassa* and *N. sitophila; Neocallimastix sp.; Orpinomyces sp.; Penicillium spp; Phanerochaete sp.; Phlebia sp.; Piromyces sp.; Pseudomonas sp.; Rhizopus sp.; Schizophyllum sp.; Streptomyces spp; Trametes sp.;* and *Trichoderma sp.,* including *T. reesei, T. longibrachiatum* and *T. viride;* and *Zygorhynchus sp.* Similarly, it is envisioned that a phytase and/or DNA encoding a phytase as described herein may be derived from bacteria such as *Streptomyces* sp., including *S. olivochromogenes*; specifically fiber degrading ruminal bacteria such as *Fibrobacter succinogenes;* and in yeast including *Candida torresii; C. parapsllosis; C. sake; C. zeylanoides; Pichia minuta; Rhodotorula glutinis; R. mucilaginosa;* and *Sporobolomyces holsaticus.*

In one preferred embodiment, the phytase and/or polynucleotides encoding the phytase according to the present invention is/are derived from (i) a grain-spoilage fungus, such as *Penicillium hordei, Penicillium piceum*, or *Penicillium brevi-compactum;* or (ii) an ectomycorrhizal fungus associated with tree roots, e.g., *Laccaria laccata, Laccaria rufus, Paxillus involutus, Hebeloma crustuliniforme, Amanita rubescens,* or *Amanita muscaria.* According to a preferred embodiment, the phytase and/or polynucleotide encoding the phytase of the present invention is in a purified form, i.e., present in a particular composition in a higher or lower concentration than exists in a naturally occurring or wild type organism or in combination with components not normally present upon expression from a naturally occurring or wild type organism.

The invention encompasses phytate hydrolyzing proteins and peptides comprising at least 55% identity, preferably at least 60% identity, more preferably at least 65% identity, still more preferably at least 70% identity, yet more preferably at least 75% identity, even more preferably at least 80% identity, again more preferably at least 85% identity, yet again more preferably at least 90% identity, and most preferably at least 95% up to about 100% identity to the phytate hydrolyzing enzyme having the amino acid sequence disclosed in Figures 2, 3, 5, 6, 8, 10 or 11.

The invention further encompasses polynucleotides, e.g., DNA, which encode phytate hydrolyzing enzymes derived from fungal sources, such as *Penicillium sp.*, which polynucleotides include a sequence having at least 65% identity, at least 70% identity, at least 75% identity, at least 80% identity, at least 85% identity, at least 90% identity and at least 95% identity to the polynucleotide sequence disclosed in any one of Figures 1, 4, 7 and 9, as long as the enzyme encoded by the polynucleotide is capable of catalyzing the hydrolysis of phytate and releasing inorganic phosphate. In a preferred embodiment, the polynucleotide encoding the phytate hydrolyzing enzyme has the polynucleotide sequence as shown in any one of Figures 1, 4, 7 and 9, or is capable of hybridizing to the polynucleotide sequence as shown in any one of Figures 1, 4, 7 and 9 or its complement, or is complementary to the polynucleotide sequence as shown in any one of Figures 1, 4, 7 and 9. As will be understood by the skilled artisan, due to the degeneracy of the genetic code, a variety of polynucleotides can encode the phytate hydrolyzing enzyme disclosed in any one of Figures 2, 3, 5, 6, 8, 10 and 11. The present invention encompasses all such polynucleotides.

III. Obtaining polynucleotides encoding a phytate hydrolyzing enzyme

The nucleic acid sequence encoding a phytate hydrolyzing enzyme may be obtained by standard procedures known in the art from, for example, cloned DNA (*e.g.*, a DNA "library"), by chemical synthesis, by cDNA cloning, by PCR, or by the cloning of genomic DNA, or fragments thereof, purified from a desired cell, such as a fungal species (*See*, for example, Sambrook *et al.*, 2001, Molecular Cloning, A Laboratory Manual, 3d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York; Glover, DM and Hames, BD (Eds.), 1995, DNA Cloning 1: A Practical Approach and DNA Cloning 2: A Practical Approach, Oxford University Press, Oxford). Nucleic acid sequences derived from genomic DNA may contain regulatory regions in addition to coding regions.

In the molecular cloning of the gene from genomic DNA, DNA fragments are generated, some of which will comprise at least a portion of the desired gene. The DNA may be cleaved at specific sites using various restriction enzymes. Alternatively, one may use DNAse in the presence of manganese to fragment the DNA, or the DNA can be physically sheared, as for example, by sonication. The linear DNA fragments can then be separated according to size by standard techniques, including but not limited to, agarose and polyacrylamide gel electrophoresis, PCR and column chromatography.

Once nucleic acid sequence fragments are generated, identification of the specific DNA fragment encoding a phytate hydrolyzing enzyme may be accomplished in a number of ways. For example, a phytate hydrolyzing enzyme encoding gene of the present invention or its specific RNA, or a fragment thereof, such as a probe or primer, may be isolated and labeled and then used in hybridization assays to detect a generated gene. (Benton, W. and Davis, R., 1977, *Science 196*:180; Grunstein, M. and Hogness, D., 1975, *Proc. Natl. Acad. Sci. USA 72*:3961). Those DNA fragments sharing substantial sequence similarity to the probe will hybridize under medium to high stringency.

The present invention encompasses phytate hydrolyzing enzymes derived from fungal species (esp., *Penicillium*, *Fusarium* and *Humicola* species) which are identified through nucleic acid sequence hybridization techniques using one of the sequences disclosed in Figures 1, 4, 7 and 9, or a suitable portion or fragment thereof (e.g., at least about 10-15 contiguous nucleotides), as a probe or primer and screening nucleic acid sequences of either genomic or cDNA origin. Nucleic acid sequences encoding phytate hydrolyzing enzymes derived from fungal species and having at least 65% identity to the sequence of one of Figures 1, 4, 7 and 9 or a portion or fragment thereof can be detected by DNA-DNA or DNA-RNA hybridization or amplification using probes, portions or fragments of the disclosed sequences. Accordingly, the present invention provides a method for the detection of nucleic acid sequences encoding a phytate hydrolyzing enzyme encompassed by the present invention which comprises hybridizing part or all of a nucleic acid sequence of Figure 1, 4, 7 or 9 with a nucleic acid sequence of either genomic or cDNA origin.

Also included within the scope of the present invention are polynucleotide sequences that are capable of hybridizing to the nucleotide sequence disclosed in Figures 1, 4, 7 or 9 under conditions of medium to high stringency. In one embodiment, hybridization conditions are based on the melting temperature (Tm) of the nucleic acid sequence binding complex, as taught in Berger and Kimmel (1987, Guide to Molecular Cloning Techniques, Methods in Enzymology, Vol 152, Academic Press, San Diego CA) incorporated herein by reference, and confer a defined stringency. In this embodiment, "maximum stringency" typically occurs at about Tm-5°C (5°C below the Tm of the probe); "high stringency" at about 5°C to 10°C below Tm; "medium" or "intermediate stringency" at about 10°C to 20°C below Tm; and "low stringency" at about 20°C to 25°C below Tm. A maximum stringency hybridization can be used to identify or detect identical or near-identical polynucleotide sequences, while an intermediate or low stringency hybridization can be used to identify or detect polynucleotide sequence homologs.

The process of amplification as carried out in polymerase chain reaction (PCR) technologies is described in Dieffenbach CW and GS Dveksler (1995, PCR Primer, a Laboratory Manual, Cold Spring Harbor Press, Plainview NY). A nucleic acid sequence of at least about 10 nucleotides and as many as about 60 nucleotides from the sequences of Figure 1, 4, 7 or 9, preferably about 12 to 30 nucleotides, and more preferably about 25 nucleotides can be used as a probe or PCR primer.

A preferred method of isolating a nucleic acid sequence construct of the invention from a cDNA or genomic library is by use of polymerase chain reaction (PCR) using degenerate oligonucleotide probes prepared on the basis of the amino acid sequence of the protein having the amino acid sequence shown in any one of Figures 2, 3, 5, 6, 8, 10 and 11. For instance, the PCR may be carried out using the techniques described in US Patent No. 4,683,202.

In view of the above, it will be appreciated that the polynucleotide sequences provided in Figures 1, 4, 7 and 9 are useful for obtaining identical or homologous fragments of polynucleotides from other species, and particularly from fungi (e.g., the grain-spoilage fungi, or the Ectomycorrhizae) which encode enzymes having phytase activity.

IV. Obtaining derivative or variant phytate hydrolyzing enzymes

In one embodiment, the phytase proteins are derivative or variant phytase as compared to the wild-type sequence. That is, as outlined more fully below, the derivative phytase peptide will contain at least one amino acid substitution, deletion or insertion, with amino acid substitutions being particularly preferred. The amino acid substitution, insertion or deletion may occur at any residue within the phytase peptide.

Also included in an embodiment of phytase proteins of the present invention are amino acid sequence variants. These variants fall into one or more of three classes: substitutional, insertional or deletional variants. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the phytase protein, using cassette or PCR mutagenesis or other techniques well known in the art, to produce DNA encoding the variant, and thereafter expressing the DNA in recombinant cell culture as outlined above. However, variant phytase protein fragments having up to about 100-150 residues may be prepared by in vitro synthesis using established techniques. Amino acid sequence variants are characterized by the predetermined nature of the variation, a feature that sets them apart from naturally occurring allelic or interspecies variation of the phytase protein amino acid sequence. The variants typically exhibit the same qualitative biological activity as the naturally occurring analogue, although variants can also be selected which have modified characteristics as will be more fully outlined below.

While the site or region for introducing an amino acid sequence variation is predetermined, the mutation per se need not be predetermined. For example, in order to optimize the performance of a mutation at a given site, random mutagenesis may be conducted at the target codon or region and the expressed variants screened for the optimal combination of desired activity. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example, M13 primer mutagenesis and PCR mutagenesis. Screening of the mutants is done using assays of phytase protein activities.

Amino acid substitutions are typically of single residues; insertions usually will be on the order of from about 1 to 20 amino acids, although considerably larger insertions may be tolerated, and may occur internally or at either terminus of the encoded protein. Deletions range from about 1 to about 20 residues, although in some cases deletions may be much larger.

Substitutions, deletions, insertions or any combination thereof may be used to arrive at a final derivative. Generally these changes are done on a few amino acids to minimize the alteration of the molecule. However, larger changes may be tolerated in certain circumstances. When small alterations in the characteristics of the phytase are desired, substitutions are generally made in accordance with the following chart of conservative substitution residues:

Chart I

Original Residue	Exemplary Substitutions
Ala	Ser
Arg	Lys
Asn	Gln, His
Asp	Glu
Cys	Ser
Gln	Asn
Glu	Asp
Gly	Pro
His	Asn, Gln
Ile	Leu, Val
Leu	Ile, Val
Lys	Arg, Gln, Glu
Met	Leu, Ile
Phe	Met, Leu, Tyr
Ser	Thr
Thr	Ser
Trp	Tyr
Tyr	Trp, Phe
Val	Ile, Leu

Substantial changes in function or immunological identity are made by selecting substitutions that are less conservative than those shown in Chart I. For example, substitutions may be made which more significantly affect: the structure of the polypeptide backbone in the area of the alteration, for example the alpha-helical or beta-sheet structure; the charge or hydrophobicity of the molecule at the target site; or the bulk of the side chain. The substitutions which in general are expected to produce the greatest changes in the polypeptide's properties are those in which (a) a hydrophilic residue, e.g. seryl or threonyl is substituted for (or by) a hydrophobic residue, e.g. leucyl, isoleucyl, phenylalanyl, valyl or alanyl; (b) a cysteine or proline is substituted for (or by) any other residue; (c) a residue having an electropositive side chain, e.g. lysyl, arginyl, or histidyl, is substituted for (or by) an electronegative residue, e.g. glutamyl or aspartyl; or (d) a residue having a bulky side chain, e.g. phenylalanine, is substituted for (or by) one not having a side chain, e.g. glycine.

The variants typically exhibit the same qualitative biological activity and may elicit the same immune response as the naturally-occurring analogue, although variants also are selected to modify the characteristics of the phytase proteins as needed. Alternatively, the variant may be designed such that the biological activity of the phytase is altered. For example, glycosylation sites may be altered or removed. Such alterations may result in altered immunogenicity, as well.

Covalent modifications of phytase polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid residues of a phytase polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N-or C-terminal residues of a phytase polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking a phytase to another protein. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithio]propioimidate.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl, threonyl or tyrosyl residues, methylation of the α-amino groups of lysine, arginine, and histidine side chains [T.E. Creighton, Proteins: Structure and Molecular Properties, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the phytase polypeptide included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. "Altering the native glycosylation pattern" is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native phytase, and/or adding one or more glycosylation sites that are not present in the native polypeptide.

Addition of glycosylation sites to polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequence phytase polypeptide (for O-linked glycosylation sites). The phytase amino acid sequence may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the phytase polypeptide at preselected bases such that codons are generated that will translate into the desired amino acids.

Another means of increasing the number of carbohydrate moieties on the phytase polypeptide is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330 published 11 September 1987, and in Aplin and Wriston, Crit. Rev. Biochem., pp. 259-306 (1981).

Removal of carbohydrate moieties present on the phytase may be accomplished chemically or enzymatically or by mutational substitution of codons encoding for amino acid residues that serve as targets for glycosylation. Chemical deglycosylation techniques are known in the art and described, for instance, by Hakimuddin, et al., Arch. Biochem. Biophys., 259:52 (1987) and by Edge et al., Anal. Biochem., 118:131 (1981). Enzymatic cleavage of carbohydrate moieties on polypeptides can be achieved by the use of a variety of endo-and exo-glycosidases as described by Thotakura et al., Meth. Enzymol., 138:350 (1987).

Another type of covalent modification of phytase comprises linking the phytase polypeptide to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes, in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337.

Phytases of the present invention may also be modified to form chimeric molecules comprising a phytase polypeptide fused to another, heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of a phytase polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino-or carboxyl-terminus of the phytase polypeptide. The presence of such epitope-tagged forms of a phytase can be detected using an antibody against the tag polypeptide. Also, provision of the epitope tag enables the phytase to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitope tag. In preferred embodiment, the chimeric molecule may comprise a fusion of a phytase polypeptide with an initial sequence or signal polypeptide, such as a secretion signal, of a different phytase or other protein. The fusion may involve the addition of a sequence from a protein, such as a phytase, which is native to the host cell in which the phytase is being expressed. Specific examples of this are provided in the Examples section, below.

Various tag polypeptides and their respective antibodies are well known in the art. Examples include poly-histidine (poly-his) or poly-histidine-glycine (poly-his-gly) tags; the flu HA tag polypeptide and its antibody 12CA5 [Field et al., Mol. Cell. Biol., 8:2159-2165 (1988)]; the c-myc tag and the 8F9, 3C7, 6E10, G4, B7 and 9E10 antibodies thereto [Evan et al., Molecular and Cellular

Biology, 5:3610-3616 (1985)]; and the Herpes Simplex virus glycoprotein D (gD) tag and its antibody [Paborsky et al., Protein Engineering, 3(6):547-553 (1990)]. Other tag polypeptides include the Flag-peptide [Hopp et al., BioTechnology, 6:1204-1210 (1988)]; the KT3 epitope peptide [Martin et al., Science, 255:192-194 (1992)]; tubulin epitope peptide [Skinner et al., J. Biol. Chem., 266:15163-15166 (1991)]; and the T7 gene 10 protein peptide tag [Lutz-Freyermuth et al., Proc. Natl. Acad. Sci. USA, 87:6393-6397 (1990)].

Also included with the definition of phytase in one embodiment are other phytase proteins from other organisms, which are cloned and expressed as outlined below. Thus, probe or degenerate polymerase chain reaction (PCR) primer sequences may be used to find other related phytases from fungi or other organisms. As will be appreciated by those in the art, particularly useful probe and/or PCR primer sequences include the highly conserved amino acid sequences and the known binding or catalytic sequences. For example, the phosphate binding region of phytase produced in various fungi is highly conserved. As is generally known in the art, preferred PCR primers are from about 15 to about 35 nucleotides in length, with from about 20 to about 30 being preferred, and may contain inosine as needed. The conditions for the PCR reaction are well known in the art.

V. Expression and recovery of phytate hydrolyzing enzymes

The polynucleotide sequences of the present invention may be expressed by operatively linking them to an expression control sequence in an appropriate expression vector and employed in that expression vector to transform an appropriate host according to techniques well established in the art. The polypeptides produced on expression of the DNA sequences of this invention can be isolated from the fermentation of cell cultures and purified in a variety of ways according to well established techniques in the art. One of skill in the art is capable of selecting the most appropriate isolation and purification techniques.

More particularly, the present invention provides host cells, expression methods and systems for the production of phytate hydrolyzing enzymes derived from microorganisms, such as *Penicillium*, *Fusarium* and *Humicola* species. Once a nucleic acid sequence encoding a phytate hydrolyzing enzyme of the present invention is obtained, recombinant host cells containing the nucleic acid sequence may be constructed using techniques well known in the art. Molecular biology techniques are disclosed in Sambrook et al., Molecular Biology Cloning: A Laboratory Manual, Second Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989) and Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, 3d Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (2001).

In one embodiment, nucleic acid sequences encoding phytate hydrolyzing enzymes derived from *Penicillium*, *Fusarium* and *Humicola* species and having at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% and at least 95% identity to the nucleic acid sequence of any one of Figures 1, 4, 7 and 9 or a functional derivative thereof, or which is capable of hybridizing under conditions of intermediate to high stringency to the nucleic acid sequence of any one of Figures 1, 4, 7 and 9, or which is complementary to the nucleic acid sequence of any one of Figures 1, 4, 7 and 9 is obtained and transformed into a host cell using appropriate vectors.

The nucleic acid sequences encoding phytate hydrolyzing enzymes can include a leader sequence capable of providing for the secretion of the encoded phytase. Depending on whether the phytase is to be expressed intracellularly or is secreted, a DNA sequence or expression vector of the invention can be engineered such that the mature form of the phytase is expressed with or without a natural phytase signal sequence or a signal sequence which functions in a fungus (e.g., *Aspergillus niger*), other prokaryotes or eukaryotes. Expression can also be achieved by either removing or partially removing said signal sequence.

A variety of vectors and transformation and expression cassettes suitable for the cloning, transformation and expression in fungus, yeast, bacteria, insect and plant cells are known by those of skill in the art. Typically, the vector or cassette contains sequences directing transcription and translation of the nucleic acid sequence, a selectable marker, and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region 5' of the gene which harbors transcriptional initiation controls and a region 3' of the DNA fragment which controls transcriptional termination. These control regions may be derived from genes homologous or heterologous to the host as long as the control region selected is able to function in the host cell.

Initiation control regions or promoters, which are useful to drive expression of the phytate hydrolyzing enzymes in a host cell are known to those skilled in the art. A nucleic acid sequence encoding the phytate hydrolyzing enzyme is linked operably through initiation codons to selected expression control regions for effective expression of such enzyme. Once suitable cassettes are constructed, they are used to transform the host cell.

In cases where plant expression vectors are used, the expression of a sequence encoding phytase may be driven by any of a number of promoters. For example, viral promoters such as the 35S and 19S promoters of CaMV (Brisson et al (1984) Nature 310:511-514) may be used alone or in combination with the omega leader sequence from TMV (Takamatsu et al (1987) EMBO J 6:307-311). Alternatively, plant promoters such as the small subunit of RUBISCO (Coruzzi et al (1984) EMBO J 3:1671-1680; Broglie et al (1984) Science 224:838-843); or heat shock promoters (Winter J and Sinibaldi RM (1991) Results Probl Cell Differ 17:85-105) may be used. These constructs can be introduced into plant cells by direct DNA transformation or pathogen-mediated transfection. For reviews of such techniques, see Hobbs S or Murry LE (1992) in McGraw Hill Yearbook of Science and Technology , McGraw Hill, New York, N.Y., pp 191-196; or Weissbach and Weissbach (1988) Methods for Plant Molecular Biology, Academic Press, New York, N.Y., pp 421-463.

General transformation procedures are taught in Current Protocols In Molecular Biology (3rd edition, edited by Ausubel et al., John Wiley & Sons, Inc. 1995, Chapter 9) and include calcium phosphate methods, transformation using PEG and electroporation.

For Aspergillus and Trichoderma, PEG and Calcium mediated protoplast transformation can be used (Finkelstein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi. Technology and Products (eds by Finkelstein & Bill) 113-156. Electroporation of protoplast is disclosed in Finkelestein, DB 1992 Transformation. In Biotechnology of Filamentous Fungi. Technology and Products (eds by Finkelstein & Bill) 113-156. Microprojection bombardment on conidia is described in Fungaro et al. (1995) Transformation of Aspergillus nidulans by microprojection bombardment on intact conidia, FEMS Microbiology Letters 125 293-298. Agrobacterium mediated transformation is disclosed in Groot et al. (1998) Agrobacterium tumefaciens-mediated transformation of filamentous fungi, Nature Biotechnology 16 839-842 and U.S. Patent No. 6,255,115. For transformation of Saccharomyces, lithium acetate mediated transformation and PEG and calcium mediated protoplast transformation as well as electroporation techniques are known by those of skill in the art.

Host cells which contain the coding sequence for a phytate hydrolyzing enzyme of the present invention and express the protein may be identified by a variety of procedures known to those of skill in the art. These procedures include, but are not limited to, DNA-DNA or DNA-RNA hybridization and protein bioassay or immunoassay techniques which include membrane-based, solution-based, or chip-based technologies for the detection and/or quantification of the nucleic acid sequence or protein.

It should also be noted that the invention contemplates in vitro expression of the phytase enzymes described herein.

In preferred embodiments of the invention, phytase is produced in fungal cells. In one embodiment of the present invention, a polynucleotide sequence encoding a phytate hydrolyzing enzyme derived from *Penicillium chrysogenum* (deposit No. NRRL 1951) is isolated and expressed in *Aspergillus niger*, and in another embodiment is expressed in *Aspergillus nidulans*. In another embodiment, a polynucleotide sequence encoding a phytate hydrolyzing enzyme derived from *Fusarium javanicum* (deposit No. CBS 203.32) or *Fusarium vertisillibodes* is isolated and expressed. In yet another embodiment, a polynucleotide sequence encoding a phytate hydrolyzing enzyme derived from *Humicola grisea* (deposit No. ATCC 22081) is isolated and expressed. The expressed phytase can then be recovered, e.g., as described below.

In preferred embodiments of the invention, the phytase is expressed in plants. Transgenic plant, as used herein, refers to a plant that contains recombinant genetic material not normally found in plants of this type and which has been introduced into the plant in question (or into progenitors of the plant) by human manipulation. Thus, a plant that is grown from a plant cell into which recombinant DNA is introduced by transformation is a transgenic plant, as are all offspring of that plant that contain the introduced transgene (whether produced sexually or asexually). It is understood that the term transgenic plant encompasses the entire plant and parts of said plant, for instance grains, seeds, flowers, leaves, roots, fruit, pollen, stems, etc.

The present invention is applicable to both dicotyledonous plants (e.g. tomato, potato, soybean, cotton, tobacco, etc.) and monocotyledonous plants, including, but not limited to graminaceous monocots such as wheat (*Triticum* spp.), rice (*Oryza* spp.), barley (*Hordeum* spp.), oat (*Avena* spp.), rye (*Secale* spp.), corn (*Zea* mays), sorghum (*Sorghum* spp.) and millet (*Pennisetum* spp). For example, the present invention can be employed with barley genotypes including, but not limited to Morex, Harrington , Crystal, Stander, Moravian III, Galena, Salome, Steptoe, Klages, Baronesse, and with wheat genotypes including, but not limited to Yecora Rojo, Bobwhite, Karl and Anza. In general, the invention is particularly useful in cereals.

Standard molecular biology methods and plant transformation techniques can be used to produce transgenic plants that produce seeds containing phytase protein. The following description provides general guidance as to the selection of particular constructs and transformation procedures.

The present invention utilizes recombinant constructs that are suitable for obtaining expression of phytase in plant seeds relative to non-transformed plant seeds. In their most basic form, these constructs may be represented as Pr-Ph, wherein Pr is a seed-specific promoter and Ph is a nucleic acid sequence encoding phytase. In another embodiment, a peptide signal sequence that targets expression of the phytase polypeptide to an intracellular body may be employed. Such constructs may be represented as Pr-SS-Ph, wherein SS is the signal peptide. Nucleic acid molecules that may be used as the source of each of these components are described in the Definitions section above.

Each component is operably linked to the next. For example, where the construct comprises the hordein D-promoter (P), the hordein D-signal sequence (SS) encoding the hordein signal peptide, and an open reading frame encoding a phytase (Ph), the hordein promoter is linked to the 5' end of the sequence encoding the hordein signal sequence, and the hordein signal sequence is operably linked to the 5' end of the phytase open reading frame, such that C terminus of the signal peptide is joined to the N-terminus of the encoded protein.

The construct will also typically include a transcriptional termination region following the 3' end of the encoded protein ORF. Illustrative transcriptional termination regions include the *nos* terminator from *Agrobacterium* Ti plasmid and the rice alpha-amylase terminator.

Standard molecular biology methods, such as the polymerase chain reaction, restriction enzyme digestion, and/or ligation may be employed to produce these constructs comprising any nucleic acid molecule or sequence encoding a phytase protein or polypeptide.

Introduction of the selected construct into plants is typically achieved using standard transformation techniques. The basic approach is to: (a) clone the construct into a transformation vector; which (b) is then introduced into plant cells by one of a number of techniques (e.g., electroporation, microparticle bombardment, *Agrobacterium* infection); (c) identify the transformed plant cells; (d) regenerate whole plants from the identified plant cells, and (d) select progeny plants containing the introduced construct. Preferably all or part of the transformation vector will stably integrate into the genome of the plant cell. That part of the transformation vector which integrates into the plant cell and which contains the introduced Pr-Ph or Pr-SS-Ph sequence (the introduced " phytase transgene") may be referred to as the recombinant expression cassette.

Selection of progeny plants containing the introduced transgene may be made based upon the detection of phytase expression in seeds, or upon enhanced resistance to a chemical agent (such as an antibiotic) as a result of the inclusion of a dominant selectable marker gene incorporated into the transformation vector.

Successful examples of the modification of plant characteristics by transformation with cloned nucleic acid sequences are replete in the technical and scientific literature. Selected examples, which serve to illustrate the knowledge in this field of technology include:

U.S. Patent No.5,571,706 ("Plant Virus Resistance Gene and Methods");
U.S. Patent No.5,677,175 ("Plant Pathogen Induced Proteins");
U.S. Patent No.5,510,471 ("Chimeric Gene for the Transformation of Plants");
U.S. Patent No.5,750,386 ("Pathogen-Resistant Transgenic Plants");
U.S. Patent No.5,597,945 ("Plants Genetically Enhanced for Disease Resistance");
U.S. Patent No.5,589,615 ("Process for the Production of Transgenic Plants with Increased Nutritional Value Via the Expression of Modified 2S Storage Albumins");
U.S. Patent No.5,750,871 ("Transformation and Foreign Gene Expression in *Brassica* Species");
U.S. Patent No. 5,268,526 ("Over expression of Phytochrome in Transgenic Plants");
U.S. Patent No. 5,780,708 ("Fertile Transgenic Com Plants");
U.S. Patent No. 5,538,880 ("Method For Preparing Fertile Transgenic Corn Plants");
U.S. Patent No. 5,773,269 ("Fertile Transgenic Oat Plants");
U.S. Patent No. 5,736,369 ("Method For Producing Transgenic Cereal Plants");
U.S. Patent No. 5,610,049 ("Methods For Stable Transformation of Wheat").

These examples include descriptions of transformation vector selection, transformation techniques and the construction of constructs designed to express an introduced transgene.

The transgene-expressing constructs of the present invention may be usefully expressed in a wide range of higher plants to obtain seed- or grain-specific expression of selected polypeptides. The invention is expected to be particularly applicable to monocotyledonous cereal plants including barley, wheat, rice, rye, maize, triticale, millet, sorghum, oat, forage, and turf grasses. In particular, the transformation methods described herein will enable the invention to be used with genotypes of barley including Morex, Harrington, Crystal, Stander, Moravian III, Galena, Golden Promise, Steptoe, Klages and Baronesse, and commercially important wheat genotypes including Yecora Rojo, Bobwhite, Karl and Anza.

The invention may also be applied to dicotyledenous plants, including, but not limited to, soybean, sugar beet, cotton, beans, rape/canola, alfalfa, flax, sunflower, safflower, brassica, cotton, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers; and tree fruits such as citrus, apples, pears, peaches, apricots, and walnuts.

A number of recombinant vectors suitable for stable transformation of plant cells or for the establishment of transgenic plants have been described including those described in Weissbach and Weissbach, (1988), and Gelvin *et al.*, J. Bacteriol. 172(3):1600-1608 (1990). Typically, plant transformation vectors include one or more ORFs under the transcriptional control of 5' and 3' regulatory sequences and a dominant selectable marker with 5' and 3' regulatory sequences. The selection of suitable 5' and 3' regulatory sequences for constructs of the present invention is discussed above. Dominant selectable marker genes that allow for the ready selection of transformants include those encoding antibiotic resistance genes (e.g., resistance to hygromycin, kanamycin, bleomycin, G418, streptomycin or spectinomycin) and herbicide resistance genes (e.g, phosphinothricin acetyltransferase).

Methods for the transformation and regeneration of both monocotyledonous and dicotyledonous plant cells are known, and the appropriate transformation technique will be determined by the practitioner. The choice of method will vary with the type of plant to be transformed; those skilled in the art will recognize the suitability of particular methods for given plant types. Suitable methods may include, but are not limited to: electroporation of plant protoplasts; liposome-mediated transformation; polyethylene glycol (PEG) mediated transformation; transformation using viruses; micro-injection of plant cells; micro-projectile bombardment of plant cells; vacuum infiltration; and *Agrobacterium* mediated transformation. Typical procedures for transforming and regenerating plants are described in the patent documents listed at the beginning of this section.

Following transformation, transformants are preferably selected using a dominant selectable marker. Typically, such a marker will confer antibiotic or herbicide resistance on the seedlings of transformed plants, and selection of transformants can be accomplished by exposing the seedlings to appropriate concentrations of the antibiotic or herbicide. After transformed plants are selected and grown to maturity to allow seed set, the seeds can be harvested and assayed for expression of phytase.

The phytase of the invention can be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (*e.g.* Triton-X 100) or by enzymatic cleavage. Cells employed in expression of phytase can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents. It may be desired to purify the phytase from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reverse phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex G-75; protein A Sepharose columns to remove contaminants; and metal chelating columns to bind epitope-tagged forms of the phytase. Various methods of protein purification may be employed and such methods are known in the art and described for example in Deutscher, Methods in Enzymology, 182 (1990); Scopes, Protein Purification: Principles and Practice, Springer-Verlag, New York (1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular form of phytase produced.

In a preferred embodiment, the phytase(s) is/are produced in transgenic non-human animals. Methods of producing such transgenic animals are described, for example, in U.S. Patent No. 6,291,740. Methods for the successful production of transgenic bovine (e.g., U.S. Patent Nos. 6,080,912 and 6,066,725), swine (e.g., U.S. Patent Nos. 6,271,436 and 5,942,435), goats (e.g., U.S. Patent No. 5,907,080) and fish (e.g., U.S. Patent No. 5,998,697) are available in the art. Furthermore, organ-specific expression, particularly expression in milk produced by the transgenic animals, is within the skill of the ordinary artisan (e.g., e.g., U.S. Patent Nos. 6,268,545 and 6,262,336). The disclosure of each of these patents is incorporated herein in its entirety.

VI. Assaying for phytase activity

Assays for phytase activity are well known in the art. Perhaps the most widely used is the classic assay for liberation of inorganic phosphate developed by Fiske and SubbaRow, *Journal of Biological Chemistry* 66:375-392 (1925). A variation of this method is found in Mitchell et al., *Microbiol.* 143:245-252 (1997). A preferred method is described in Food Chemicals Codex, 4th Edition, Committee on Food Chemicals Codex, Institute of Medicine, National Academy Press, Washington, DC, 1996 at pages 809-810. Each of these references are incorporated herein.

Generally, the assay involves allowing a measured weight or volume of a phytase sample to react with phytate in solution for a measured period of time. The reaction is stopped and a color solution containing ammonium molybdate (AM) is added to the reaction solution. Colorimetry is then performed using a spectrophotometer and compared to controls of known concentration of inorganic phosphate (P_i) and/or controls produced by reactions with enzymes having known phytase activity. A Unit of activity is determined as the amount of enzyme sample required to liberate 1 µmol P_i per minute from phytate under defined reaction conditions.

Enzyme reactions are frequently run at pH 5.5 and 37 □C. However, pH and temperature conditions may be varied to determine optimum reaction conditions and tolerances for a given phytase. When different reaction conditions are tested, Units of activity should still be related to a single specific set of reaction conditions.

The reaction may be stopped and then the color solution added, or a stop/color solution may be used that both arrests the enzyme activity and adds a product whose spectral absorbance is measurably affected by the concentration of P_i in a predictable and calculatable manner. As discussed above, the color solutions generally contain AM. Various examples of such solutions are available in the relevant literature. In U.S. Patent No. 6,039,942, the reaction is stopped using trichloroactetate (TCA) and the color solution added thereafter contained ferrous sulfate and AM. In other examples wherein the reaction was first stopped with TCA, different color solution contained sulfuric acid, AM and ascorbic acid (U.S. Patent No. 6,221,644) and sulfuric acid, AM and ferrous sulfate (U.S. Patent No. 6,190,897). In other cases, the color and stop solution are the same. For example, in both U.S. Patent Nos. 6,139,902 and 6,261,592, the solution contained sulfuric acid, AM and acetone, after which a solution containing acetic acid was added. In a preferred embodiment, the color/stop solution contains ammonium vanadate, AM and nitric acid (*see* <u>Food Chemicals Codex</u>, above).

Wavelength-specific absorption by the final solution, containing the reaction solution and stop/color solution(s), is measured using a spectrophotometer. Many such instruments are available and their use is routine in the art. The wavelength used for absorption measurement can vary with the components of the color solution. For example, the references cited above measured absorbance at 380, 415, 690, 700 or 750 nm. Any of these may provide adequate indication of P_i concentration in these solutions. However, the wavelength used should generally be the one described in a given protocol. The skilled artisan can easily determine empirically which wavelength provides optimum discrimination of differences in P_i concentration by comparing the linearity of absorption change between serially diluted control solutions of known P_i concentration at different wavelengths.

VII. Applications of phytate hydrolyzing enzymes

The phytase and derivatives thereof as taught herein can be used in a variety of applications where it is desirable to separate phosphorous from phytate. Several exemplary applications are set forth below.

For example, the invention provides for the use of cells or spores capable of producing phytase according to the invention as a probiotic or direct fed microbial product. Preferred embodiments for said uses are phytase-producing *Aspergillus* sp. of the invention.

In addition, the invention contemplates the use of phytase as described herein in food or animal feed.

The present invention provides food or animal feed including phytase as described herein. Preferably, said food or animal feed comprises phytase as an additive which is active in the digestive tract, preferably the crop and/or small intestine, of livestock, such as poultry and swine, and aquatic farm animals including fish and shrimp. Said additive is also preferably active in food or feed processing.

In an alternative embodiment, phytase or phytase producing organisms are added as a pretreatment to food or animal feed, such as in the processing of the food or feed. In this embodiment, the phytase is active prior to consumption of the food or feed, but may or may not be active at the time the food or animal feed is consumed.

Compositions comprising polypeptides or proteins possessing phytase activity may be prepared in accordance with methods known in the art and may be in the form of a liquid or a dry composition. The polypeptide to be included in the composition may be stabilized in accordance with methods known in the art.

The invention additionally provides food or animal feed comprising cells, spores or plant parts, including seeds, capable of expressing phytase as described herein.

Still further, the present invention contemplates a method for the production of a food or animal feed, characterized in that phytase according to the invention is mixed with said food or animal feed. Said phytase is added as a dry product or as a liquid, before or after processing. According to one embodiment, wherein a dry powder is used, the enzyme is diluted as a liquid onto a dry carrier such as milled grain.

Liquid compositions need not contain anything more than the phytase enzyme, preferably in a purified form. Usually, however, a stabilizer such as glycerol, sorbitol or mono propylene glycol is also added. The liquid composition may also comprise one or more other additives, such as salts, sugars, preservatives, pH-adjusting agents (i.e., buffering agents), proteins, or phytate (a phytase substrate). Typical liquid composition are aqueous or oil-based slurries.

The liquid compositions can be added to a food or feed after an optional pelleting thereof. Dry compositions may be spray-dried compositions, in which case the composition need not contain anything more than the enzyme in a dry form. Usually, however, dry compositions are so-called granulates which may readily be mixed with for example food or feed components, or more preferably, form a component of a pre-mix. The particle size of the enzyme granulates preferably is compatible with that of the other components of the mixture. This provides a safe and convenient means of incorporating enzymes into for example an animal feed.

Agglomeration granules are prepared using agglomeration techniques in a high shear mixer (e.g., Lodige) during which a filler material and the enzyme are co-agglomerated to form granules. Absorption granulates are prepared by having cores of a carrier material to adsorb/be coated by the enzyme.

Typical filler materials are salts such as disodium sulphate. Other fillers are kaolin, talc, magnesium aluminium silicate and cellulose fibers. Optionally, binders such as dextrins are also included in agglomeration granules.

Typical carrier materials are starch, e.g., in the form of cassava, corn, potato, rice and wheat. Salts may also be used.

Optionally, the granulates are coated with a coating mixture. Such mixture comprises coating agents, preferably hydrophobic coating agents, such as hydrogenated palm oil and beef tallow, and if desired other additives, such as calcium carbonate or kaolin.

Additionally, phytase compositions may contain other substituents such as coloring agents, aroma compounds, stabilizers, vitamins, minerals other feed or food enhancing enzymes and the like. This is so in particular for the so-called pre-mixes.

A “feed” and a “food,” respectively, means any natural or artificial diet, meal or the like or components of such meals intended or suitable for being eaten, taken in, digested, by an animal and a human being, respectively.

A "food or feed additive" is an essentially pure compound or a multi component composition intended for or suitable for being added to food or feed. It usually comprises one or more compounds such as vitamins, minerals or feed enhancing enzymes and suitable carriers and/or excipients, and it is usually provided in a form that is suitable for being added to animal feed.

The phytases of the invention can also be used in poultry food to improve egg shell quality (reduction of losses due to breaking), see for example, The Merck Veterinary Manual (Seventh Edition, Merck & Co., Inc., Rahway, N.J., USA, 1991, page 1268); Jeroch et al. Bodenkultur Vo. 45(4):361-368 (1994); Poultry Science, 75(1):62-68 (1996); Canadian Journal of Animal Science 75(3):439-444 (1995); Poultry Science 74(5):784-787 (1995) and Poultry Science 73(10):1590-1596 (1994).

An effective amount of the polypeptide in food or feed is typically from about 10 to 50,000 U/kg feed or food; preferably from about 10 to 15,000, more preferably from about 10 to 10,000, in particular from about 100 to 5,000, especially from about 100 to about 2,000 U/kg feed or food.

The present invention also provides a method for the production of a food or animal feed, characterized in that cells, plant parts, including seeds, and/or spores capable of expressing phytase according to the invention are added to said food or animal feed. Such cells or spores, may be of any origin, bacterial, plant, or animal.

Further, the present invention provides for the use of the phytase described herein with or without accessory phosphatases in the production of inositol and inorganic phosphate, and phytate intermediates.

Also provided is a method for the reduction of levels of phosphorous in animal manure, characterized in that an animal is fed an animal feed according to the invention in an amount effective in converting phytate contained in said animal feed.

In one embodiment, the transgene protein, for example phytase expressed in plants, especially seeds or grains, using the methods described herein, is used in the production and synthesis of phytase. The phytase transgene expressed by the recombinant nucleic acid of the invention may be harvested at any point after expression of the protein has commenced. When harvesting from the seed or grain or other part of a plant for example, it is not necessary for the seed or grain or other part of the plant to have undergone maturation prior to harvesting. For example, transgene expression may occur prior to seed or grain maturation or may reach optimal levels prior to seed or grain maturation. The transgene protein may be isolated from the seeds or grain, if desired, by conventional protein purification methods. For example, the seed or grain can be milled, then extracted with an aqueous or organic extraction medium, followed by purification of the extracted phytase protein. Alternatively, depending on the nature of the intended use, the transgene protein may be partially purified, or the seed or grain may be used directly without purification of the transgene protein for food or animal feed, food processing or other purposes.

Alpha-amylases break down starch 1-4 linkages. Amylases are enzymes fundamental to the brewing and baking industries. Amylases are required to break down starch in malting and in certain baking procedures carried out in the absence of added sugars or other carbohydrates. Obtaining adequate activity of these enzymes is problematic especially in the malting industry. It has been known for some time that phytate has an inhibitory effect on amylases. A method of adequately increasing the activity of amylases with a physiologically acceptable system, leads to more rapid malting methods and, owing to increased sugar availability, to alcoholic beverages such as beers with reduced carbohydrate content.

Accordingly, seeds or grains with phytase expression provide advantages in the production of malt and beverages produced by a fermentation process. Enhanced activity of amylases in grain increases the speed and efficiency of germination, important in malting, where malt is produced having increased enzymatic activity resulting in enhanced hydrolysis of starch to fermentable carbohydrates, thereby, improving the efficiency of fermentation in the production of alcoholic beverages, for example, beer and scotch whiskey. Enhanced fermentation processes also find use in the production of alcohols that are not intended for human consumption, i.e., industrial alcohols.

The phytase and phytate-derived intermediates of the invention also find use in many other agricultural, industrial, medical and nutritional applications. For example phytase and phytate-derived intermediates can be used in grain wet milling. Phytate is used in cleaning products, rust removal products and in the removal of metals and other polycations from such diverse materials as waste products and carbonated beverages. Phytate and phytases may be used in the isolation and recovery of rare metals. Phytase may be used to produce lower phosphate homologs of phytate, which may be used in dentifrice and other dental care products as well as potential treatments or preventatives of bone resorption (e.g., in osteoporosis) and renal calculi (kidney stones). Phytate and derivatives have found use in the production of tofu, and chelation of minerals (e.g., iron, zinc, calcium or magnesium)with phytate, followed by release with addition of phytase may provide a unique means of providing these nutrients. Phytases may be used in the production of inositol from phytate its use in food products. Phytases may also be used in the chemical and biochemical synthesis of phosphate containing materials. Phytase, phytate and lower phosphate phytate derivatives find many other uses in personal care products, medical products and food and nutritional products, as well as various industrial applications, particularly in the cleaning, textile, lithographic and chemical arts.

The following examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. The skilled artisan will appreciate that the methods disclosed may be applied to any number of different species, including to obtain all sequences disclosed herein. All patent and literature references cited in the present specification are hereby incorporated by reference in their entirety.

EXAMPLES

Example 1

Preparation of Genomic DNA Encoding Phytases

Genomic DNA was prepared for several different microorganisms for the purpose of undertaking a PCR reaction to determine whether phytases are encoded by the DNA for a particular organism.

Genomic DNA is obtained from *Penicillium chrysogenum* (deposit no. NRRL 1951); *Fusarium javanicum* (deposit no. CBS 203.32); *Fusarium vertisillibodes*; *Humicola grisea var. thermoidia* deposit no. CBS 225.63 or ATCC 22081; and *Emericella desertorum* deposit no. CBS 653.73 and isolated according to standard methods.

Alignments were performed for several known phytase sequences, including those from *Aspergillus Niger/Aspergillus ficum*, *Aspergillus terreus* 59, *Aspergillus terreus* 60, *Aspergillus fumigatus*, *Aspergillus niger*, *Emericella nidulans*, *Talaromyces thermophylus* and *Myceliopthora thermophila*. From these, several "boxes" were identified as being largely conserved, and from these primers were developed.

The following DNA primers were constructed for use in amplification of phytase genes from the libraries constructed from the various microorganisms. All symbols used herein for protein and DNA sequences correspond to IUPAC IUB Biochemical Nomenclature Commission codes.

BOX1: primers coding for (V/L)L(A/S)RHGAR

forward primer	BTIYTIKCIMGICAYGGIHCIMG
forward primer	BTIYTIAGYMGICAYGGIHCIMG

BOX2: primers coding for NNTL(D/E/H)

forward primer	AAYAAYACIYTISA
reverse primer	TSIARIGTRTTRTT

BOX3: primers coding for LSPFC

forward primer	YTTTCICCITTYTGY
forward primer	YTIAGYCCITTYTGY
reverse primer	RCARAAIGGIGAIAR
reverse primer	RCARAAIGGRCTIAR

BOX4: primers coding for G(N/S)PLGP

forward primer	GGIWVICCIYTIGGICC

reverse primer	CCIARIGGIBWICC

BOX5: primers coding for DFSHD

forward primer	GAYTTYTCICAYGAY
forward primer	GAYTTYAGYCAYGAY
reverse primer	RTCRTGIGARAARTC
reverse primer	RTCRTGRCTRAARTC

BOX6: primers coding for VR(A/V)I(I/V)NDR

reverse primer	CKRTCRTTIAYIARIRCICKIAC

Boxes were also developed according to the methods of Pasamontes et al. *Appl. Evir. Microbiol.* 63(5):1696-1700 (1997) (expressly incorporated herein) to provide the following primers.

BOX2.5: coding for MDMCSFD

forward primer	ATGGAYATGTGYTCNTTYGA

BOX4□: coding for YGHGAG

reverse primer	TTRCCRGCRCCRTGNCCRTA

PCR is performed on a standard PCR machine such as the PTC-150 Mini Cycler from MJ Research Inc. (Watertown, MA) or an Eppendorf Mastercycler (Hamburg, Germany). In the experiments described below, PCR was performed using a Hybaid Touchdown thermocycler (Middlesex, UK).

PCR conditions for Pwo polymerase (Boehringer Mannheim, Cat # 1644-947) comprise a 100 microliter solution made of 10 microliter of 10X reaction buffer (10X reaction buffer comprising 100mM Tris HCl, pH 8-8.5; 250 mM KCl; 50 mM $(NH_4)_2SO_4$; 20 mM $MgSO_4$); 0.2 mM each of dATP, dTTP, dGTP, dCTP (final concentration), 1 microliter of 100 nanogram/microliter genomic DNA, 1 microliter of PWO at 1 unit per microliter, 500 mM primers (final concentration) and water to 100 microliters. The solution is overlaid with mineral oil.

Two approaches were developed for amplification of phytase genes from the genomic DNA:

A) A first PCR is run using BOX1 and BOX6 primers; the products are run on an agarose gel and approximately 1 kb fragments are isolated and run in a second PCR using nested primers. For the second PCR run, best results were obtained using primers from BOX1-BOX5 or from BOX5-BOX6 or BOX2.5/BOX4'.

Protocol A:

PCR1: - 2' at 94°C (1 cycle)
- 45" at 94°C; 1'30" at 40°C; 1'30" at 72°C (30 cycles)
- 7' at 72°C (1 cycle)
- hold at 4°C

Fragments were put on a 1% low melting gel and fragments around the expected size (0.0-1.2 kb) were sliced from the gel, isolated and used as a template for the second PCR run (PCR2). PCR 2 followed the same cycling protocol as PCR1.

B) Touchdown PCR was performed using BOX2.5/BOX4' primers. Using this technique, a specific fragment could be isolated, cloned into a TOPO vector (Invitrogen Corp., Carlsbad, CA), and sequenced without further processing.

Protocol B:
- 3' at 95°C (1 cycle)
- 1' at 95°C; 1' at 60°C, decreasing to 50°C; 30" at 72°C (20 cycles, so that the temperature dropped 0.5°C each cycle in the annealing step)
- 1' at 95°C; 1□ at 50°C; 30" at 72°C (10 cycles)
- hold at 4°C

From the sequenced fragments, it was possible to use the RAGE technique (rapid amplification of genomic ends) to rapidly obtain the sequence of the full length gene. Using the GenomeWalker□ Kit from Clontech Laboratories, Inc (Palo Alto, CA) and manufacturer's protocol (GenomeWalker□ Kits User Manual, published November 10, 1999, expressly incorporated herein), adapter ligations were derived from the fragment sequences to further determine upstream gene sequence. Sequences of phytase genes were determined from chromosomal DNA of various species.

Figure 22 shows the phytase polynucleotide sequence of *E. desertorum* obtained by the above methods and the sequences therein from which adapter ligations (primers GSP1rev:fyt037 and GSP2rev:fyt036) were derived to obtain the upstream sequences encoding this phytase (*see* Figure 17A).

Figure 23 shows the phytase sequence of *F. javanicum* obtained by the above methods and the sequences therein from which adapter ligations (primers GSP1rev:fyt039 and GSP2rev:fyt038) were derived to obtain the upstream sequences encoding this phytase (*see* Figures 18A-18B).

Example 5

Evidence of phytate hydrolyzing activity in liquid culture

A selected fungal species is grown in defined media containing various concentrations of inorganic phosphate, and growth characteristics and phytase production are assayed and compared. Spore suspensions are used ($2x10^6$ spores/ml final con) to inoculate a minimal media (Vogels) where the phosphate concentration is altered to see how this will affect growth and phytase production. Cultures are grown in 50ml of medium in shake flask culture at 25°C to 30°C. Cultures are harvested at 24, 48, 72 and 96 hours. Culture supernatants are assayed for phytase activity using the method of Fiske and SubbaRow, *Journal of Biological Chemistry* 66:375-392 (1925). Growth may be determined by dry weight or OD readings.

5A. Effect of different media conditions on growth and morphology

A series of fungal growth curves are produced to look at the effect of available P in the medium on growth and phytase production. In some instances, when the P level is reduced, morphological changes in the growth of the fungus are observed which are associated with a stressed condition (e.g., mycelial fragmentation, pelleting, heterogeneous growth and an overall appearance of a pale yellow color). This physiological strain may be related to the appearance of phytase activity at a point in the growth curve, for example approaching late exponential phase. Morphological evidence of phytic acid utilization may be observed in cultures of low P (e.g., 0.57mM) supplemented after 24 hours growth with 1mM phytate as a phosphorus source. The morphological changes seen without added phytate may not be apparent, indeed the supplement samples may resemble cultures in media of higher P which were not limiting. This response would indicate that a phytic acid specific hydrolyzing activity was being produced so that P could be supplied to the growing fungus. As a caveat, it is possible that higher concentrations of phytate (e.g., 5 mM) supplementing the cultures result in a lack of cell growth. Such a result would suggest that the high level of phytate in the medium chelates essential minerals resulting in a medium that cannot support fungal growth and nutrition.

In an exemplary study, the fungus is grown in media containing

- High phosphate (1.14 mM)
- Low phosphate (0.57 mM)
- Low phosphate plus 1mM supplemented phytate.

Growth is monitored over 0, 24, 48, 72 and 96 hours by dry weight measurements, and the morphological characteristics in response to the different media conditions are also observed. In a situation where phytate hydrolyzing activity which allows the fungus to access phosphate from phytate, and so circumvent phosphate starvation stresses that the culture may otherwise experience, the major observations that would be expected are:

1. Good growth in high phosphate, consistent fungal morphology indicative of healthy culture.

2. Markedly poorer growth in low phosphate condition, fungal morphology heterogenous with evidence of clumping and mycelial fragmentation. The culture may have a sickly yellow appearance.

3. Similar cultures as for (2), when supplemented with phytate (the substrate), no longer appear to be under the same physiological stress. Biomass growth is similar to condition (1) and the fungal morphology is the same as for the high phosphate condition.

4. Growth curves and photographic evidence support these observations.

5B. Phytase activity in culture supernatants

Phytase activity in the supernatants of fungi growing on media with variable levels of inorganic P can be measured. Supernatant samples are used to compare activities at a specified time post inoculation. Phytase activity may be expressed as the number of mmoles P released per minute per ml culture supernatant. Sample activities are calculated from triplicate culture flasks where supernatants are assayed for phytase in duplicate. Activities are shown as mean_SD. Along with the observations above, a clear physiological stress associated with cultures where phosphate is limited, which adversely affected growth, may be observed and linked to the appearance of phytase activity.

5C. Concentration of culture supernatants

Additional evidence of phytase activity can be expected from concentrated supernatant (concentrated protein). For example, concentrated protein samples can be obtained from:

1. Cultures of fungus from conditions of stress and low phosphate (where phytase is expected to be expressed),
2. Cultures of fungus of high phosphate and no stress, where phytase is not expected to be produced, and
3. Cultures supplemented with low phosphate and supplemental phytate.

Silver stained SDS-PAGE gels of these concentrated protein samples are expected to show a protein profile demonstrating the appearance of a protein band (putative phytase band) in concentrated protein from condition 1 (above) which is not present in condition 2. A similar appearance of this band is also expected in condition 3, albeit at a lower level. Based on the amino acid sequence of a specific phytase, and on whether it appears to be an extracellular enzyme, the size of the protein may be approximated. It should be noted, however, that glycosylation modification on the extracellular enzyme may increase the MW.

Example 6

PCR amplification of phytase gene fragments

6A. Degenerate primer design

Based on alignments of published phytase amino acid sequences, a range of degenerate primers are designed against conserved structural and catalytic regions. Such regions included those that are highly conserved among the phytases, as well as those known to be important for enzyme structure and function.

For example, amino acid sequences for published phytases are aligned. It should be noted that many phytase sequences are publicly available from GenBank, and each is incorporated herein by reference.

Particular regions are chosen to meet the criteria above, and a range of forward and reverse primers designed from the amino acid sequences. Using the genetic code for codon usage, degenerate nucleotide PCR primers are synthesized.

As another example, primers are designed from the published amino acid sequence for different phytases from a single species (e.g., *A. niger*). These primers may be designed as follows:

1. Primer 1: Forward (5'-3') primer from, for example, the phosphate binding domain of a phytase, which should be essential for catalytic activity.

2. Primer 2: Reverse primer from a central phytase region which seems to be conserved relatively well.

All primers may be synthesized in the 5'-3' direction. The standard genetic code is used to change from amino acid to triplet codon, and standard IUB code for mixed base sites are used (e.g. to designate I for A/C/T/G).

As can be seen from the alignment of sequences for *A. niger* PhyA and PhyB, the phosphate-binding domain is well conserved with only a single amino acid difference between PhyA (RHG<u>A</u>RYP; van Hartingsveldt *et al.*, 1993) and PhyB (RHG<u>E</u>RYP; Piddington *et al.*, 1993). A degenerate primer may be designed complementary to this region in the PhyA version of the sequence only, i.e. using RHGARYPT as the basis for primer design. This would be to bias the primer towards a PhyA type phosphate binding domain. A second conserved region, which may serve as the basis for primer 2 for *A. niger*-derived primers, occurs in the middle of the PhyA and PhyB amino acid sequence. This conserved central phytase-specific domain in PhyA (FTHDEWI) corresponds to amino acids 285-291. In PhyB, the amino acid sequence (FTQDEWV) corresponds to amino acids 280-286.

Degenerate primers developed as described above may be used to amplify a phytase encoding region from other species by PCR, as described next.

6B. PCR amplification of phytase gene fragments

Genomic DNA from a species of interest may be used as a template for PCR amplification of putative phytase gene fragments using combinations of primers made as described above. PCR is carried out using the PCR Ready-to-go Beads from Amersham Pharmacia. Conditions are determined by individual experiments, but typically thirty cycles are run in a Techne thermal cycler. Successful amplification is verified by electrophoresis of the PCR reaction on a 1% agarose gel. A PCR phytase product that is amplified by the primers may be anticipated by a correct expected size. The product is then purified by gel extraction using the Qiaquick Spin Gel Extraction kit from Qiagen. The purified PCR product is ligated into the commercial pGEM-T Easy vector System (Promega Corporation) to facilitate cloning. Ligation reactions are incubated at 4^0C overnight in a total volume of 10 ml containing 0.1 volumes of 10 x ligase buffer and 1 ml (1 $U.ml^{-1}$) of T4 DNA ligase. Typically insert DNA is used in the reaction in a 1-4:1 molar ratio of insert to vector DNA. A 100 ml aliquot of $CaCl_2$ competent *E.coli* XL-1 Blue cells are removed from -80°C storage and thawed on ice for transformation. 3ml of ligation mix is added to the cells and the mixture incubated on ice for 20 min. The cells are then heat shocked at 42°C for 1 min. and returned to ice for 5 min. The transformation mixture is added to 0.9mL of L-broth, and the cells incubated with shaking and without selection to allow expression of the ampicillin resistance gene product before selection is applied (37°C, 1 h). Aliquots of 200, 300 and 400 ml of this culture are then spread directly on selective agar plates. Plates are incubated at 37°C overnight. Colonies containing recombinant plasmids are visualized using blue/white selection. For rapid screening of recombinant transformants, plasmid DNA is prepared from cultures of putative positive (white) colonies. DNA is isolated by the method of Birnboim and Doly following the protocol in Sambrook et al (1989). The presence of the correct insert (650bp) in the recombinant plasmid is confirmed by restriction analysis. DNA is digested with restriction enzymes (e.g., Not1-pPst1) overnight at 37°C, and digest products visualized by agarose gel electrophoresis. A number of clones may contain the correct sized insert and can be selected for manual sequencing to see if the insert is a phytase gene fragment. Inserts are sequenced using the dideoxy chain termination method of Sanger *et al* (1977) with a modified form of T7 DNA polymerase (Sequenase version 2.0). The reactions are carried out using reagents supplied in the Sequenase version 2.0 kit (Amersham Life Science-United States Biochemical Corporation), following the manufacturer's protocol. Partial sequence from the ends clones may indicate that a phytase gene fragment had been cloned. Full sequencing of the double-stranded inserts is performed on plasmid DNA from these clones.

6C. Sequence analysis

The sequences are analyzed by BLAST and protein translation sequence tools. BLAST comparison at the nucleotide level may show various levels of homology to published phytase sequences. Initially, nucleotide sequences are submitted to BLAST (Basic BLAST version 2.0) by accessing the BLAST database on the world wide web. The web site used is at http://ncbi.nlm.nih.gov/cgi-bin/BLAST. The program chosen is **blastn**, and the database chosen is **nr**. Standard/default parameter values are employed. Sequence data for putative gene fragments are entered as **sequence in FASTA format** and the query submitted to BLAST to compare these sequences to those already in the database.

The sequences are then subjected to a DNA-to-protein translation tool called Protein machine. This tool is also available on the web at http://medkem.gu.se/edu/translat.html. Another suitable translation tool is known as Translation Machine, available on the web at http://www2.ebi.ac.uk/translate/. The DNA sequences of putative phytase gene fragments are inserted into the analysis block, and the standard genetic code is used as the basis for the translation. Translations are carried out in all three frames and on forward and reverse strands. The translated amino acid sequence is delivered on the screen by the analysis tool as amino acid sequence in one letter code. Ideally, analysis of the amino acid sequence will show that the fragment contains both correct ends (as used to design the primers), contains the essential P binding motif and perhaps other residues which are also present in published phytase sequences. From this, it may be concluded that the fragment cloned is a phytase gene fragment.

Sequence alignments and analysis of those alignments is carried out at the nucleotide and amino acid level using the ALIGN program (Alignment Editor Version 4/97; Dominick Hepperle, Fontanestr. 9c, D016775, Neuglobsow, Germany). In performing the analysis, subject sequences are pasted in, and the PHYLIP Interleaved format employed. The homology analysis is carried out using the "*Analyze*" section of the program, and specifically the option entitled "*Distance Analysis*." This calculates % homologies and the number of different sites between species, using a minimum of two amino acid sequences (i.e., two "species"). Minimal and maximal homologies are calculated as %. The basis for homology analysis is done as % identity, on the calculation of "number of identical amino acids (or bases) divided by the total number of amino acids (or bases) multiplied by 100" to give a percentage value. Amino acid sequences are placed into the ALIGN program along with published phytase sequences and a manual alignment at the amino acid level is carried out. From this, the deduced translation for the PCR product obtained using degenerate primers may be obtained.

Example 7

Southern analysis for library production

Genomic DNA from different species is digested with a range of restriction enzymes overnight at 37°C. Successfully digested DNA is run out on a 1% agarose gel in preparation for transfer to the nylon membrane. After completion of electrophoresis, the agarose gel is soaked for 10 min. in 0.2M HCl to depurinate the DNA and then rinsed briefly in ddH_2O. The DNA is transferred to the Hybond□-N+ membrane (Amersham International PLC) by alkali capillary blotting. The blot is set up so that the nylon filter is sandwiched between the gel and a stack of absorbent paper towels. A wick of Whatman 3MM paper (Schleicher and Schuell, Dassel, Germany) is prepared on a glass plate over a reservoir of transfer buffer (0.4M NaOH). The gel is inverted on the wick, taking care to avoid the formation of air bubbles, and surrounded by strips of Nescofilm to prevent the blotting action of the paper towels from by-passing the gel at its edges. The gel is covered with an equal sized piece of Hybond□-N+ membrane which had been cut in the corner to match the gel and pre-wetted in 3xSSC. Next, 3-5 pieces of 3MM paper are placed on top of the filter and the blot completed by adding a 10 cm stack of blotting paper followed by a 0.5kg weight. The blot is left for 8-24 h to transfer the DNA. The membrane is then washed briefly in 2xSSC at RT and baked in a vacuum oven at 80°C to fix the DNA to the membrane. An isolated fragment from the procedures above is used to probe the Southern blot. It is firstly labeled with ^{32}P isotope by use of the High Prime DNA Labeling Kit (Boehringer Mannheim). Denatured fragment is added into a random primed labeling reaction which incorporates radio-labeled adenine. The Southern blot is prehybridised for 1 hour at 42°C in 12 mL of Easy-Hyb buffer (Boehringer Mannheim) in a hybridization tube. Radiolabeled probe is denatured and added to 5mL of Easy-Hyb hybridization buffer and left to hybridize overnight at 42°C. Following hybridization, the blot is washed by incubation in 40 mL 3xSSC, 0.1%SDS for 15min at 42°C. This low stringency wash is repeated with fresh wash solution. After stringency washing, the lot is rinsed in 3xSSC, sealed in clear plastic and exposed to x-ray film. This is left for 2 hours and the film developed.

Strong hybridizing bands may be observed for a given species digest. Such results indicate that the fragment can be used as a probe for library screening.

Example 8

Isolation of a polynucleotide sequence from the genome of a species of interest encoding a phytase

8A. genomic library generation and screening

Following the Southern hybridization analysis, a partial genomic library may be made in order to try and clone a full-length phytase gene. A size restricted plasmid library targeting a digestion fragment (as estimated from Southern analysis) is generated. Digested genomic DNA is run out on a 1.25% agarose gel. The digested fragments of a preferred approximate size are extracted from the gel, and purified by Glass-Max (Gibco-BRL, Scotland). Purified genomic fragments are used in a shotgun ligation reaction with restriction nuclease linearized pSK II Bluescript vector (Stratagene). The vector is first dephosphorylated before ligation, and the ligation reaction is carried out at 14°C overnight. The library is produced by transformation of E.coli XL-10 Gold ultracompetent cells (Stratagene). 100 ml aliquots cells are removed from -80°C storage and thawed on ice for transformation. 4mL of b-mercaptoethanol is added to the cells on ice. 3ml of ligation mix is added to the mixture and the mixture incubated on ice for 20 min. The cells are then heat shocked at 42□C for 30 sec and returned to ice for 2 min. The transformation mixture is added to 0.9mL of NZY-broth, and the cells incubated with shaking and without selection to allow expression of the ampicillin resistance gene. The transformed cells are plated out on blue/white selection LB-agar plates, and left to incubate overnight at 37°C. The colonies are lifted onto nitrocellulose filters by the method of Maniatis (10% SDS – lysis, 3min; 1.5M NaOH-denaturation, 5min; 1.5M TricHCl – neutralisation, 5min; 3xSSC – rinse, 5min). The filters are then baked for 2 hours at 80°C under vacuum to fix the DNA. The library is screened with ^{32}P radiolabeled 636bp probe in the same manner as for Southern hybridization. After hybridization the filters are washed twice in 3xSSC, 0.1%SDS, 42°C, 15min. The filters are then rinsed in 3xSSC, sealed in plastic and exposed to X-ray film overnight at -80°C. Positive hybridizing spots are identified on the film. These are aligned to the agar plates containing the transformants. The hybridizing spots are matched up single colonies on the agar plates. All colonies in the radius of the hybridizing spot are picked up using sterile loops and used to inoculate 2 mL of Luria broth. The cultures are grown at 37°C for 2 hours. Dilutions of the cultures are made from 10^{-1} to 10^{-5} and 100mL of each sample is plated out on LB-amp agar plates and incubated overnight at 37°C. The plates which have between 10 and 150 colonies on them are chosen to go forward for a secondary screen. Colony lifts are done as before, and filters are processed using the same procedures. Fresh ^{32}P labeled probe is prepared, and the filters screened in the same way as outlined previously. Stringency washes are carried out using 2xSSC, 0.1%SDS at 42°C for 15 min. Filters are then rinsed in 2xSSC, sealed in plastic and exposed to X-ray film for 2 hours. The developed film should shows hybridizing spots, consistent with amplification of the positive colonies from the primary screen. The film is then aligned to the plates, and the spots co-ordinated to see if they corresponded to single isolated colonies. The best positives that match up to single colonies are picked and used to inoculate Luria broth for plasmid DNA preparations. Plasmid DNA is purified by Qiaspin Mini-Prep kit (Qiagen) and restriction analysis carried out to estimate the size of the inserts. All clones giving the same restriction profile can be used to suggest an insert size. Clones may be partially sequenced to determine if they are the correct gene/gene fragment. The full sequence of these clones is then determined.

8B. Percentage identity comparison between fungal phytases

The deduced polypeptide product of the cloned phytase gene fragment is used for homology analysis with published phytases. The analysis shows percent identities and, together with analysis of the translated sequence, may provided evidence that the gene fragment cloned is a homolog of a specific phytase.

8C. Generation and screening of a restriction enzyme-based size-restricted genomic library to isolate remainder of phytase gene

In order to isolate the remaining portion of a gene, a second restriction enzyme may be used to generate a second partial genomic library, and fragments may then be subcloned together. The restriction endonuclease recognition sites present within a cloned phytase sequence are identified using Webcutter. Of particular interest are sites for enzymes that are used in the Southern analysis discussed above. Very large fragments (e.g., 8 Kb), would be difficult to clone in a plasmid-based library. a low degree of hybridization with a specific restriction enzyme band argues against use of such in a library screen, and the presence of two bands in a restriction enzyme lane is likely to complicate the screening process. The library is made as before in pBluecript SKII, and screened using the same probe. A selection of positive hybridizing colonies are chosen and aligned to colonies on the plates. Matching colonies are picked for plasmid DNA preparations. Restriction analysis may show how many clones have inserts. These clones are then fully sequenced.

8D. Amplification of contiguous phytase gene for heterologous expression

A composite phytase sequence is produced from genomic clones and used to design a number of upstream and downstream primers which could be used to amplify a contiguous phytase gene sequence. PCR amplification is also designed to facilitate cloning and expression of the complete phytase gene in to a heterologous expression vector (e.g., pGAPT-PG, a 5.1Kb construct provided by Genencor International, Inc.) . Restriction enzyme sites within the multiple cloning site of the vector which are not present within the phytase gene sequence are determined. A number of 5' and 3' flanking primers may be designed using the phytase gene sequence, and modified to include the restriction enzyme recognition sites for these enzymes.

Restriction enzyme recognition sites are designed into the primer sequences to facilitate cloning into the expression vector. The upstream and downstream flanking regions used to design the primers are arbitrarily chosen at approximately 100bp upstream from the ATG (start) codon and downstream from the TAG (stop) codon respectively. The gene sequence used is also chosen to contain as equal balance of bases as possible.

Amplification of the phytase gene by PCR may be done using genomic DNA combinations of primers. PCR should amplify a region corresponding to the full-length phytase gene. The desired product produced by amplification with the primers is cloned into a vector and several clones which contain the correct size of insert are selected for sequencing. Homology analysis of the clone sequences is then performed and a full length phytase sequence determined.

PCR amplification genomic DNA is carried out using a combination of 5' primers and 3' primers, and using a high fidelity DNA polymerase, Pfu, to minimize error for expression of the phytase gene. This polymerase is *Pfu* DNA polymerase (Stratagene) and comes as part of the *Pfu* DNA polymerase kit for PCR. For these reactions, reaction buffer, dNTPs, target DNA and primers are mixed together, and 2.5 units of *Pfu* polymerase added in a final reaction volume of 50_L. After amplification, a 5_L aliquot of the reaction mixture is analyzed by gel electrophoresis. Selected fragments are cloned directly into the vector pCR-Blunt II TOPO (Invitrogen) , and a select number of clones analyzed to confirm the presence of the correct insert. (Blunt-ended PCR products that are generated by Pfu DNA polymerase are cloned into the Zero Blunt_ TOPO_PCR cloning kit (Invitrogen). This vector contains a MCS site and a kanamycin gene for antibiotic resistance, but also allows selection based on disruption of the lethal E.coli gene ccdb, as opposed to blue-white selection. Purified PCR product (50-200ng) is added to 1_L of pCR-BluntII-TOPO vector and the reaction volume made up to 5_L with sterile water. This is mixed gently and left to incubate for 5 min at room temperature. 1_L of 6 x TOPO Cloning Stop Solution is added, and the reaction left on ice or frozen at -20°C for up to 24 hours for transformation.) The integrity of the engineered restriction sites are also confirmed by this analysis. A number of clones are prepared and sequenced. Sequence analysis may confirm the presence of a full-length phytase gene. This gene may then be taken forward for expression in a heterologous system, and subsequent biochemical characterisation of the enzyme.

8E. Analysis of phytase sequence

An alignment is made of the isolated sequence and published phytases and homology analysis done, on a % identity basis.

Example 9

Cloning, expression and characterization of the phytase

Over-expression of the phytase gene in a heterologous host may be done to produce enough protein to carry out characterization of the enzyme.

9A. Cloning of phytase gene into expression vector and transformation in to a host

The full-length phytase gene is amplified with a high-fidelity DNA polymerase, is produced using primers that are engineered to contain two restriction enzyme sites (e.g., EcoRV and AgeI). These sites are used to facilitate cloning into the expression vector (e.g., pGAPT-PG). The phytase clones are digested with the enzymes to produce a single insert fragment. The vector is also digested with these enzymes and linearize. The phytase gene fragment is ligated to the expression vector, and a number of transformants produced. A selection of these clones is analyzed to confirm the presence of the insert. The phytase clones are then used to transform swollen spores of A. nidulans by electroporation.

The transformation of host such as *A. niger* strain FGSC A767 and *A. nidulans* FGSC A1032 by electroporation is adapted from the protocol of O. Sanchez and J. Aguirre developed for A. nidulans. 50 mL of YG medium (0.5% yeast extract, 2% glucose, supplemented with 10mM uridine and 10mM uracil) is inoculated at 10^7 spores/mL with appropriate spore suspension. The cultures are grown for 4 hr at 25□C at 300 rpm on rotary shaker. Swollen spores are collected by centrifugation at 4000rpm for 5 min at 4□C. Spores are resuspended in 200 mL ice-cold sterile water and centrifuged at 4000 rpm for 5 min at 4□C. The supernatant is poured off and the spores are resuspended in 12.5 ml YED media pH 8.0 (1% yeast extract, 1% glucose, 20mM HEPES) and incubated for 60 min at 30°C at 100 rpm on rotary shaker. The spores are collected by centrifugation at 4000rpm for 5 min, then resuspended in 1 mL of ice-cold EB buffer (10mM tris-HCl, pH 7.5, 270mM sucrose, 1mM Lithium acetate) at a concentration of 10^9 conidia.mL^{-1} and kept on ice. 50_L of the swollen spore suspension is mixed with 1 to 2 µg DNA in a total volume of 60 µl in sterile Eppendorf and kept on ice for 15 min. The suspension is transferred to 0.2 cm electroporation cuvette. Electroporation is carried out in a BioRad electroporation device (settings 1 kV, 400 W, 25 µF). 1 mL of ice-cold YED is added to the suspension after electroporation, and the combined mix is transferred to a pre-chilled sterile 15 mL Falcon tube and kept on ice for 15 min. This is then incubate at 30□C for 90 min at 100 rpm on rotary shaker, with the tubes in a horizontal position. The spores are plated out and transformants are observed after 36-48 hours.

Circular plasmid DNA may be used. *A. niger* strain FGSC A767 and *A. nidulans* strain FGSC A1032 can be obtained from the Fungal Genetics Stock Center, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas, USA.

9B. Preliminary characterization of transformants

Transformants are selected for further analysis. Spores from each of these transformants are used to inoculate selective media, and spore suspensions of each clone are made. These are used to inoculate liquid cultures of the transformants which are screened for phytase activity. Cultures are grown over 72 hours, and the supernatants collected. Samples are desalted in PD-10 columns, and the protein samples eluted in 0.25 M sodium acetate. Phytase assays are carried out in the standard conditions (pH 5.5, 37oC for 30 min). Clones are identified having phytase activity. These are taken forward for further analysis.

9C. Time of maximal expression of phytase in liquid culture

In order to assess when the level of phytase production is at its highest for subsequent biochemical characterisation, a series of liquid cultures of clones are generated over a 2-day to 7-day period. Cultures are inoculated with spore suspension of the appropriate transformants, and harvested at each day over this period. Culture supernatants are processed as standard, and the desalted culture supernatant is assayed under standard phytase conditions. The time point of highest phytase activity is then determined

Liquid cultures are harvested at each time point, desalted and eluted in 0.25 mM sodium acetate pH 5.5. Phytase assays are carried out under standard conditions (pH 5.5, 37oC, 30 min) in duplicate. Activity is expressed in phytase units per mL of culture supernatant (µmoles of Pi released min-1 mL-1).

Untransformed host may also be assayed across these time-points as a control. Protein samples from selected supernatant samples (day 4 and day 6), both before and after desalting are analyzed by SDS-PAGE to determine levels of secretion.

9D. Southern analysis of transformants

Although there may be evidence that the phytase gene has been successfully cloned into the expression vector, and that expression of an active enzyme had been achieved, molecular evidence may also be obtained. Genomic DNA preparations are made from the transformed host, and from the original untransformed host. The DNA is digested with a restriction enzyme, preferably one where there is no internal site within the phytase gene, and Southern hybridization analysis of the transformants is carried out. The Southern blots are analyzed with a phytase probe from species under investigation. Single strong hybridizing bands seen for the transformants under conditions of medium to high stringency (3xSSC) indicate successful cloning. If there is no evidence of any other hybridizing bands, it can be concluded that a single-copy of the phytase gene is present in the transformed host. A lack of hybridizing bands in the untransformed sample indicates that there is no homology between the phytase of interest and any phytases present in the host genome.

9E. Biochemical characterization of a phytase

To prove that the cloned gene represents a specific phytase activity, and to characterize that activity, a range of biochemical analyses are carried out on the over-expressed enzyme. Preliminary characterization may indicate that the gene is producing a phytic-acid hydrolyzing activity. This analysis can be extended to examine activity at different pHs, temperatures and against different substrates.

Transformants are taken forward for these analyses, and cultures are harvested at optimum expression time, as determined above. With phytic acid as the substrate, the pH effect on enzyme activity can be shown. The purified enzyme sample is desalted from culture supernatant, and eluted in 0.025mM sodium acetate pH 5.0. This is then added to substrate which is made in solutions of the following buffers: pH 3.0: 0.4M glycine-HCl , pH 4.0: 0.4M Sodium acetate, pH 5.0: 0.4M Sodium acetate, pH 6.0: 0.4M imidazole-HCl, pH 7.0: 0.4M Tris-HCl , pH 8.0: 0.4M Tris-HCl pH 9.0: 0.4M Tris-HCl. An optimum pH for the phytase activity may be determined, as well. Little activity seen when

4-nitrophenyl-phosphate is used as the substrate indicates a high level of specificity for the phytic-acid substrate.

The temperature profile of the enzyme is characterized using pH 5.0 buffer, over a range of temperatures, using phytic acid as the substrate. The phytase temperature activity range and optimum activity temperature can be determined.

Preliminary stability studies may also be carried out on the phytase. Samples of the protein are left at –20°C, 4°C, and 37°C overnight and then assayed under standard conditions. Samples may also be exposed to high temperature (e.g., 80-105□C for 5-25 minutes) to determine the thermostability of the phytase activity. Residual activity is based on comparison to phytase activity determinations taken from the samples before exposure to each condition. Samples may be assayed afterwards in the same assay conditions.

Of course, it should be understood that a wide range of changes and modifications can be made to the preferred embodiment described above. It is therefore intended that the foregoing detailed description be understood in the context of the following claims, including all equivalents, which are intended to define the scope of this invention.

CLAIMS

1. An isolated polynucleotide comprising a nucleotide sequence (i) having at least 55% identity to a nucleotide sequence as disclosed in Figure 1 or 19A-19C, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 1 or 19A-19C under conditions of high stringency, or (iii) being complementary to a nucleotide sequence having at least 85% identity to a nucleotide sequence as disclosed in Figure 1 or 19A-19C.

2. An expression construct comprising the polynucleotide of claim 2.

3. A vector including the expression construct of claim 2.

4. A host cell transformed with the vector of claim 3.

5. An isolated polyncucleotide encoding an enzyme having phytase activity, wherein said enzyme includes an amino acid sequence having at least 70% identity to an amino acid sequence as disclosed in Figures 2, 3 or 19A-19C.

6. Food or animal feed including an enzyme having phytase activity, wherein said enzyme comprises an amino acid sequence having at least 70% identity to an amino acid sequence as disclosed in Figure 2, 3 or 19A-19C.

7. An isolated phytase enzyme wherein said enzyme is obtained from a Penicillium chrysogenum, and has the following physiochemical properties: (1) Molecular weight: between about 49 and 51 kDa (non-glycosylated); and (2) Substrate: phytate.

8. A method of producing an enzyme having phytase activity, comprising:

(a) providing a host cell transformed with an expression vector comprising a polynucleotide as defined in claim 1;

(b) cultivating said transformed host cell under conditions suitable for said host cell to produce said phytase; and

(c) recovering said phytase.

9. The method of claim 8, wherein said host cell is an Aspergillus species.

10. A purified enzyme having phytase activity, produced by the method of claim 9.

11. A method of separating phosphorous from phytate, comprising:

treating said phytate with an enzyme comprising an amino acid sequence having at least 70% identity to an amino acid sequence as disclosed in Figure 2, 3 or 19A-19C.

12. A method of separating phosphorous from phytate, comprising: treating said phytate with an enzyme as defined in claim 8.

13. An isolated polynucleotide encoding an enzyme, said enzyme comprisingan an amino acid sequence having at least 55% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21.

14. An isolated polynucleotide including a nucleotide sequence (i) having at least 55% identity to a nucleotide sequence as disclosed in Figure 4, 7, 18A-18C, and 21, or (ii) being capable of hybridizing to a probe derived from the nucleotide sequence disclosed in Figure 4, 7, 18A-18C, and 21 under conditions of intermediate to high stringency, or (iii) being complementary to a nucleotide sequence having at least 55% identity to a nucleotide sequence as disclosed in Figure 4, 7, 18A-18C, and 21.

15. An expression construct including a polynucleotide according to claim 13 or claim 14.

16. A vector including the expression construct of claim 15.

17. A host cell transformed with the vector of claim 16.

18. Food or animal feed including an enzyme having phytase activity, wherein said enzyme includes an amino acid sequence having at least 55% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21.

19. A method of producing an enzyme having phytase activity, comprising:

(a) providing a host cell transformed with an expression vector comprising a polynucleotide as defined in claim 13 or claim 14;

(b) cultivating said transformed host cell under conditions suitable for said host cell to produce said phytase; and

(c) recovering said phytase.

20. The method of claim 19, wherein said host cell is an Aspergillus species.

21. A purified enzyme having phytase activity, produced by the method of claim 20.

22. A method of separating phosphorous from phytate, comprising:

treating said phytate with an enzyme (i) having phytate hydrolyzing activity and (ii) including an amino acid sequence having at least 55% identity to an amino acid sequence as disclosed in Figure 5, 6, 8, 18A-18C or 21.

FIG. 1

TGCACTACTGTCGATGGCGGTTACCAATGCAATTCCGAGCTCTCACACAAGTGGGGCCAGTATTCGCCCTATTTCTCTCT
TTCCGAAGAATCATCCATCTCGAATGAGGTACCTCATGATTGTCAGATCACTTTTGCTCAAGTGATCTCCCGTCATGGTG
CTCGATTCCCGTCCGCGAAGAAGAGCAAGGTATATGCCAAGCTCATTGAAAATATCCAAGCGAACGCGACTGCATACAAT
GGCAACACGAAGTTCCTCCGCTCATACAAGTACACCATGGGCGGTGATGATTTGGTACCCTTCGGAGTGAACCAGACGGT
GGACTCGGGGACCAAATTCTACCAGCGCTACGAGGCGTTGGCGAAGAAAGCTGTGCCCTTCATTCGGTCATCTGACTCAG
GGCGGGTTGTGGCTTCAGGCGTGAACTTTATCAAGGGATTCCAGCAGGCAAAGTTGGATGATAAAAATGCCAATCACCGT
CAGCCAAGCCCCAAAACCAACGTCATCATCTCAGAAGAGTCTGGCACCAACAACACTCTGAACCACAGCGAGATCTGTCC
TAAGTTCGAAGACAATGAGCTGGGCGACAAGGTCGAAGAAAAATACATGAAAATCTTTGTGCCGCCCATCCGAGCTCGTC
TCGAGGCCGATCTCCCTGGCGTTAAACTTGAAGACATCGATGTTGTCAGTCTGATGGACATCTGCCCTTTCGAGACAGTG
TCTTCAAGTGACGACGCAGCCGAGCTATCTCCATTCTGCGACCTCTTCACCCCGACCGAATGGAGCCAATATGACTACCT
CCAGTCGTTAAGCAAGTACTATGGTTATGGCGCCGGCAATCCTCTCGGCCCGACCCAGGGTGTCGGTTTCGTAAACGAAC
TGATTGCCCGACTCACTCGCCACCCAGTGAGAGACCACACAAGCACAAACCGTGCGCTCGATGCCCCCGGCGCTGCGACA
TTCCCCCTCAACTACACCATGTATGCCGACTTCACGCATGACAACGGAATGATCCCGTTCTTCTTTGCTTTGGGGCTGTA
CAACGGCACCGCTCCACTCTCGCTCACCCACGTCCAGTCTCCTAGCCAAACAGACGGGTTCTCATCCGCCTGGACAGTCC
CCTTCGGTGCTCGGGCTTATGTTGAGATGATGCAATGTCGTCGGGAACCTGAGCCGCTCGTGCGAGTCCTCGTTAATGAC
CGTGTTATTCCGCTGCACGGTTGCCCGGTGGATAAACTTGGCCGTTGTCGCCGTCGTGATTTCGTGAAAGGGCTTACTTT
CGCACGCTCTGGCGGCGACTGGGCCAGGTGTTATAAA

FIG. 2

CTTVDGGYQCNSELSHKWGQYSPYFSLSEESSISNEVPHDCQITFAQVISRHGARFPSAKKSKVYAKLIENIQANATAYN
GNTKFLRSYKYTMGGDDLVPFGVNQTVDSGTKFYQRYEALAKKAVPFIRSSDSGRVVASGVNFIKGFQQAKLDDKNANHR
QPSPKTNVIISEESGTNNTLNHSEICPKFEDNELGDKVEEKYMKIFVPPIRARLEADLPGVKLEDIDVVSLMDICPFETV
SSSDDAAELSPFCDLFTPTEWSQYDYLQSLSKYYGYGAGNPLGPTQGVGFVNELIARLTRHPVRDHTSTNRALDAPGAAT
FPLNYTMYADFTHDNGMIPFFFALGLYNGTAPLSLTHVQSPSQTDGFSSAWTVPFGARAYVEMMQCRREPEPLVRVLVND
RVIPLHGCPVDKLGRCRRRDFVKGLTFARSGGDWARCYK

FIG. 3

ASRNQSTCTTVDGGYQCNSELSHKWGQYSPYFSLSEESSISNEVPHDCQITFAQVISRHGARFPSAKKSKVYAKLIENIQ
ANATAYNGNTKFLRSYKYTMGGDDLVPFGVNQTVDSGTKFYQRYEALAKKAVPFIRSSDSGRVVASGVNFIKGFQQAKLD
DKNANHRQPSPKTNVIISEESGTNNTLNHSEICPKFEDNELGDKVEEKYMKIFVPPIRARLEADLPGVKLEDIDVVSLMD
ICPFETVSSSDDAAELSPFCDLFTPTEWSQYDYLQSLSKYYGYGAGNPLGPTQGVGFVNELIARLTRHPVRDHTSTNRAL
DAPGAATFPLNYTMYADFTHDNGMIPFFFALGLYNGTAPLSLTHVQSPSQTDGFSSAWTVPFGARAYVEMMQCRREPEPL
VRVLVNDRVIPLHGCPVDKLGRCRRRDFVKGLTFARSGGDWARCYK

FIG. 4

TGTGAGTATGACGGGAGCTGTAATGACATCTCTCGGCTCTGGGGACAGTACTCTGCATACTTCCCAATCCCGTCTGAGCT
TGATGCCTCAACACCAGACGATTGTGATGTGACTTTTGCACTCGTCTTGTCCCGCCATGGAGCCAGGTACCCAACGGACA
GCAAGTCTGCAGCATACAACGCTACCATTGCCCGCATTCAAAAGTCTGCTACCATGTACGGCAAGAACTACAAGTGGCTT
AAGGAGTATACCTACAGTCTCGGCGCTGAAGACCTGACTGAGTTTGGCCAGCGGCAGATGGTCGACTCTGGTAGGGCCTT
TTATGAGCGGTACATGAGTCTCGCTGAGAAGACTGAGCCTTTTGTTCGGGCATCGGGCTCAGATCGGGTCATCATGTCGT
CTTACAATTTTACGCAAGGCTTTTACGCATCGCGAGGAGAGTCTGGAGACGATTATACTCAGGATGTTCTTATCATCCCT
GAAGAACCTGGCATCAACAACACCATGTTGCATGGATCGTGCGCCTCATTCGAAAGCGACAGAGTTCCTAAAGACGCAGA
TGAAAAGGCCGAGGTTGCATGGGGAGCAAGATTCCTCCCCGAGATTCGAAATAGGTTGAACCACCACCTGCCAGGAGTCA
ACCTGACGCTGGAGGAAACCATCTACATGATGGACATGTGTCCGTTCCTCGCGGCTGACACACCTGATGGCGCTGGTCAC
TCGAGGTTCTGCGACCTCTTCACCAAGGCAGACTGGCGAAGTTACGACTACTACATGACTCTGAGCAAGTTCTACAAGTT
TGGCAATGGCAATGCCATGGGACCGACACAAGGTGTTGGATATGTCAACGAACTCATCTCACGCTTGACTGGGAAGCCTG
TTGACGACCACACCACGACCAACAGCACATTGGACTCATCGCCAAAGACGTTCCCTCTTGACAGGGCTCTATATGCGGAT
TTTAGCCACGACAACAGCATGGTCTCCATCTTCTCAGCACTGGGCTTGTACAACTCGACTACCCTGCTACCAAAGGACCA
TATTGTGCCCGCGATCAAGGCGCACGGCTACTCATCGACATGGGTAGTCCCCTTTGGAGCCAGAATGTACGTCGAGAAGC
TCGAGTGTGGTGCCAGCAGGAATGAAAAGAGAGACGAGTACGTGCGAGTCCTGGTCAACGACCGAGTGATGTCGCTCGAA
ACCTGCGGAGGCGACGAGTACGGGCTCTGCAGACTAGAAAACTTTGTGGAGAGTCTGTCGTTTGCCGCCTCGGGAGGAAA
CTGGGATCAATGCGGTGGA

FIG. 5

CEYDGSCNDISRLWGQYSAYFPIPSELDASTPDDCDVTFALVLSRHGARYPTDSKSAAYNATIARIQKSATMYGKNYKWL
KEYTYSLGAEDLTEFGQRQMVDSGRAFYERYMSLAEKTEPFVRASGSDRVIMSSYNFTQGFYASRGESGDDYTQDVLIIP
EEPGINNTMLHGSCASFESDRVPKDADEKAEVAWGARFLPEIRNRLNHHLPGVNLTLEETIYMMDMCPFLAADTPDGAGH
SRFCDLFTKADWRSYDYYMTLSKFYKFGNGNAMGPTQGVGYVNELISRLTGKPVDDHTTTNSTLDSSPKTFPLDRALYAD
FSHDNSMVSIFSALGLYNSTTLLPKDHIVPAIKAHGYSSTWVVPFGARMYVEKLECGASRNEKRDEYVRVLVNDRVMSLE
TCGGDEYGLCRLENFVESLSFAASGGNWDQCGG

FIG. 6

ASRNQSTCEYDGSCNDISRLWGQYSAYFPIPSELDASTPDDCDVTFALVLSRHGARYPTDSKSAAYNATIARIQKSATMY
GKNYKWLKEYTYSLGAEDLTEFGQRQMVDSGRAFYERYMSLAEKTEPFVRASGSDRVIMSSYNFTQGFYASRGESGDDYT
QDVLIIPEEPGINNTMLHGSCASFESDRVPKDADEKAEVAWGARFLPEIRNRLNHHLPGVNLTLEETIYMMDMCPFLAAD
TPDGAGHSRFCDLFTKADWRSYDYYMTLSKFYKFGNGNAMGPTQGVGYVNELISRLTGKPVDDHTTTNSTLDSSPKTFPL
DRALYADFSHDNSMVSIFSALGLYNSTTLLPKDHIVPAIKAHGYSSTWVVPFGARMYVEKLECGASRNEKRDEYVRVLVN
DRVMSLETCGGDEYGLCRLENFVESLSFAASGGNWDQCGG

FIG. 7

GCGGATTTTAGGCACGATAATAGTCTGACCTCGATATACGCTGCTCTTGGTCTGTATAACGGCACAAAGCAACTATCCAA
ATCGAGGATAGAATCGACAAACCAGACAAATGGCTATTCTGCTGGCTGGACAGTTCCATTTGGAGCAAGGGCGTATGTTG
AGATGATGCAATGCCCCTCGGGGGATGAACCTCTGATTCGAGTTCTGGTGAACGATCGCGTCAT

FIG. 8

ADFRHDNSLTSIYAALGLYNGTKQLSKSRIESTNQTNGYSAGWTVPFGARAYVEMMQCPSGDEPLIRVLVNDRV

FIG. 9

TGCGACTCTGTCGACAGAGGCTTCTGGTGCGCCGCCGACATCTCCCACTCCTGGGGACAGTACTCACCATACTTCTCCGT
CCCCTCTGACATTGACCCGGGTTTCCCCAAGGGCTGCAATGTGACGTTCGCACAGGTCCTCTCACGCCACGGCGCCCGCG
CCCCAACTACGGGCCGGGCCGCCTACTACGTCGACGTGATTGACCGCGTCCAGCGTCAGGCGACCTCGTACGGCCCCGGC
CACGCGTTCCTGCGCTCCTACCGCTACACCCTCGGCGCCAACGAGCTTACCCCGATGGGAGAGCGGCAGCTGGCGTATTC
CGGCGCAAGGTTTTACCATCGCTATCGCGAACTTGCGCGCGTCGAGGCGCCCTTCGTGCGGTCCAGTGGCGTAAGCCGCG
TTGTAGCCTCAGCTGTCAATTTCACCCAGGGCTTCCACCAGGCGCGGCTCGCCGACCGCGGCGCCACGTTGCCCCCGCCA
ACACTGCCCTATGACATGGTGATCATCTCGTCAGACGACACCGCCAACAACACCTTGCACCACGGTCTCTGCACGGTCTT
CGAGGAGGGGCCCTATGCCGACATTGGCGACAAGGCGCAGAAAGAATACCTCTCCAAGTTTGTCGGTCCCATCGTGGAGC
GCATTAACGCGCAGCTGCCCGGCGCGAATCTCAACGCGACGGACATCATCGCGCTGATGGACCTGTGCCCGTTCGAGACG
GTCGCGTTCCCAGAAGGCACGAAGCTGTCGCCCTTCTGCCGGCTCTTCACGGCCGCCGAATGGCGGGCCTACGACCGGTA
CCAGGACGTCGGCAAATGGTTCGGCTACGGCCCGGGCAATCCGCTCGGCCCGACTCAGGGGGTCGGGTTCGTCAACGAGC
TGATCGCGCGGCTGTCCGGCCAGCCGGTGAGCGATGGGACCAGCACGAACCGCACGCTGGATGAGAACCCGGAGACCTTC
CCGCTCGGGAGGAGGCTGTATGCGGATTTCAGCCATGATAACGACATGGTGGGCATCCTCAGCGCCTTGGGGTTGTGGGA
CAACCATGAAGAACCTGGGAATGAAATGCCCGCTGAGGGGGAGGAGGACGACAATGGTCGGTTCTCGACTGCTAGGGCCG
TGCCGTTCGGGGCGCGGGTGTATGTCGAAAAGCTGCGGTGTGGGGGATCGGAGGAGGATGAAGAAATGGTGCGCGTGTTG
GTCAATGACCGGGTGATGCCCCTTGCACAGTGCGGAGGGGACAAGAGGGGAATGTGCACCCTCAGCCGGTTCGTTGAAAG
CTTGAAGTTTGCGCGGAACAACGGGAGGTGGGACATGTGTTTTGAA

FIG. 10

CDSVDRGFWCAADISHSWGQYSPYFSVPSDIDPGFPKGCNVTFAQVLSRHGARAPTTGRAAYYVDVIDRVQRQATSYGPG
HAFLRSYRYTLGANELTPMGERQLAYSGARFYHRYRELARVEAPFVRSSGVSRVVASAVNFTQGFHQARLADRGATLPPP
TLPYDMVIISSDDTANNTLHHGLCTVFEEGPYADIGDKAQKEYLSKFVGPIVERINAQLPGANLNATDIIALMDLCPFET
VAFPEGTKLSPFCRLFTAAEWRAYDRYQDVGKWFGYGPGNPLGPTQGVGFVNELIARLSGQPVSDGTSTNRTLDENPETF
PLGRRLYADFSHDNDMVGILSALGLWDNHEEPGNEMPAEGEEDDNGRFSTARAVPFGARVYVEKLRCGGSEEDEEMVRVL
VNDRVMPLAQCGGDKRGMCTLSRFVESLKFARNNGRWDMCFE

FIG. 11

ASRNQSTCDSVDRGFWCAADISHSWGQYSPYFSVPSDIDPGFPKGCNVTFAQVLSRHGARAPTTGRAAYYVDVIDRVQRQ
ATSYGPGHAFLRSYRYTLGANELTPMGERQLAYSGARFYHRYRELARVEAPFVRSSGVSRVVASAVNFTQGFHQARLADR
GATLPPPTLPYDMVIISSDDTANNTLHHGLCTVFEEGPYADIGDKAQKEYLSKFVGPIVERINAQLPGANLNATDIIALM
DLCPFETVAFPEGTKLSPFCRLFTAAEWRAYDRYQDVGKWFGYGPGNPLGPTQGVGFVNELIARLSGQPVSDGTSTNRTL
DENPETFPLGRRLYADFSHDNDMVGILSALGLWDNHEEPGNEMPAEGEEDDNGRFSTARAVPFGARVYVEKLRCGGSEED
EEMVRVLVNDRVMPLAQCGGDKRGMCTLSRFVESLKFARNNGRWDMCFE

FIG. 12

ATGGGCGTCTCTGCTGTTCTACTTCCTTTGTATCTCCTAGCTGGGTATGCTAAGCACCGCTATCTAAGTCTGATAAGGAC
CCTCTCTGCCGAGGGCCCCTGAAGCTCGGACTGTGTGGGACTACTGATCGCTGACAATCTGTGCAGAGTCACCTCCGGAC
TGGCAGTCCCCGCCTCGAGAAATCAATCCACT

FIG. 13

MGVSAVLLPLYLLAGVTSGLAVPASRNQST

FIG. 14A

```
                      .    .   .    .   .
A. niger            1 ASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANESVISPEVPAGCRV 50
                      ||||||.| ||| |||| || || ||||.|:|||. || || |||  |.:
P. chrysogenum      1 ASRNQSTCTTVDGGYQCNSELSHKWGQYSPYFSLSEESSISNEVPHDCQI 50

                               .         .         .         .         .
                   51 TFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSL 100
                      |||||:||||||:|.  | | |. ||| || ||| :.|   ||:.| |.:
                   51 TFAQVISRHGARFPSAKKSKVYAKLIENIQANATAYNGNTKFLRSYKYTM 100

                               .         .         .         .         .
                  101 GADDLTPFGEQELVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKF 150
                      | ||| |||  : |.|| |||||||.| :  ||||||| | ||:|||  |
                  101 GGDDLVPFGVNQTVDSGTKFYQRYEALAKKAVPFIRSSDSGRVVASGVNF 150

                               .         .         .         .         .
                  151 IEGFQSTKLKDPRAQPGQSSPKIDVVISEASSSNNTLDPG.TCTVFEDSE 199
                      |.|||  || |  |   | ||| .|:||| | .||||.    |  |||.|
                  151 IKGFQQAKLDDKNANHRQPSPKTNVIISEESGTNNTLNHSEICPKFEDNE 200

                               .         .         .         .         .
                  200 LADTVEANFTATFVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTISTS 249
                      | | ||  :   ||| || ||| || || | | :|  |||.| |:|:|.|
                  201 LGDKVEEKYMKIFVPPIRARLEADLPGVKLEDIDVVSLMDICPFETVSSS 250

                               .         .         .         .         .
                  250 TVDTKLSPFCDLFTHDEWINYDYLQSLKKYYGHGAGNPLGPTQGVGYANE 299
                          .|||||||||  ||  ||||||| ||||:|||||||||||||: ||
                  251 DDAAELSPFCDLFTPTEWSQYDYLQSLSKYYGYGAGNPLGPTQGVGFVNE 300

                               .         .         .         .         .
                  300 LIARLTHSPVHDDTSSNHTLDS.SPATFPLNSTLYADFSHDNGIISILFA 348
                      ||||||  || | ||.|  ||.   |||||| |:||||.||||.|   ||
                  301 LIARLTRHPVRDHTSTNRALDAPGAATFPLNYTMYADFTHDNGMIPFFFA 350

                               .         .         .         .         .
                  349 LGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQAEQEPL 398
                      ||||||| ||| | |:. .||||||||||||| .| |||||||. | |||
                  351 LGLYNGTAPLSLTHVQSPSQTDGFSSAWTVPFGARAYVEMMQCRREPEPL 400

                               .         .         .         .    .
                  399 VRVLVNDRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAECFA 444
                      |||||||||:|||||||| |||| |  ||:||.||||||||| |:
                  401 VRVLVNDRVIPLHGCPVDKLGRCRRRDFVKGLTFARSGGDWARCYK 446
```

FIG. 14B

```
(P.c.): 1   ASRNQSTCTTVDGGYQCNSELSHKWGQYSPYFSLSEESSISNEVPHDCQITFAQVISRHG 60
            +S    +C TVD GYQC+   SH WGQYSP+FSL +E S+S+++P DC+IT  QV+SRHG
(A.f.): 407 SSAGSKSCDTVDLGYQCSPATSHLWGQYSPFFSLEDELSVSSKLPKDCRITLVQVLSRHG 586

      : 61  ARFPSAKKSKVYAKLIENIQANATAYNGNTKFLRSYKYTMGGDDLVPFGVNQTVDSGTKF 120
            AR+P++ KSK Y KL+  IQANAT + G   FL++Y YT+G DDL PFG  Q V+SG KF
      : 587 ARYPTSSKSKKYKKLVTAIQANATDFKGKFAFLKTYNYTLGADDLTPFGEQQLVNSGIKF 766

      : 121 YQRYEALAKKAVPFIRSSDSGRVVASGVNFIKGFQQAKLDDKNANHRQPSPKTNVIISEE 180
            YQRY+ALA+  VPFIR+S S RV+ASG  FI+GFQQAKL D  A +R  +P  +VII E
      : 767 YQRYKALARSVVPFIRASGSDRVIASGEKFIEGFQQAKLADPGATNR-AAPAISVIIPES 943

      : 181 SGTNNTLNHSEICPKFEDNELGDKVEEKYMKIFVPPIRARLEADLPGVKLEDIDVVSLMD 240
               NNTL+H  +C KFE ++LGD+V   +  +F P IRAR E  LPGV L D DVVSLMD
      : 944 ETFNNTLDHG-VCTKFEASQLGDEVAANFTALFAPDIRARAEKHLPGVTLTDEDVVSLMD 1120

      : 241 ICPFETVSSSDDAAELSPFCDLFTPTEWSQYDYLQSLSKYYGYGAGNPLGPTQGVGFVNE 300
            +C F+TV+ + DA++LSPFC LFT  EW +Y+YLQSL KYYGYGAGNPLGP QG+GF NE
      : 1121MCSFDTVARTSDASQLSPFCQLFTHNEWKKYNYLQSLGKYYGYGAGNPLGPAQGIGFTNE 1300

      : 301 LIARLTRHPVRDHTSTNRALDAPGAATFPLNYTMYADFTHDNGMIPFFFALGLYNGTAPL 360
            LIARLTR PV+DHTSTN  L     ATFPLN TMY DF+HDN M+  FFALGLYNGT PL
      : 1301LIARLTRSPVQDHTSTNSTL-VSNPATFPLNATMYVDFSHDNSMVSIFFALGLYNGTEPL 1477

      : 361 SLTHVQSPSQTDGFSSAWTVPFGARAYVEMMQCRREPEPLVRVLVNDRVIPLHGCPVDKL 420
            S T V+S  + DG+S++W VPFGARAY E MQC+ E EPLVR L+NDRV+PLHGC VDKL
      : 1478SRTSVESAKELDGYSASWVVPFGARAYFETMQCKSEKEPLVRALINDRVVPLHGCDVDKL 1657

      : 421 GRCRRRDFVKGLTFARSGGDWARCY 445
            GRC+  DFVKGL++ARSGG+W  C+
      : 1658GRCKLNDFVKGLSWARSGGNWGECF 1732
```

FIG. 14C

```
(P.c.): 4   NQSTCTTVDGGYQCNSELSHKWGQYSPYFSLSEESSISNEVPHDCQITFAQVISRHGARF 63
            N S CT+VD GYQC  ELSHKWG Y+PYFSL +ES    +VP DC ITF QV++RHGAR
(A.t.): 411 NHSDCTSVDRGYQCFPELSHKWGLYAPYFSLQDESPFPLDVPDDCHITFVQVLARHGARS 590

      : 64  PSAKKSKVYAKLIENIQANATAYNGNTKFLRSYKYTMGGDDLVPFGVNQTVDSGTKFYQR 123
            P+  K+K YA  I  IQ NATA  G   FL+SY Y+MG ++L PFG NQ  D G +FY+R
      : 591 PTDSKTKAYAATIAAIQKNATALPGKYAFLKSYNYSMGSENLNPFGRNQLQDLGAQFYRR 770

      : 124 YEALAKKAVPFIRSSDSGRVVASGVNFIKGFQQAKLDDKNANHRQPSPKTNVIISEESGT 183
            Y+ L +   PF+R++DS RV  S   F++GFQ A+  D +AN  QPSP+ +V+I E +
      : 771 YDTLTRHINPFVRAADSSRVHESAEKFVEGFQNARQGDPHANPHQPSPRVDVVIPEGTAY 950

      : 184 NNTLNHSEICPKFEDNELGDKVEEKYMKIFVPPIRARLEADLPGVKLEDIDVVSLMDICP 243
            NNTL HS IC  FE + +GD   + +  +F P I  RLEADLPGV+L   DVV+LM +CP
      : 951 NNTLEHS-ICTAFEASTVGDAAADNFTAVFAPAIAKRLEADLPGVQLSADDVVNLMAMCP 1127

      : 244 FETVSSSDDAAELSPFCDLFTPTEWSQYDYLQSLSKYYGYGAGNPLGPTQGVGFVNELIA 303
            FETVS +DDA  LSPFCDLFT  EW+QY+YL SL KYYGYG GNPLGP QGVG+ NELIA
      : 1128FETVSLTDDAHTLSPFCDLFTAAEWTQYNYLLSLDKYYGYGGGNPLGPVQGVGWANELIA 1307

      : 304 RLTRHPVRDHTSTNRALDAPGAATFPLNYTMYADFTHDNGMIPFFFALGLYNGTAPLSLT 363
            RLTR PV DHT  N  LDA   ATFPLN T+YADF+HD+ ++  F+ALGLYNGT PLS T
      : 1308RLTRSPVHDHTCVNNTLDA-NPATFPLNATLYADFSHDSNLVSIFWALGLYNGTKPLSQT 1484

      : 364 HVQSPSQTDGFSSAWTVPFGARAYVEMMQCRREPEPLVRVLVNDRVIPLHGCPVDKLGRC 423
             V+  ++TDG+++AWTVPF ARAY+EMMQCR E +PLVRVLVNDRV+PLHGC VD LGRC
      : 1485TVEDITRTDGYAAAWTVPFAARAYIEMMQCRAEKQPLVRVLVNDRVMPLHGCAVDNLGRC 1664

      : 424 RRRDFVKGLTFARSGGDWARCY 445
            +R DFV+GL+FAR+GG+WA C+
      : 1665KRDDFVEGLSFARAGGNWAECF 1730
```

FIG. 14D

```
(P.c.): 7   TCTTVDGGYQCNSELSHKWGQYSPYFSLSEESSISNEVPHDCQITFAQVISRHGARFPSA 66
            +C T DGGYQC   +SH WGQYSPYFS+ +ES+IS +VPH C++TF QV+SRHGAR+P+
(E.n.): 293 SCNTADGGYQCFPNVSHVWGQYSPYFSIEQESAISEDVPHGCEVTFVQVLSRHGARYPTE 472

      : 67  KKSKVYAKLIENIQANATAYNGNTKFLRSYKYTMGGDDLVPFGVNQTVDSGTKFYQRYEA 126
             KSK Y+ LIE IQ NAT++ G   FL SY YT+G DDL  FG NQ VDSG KFY+RY+
      : 473 SKSKAYSGLIEAIQKNATSFWGQYAFLESYNYTLGADDLTIFGENQMVDSGAKFYRRYKN 652

      : 127 LAKKAVPFIRSSDSGRVVASGVNFIKGFQQAKLDDKNANHRQPSPKTNVIISEESGTNNT 186
            LA+K  PFIR+S S RVVAS   FI GF++A+L D  +  ++ +P  NVII E  G NNT
      : 653 LARKNTPFIRASGSDRVVASAEKFINGFRKAQLHDHGS--KRATPVVNVIIPEIDGFNNT 826

      : 187 LNHSEICPKFEDNELGDKVEEKYMKIFVPPIRARLEADLPGVKLEDIDVVSLMDICPFET 246
            L+HS  C  FE++E  D++E  +  I  PPIR RLE DLPG+KL + +V+ LMD+C F+T
      : 827 LDHS-TCVSFENDERADEIEANFTAIMGPPIRKRLENDLPGIKLTNENVIYLMDMCSFDT 1003

      : 247 VSSSDDAAELSPFCDLFTPTEWSQYDYLQSLSKYYGYGAGNPLGPTQGVGFVNELIARLT 306
            ++ +    ELSPFC +FT  EW QYDYLQSLSKYYGYGAG+PLGP QG+GF NELIARLT
      : 1004MARTAHGTELSPFCAIFTEKEWLQYDYLQSLSKYYGYGAGSPLGPAQGIGFTNELIARLT 1183

      : 307 RHPVRDHTSTNRALDAPGAATFPLNYTMYADFTHDNGMIPFFFALGLYNGTAPLSLTHVQ 366
            + PV+D+TSTN  LD+   ATFPL+  +YADF+HDN MI  FFA+GLYNGT PLS+  V+
      : 1184QSPVQDNTSTNHTLDS-NPATFPLDRKLYADFSHDNSMISIFFAMGLYNGTQPLSMDSVE 1360

      : 367 SPSQTDGFSSAWTVPFGARAYVEMMQCRREPEPLVRVLVNDRVIPLHGCPVDKLGRCRRR 426
            S  + DG++++WTVPFGARAY E+MQC ++ EPLVRVLVNDRV+PLHGC VDK GRC
      : 1361SIQEMDGYAASWTVPFGARAYFELMQCEKK-EPLVRVLVNDRVVPLHGCAVDKFGRCTLD 1537

      : 427 DFVKGLTFARSGGDWARCY 445
            D+V+GL FARSGG+W  C+
      : 1538DWVEGLNFARSGGNWKTCF 1594
```

FIG. 15A

```
A. niger       1 ASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANESVISPEVPAGCRV 50
                 ||||||.|:         .: | |||||. :| : .|  :    |  | |
F. javanicum   1 ASRNQSTCEYDGS....CNDISRLWGQYSAYFPIPSE..LDASTPDDCDV 44

                          .         .         .         .         .
              51 TFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSL 100
                 ||| ||||||||||||||   |.| |  ||..|| :   | .|| | |||
              45 TFALVLSRHGARYPTDSKSAAYNATIARIQKSATMYGKNYKWLKEYTYSL 94

                          .         .         .         .         .
             101 GADDLTPFGEQELVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKF 150
                 ||:||| ||:.::|.||  ||:|| ||     ||:|.||| ||| |   |
              95 GAEDLTEFGQRQMVDSGRAFYERYMSLAEKTEPFVRASGSDRVIMSSYNF 144

                          .         .         .         .         .
             151 IEGFQSTKLKDPRAQPGQSSPKIDVVISEASSSNNTLDPGTCTVFEDSEL 200
                  :||  ..     | : |    .  .:| |    |||:  |.|  ||   .
             145 TQGFYAS.....RGESGDDYTQDVLIIPEEPGINNTMLHGSCASFESDRV 189

                          .         .         .         .         .
             201 A....DTVEANFTATFVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTI 246
                      :  |  . | |.| || || . | || ||  |  |:|||| |
             190 PKDADEKAEVAWGARFLPEIRNRLNHHLPGVNLTLEETIYMMDMCPFLAA 239

                          .         .         .         .         .
             247 STSTVDTKLSPFCDLFTHDEWINYDYLQSLKKYYGHGAGNPLGPTQGVGY 296
                  |       | ||||||  :| .|||  .| |:|  |  ||.:||||||||
             240 DTPD.GAGHSRFCDLFTKADWRSYDYYMTLSKFYKFGNGNAMGPTQGVGY 288

                          .         .         .         .         .
             297 ANELIARLTHSPVHDDTSSNHTLDSSPATFPLNSTLYADFSHDNGIISIL 346
                  ||||.|||  || | |..| |||||| ||||.  ||||||||| .:||
             289 VNELISRLTGKPVDDHTTTNSTLDSSPKTFPLDRALYADFSHDNSMVSIF 338

                          .         .         .         .         .
             347 FALGLYNGTKPLSTTTVENITQTDGFSSAWTVPFASRLYVEMMQCQA... 393
                  ||||||  |  |    :    .  |:|| | ||| .|:||| ::| |
             339 SALGLYNSTTLLPKDHIVPAIKAHGYSSTWVVPFGARMYVEKLECGASRN 388

                          .         .         .         .         .
             394 .EQEPLVRVLVNDRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAEC 442
                  ..:  |||||||||. |  |  |  | |  :.||  |||| |||.| :|
             389 EKRDEYVRVLVNDRVMSLETCGGDEYGLCRLENFVESLSFAASGGNWDQC 438

             443 FA 444

             439 GG 440
```

FIG. 15B

```
F. javanicum     1 ......................ASRNQSTCEYDGSCN...DISRLWGQYS 25
                                           .| |    ||      .:| .|||||
E. nidulans      1 MAFFTVALSLYYLLSRVSAQAPVVQNHSCNTADGGYQCFPNVSHVWGQYS 50

                            .         .         .         .         .
                26 AYFPIPSE..LDASTPDDCDVTFALVLSRHGARYPTDSKSAAYNATIARI 73
                    || |  |  :    |  |:|||  |||||||||||:||| ||.  |  |
                51 PYFSIEQESAISEDVPHGCEVTFVQVLSRHGARYPTESKSKAYSGLIEAI 100

                            .         .         .         .         .
                74 QKSATMYGKNYKWLKEYTYSLGAEDLTEFGQRQMVDSGRAFYERYMSLAE 123
                   ||.|| :   | .|. | |.|||:||| ||: ||||||  || || .||
               101 QKNATSFWGQYAFLESYNYTLGADDLTIFGENQMVDSGAKFYRRYKNLAR 150

                            .         .         .         .         .
               124 KTEPFVRASGSDRVIMSSYNFTQGF.YASRGESGDDYTQDV..LIIPEEP 170
                   |  ||:||||||||: |.  |  ||  |   : |      |  .||||
               151 KNTPFIRASGSDRVVASAEKFINGFRKAQLHDHGSKRATPVVNVIIPEID 200

                            .         .         .         .         .
               171 GINNTMLHGSCASFESDRVPKDADEKAEVAWGARFLPEIRNRLNHHLPGV 220
                   | |||: | .| |||.|    :  :. | .  |   | || || . |||:
               201 GFNNTLDHSTCVSFEND....ERADEIEANFTAIMGPPIRKRLENDLPGI 246

                            .         .         .         .         .
               221 NLTLEETIYMMDMCPF.LAADTPDGAGHSRFCDLFTKADWRSYDYYMTLS 269
                    || |  ||:|||| |   | |  |   | || :||. :|  |||  .||
               247 KLTNENVIYLMDMCSFDTMARTAHGTELSPFCAIFTEKEWLQYDYLQSLS 296

                            .         .         .         .         .
               270 KFYKFGNGNAMGPTQGVGYVNELISRLTGKPVDDHTTTNSTLDSSPKTFP 319
                   |:| :| |. :|| ||:|: ||||.|||  || |.|.|| ||||.| |||
               297 KYYGYGAGSPLGPAQGIGFTNELIARLTQSPVQDNTSTNHTLDSNPATFP 346

                            .         .         .         .         .
               320 LDRALYADFSHDNSMVSIFSALGLYNSTTLLPKDHIVPAIKAHGYSSTWV 369
                   ||| |||||||||||:||| |:|||| |  |  | :    .  ||...|
               347 LDRKLYADFSHDNSMISIFFAMGLYNGTQPLSMDSVESIQEMDGYAASWT 396

                            .         .         .         .         .
               370 VPFGARMYVEKLECGASRNEKRDEYVRVLVNDRVMSLETCGGDEYGLCRL 419
                   |||||| | | ::|     ||::  |||||||||. |  |  |.:| | |
               397 VPFGARAYFELMQC.....EKKEPLVRVLVNDRVVPLHGCAVDKFGRCTL 441

                            .         .
               420 ENFVESLSFAASGGNWDQCGG. 440
                   :..|| |.|| |||||  |
               442 DDWVEGLNFARSGGNWKTCFTL 463
```

FIG. 15C

```
(F.j.): 17      ISRLWGQYSAYFPIPSELDASTPDDCDVTFALVLSRHGARYPTDSKSAAYNATIARIQKS 76
                IS  WGQYS YF +PSELDAS PDDC+VTFA VLSRHGAR PT  ++A+Y   I RI
(M.t.): 2379    ISHFWGQYSPYFSVPSELDASIPDDCEVTFAQVLSRHGARAPTLKRAASYVDLIDRIHHG 2558

      : 77      ATMYGKNYKWLKEYTYSLGAEDLTEFGQRQMVDSGRAFYERYMSLAEKTEPFVRASGSDR 136
                A  YG  Y++L+ Y Y+LGA++LT  GQ+QMV+SG  FY RY +LA K+ PFVR +G DR
      : 2559    AISYGPGYEFLRTYDYTLGADELTRTGQQQMVNSGIKFYRRYRALARKSIPFVRTAGQDR 2738

      : 137     VIMSSYNFTQGFY----ASRGES-GDDYTQDVLIIPEEPGINNTMLHGSCASFESDRVPK 191
                V+ S+ NFTQGF+    A RG +       D+++IPE  G NNT+ +  C +FE
      : 2739    VVHSAENFTQGFHSALLADRGSTVRPTLPYDMVVIPETAGANNTLHNDLCTAFEEGPYST 2918

      : 192     DADEKAEVAWGARFLPEIRNRLNHHLPGVNLTLEETIYMMDMCPF--LAADTPD------ 243
                  D+ A+  + + F   I  R+N +LPG NLT  +T+ +MD+CPF  +A+ + D
      : 2919    IGDD-AQDTYLSTFAGPITARVNANLPGANLTDADTVALMDLCPFETVASSSSDPATADA 3095

      : 244     GAGHSR----FCDLFTKADWRSYDYYMTLSKFYKFGNGNAMGPTQGVGYVNELISRLTGK 299
                G G+ R    FC LF++++WR+YDY  ++ K+Y +G GN +GPTQGVG+VNEL++RL G
      : 3096    GGGNGRPLSPFCRLFSESEWRAYDYLQSVGKWYGYGPGNPLGPTQGVGFVNELLARLAGV 3275

      : 300     PVDDHTTTNSTLDSSPKTFPLDRALYADFSHDNSMVSIFSALGLYNSTTLLPKDHIVPAI 359
                PV D T+TN TLD  P+TFPL R LYADFSHDN M+ +  ALG Y+    L K
      : 3276    PVRDGTSTNRTLDGDPRTFPLGRPLYADFSHDNDMMGVLGALGAYDGVPPLDKTARRDPE 3455

      : 360     KAHGYSSTWVVPFGARMYVEKLEC---------GASRNEKRDEYVRVLVNDRVMSLETCG 410
                +  GY+++W VPF AR+YVEK+ C         G  R EK +E VRVLVNDRVM+L+ CG
      : 3456    ELGGYAASWAVPFAARIYVEKMRCSGGGGGGGGGEGRQEKDEEMVRVLVNDRVMTLKGCG 3635

      : 411     GDEYGLCRLENFVESLSFAASGGNWDQC 438
                 DE G+C LE F+ES++FA   G WD C
      : 3636    ADERGMCTLERFIESMAFARGNGKWDLC 3719
```

FIG. 16A

```
H. grisea            1 ..................ASRNQSTCDSVDRGFWCAADISHSWGQYSPYF 32
                                                ||. | || |   ||| ||||||||
M. thermophila       1 MTGLGVMVVMVGFLAIASLQSESRPCDTPDLGFQCGTAISHFWGQYSPYF 50

                                .         .         .         .         .
                    33 SVPSDIDPGFPKGCNVTFAQVLSRHGARAPTTGRAAYYVDVIDRVQRQAT 82
                       ||||::|   |  | ||||||||||||||||  ||| |||.|||:   |
                    51 SVPSELDASIPDDCEVTFAQVLSRHGARAPTLKRAASYVDLIDRIHHGAI 100

                                .         .         .         .         .
                    83 SYGPGHAFLRSYRYTLGANELTPMGERQLAYSGARFYHRYRELARVEAPF 132
                       |||||: |||.| |||||.|||  |:.|:  || :|| ||| |||   ||
                   101 SYGPGYEFLRTYDYTLGADELTRTGQQQMVNSGIKFYRRYRALARKSIPF 150

                                .         .         .         .         .
                   133 VRSSGVSRVVASAVNFTQGFHQARLADRGATLPPPTLPYDMVIISSDDTA 182
                       ||..|  ||| || ||||||| | |||||.|.  ||||||||:|     |
                   151 VRTAGQDRVVHSAENFTQGFHSALLADRGSTV.RPTLPYDMVVIPETAGA 199

                                .         .         .         .         .
                   183 NNTLHHGLCTVFEEGPYADIGDKAQKEYLSKFVGPIVERINAQLPGANLN 232
                       |||||. ||| ||||||. ||| ||  ||| | |||  |:|| ||||||
                   200 NNTLHNDLCTAFEEGPYSTIGDDAQDTYLSTFAGPITARVNANLPGANLT 249

                                .         .         .         .         .
                   233 ATDIIALMDLCPFETVAFP............EGTKLSPFCRLFTAAEWRA 270
                         | :||||||||||||               |  ||||||||. .||||
                   250 DADTVALMDLCPFETVASSSSDPATADAGGGNGRPLSPFCRLFSESEWRA 299

                                .         .         .         .         .
                   271 YDRYQDVGKWFGYGPGNPLGPTQGVGFVNELIARLSGQPVSDGTSTNRTL 320
                       ||  | ||||:||||||||||||||||||||:|||.| || |||||||||
                   300 YDYLQSVGKWYGYGPGNPLGPTQGVGFVNELLARLAGVPVRDGTSTNRTL 349

                                .         .         .         .         .
                   321 DENPETFPLGRRLYADFSHDNDMVGILSALGLWDNHEEPGNEMPAEGEED 370
                       | .| |||||| |||||||||||.|:| ||| :|    |  :  |  : :
                   350 DGDPRTFPLGRPLYADFSHDNDMMGVLGALGAYDG..VPPLDKTARRDPE 397

                                .         .         .         .         .
                   371 DNGRFSTARAVPFGARVYVEKLRCGG............SEEDEEMVRVLVN 409
                       : | :. . |||| ||:||||:|| |            |.||||||||||
                   398 ELGGYAASWAVPFAARIYVEKMRCSGGGGGGGGGEGRQEKDEEMVRVLVN 447

                                .         .         .         .
                   410 DRVMPLAQCGGDKRGMCTLSRFVESLKFARNNGRWDMCFE 449
                       |||| |  || |.|||||| ||:||: ||| ||:||:||
                   448 DRVMTLKGCGADERGMCTLERFIESMAFARGNGKWDLCFA 487
```

FIG. 16B

```
A. niger      1 ASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSLANESVISPEVPAGCRV 50
                ||||||.||.||.|: | .: || ||||.|:||. .:  | |  | || |
H. grisea     1 ASRNQSTCDSVDRGFWCAADISHSWGQYSPYFSVPSD..IDPGFPKGCNV 48
                         .         .         .         .         .
             51 TFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLKTYNYSL 100
                |||||||||||| ||  :   |  .|: :|. ||.:   :|||:.| |.|
             49 TFAQVLSRHGARAPTTGRAAYYVDVIDRVQRQATSYGPGHAFLRSYRYTL 98
                         .         .         .         .         .
            101 GADDLTPFGEQELVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKF 150
                ||.:||| ||.:|  || :|| ||  | |   ||:|||| |||:||   |
             99 GANELTPMGERQLAYSGARFYHRYRELARVEAPFVRSSGVSRVVASAVNF 148
                         .         .         .         .         .
            151 IEGFQSTKLKDPRAQ.PGQSSPKIDVVISEASSSNNTLDPGTCTVFED.. 197
                 :||   :| |  |  |  . |   |:||   ..||||  | |||||:
            149 TQGFHQARLADRGATLPPPTLPYDMVIISSDDTANNTLHHGLCTVFEEGP 198
                         .         .         .         .         .
            198 .SELADTVEANFTATFVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTI 246
                 .:: |  :  : . ||  | :|:   | |  |  |::  |||:| |:|:
            199 YADIGDKAQKEYLSKFVGPIVERINAQLPGANLNATDIIALMDLCPFETV 248
                         .         .         .         .         .
            247 STSTVDTKLSPFCDLFTHDEWINYDYLQSLKKYYGHGAGNPLGPTQGVGY 296
                .     ||||||| |||  ||  ||  | . |::|:| |||||||||||:
            249 AFPE.GTKLSPFCRLFTAAEWRAYDRYQDVGKWFGYGPGNPLGPTQGVGF 297
                         .         .         .         .         .
            297 ANELIARLTHSPVHDDTSSNHTLDSSPATFPLNSTLYADFSHDNGIISIL 346
                 |||||||.  || | ||.| ||| .| ||||   ||||||||| .: ||
            298 VNELIARLSGQPVSDGTSTNRTLDENPETFPLGRRLYADFSHDNDMVGIL 347
                         .         .         .         .         .
            347 FALGLY.NGTKPLSTTTVENITQTDG.FSSAWTVPFASRLYVEMMQC..Q 392
                 ||||: |  .| .    |     .| ||.|  ||| .|.||| :.|
            348 SALGLWDNHEEPGNEMPAEGEEDDNGRFSTARAVPFGARVYVEKLRCGGS 397
                         .         .         .         .         .
            393 AEQEPLVRVLVNDRVVPLHGCPVDALGRCTRDSFVRGLSFARSGGDWAEC 442
                 | | :|||||||||.||  |  |  | ||   ||  | |||. | |  |
            398 EEDEEMVRVLVNDRVMPLAQCGGDKRGMCTLSRFVESLKFARNNGRWDMC 447

            443 FA 444
                |
            448 FE 449
```

FIG. 16C

```
(H.g.): 8     CDSVDRGFWCAADISHSWGQYSPYFSVPSDIDPGFPKGCNVTFAQVLSRHGARAPTTGRA 67
              CD+ D GF C   ISH WGQYSPYFSVPS++D   P  C VTFAQVLSRHGARAPT  RA
(M.t.): 2340  CDTPDLGFQCGTAISHFWGQYSPYFSVPSELDASIPDDCEVTFAQVLSRHGARAPTLKRA 2519

      : 68    AYYVDVIDRVQRQATSYGPGHAFLRSYRYTLGANELTPMGERQLAYSGARFYHRYRELAR 127
              A YVD+IDR+   A SYGPG+ FLR+Y YTLGA+ELT  G++Q+  SG +FY RYR LAR
      : 2520  ASYVDLIDRIHHGAISYGPGYEFLRTYDYTLGADELTRTGQQQMVNSGIKFYRRYRALAR 2699

      : 128   VEAPFXXXXXXXXXXXXXXNFTQGFHQARLADRGATLPPPTLPYDMVIISSDDTANNTLH 187
                 PF              NFTQGFH A LADRG+T+  PTLPYDMV+I     ANNTLH
      : 2700  KSIPFVRTAGQDRVVHSAENFTQGFHSALLADRGSTV-RPTLPYDMVVIPETAGANNTLH 2876

      : 188   HGLCTVFEEGPYADIGDKAQKEYLSKFVGPIVERINAQLPGANLNATDIIALMDLCPFET 247
              + LCT FEEGPY+ IGD AQ  YLS F GPI  R+NA LPGANL   D +ALMDLCPFET
      : 2877  NDLCTAFEEGPYSTIGDDAQDTYLSTFAGPITARVNANLPGANLTDADTVALMDLCPFET 3056

      : 248   VAFP------------EGTKLSPFCRLFTAAEWRAYDRYQDVGKWFGYGPGNPLGPTQGV 295
              VA               G  LSPFCRLF+ +EWRAYD  Q VGKW+GYGPGNPLGPTQGV
      : 3057  VASSSSDPATADAGGGNGRPLSPFCRLFSESEWRAYDYLQSVGKWYGYGPGNPLGPTQGV 3236

      : 296   GFVNELIARLSGQPVSDGTSTNRTLDENPETFPLGRRLYADFSHDNDMVGILSALGLWDN 355
              GFVNEL+ARL+G PV DGTSTNRTLD +P TFPLGR LYADFSHDNDM+G+L ALG +D
      : 3237  GFVNELLARLAGVPVRDGTSTNRTLDGDPRTFPLGRPLYADFSHDNDMMGVLGALGAYDG 3416

      : 356   HEEPGNEMPAEGEEDDNGRFSTARAVPFGARVYVEKLRCGG-----------SEEDEEMV 404
                 P  +  A  + ++ G ++ + AVPF AR+YVEK+RC G            E+DEEMV
      : 3417  --VPPLDKTARRDPEELGGYAASWAVPFAARIYVEKMRCSGGGGGGGGGEGRQEKDEEMV 3590

      : 405   RVLVNDRVMPLAQCGGDKRGMCTLSRFVESLKFARNNGRWDMCF 448
              RVLVNDRVM L  CG D+RGMCTL RF+ES+ FAR NG+WD+CF
      : 3591  RVLVNDRVMTLKGCGADERGMCTLERFIESMAFARGNGKWDLCF 3722
```

FIG. 17A

ATGGTTCTTTTCACGGTCTCCCTTTCGCTGTACTACCTACTTACGAGGTGAGATCTCTACAGTAGCTG
CTTGTTTAGTTGAGTTGGTACTTACCTACACAGCGTCTCTGCTCAGGCCGTGGTGGCGCAGGAATAT
TCATGTAATTCGGCCGACGCTGGGTATCAATGTTTCCCCAATGTCTCGCACGTCTGGGGCCAGTACT
CGCCGTACTTCTCACTCGAGCATGAGTCTGCCATTTCTCAGGACGTGCCTCATGGCTGTGAGGTTAC
CTTCGTGCAGGTGCTCTCGCGACATGGGGCTAGATATcCTTCGGAGTCAAAAAGCAAGGCGTATGCG
AAGTTGATTGACGCTATCAAGAAGAATGCTACTTCGTTTTCGGGACAGTATGCTTTTCTGGAGAGTT
ATAATTATACTCTCGGCGCGGAAGACTTGACTACTTTTGGTGAGAACCAGATGGTCGACTCGGGTGC
CAAGTTTTACCGGCGGTATAAGAATTTGGCCAGGAAAAATACTCCATTCATACGTGCATCAGGGTCT
GACCGTGTCGTTGCGTCCGCGGAGAAGTTTATTGACGGACTTCGAGACGCCCAGACCCACGACCAG
GGCTCCAAACGTGTTGCCCCAGTTGTCAATGTGGTTATCCCTGAAACTGATGGATTTAACAACACCC
TGGATCATAGCACTTGCGTGTCTTTTGAGAATGATGAGCGGGCGGACGAAATTGAAGCCAACTTCGC
CGCGATCATTGGACCTCCGAtTCGCAAACGTCTGGAAAACGACCTTCCTGGCGTTGAGCTTACAAAT
GAGCATGTGGAATACTTGATGGATATGTgctcgttcgacaccatggcgcgcaccgcccatggaaccgagctgtctccatt
ctgcgccatcttcactgaaaaggagtggctgcagtacgacTACCTACAATCTCTGtCAAAGTACTACGGCTACGGTGC
CGGGAACCCCCTTGGCCCAGCTCAGGGAATTGGCTTCACCAACGAGCTGATTGCcCGACTGA
CGCAGTCGCCTGTCCAGGACAACACGAGCACCAACCACACTCTAGACTCTGACCCGGCCACGTTCC
CCCTCGACAGGAAGCTCTACGCCGACTTCTCCCACGACAATAACATGATTTCTATATTCTTCGCCAT
GGGCCTGTACAACGGCACCCAGCCGCTGTCCATGGACACTGTGGAGTCGATTGAGGAGATGGATGG
CTACGCGGCGTCTTGGACTGTCCCGTTTGGTGCGAGGGCTTACTTTGAGGTGATGCAGTGCCAAAAA
AAGAAGGAGCCACTTGTGCGGGTATTAGTGAATGATCGCGTTGTTCCTCTCCATGGCTGTGCTGTTG
ACAAGCTCGGACGATGCACTTTGGACGATTGGGTCGAGGGCTTGAGTTTTGCGAGGGCCGGTGGGA
ACTGGAAGGCTTGTTTTACTGCCTAA

FIG. 17B

MVLFTVSLSLYYLLTSVSAQAVVAQEYSCNSADAGYQCFPNVSHVWGQYSPYFSLEHESAISQDVPHGCE
VTFVQVLSRHGARYPSESKSKAYAKLIDAIKKNATSFSGQYAFLESYNYTLGAEDLTTFGENQMVDSGAKF
YRRYKNLARKNTPFIRASGSDRVVASAEKFIDGLRDAQTHDQGSKRVAPVVNVVIPETDGFNNTLDHSTCV
SFENDERADEIEANFAAIIGPPIRKRLENDLPGVELTNEHVEYLMDMCSFDTMARTAHGTELSPFCAIFTEKE
WLQYDYLQSLSKYYGYGAGNPLGPAQGIGFTNELIARLTQSPVQDNTSTNHTLDSDPATFPLDRKLYADFS
HDNNMISIFFAMGLYNGTQPLSMDTVESIEEMDGYAASWTVPFGARAYFEVMQCQKKKEPLVRVLVNDR
VVPLHGCAVDKLGRCTLDDWVEGLSFARAGGNWKACFTA.

FIG. 18A

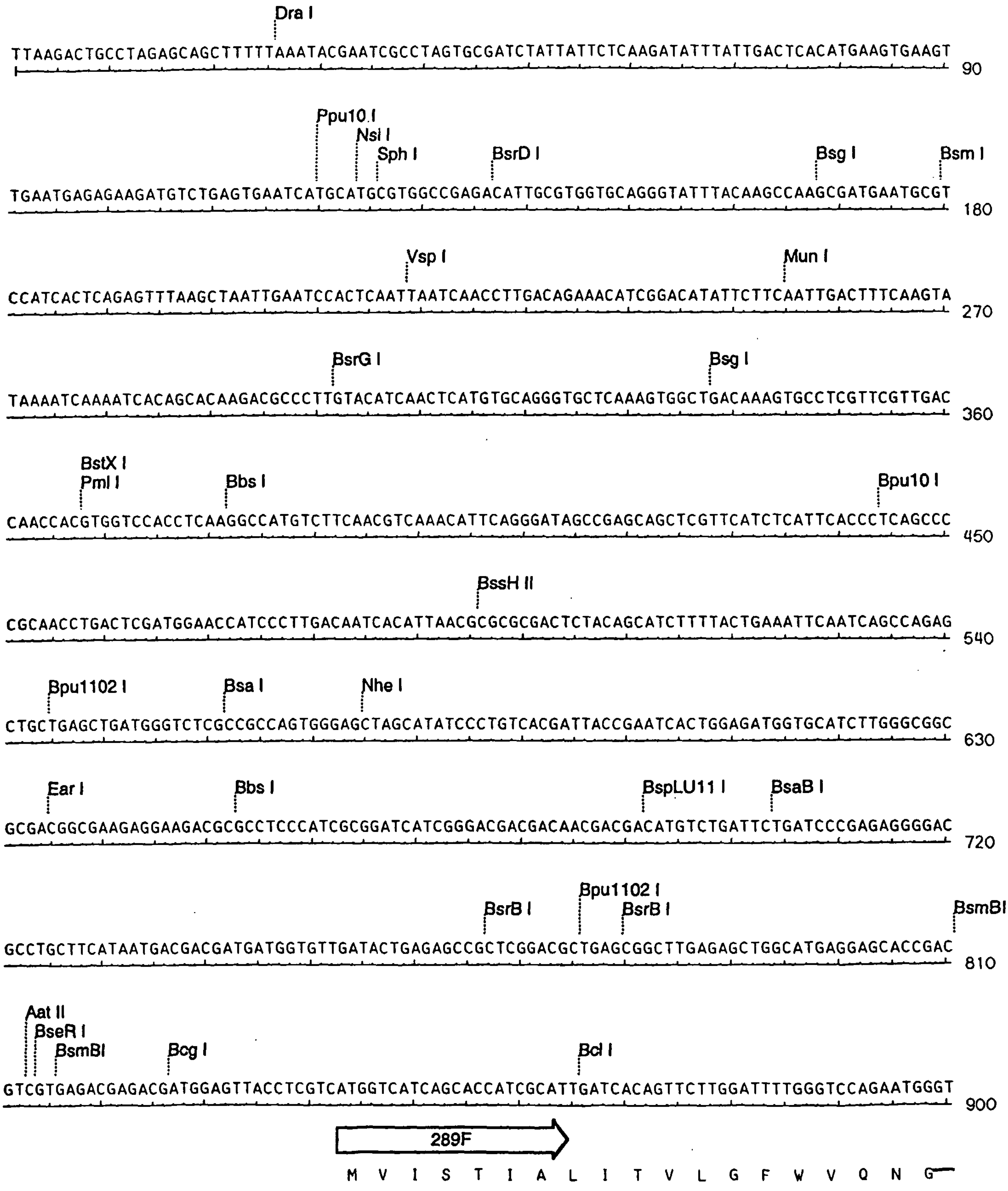
Dra I
TTAAGACTGCCTAGAGCAGCTTTTTAAATACGAATCGCCTAGTGCGATCTATTATTCTCAAGATATTTATTGACTCACATGAAGTGAAGT
90
Ppu10 I
Nsi I
Sph I
BsrD I
Bsg I
Bsm I
TGAATGAGAGAAGATGTCTGAGTGAATCATGCATGCGTGGCCGAGACATTGCGTGGTGCAGGGTATTTACAAGCCAAGCGATGAATGCGT
180
Vsp I
Mun I
CCATCACTCAGAGTTTAAGCTAATTGAATCCACTCAATTAATCAACCTTGACAGAAACATCGGACATATTCTTCAATTGACTTTCAAGTA
270
BsrG I
Bsg I
TAAAATCAAAATCACAGCACAAGACGCCCTTGTACATCAACTCATGTGCAGGGTGCTCAAAGTGGCTGACAAAGTGCCTCGTTCGTTGAC
360
BstX I
Pml I
Bbs I
Bpu10 I
CAACCACGTGGTCCACCTCAAGGCCATGTCTTCAACGTCAAACATTCAGGGATAGCCGAGCAGCTCGTTCATCTCATTCACCCTCAGCCC
450
BssH II
CGCAACCTGACTCGATGGAACCATCCCTTGACAATCACATTAACGCGCGCGACTCTACAGCATCTTTTACTGAAATTCAATCAGCCAGAG
540
Bpu1102 I
Bsa I
Nhe I
CTGCTGAGCTGATGGGTCTCGCCGCCAGTGGGAGCTAGCATATCCCTGTCACGATTACCGAATCACTGGAGATGGTGCATCTTGGGCGGC
630
Ear I
Bbs I
BspLU11 I
BsaB I
GCGACGGCGAAGAGGAAGACGCGCCTCCCATCGCGGATCATCGGGACGACGACAACGACGACATGTCTGATTCTGATCCCGAGAGGGGAC
720
Bpu1102 I
BsrB I
BsrB I
BsmBI
GCCTGCTTCATAATGACGACGATGATGGTGTTGATACTGAGAGCCGCTCGGACGCTGAGCGGCTTGAGAGCTGGCATGAGGAGCACCGAC
810
Aat II
BseR I
BsmBI
Bcg I
Bcl I
GTCGTGAGACGAGACGATGGAGTTACCTCGTCATGGTCATCAGCACCATCGCATTGATCACAGTTCTTGGATTTTGGGTCCAGAATGGGT
900
289F
M V I S T I A L I T V L G F W V Q N G

FIG. 18B

```
                   BsaB I     Dra I
GAGTTATATGAGTTGATGCTCATCTTTTAAATCAAACTGACACGCCTGATAGAACTCGACCGGCTGGGTGTGAGTATGACGGGAGCTGTA  990
                                                   T  R  P  A  G  C  E  Y  D  G  S  C

                                   Sca I
ATGACATCTCTCGGCTCTGGGGACAGTACTCTGCATACTTCCCAATCCCGTCTGAGCTTGATGCCTCAACACCAGACGATTGTGATGTGA  1080
N  D  I  S  R  L  W  G  Q  Y  S  A  Y  F  P  I  P  S  E  L  D  A  S  T  P  D  D  C  D  V

                         Nco I     Acc65 I                Pst I          BsrD I   Bsm I
CTTTTGCACTCGTCTTGTCCCGCCATGGAGCCAGGTACCCAACGGACAGCAAGTCTGCAGCATACAACGCTACCATTGCCCGCATTCAAA  1170
T  F  A  L  V  L  S  R  H  G  A  R  Y  P  T  D  S  K  S  A  A  Y  N  A  T  I  A  R  I  Q

                                                                                       Bal I
                                  Afl II    Bst1107 I                      Bbs I       Eco57 I
AGTCTGCTACCATGTACGGCAAGAACTACAAGTGGCTTAAGGAGTATACCTACAGTCTCGGCGCTGAAGACCTGACTGAGTTTGGCCAGC  1260
K  S  A  T  M  Y  G  K  N  Y  K  W  L  K  E  Y  T  Y  S  L  G  A  E  D  L  T  E  F  G  Q

         Sal I                     BsrB I                     Bbs I
GGCAGATGGTCGACTCTGGTAGGGCCTTTTATGAGCGGTACATGAGTCTCGCTGAGAAGACTGAGCCTTTTGTTCGGGCATCGGGCTCAG  1350
R  Q  M  V  D  S  G  R  A  F  Y  E  R  Y  M  S  L  A  E  K  T  E  P  F  V  R  A  S  G  S

                                                     Nru I    BsmBI     BseR I
ATCGGGTCATCATGTCGTCTTACAATTTTACGCAAGGCTTTTACGCATCGCGAGGAGAGTCTGGAGACGATTATACTCAGGATGTTCTTA  1440
D  R  V  I  M  S  S  Y  N  F  T  Q  G  F  Y  A  S  R  G  E  S  G  D  D  Y  T  Q  D  V  L

                                   Eco57 I
TCATCCCTGAAGAACCTGGCATCAACAACACCATGTTGCATGGATCGTGCGCCTCATTCGAAAGCGACAGAGTTCCTAAAGACGCAGATG  1530
I  I  P  E  E  P  G  I  N  N  T  M  L  H  G  S  C  A  S  F  E  S  D  R  V  P  K  D  A  D

                                                                               BspM I
AAAAGGCCGAGGTTGCATGGGGAGCAAGATTCCTCCCCGAGATTCGAAATAGGTTGAACCACCACCTGCCAGGAGTCAACCTGACGCTGG  1620
E  K  A  E  V  A  W  G  A  R  F  L  P  E  I  R  N  R  L  N  H  H  L  P  G  V  N  L  T  L

                          BspLU11 I
                 Xcm I       PshA I                                                           Ear I
AGGAAACCATCTACATGATGGACATGTGTCCGTTCCTCGCGGCTGACACACCTGATGGCGCTGGTCACTCGAGGTTCTGCGACCTCTTCA  1710
E  E  T  I  Y  M  M  D  M  C  P  F  L  A  A  D  T  P  D  G  A  G  H  S  R  F  C  D  L  F
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FIG. 18C

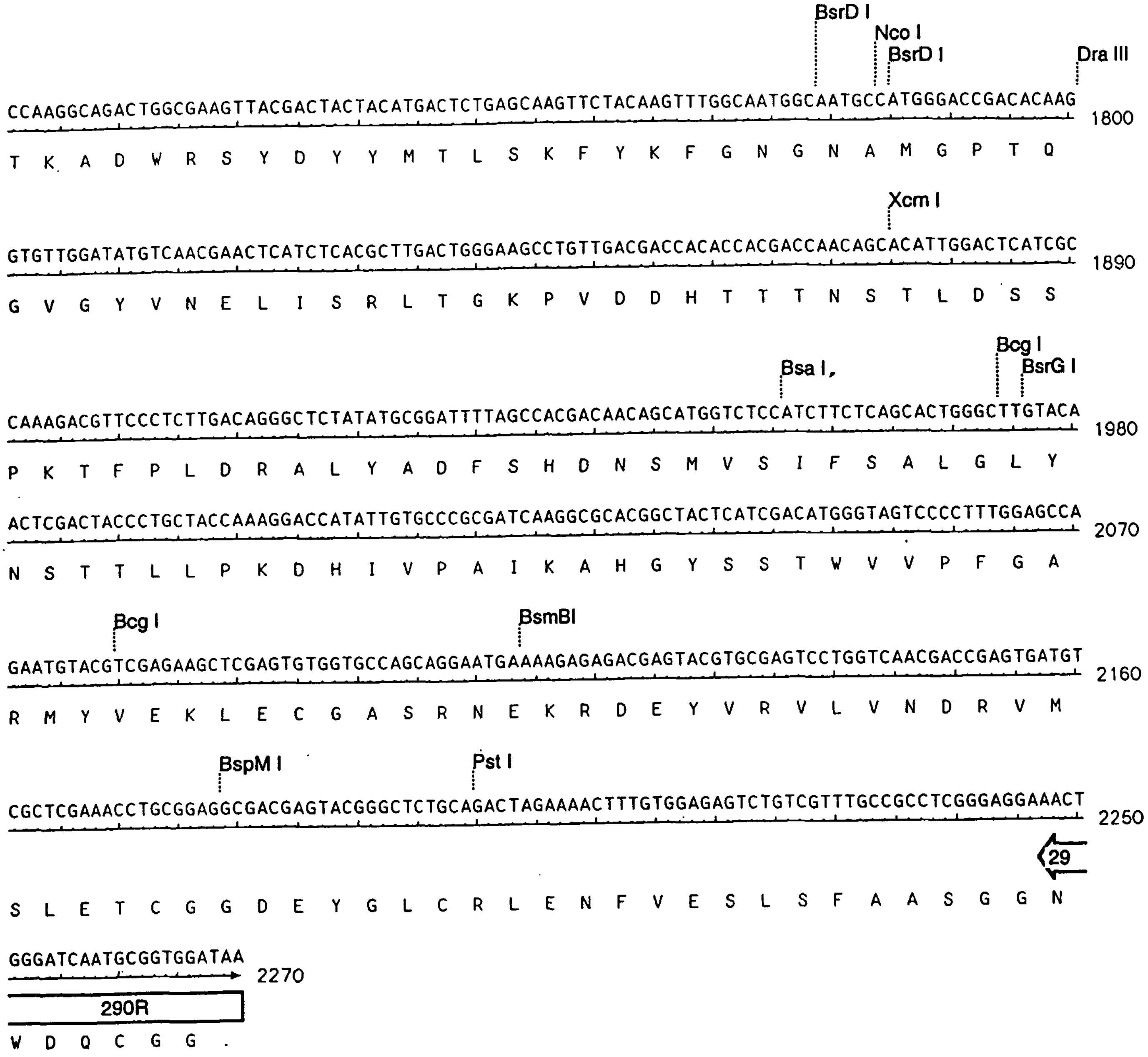
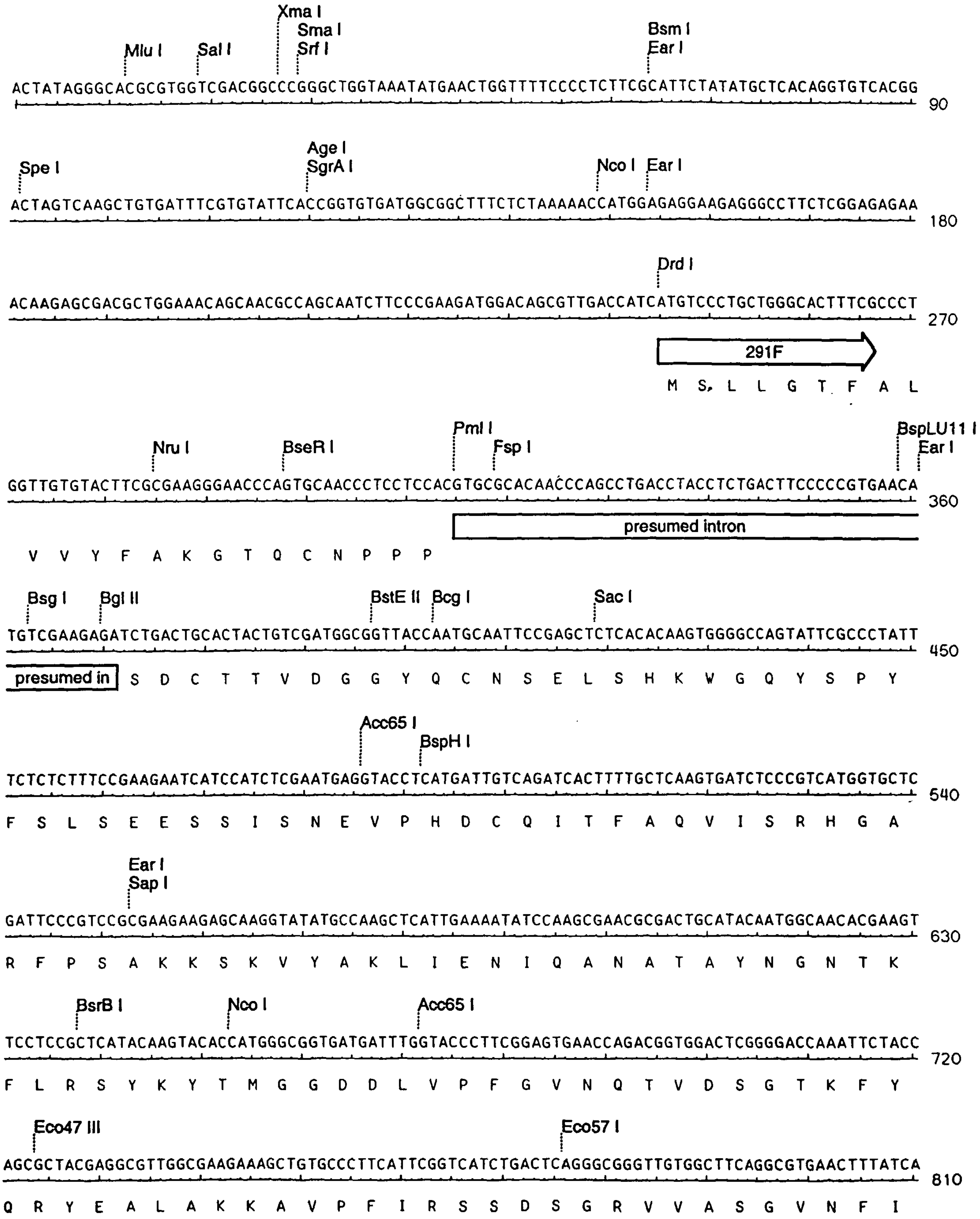

BsrD I
Nco I
BsrD I
Dra III
CCAAGGCAGACTGGCGAAGTTACGACTACTACATGACTCTGAGCAAGTTCTACAAGTTTGGCAATGGCAATGCCATGGGACCGACACAAG 1800
T K A D W R S Y D Y Y M T L S K F Y K F G N G N A M G P T Q
Xcm I
GTGTTGGATATGTCAACGAACTCATCTCACGCTTGACTGGGAAGCCTGTTGACGACCACACCACGACCAACAGCACATTGGACTCATCGC 1890
G V G Y V N E L I S R L T G K P V D D H T T T N S T L D S S
Bsa I,
Bcg I
BsrG I
CAAAGACGTTCCCTCTTGACAGGGCTCTATATGCGGATTTTAGCCACGACAACAGCATGGTCTCCATCTTCTCAGCACTGGGCTTGTACA 1980
P K T F P L D R A L Y A D F S H D N S M V S I F S A L G L Y
ACTCGACTACCCTGCTACCAAAGGACCATATTGTGCCCGCGATCAAGGCGCACGGCTACTCATCGACATGGGTAGTCCCCTTTGGAGCCA 2070
N S T T L L P K D H I V P A I K A H G Y S S T W V V P F G A
Bcg I
BsmBI
GAATGTACGTCGAGAAGCTCGAGTGTGGTGCCAGCAGGAATGAAAAGAGAGACGAGTACGTGCGAGTCCTGGTCAACGACCGAGTGATGT 2160
R M Y V E K L E C G A S R N E K R D E Y V R V L V N D R V M
BspM I
Pst I
CGCTCGAAACCTGCGGAGGCGACGAGTACGGGCTCTGCAGACTAGAAAACTTTGTGGAGAGTCTGTCGTTTGCCGCCTCGGGAGGAAACT 2250
29
S L E T C G G D E Y G L C R L E N F V E S L S F A A S G G N
GGGATCAATGCGGTGGATAA 2270
290R
W D Q C G G .

FIG. 19A



ACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGTAAATATGAACTGGTTTTCCCCTCTTCGCATTCTATATGCTCACAGGTGTCACGG 90

ACTAGTCAAGCTGTGATTTCGTGTATTCACCGGTGTGATGGCGGCTTTCTCTAAAAACCATGGAGAGGAAGAGGGCCTTCTCGGAGAGAA 180

ACAAGAGCGACGCTGGAAACAGCAACGCCAGCAATCTTCCCGAAGATGGACAGCGTTGACCATCATGTCCCTGCTGGGCACTTTCGCCCT 270

M S L L G T F A L

GGTTGTGTACTTCGCGAAGGGAACCCAGTGCAACCCTCCTCCACGTGCGCACAACCCAGCCTGACCTACCTCTGACTTCCCCCGTGAACA 360

V V Y F A K G T Q C N P P P

TGTCGAAGAGATCTGACTGCACTACTGTCGATGGCGGTTACCAATGCAATTCCGAGCTCTCACACAAGTGGGGCCAGTATTCGCCCTATT 450

S D C T T V D G G Y Q C N S E L S H K W G Q Y S P Y

TCTCTCTTTCCGAAGAATCATCCATCTCGAATGAGGTACCTCATGATTGTCAGATCACTTTTGCTCAAGTGATCTCCCGTCATGGTGCTC 540

F S L S E E S S I S N E V P H D C Q I T F A Q V I S R H G A

GATTCCCGTCCGCGAAGAAGAGCAAGGTATATGCCAAGCTCATTGAAAATATCCAAGCGAACGCGACTGCATACAATGGCAACACGAAGT 630

R F P S A K K S K V Y A K L I E N I Q A N A T A Y N G N T K

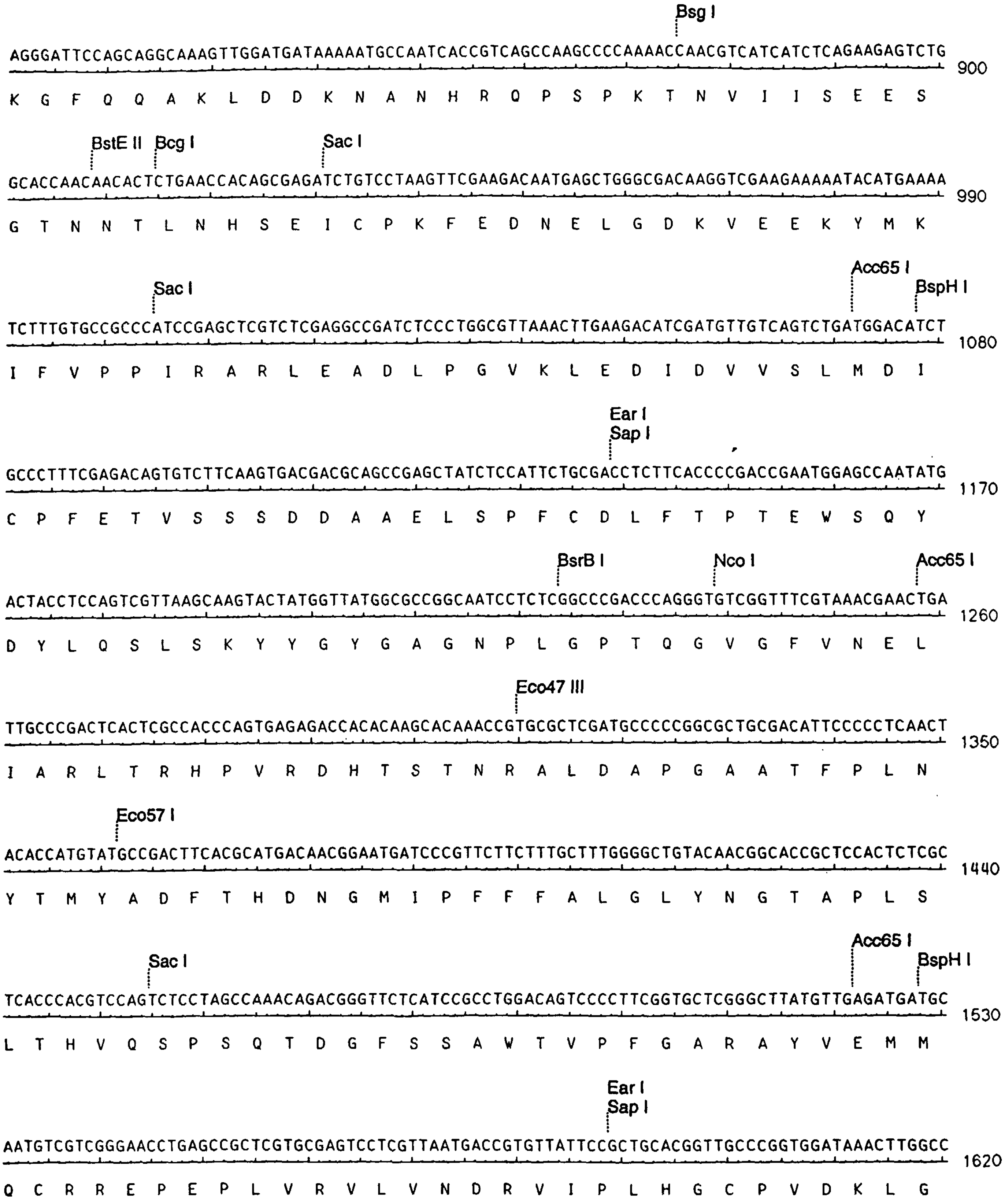
TCCTCCGCTCATACAAGTACACCATGGGCGGTGATGATTTGGTACCCTTCGGAGTGAACCAGACGGTGGACTCGGGGACCAAATTCTACC 720

F L R S Y K Y T M G G D D L V P F G V N Q T V D S G T K F Y

AGCGCTACGAGGCGTTGGCGAAGAAAGCTGTGCCCTTCATTCGGTCATCTGACTCAGGGCGGGTTGTGGCTTCAGGCGTGAACTTTATCA 810

Q R Y E A L A K K A V P F I R S S D S G R V V A S G V N F I

FIG. 19B



```
AGGGATTCCAGCAGGCAAAGTTGGATGATAAAAATGCCAATCACCGTCAGCCAAGCCCCAAAACCAACGTCATCATCTCAGAAGAGTCTG 900
K  G  F  Q  Q  A  K  L  D  D  K  N  A  N  H  R  Q  P  S  P  K  T  N  V  I  I  S  E  E  S

GCACCAACAACACTCTGAACCACAGCGAGATCTGTCCTAAGTTCGAAGACAATGAGCTGGGCGACAAGGTCGAAGAAAAATACATGAAAA 990
G  T  N  N  T  L  N  H  S  E  I  C  P  K  F  E  D  N  E  L  G  D  K  V  E  E  K  Y  M  K

TCTTTGTGCCGCCCATCCGAGCTCGTCTCGAGGCCGATCTCCCTGGCGTTAAACTTGAAGACATCGATGTTGTCAGTCTGATGGACATCT 1080
I  F  V  P  P  I  R  A  R  L  E  A  D  L  P  G  V  K  L  E  D  I  D  V  V  S  L  M  D  I

GCCCTTTCGAGACAGTGTCTTCAAGTGACGACGCAGCCGAGCTATCTCCATTCTGCGACCTCTTCACCCCGACCGAATGGAGCCAATATG 1170
C  P  F  E  T  V  S  S  S  D  D  A  A  E  L  S  P  F  C  D  L  F  T  P  T  E  W  S  Q  Y

ACTACCTCCAGTCGTTAAGCAAGTACTATGGTTATGGCGCCGGCAATCCTCTCGGCCCGACCCAGGGTGTCGGTTTCGTAAACGAACTGA 1260
D  Y  L  Q  S  L  S  K  Y  Y  G  Y  G  A  G  N  P  L  G  P  T  Q  G  V  G  F  V  N  E  L

TTGCCCGACTCACTCGCCACCCAGTGAGAGACCACACAAGCACAAACCGTGCGCTCGATGCCCCCGGCGCTGCGACATTCCCCCTCAACT 1350
I  A  R  L  T  R  H  P  V  R  D  H  T  S  T  N  R  A  L  D  A  P  G  A  A  T  F  P  L  N

ACACCATGTATGCCGACTTCACGCATGACAACGGAATGATCCCGTTCTTCTTTGCTTTGGGGCTGTACAACGGCACCGCTCCACTCTCGC 1440
Y  T  M  Y  A  D  F  T  H  D  N  G  M  I  P  F  F  F  A  L  G  L  Y  N  G  T  A  P  L  S

TCACCCACGTCCAGTCTCCTAGCCAAACAGACGGGTTCTCATCCGCCTGGACAGTCCCCTTCGGTGCTCGGGCTTATGTTGAGATGATGC 1530
L  T  H  V  Q  S  P  S  Q  T  D  G  F  S  S  A  W  T  V  P  F  G  A  R  A  Y  V  E  M  M

AATGTCGTCGGGAACCTGAGCCGCTCGTGCGAGTCCTCGTTAATGACCGTGTTATTCCGCTGCACGGTTGCCCGGTGGATAAACTTGGCC 1620
Q  C  R  R  E  P  E  P  L  V  R  V  L  V  N  D  R  V  I  P  L  H  G  C  P  V  D  K  L  G
```

FIG. 19C


BsrB I
Nco I
GTTGTCGCCGTCGTGATTTCGTGAAAGGGCTTACTTTCGCACGCTCTGGCGGCGACTGGGCCAGGTGTTATAAATAG
1697
R C R R R D F V K G L T F A R S G G D W A R C Y K .
292R

FIG. 20A

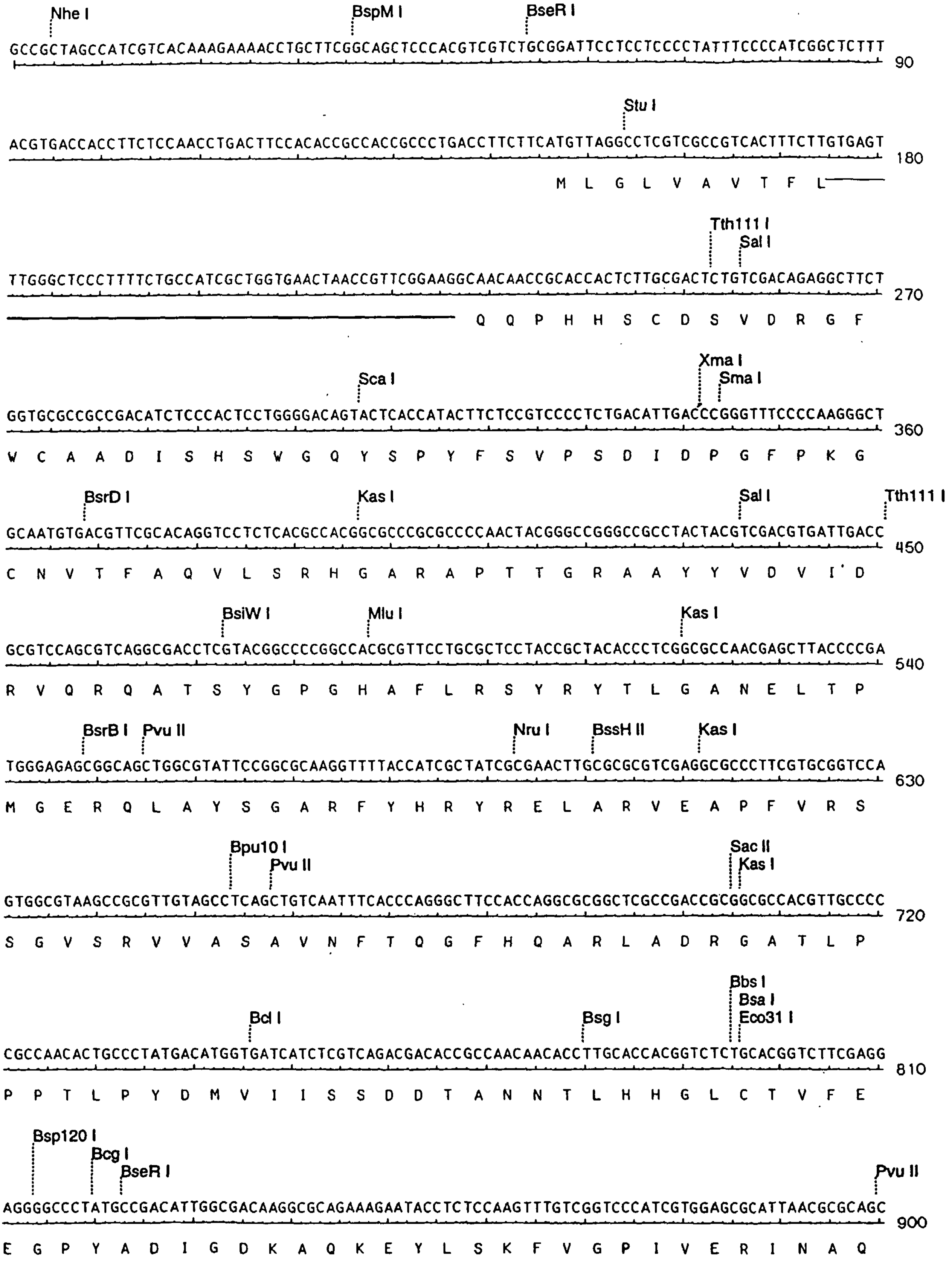

Nhe I
BspM I
BseR I
GCCGCTAGCCATCGTCACAAAGAAAACCTGCTTCGGCAGCTCCCACGTCGTCTGCGGATTCCTCCTCCCCTATTTCCCCATCGGCTCTTT
90
Stu I
ACGTGACCACCTTCTCCAACCTGACTTCCACACCGCCACCGCCCTGACCTTCTTCATGTTAGGCCTCGTCGCCGTCACTTTCTTGTGAGT
180
M L G L V A V T F L
Tth111 I
Sal I
TTGGGCTCCCTTTTCTGCCATCGCTGGTGAACTAACCGTTCGGAAGGCAACAACCGCACCACTCTTGCGACTCTGTCGACAGAGGCTTCT
270
Q Q P H H S C D S V D R G F
Xma I
Sca I
Sma I
GGTGCGCCGCCGACATCTCCCACTCCTGGGGACAGTACTCACCATACTTCTCCGTCCCCTCTGACATTGACCCGGGTTTCCCCAAGGGCT
360
W C A A D I S H S W G Q Y S P Y F S V P S D I D P G F P K G
BsrD I
Kas I
Sal I
Tth111 I
GCAATGTGACGTTCGCACAGGTCCTCTCACGCCACGGCGCCCGCGCCCCAACTACGGGCCGGGCCGCCTACTACGTCGACGTGATTGACC
450
C N V T F A Q V L S R H G A R A P T T G R A A Y Y V D V I D
BsiW I
Mlu I
Kas I
GCGTCCAGCGTCAGGCGACCTCGTACGGCCCCGGCCACGCGTTCCTGCGCTCCTACCGCTACACCCTCGGCGCCAACGAGCTTACCCCGA
540
R V Q R Q A T S Y G P G H A F L R S Y R Y T L G A N E L T P
BsrB I
Pvu II
Nru I
BssH II
Kas I
TGGGAGAGCGGCAGCTGGCGTATTCCGGCGCAAGGTTTTACCATCGCTATCGCGAACTTGCGCGCGTCGAGGCGCCCTTCGTGCGGTCCA
630
M G E R Q L A Y S G A R F Y H R Y R E L A R V E A P F V R S
Bpu10 I
Sac II
Pvu II
Kas I
GTGGCGTAAGCCGCGTTGTAGCCTCAGCTGTCAATTTCACCCAGGGCTTCCACCAGGCGCGGCTCGCCGACCGCGGCGCCACGTTGCCCC
720
S G V S R V V A S A V N F T Q G F H Q A R L A D R G A T L P
Bbs I
Bsa I
Bcl I
Bsg I
Eco31 I
CGCCAACACTGCCCTATGACATGGTGATCATCTCGTCAGACGACACCGCCAACAACACCTTGCACCACGGTCTCTGCACGGTCTTCGAGG
810
P P T L P Y D M V I I S S D D T A N N T L H H G L C T V F E
Bsp120 I
Bcg I
BseR I
Pvu II
AGGGGCCCTATGCCGACATTGGCGACAAGGCGCAGAAAGAATACCTCTCCAAGTTTGTCGGTCCCATCGTGGAGCGCATTAACGCGCAGC
900
E G P Y A D I G D K A Q K E Y L S K F V G P I V E R I N A Q

FIG. 20B

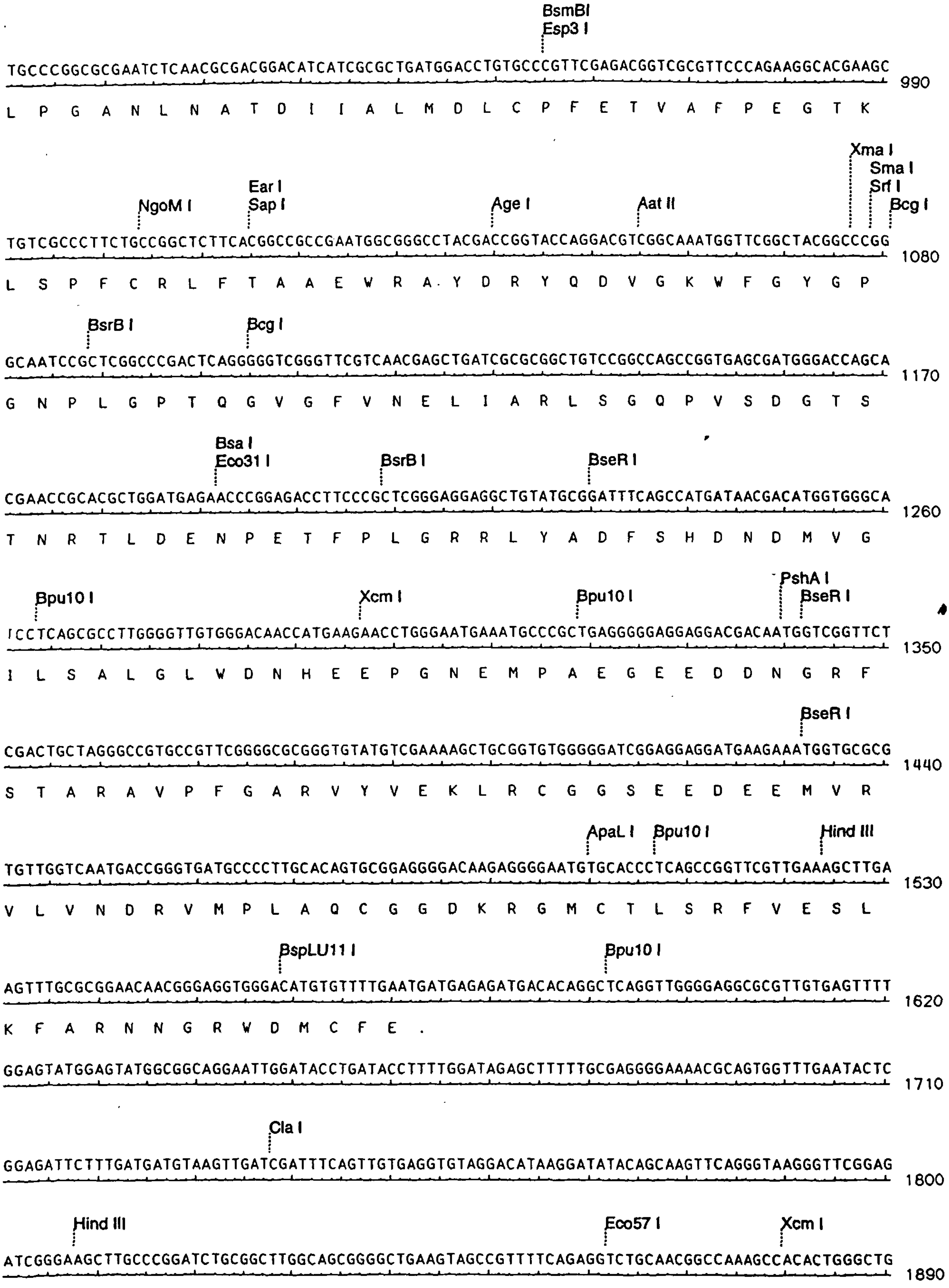
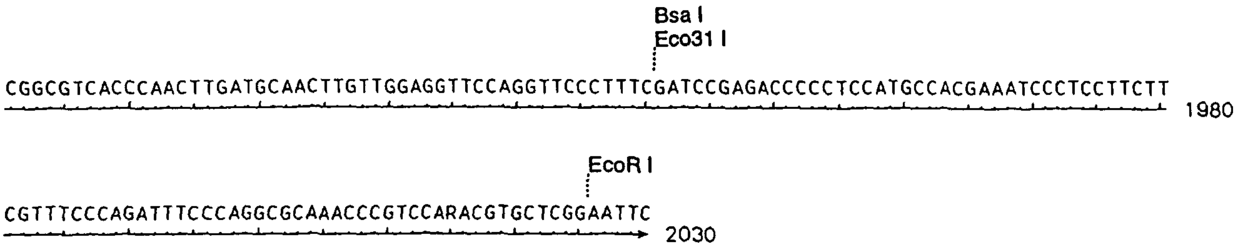

BsmBI
Esp3 I
TGCCCGGCGCGAATCTCAACGCGACGGACATCATCGCGCTGATGGACCTGTGCCCGTTCGAGACGGTCGCGTTCCCAGAAGGCACGAAGC
990
L P G A N L N A T D I I A L M D L C P F E T V A F P E G T K
NgoM I
Ear I
Sap I
Age I
Aat II
Xma I
Sma I
Srf I
Bcg I
TGTCGCCCTTCTGCCGGCTCTTCACGGCCGCCGAATGGCGGGCCTACGACCGGTACCAGGACGTCGGCAAATGGTTCGGCTACGGCCCGG
1080
L S P F C R L F T A A E W R A Y D R Y Q D V G K W F G Y G P
BsrB I
Bcg I
GCAATCCGCTCGGCCCGACTCAGGGGGTCGGGTTCGTCAACGAGCTGATCGCGCGGCTGTCCGGCCAGCCGGTGAGCGATGGGACCAGCA
1170
G N P L G P T Q G V G F V N E L I A R L S G Q P V S D G T S
Bsa I
Eco31 I
BsrB I
BseR I
CGAACCGCACGCTGGATGAGAACCCGGAGACCTTCCCGCTCGGGAGGAGGCTGTATGCGGATTTCAGCCATGATAACGACATGGTGGGCA
1260
T N R T L D E N P E T F P L G R R L Y A D F S H D N D M V G
Bpu10 I
Xcm I
Bpu10 I
PshA I
BseR I
TCCTCAGCGCCTTGGGGTTGTGGGACAACCATGAAGAACCTGGGAATGAAATGCCCGCTGAGGGGGAGGAGGACGACAATGGTCGGTTCT
1350
I L S A L G L W D N H E E P G N E M P A E G E E D D N G R F
BseR I
CGACTGCTAGGGCCGTGCCGTTCGGGGCGCGGGTGTATGTCGAAAAGCTGCGGTGTGGGGGATCGGAGGAGGATGAAGAAATGGTGCGCG
1440
S T A R A V P F G A R V Y V E K L R C G G S E E D E E M V R
ApaL I
Bpu10 I
Hind III
TGTTGGTCAATGACCGGGTGATGCCCCTTGCACAGTGCGGAGGGGACAAGAGGGGAATGTGCACCCTCAGCCGGTTCGTTGAAAGCTTGA
1530
V L V N D R V M P L A Q C G G D K R G M C T L S R F V E S L
BspLU11 I
Bpu10 I
AGTTTGCGCGGAACAACGGGAGGTGGGACATGTGTTTTGAATGATGAGAGATGACACAGGCTCAGGTTGGGGAGGCGCGTTGTGAGTTTT
1620
K F A R N N G R W D M C F E .
GGAGTATGGAGTATGGCGGCAGGAATTGGATACCTGATACCTTTTGGATAGAGCTTTTTGCGAGGGGAAAACGCAGTGGTTTGAATACTC
1710
Cla I
GGAGATTCTTTGATGATGTAAGTTGATCGATTTCAGTTGTGAGGTGTAGGACATAAGGATATACAGCAAGTTCAGGGTAAGGGTTCGGAG
1800
Hind III
Eco57 I
Xcm I
ATCGGGAAGCTTGCCCGGATCTGCGGCTTGGCAGCGGGGCTGAAGTAGCCGTTTTCAGAGGTCTGCAACGGCCAAAGCCACACTGGGCTG
1890

FIG. 20C



CGGCGTCACCCAACTTGATGCAACTTGTTGGAGGTTCCAGGTTCCCTTTCGATCCGAGACCCCCTCCATGCCACGAAATCCCTCCTTCTT

CGTTTCCCAGATTTCCCAGGCGCAAACCCGTCCARACGTGCTCGGAATTC

FIG. 21

GCGGATTTTAGGCACGATAATAGTCTGACCTCGATATACGCTGCTCTTGGTCTGTATAACGGCACAAAGCAACTATCC

A D F R H D N S L T S I Y A A L G L Y N G T K Q L S

AAATCGAGGATAGAATCGACAAACCAGACAAATGGCTATTCTGCTGGCTGGACAGTTCCATTTGGAGCAAGGGCGTATGTTGAGATGATG

K S R I E S T N Q T N G Y S A G W T V P F G A R A Y V E M M

CAATGCCCCTCGGGGGATGAACCTCTGATTCGAGTTCTGGTGAACGATCGCGTCAT

Q C P S G D E P L I R V L V N D R V I

FIG. 22

```
                                    <------GSP2rev, fy1036------
TATCCTTCGGAGTCAGAAAGCAAGGCGTATGCGAAGTTGATTGACGCTATCAAGAAGAATGCTACTTCGT     70
 Y  P  S  E  S  E  S  K  A  Y  A  K  L  I  D  A  I  K  K  N  A  T  S
<------GSP1rev, fy037---------
TTTCGGGACAGTATGCTTTTCTGGAGAGTTATAATTATACTCTCGGCGCGGAAGACTTGACTACTTTTGG    140
F  S  G  Q  Y  A  F  L  E  S  Y  N  Y  T  L  G  A  E  D  L  T  T  F  G

TGAGAACCAGATGGTCGACTCGGGTGCCAAGTTTTACCGGCGGTATAAGAATTTGGCCAGGAAAAATACT    210
  E  N  Q  M  V  D  S  G  A  K  F  Y  R  R  Y  K  N  L  A  R  K  N  T

CCATTCATACGTGCATCAGGGTCTGACCGTGTCGTTGCGTCCGCGGAGAAGTTTATTGACGGACTTCGAG    280
 P  F  I  R  A  S  G  S  D  R  V  V  A  S  A  E  K  F  I  D  G  L  R

ACGCCCAGACCCACGACCAGGGCTCCAAACGTGTTGCCCCAGTTGTCAATGTGGTTATCCCTGAAACTGA    350
D  A  Q  T  H  D  Q  G  S  K  R  V  A  P  V  V  N  V  V  I  P  E  T  D

TGGATTTAACAACACCCTGGATCATAGCACTTGCGTGTCTTTTGAGAATGATGAGCGGGCGGACGAAATT    420
  G  F  N  N  T  L  D  H  S  T  C  V  S  F  E  N  D  E  R  A  D  E  I

GAAGCCAACTTCGCCGCGATCATTGGACCTCCGATTCGCAAACGTCTGGAAAACGACCTTCCTGGCGTTG    490
 E  A  N  F  A  A  I  I  G  P  P  I  R  K  R  L  E  N  D  L  P  G  V

AGCTTACAAATGAGCATGTGGAATACTTGATGGATATGTGCTCGTTCGACACCATGGCGCGCACCGCCCA    560
E  L  T  N  E  H  V  E  Y  L  M  D  M  C  S  F  D  T  M  A  R  T  A  H

TGGAACCGAGCTGTCTCCATTCTGCGCCATCTTCACTGAAAAGGAGTGGCTGCAGTACGACTACCTACAA    630
  G  T  E  L  S  P  F  C  A  I  F  T  E  K  E  W  L  Q  Y  D  Y  L  Q

TCTCTGTCAAAGTACTACGGCTACGGTGCCGGGAACCCCCTTGGCCCAGCTCAGGGAATTGGCTTCACCA    700
 S  L  S  K  Y  Y  G  Y  G  A  G  N  P  L  G  P  A  Q  G  I  G  F  T

ACGAGCTGATTGCCCGACTGACGCAGTCGCCTGTCCAGGACAACACGAGCACCAACCACACTCTAGACTC    770
N  E  L  I  A  R  L  T  Q  S  P  V  Q  D  N  T  S  T  N  H  T  L  D  S

TGACCCGGCCACGTTCCCCCTCGACAGGAAGCTCTACGCCGACTTCTCCCACGACAATAACATGATTTCT    840
  D  P  A  T  F  P  L  D  R  K  L  Y  A  D  F  S  H  D  N  N  M  I  S

ATATTCTTCGCCATGGGCCTGTACAACGGCACCCAGCCGCTGTCCATGGACACTGTGGAGTCGATTGAGG    910
 I  F  F  A  M  G  L  Y  N  G  T  Q  P  L  S  M  D  T  V  E  S  I  E

AGATGGATGGCTACGCGGCGTCTTGGACTGTCCCGTTTGGTGCGAGGGCTTACTTTGAGGTGATGCAGTG    980
E  M  D  G  Y  A  A  S  W  T  V  P  F  G  A  R  A  Y  F  E  V  M  Q  C

CCAAAAAAAGAAGGAGCCACTTGTGCGGGTATTAGTGAATGATCGCGTTGTTCCTCTCCATGGCTGTGCT   1050
  Q  K  K  K  E  P  L  V  R  V  L  V  N  D  R  V  V  P  L  H  G  C  A

GTTGACAAGCTCGGACGATGCACTTTGGACGATTGGGTCGAGGGCTTGAGTTTTGCGAGGGCCGGTGGGA   1120
 V  D  K  L  G  R  C  T  L  D  D  W  V  E  G  L  S  F  A  R  A  G  G

ACTGGAAGGCTTGTTTTACTGCCTAA   1146
N  W  K  A  C  F  T  A  .
```

FIG. 23

```
                                    <------------GSP2rev, fyt038--------
CAGTACTCTGCATACTTCCCAATCCCGTCTGAGCTTGATGCCTCAACACCAGACGATTGTGATGTGACTT     70
 Q  Y  S  A  Y  F  P  I  P  S  E  L  D  A  S  T  P  D  D  C  D  V  T
          <----------GSP1rev, fyt039----------
TTGCACTCGTCTTGTCCCGCCATGGAGCCAGGTACCCAACGGACAGCAAGTCTGCAGCATACAACGCTAC    140
F  A  L  V  L  S  R  H  G  A  R  Y  P  T  D  S  K  S  A  A  Y  N  A  T

CATTGCCCGCATTCAAAAGTCTGCTACCATGTACGGCAAGAACTACAAGTGGCTTAAGGAGTATACCTAC    210
  I  A  R  I  Q  K  S  A  T  M  Y  G  K  N  Y  K  W  L  K  E  Y  T  Y

AGTCTCGGCGCTGAAGACCTGACTGAGTTTGGCCAGCGGCAGATGGTCGACTCTGGTAGGGCCTTTTATG    280
 S  L  G  A  E  D  L  T  E  F  G  Q  R  Q  M  V  D  S  G  R  A  F  Y

AGCGGTACATGAGTCTCGCTGAGAAGACTGAGCCTTTTGTTCGGGCATCGGGCTCAGATCGGGTCATCAT    350
E  R  Y  M  S  L  A  E  K  T  E  P  F  V  R  A  S  G  S  D  R  V  I  M

GTCGTCTTACAATTTTACGCAAGGCTTTTACGCATCGCGAGGAGAGTCTGGAGACGATTATACTCAGGAT    420
  S  S  Y  N  F  T  Q  G  F  Y  A  S  R  G  E  S  G  D  D  Y  T  Q  D

GTTCTTATCATCCCTGAAGAACCTGGCATCAACAACACCATGTTGCATGGATCGTGCGCCTCATTCGAAA    490
 V  L  I  I  P  E  E  P  G  I  N  N  T  M  L  H  G  S  C  A  S  F  E

GCGACAGAGTTCCTAAAGACGCAGATGAAAAGGCCGAGGTTGCATGGGGAGCAAGATTCCTCCCCGAGAT    560
S  D  R  V  P  K  D  A  D  E  K  A  E  V  A  W  G  A  R  F  L  P  E  I

TCGAAATAGGTTGAACCACCACCTGCCAGGAGTCAACCTGACGCTGGAGGAAACCATCTACATGATGGAC    630
  R  N  R  L  N  H  H  L  P  G  V  N  L  T  L  E  E  T  I  Y  M  M  D

ATGTGTCCGTTCCTCGCGGCTGACACACCTGATGGCGCTGGTCACTCCAGGTTCTGCGACCTCTTCACCA    700
 M  C  P  F  L  A  A  D  T  P  D  G  A  G  H  S  R  F  C  D  L  F  T

AGGCAGACTGGCGAAGTTACGACTACTACATGACTCTGAGCAAGTTCTACAAGTTTGGCAATGGCAATGC    770
K  A  D  W  R  S  Y  D  Y  Y  M  T  L  S  K  F  Y  K  F  G  N  G  N  A

CATGGGACCGACACAAGGTGTTGGATATGTCAACGAACTCATCTCACGCTTGACTGGGAAGCCTGTTGAC    840
  M  G  P  T  Q  G  V  G  Y  V  N  E  L  I  S  R  L  T  G  K  P  V  D

GACCACACCACGACCAACAGCACATTGGACTCATCGCCAAAGACGTTCCCTCTTGACAGGGCTCTATATG    910
 D  H  T  T  T  N  S  T  L  D  S  S  P  K  T  F  P  L  D  R  A  L  Y

CGGATTTTAGCCACGACAACAGCATGGTCTCCATCTTCTCAGCACTGGGCTTGTACAACTCGACTACCCT    980
A  D  F  S  H  D  N  S  M  V  S  I  F  S  A  L  G  L  Y  N  S  T  T  L

GCTACCAAAGGACCATATTGTGCCCGCGATCAAGGCGCACGGCTACTCATCGACATGGGTAGTCCCCTTT   1050
  L  P  K  D  H  I  V  P  A  I  K  A  H  G  Y  S  S  T  W  V  V  P  F

GGAGCCAGAATGTACGTCGAGAAGCTCGAGTGTGGTGCCAGCAGGAATGAAAAGAGAGACGAGTACGTGC   1120
 G  A  R  M  Y  V  E  K  L  E  C  G  A  S  R  N  E  K  R  D  E  Y  V

GAGTCCTGGTCAACGACCGAGTGATGTCGCTCGAAACCTGCGGAGGCGACGAGTACGGGCTCTGCAGACT   1190
R  V  L  V  N  D  R  V  M  S  L  E  T  C  G  G  D  E  Y  G  L  C  R  L

AGAAAACTTTGTGGAGAGTCTGTCGTTTGCCGCCTCGGGAGGAAACTGGGATCAATGCGGTGGATAA   1257
  E  N  F  V  E  S  L  S  F  A  A  S  G  G  N  W  D  Q  C  G  G  .
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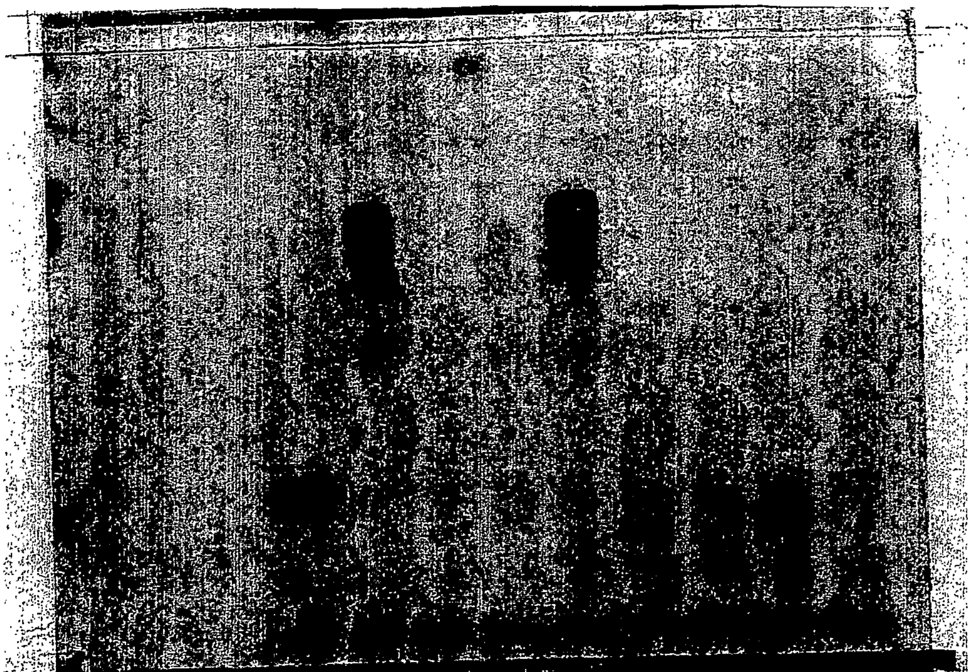

FIG. 24A

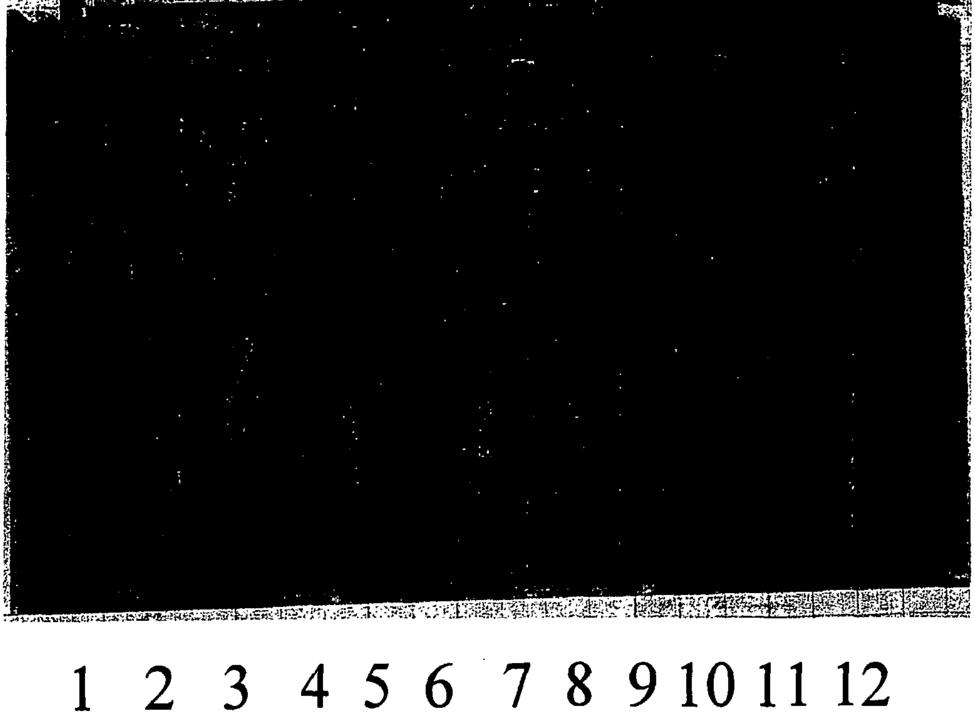

FIG. 24B