The phytase subfamily of histidine acid phosphatases: isolation of genes for two novel phytases from the fungi *Aspergillus terreus* and *Myceliophthora thermophila*

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Phytases catalyse the hydrolysis of phytate (myo-inositol hexakisphosphate) to myo-inositol and inorganic phosphate. In this study genes encoding novel phytases from two different filamentous fungi, *Aspergillus terreus* strain 9A-1 and *Myceliophthora thermophila* were isolated. The encoded PhyA phytase proteins show 60% (*A. terreus*) and 48% (*M. thermophila*) identity, respectively, to the PhyA of *Aspergillus niger* and have 21–29% identity compared to other histidine acid phosphatases. All three PhyA proteins, in contrast to the *A. niger* pH 2.5-optimum acid phosphatase, prefer phytic acid as substrate and show enzyme activity at a broad range of acidic pH values. Based on their enzyme characteristics and protein sequence homology, the phytases form a novel subclass of the histidine acid phosphatase family.

Keywords: *Aspergillus terreus*, *Myceliophthora thermophila*, gene isolation, histidine acid phosphatase, phytase

INTRODUCTION

Phytases (myo-inositol-hexakisphosphate 3-phosphohydrolase; EC 3.1.3.8) are acid phosphatase enzymes which efficiently cleave phosphate moieties from phytate (myo-inositol hexakisphosphate), thereby generating myo-inositol phosphates, myo-inositol and inorganic phosphate. Phytases belong to the family of histidine acid phosphatases, a subclass of phosphatases, all utilizing a phosphohistidine intermediate in their phosphoryl transfer reaction (van Etten, 1982). Phytate is a major phosphate storage form in plants. However, since phytate is not utilized by non-ruminants, these animals miss out on a major source of naturally occurring phosphorus. Inorganic phosphate has thus to be added to the feed to secure sufficient phosphate supply for the animal. Phytase was originally proposed as an animal feed additive to enhance the value of plant material in animal feed by liberating inorganic phosphate (Shieh & Ware, 1968). As phytase can also act as an anti-nutrient factor in animal feed by chelating minerals (DeBoland et al., 1975; Reddy et al., 1982), addition of phytase would also increase the feed value by removing this anti-nutrient factor. More recently, phytase has been seen as a way to reduce the level of phosphorus pollution that results from the excretion of phytic acid and phosphate supplements: less inorganic phosphate has to be added to feed when additional phytase is present. A number of studies have already shown that the addition of phytase enhances phosphate utilization from phytic acid and drastically reduces inorganic phosphate excretion (Nelson et al., 1971; Nasi, 1990; Simons et al., 1990).

The cloning and expression of the gene for phytase (phyA) from *Aspergillus niger* has been reported (Piddington et al., 1993; van Hartingsveldt et al., 1993). Ehrlich et al. (1993) also reported the cloning of the phyB gene from *A. niger*. However, since this protein shares over 99% amino acid sequence identity with the previously reported pH 2-5 optimum acid phosphatase encoded by the *aph* gene (Piddington et al., 1993), both may refer to the same acid phosphatase.

We are interested in novel phytases for use in animal nutrition. We identified 27 strains of fungi expressing extracellular phytase and isolated the phytase genes...

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The GenBank accession numbers for the nucleotide sequences reported in this paper are U59805 (A. terreus phyA) and U59806 (M. thermophila phyA).
from two of these strains: A. terreus strain 9A-1 and Myceliophthora thermophila. The encoded enzymes prefer phytic acid as substrate and form, together with A. niger PhyA phytase, a novel subclass of the histidine acid phosphatase family.

METHODS

Screening of strains for phytase activity. Various strains were tested for production of secreted phytase activity by growth on M3 medium containing dodecasodium phosphate (5 g l\(^{-1}\)) as the sole source of phosphate. M3 medium contained (per litre): 10 g glucose, 2 g NaNO\(_3\), 0.1 g KCl, 0.1 g MgSO\(_4\).7H\(_2\)O, 0.4 mg CuSO\(_4\), 0.8 mg FeSO\(_4\).7H\(_2\)O, 0.8 mg Na\(_2\)MoO\(_4\), 2H\(_2\)O, 8 mg ZnSO\(_4\).7H\(_2\)O, 0.04 mg Na\(_2\)B\(_4\)O\(_7\), 0.1H\(_2\)O and 0.8 mg MnSO\(_4\).H\(_2\)O. When appropriate, 1/4 g KH\(_2\)PO\(_4\), 1\(^{-1}\) and 0.68 g K\(_2\)HPO\(_4\), 1\(^{-1}\) were added. For phytase growth assays on plates, the plates were made with agarose to decrease the background level of phosphate. The following fungi showed histidine acid phosphatase family.

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**Construction of \(\lambda\) libraries and isolation of phytase genes.** A genomic library in Lambda FIX II (Stratagene) of A. terreus strain 9A-1 DNA digested with BamHI was constructed according to the manufacturer's protocols. Similarly, a genomic library of M. thermophila DNA digested with BglII was constructed. The \(\lambda\) libraries were screened using as hybridization probes the A. terreus strain 9A-1 and M. thermophila PCR fragments, respectively (see Fig. 1). Positive plaques were purified through three rounds of screening and \(\lambda\) DNA was then prepared and again tested for hybridization with the original PCR fragments. The DNA from 9A117/9A1122 was digested with PstI or BglII/XbaI and ligated into pHBluescript II SK\((+)(Stratagene). The \(\lambda\) DNA of the positive M. thermophila clone was digested with SalI and the insert ligated into pHBluescript II SK\((+)(Stratagene). Positive colonies were again identified by hybridization with the PCR fragment and the dsDNA sequence determined.

**Plasmid constructions and site-directed mutagenesis.** For each of the four proteins expression cassettes were prepared. The Ncol site 345 bp downstream of the ATG initiation codon of the M. thermophila gene was removed by site-directed mutagenesis, replacing the A by a G and creating a new Ncol (CCATGG) site at the ATG initiation codon. This resulted in an exchange of the second amino acid from Thr to Ala. The 1782 bp Ncol–SalI fragment was cloned between the 2.3 kb Kpnl–Ncol fragment of the gpdA (glyceraldehyde-3-phosphate dehydrogenase) promoter from A. nidulans (Punt et al., 1988, 1990) and the 710 bp BamHI–XbaI fragment of the trpC terminator (Mullany et al., 1985) in pUC19. Similarly, the 1956 bp Ncol–EcoRI fragment of the A. niger strain 9A-1 gene, the 1512 bp Ncol–HindIII fragment of the A. niger phyA gene and the 1623 bp Clal–SmaI fragment of the A. niger aph gene were cloned between the gpdA promoter and the trpC terminator. The Clal site in the aph gene directly preceding the ATG start codon (ATGCATATG) was introduced by site-directed mutagenesis. The A. niger phyA and aph genes used were obtained by PCR amplification. The predicted amino acid sequence of the A. niger PhyA protein used here differs in 2 aa (Q297R, S466F) and 12 aa (S14A, E66D, D89E, A106V, S130T, V155I, K171E, V236A, N292H, Q297R, S345N and V438I) from the A. niger phyase sequences published by Piddington et al. (1993) and van Hartingsveldt et al. (1993), respectively. The predicted amino acid sequence of the acid phosphatase used here was identical to the published A. niger pH 2.5-optimium acid phosphatase (Piddington et al., 1993). The expression cassettes were then isolated as Kpnl–XbaI fragments and cloned into the corresponding sites of a pUC19 derivative containing the ~2.3 kb Clal–SphiI fragment of the Neurospora crassa pyr4 gene (Oakley et al., 1987) cloned into the NarI site of pUC19. The pyr4 gene is used as a selection marker for transformation of A. niger. The resulting plasmids

**Design of degenerate PCR primers.** For isolation of phytase genes, PCR primers were made based on protein sequence comparisons of several acid phosphatases (Bajwa et al., 1984; Elliott et al., 1986; Piddington et al., 1993; van Hartingsveldt et al., 1993; Ehrlich et al., 1993). DNA sequences were selected (see below, Figs 2 and 3) which would allow the specific isolation of phytases rather than of other acid phosphatases. The N-terminal peptide MDMSFDP was used to design a 20-mer primer sequence with a degeneracy of 128. The sequence only included the first two bases of the terminal aspartic acid codon as this allows annealing with either an aspartic or glutamic acid codon. To reduce degeneracy to 32 a number of codons for serine were discarded based on a codon frequency table constructed for A. niger (not shown). The C-terminal peptide YGHGAGN was used to design a 20-mer primer sequence with a degeneracy of 1024 (only the first two bases of the asparagine codon were used). The degeneracy was reduced to 128 by discarding some of the codons for the GAG tripeptide sequence. The sequence of the two primers were as follows: primer A, 5' ATG GA(CT) ATG TG(CT) TCN TT(CT) GA 3' (sense, degeneracy = 32, Tm\(_{\text{max}}\) 60 °C, Tm\(_{\text{min}}\) 52 °C); primer B, 5' TT(AG) CC(AG) GC(AG) CC(GA) TGN CC(GA) TA 3' (antisense, degeneracy = 128, Tm\(_{\text{max}}\) 70 °C, Tm\(_{\text{min}}\) 58 °C).

**Isolation and characterization of PCR fragments.** PCR was performed using a GeneAmp kit (Perkin-Elmer Cetus) ac-
are pAT1 (containing \textit{A. terreus} strain 9A-1 \textit{phyA}), pMT1 (containing \textit{M. thermophila} \textit{phyA}), pPAN1 (containing \textit{A. niger} \textit{phyA}) and pAPAN1 (containing \textit{A. niger} \textit{aph}).

**Transformation of \textit{Aspergillus} and screening of transformants.** \textit{A. niger} strain NW205 was transformed as described by Punt & van den Hondel (1992) with some minor modifications. Small pieces of mycelium from the transformants were transferred to individual wells of 24-well plates (Falcon 3047), each well containing 1.5 ml of a 3% low melting agarose medium containing (per litre) 2 g ammonium nitrate, 5 g phytic acid (dodecasodium salt), 0.1 g KCl, 0.1 g MgSO\(_4\), 10 mM HEPES/HCl pH 5.5, 0.001% nicotinamide, 0.26 g arginine, 10 g glucose, 0.04 mg Na\(_2\)MoO\(_4\), 0.4 mg CuSO\(_4\), 0.8 g FeSO\(_4\) and 0.8 g MnSO\(_4\). After growth for 16 to 24 h at 30 °C, the mycelium was removed and the fermentation broth were dialysed overnight at 4 °C. The fermentation broth was overlaid with 1 ml of a solution containing 0.6 M H\(_2\)SO\(_4\), 0.8 mg FeSO\(_4\), 0.05 g FeSO\(_4\), and 0.03 g ZnCl\(_2\) and 0.02 g CaCl\(_2\). Determination of enzyme activity. The exact pH in the individual reactions was measured after overnight culture, diluted 1:100) to inoculate fermenter cultures for \textit{A. niger} transformants containing an additional \textit{phyA} gene, or grown for 3 d for the \textit{A. niger} cells expressing the \textit{aph} gene and used directly for determination of the enzyme activity. The fermenter medium contained (per litre): 35 g maltodextrin, 9.4 g yeast extract, 18.7 g casein hydrolysate, 2 g KH\(_2\)PO\(_4\), 2 g K\(_2\)SO\(_4\), 0.5 g MgSO\(_4\), 7H\(_2\)O, 0.05 g MnSO\(_4\), 4H\(_2\)O, 0.05 g FeSO\(_4\), 0.03 g ZnCl\(_2\) and 0.02 g CaCl\(_2\). The pH was kept at 4.5 by addition of KOH. After growth for 3 d at 30 °C, the mycelium was removed and the fermentation broth was centrifuged and sterile-filtered. Samples of the fermentation broth were dialysed overnight at 4 °C against 10 mM sodium acetate buffer pH 5.5 and diluted into buffers preadjusted to the pH used in the enzyme assay. The following buffers were used: 0.2 M glycine/HCl for pH 2.5, 0.2 M acetate/NaOH for pH values between pH 3 and 5.5, 0.05 M imidazole/HCl for the pH range between pH 6 and 6.5, and 0.2 M Tris/HCl for the pH range between 7 and 9. 'Phytase' activity was determined as described by Piddington \textit{et al.} (1993). 'Acid phosphatase' activity was determined in similar fashion using 4-nitrophenyl phosphate as substrate. The exact pH in the individual reactions was measured after mixing the substrate and the enzyme. In untransformed \textit{A. niger} strains grown under similar conditions, less than 0.1 U per ml of culture supernatant of activity against phytic acid and 4-nitrophenyl phosphate was detected.

**RESULTS**

**Isolation of phytase genes from \textit{A. terreus} strain 9A-1 and \textit{M. thermophila}**

Several fungi were tested for the presence of secreted phytase activity. In total, 27 novel strains showing phytase activity were found (for list, see Methods). All of the \textit{Aspergillus} strains tested exhibited phytase activity, while of the other fungi screened about 50% exhibited phytase activity (not shown). \textit{M. thermophila} and \textit{A. terreus} strain 9A-1 (Yamada \textit{et al.}, 1968) were selected for isolation of phytase genes using degenerate PCR primers (see Methods). The primers were chosen such that they encoded some amino acids found in acid phosphatases and phytases but also contained differences in order to increase the chances of specifically detecting phytase genes and not acid phosphatase genes. The DNA sequences of PCR fragments obtained for \textit{A. terreus} strain 9A-1 (146 bp; double-underlined in Fig. 2) and \textit{M. thermophila} (179 bp; double-underlined in Fig. 3) were determined. Each fragment encoded part of a protein having higher homology to the \textit{A. niger} phytase (van Hartingsveldt \textit{et al.}, 1993) than to known acid phosphatases, suggesting that they were fragments of a phytase gene and not of an acid phosphatase gene (see below). Genomic λ libraries of \textit{M. thermophila} and \textit{A. terreus} strain 9A-1 were constructed and screened using the PCR fragments. Restriction maps of genomic DNA prepared using the PCR fragments (Fig. 1) were used to show that the relevant parts of all positive clones are collinear with genomic DNA.

**Isolation and characterization of the \textit{phyA} gene of \textit{A. terreus} strain 9A-1**

The nucleotide sequence of the 2327 bp XbaI–PstI fragment (Fig. 1) of \textit{A. terreus} strain 9A-1 DNA was determined (Fig. 2). In the ORF three possible initiation codons (at positions 278–280, 365–367 and 374–376) were found. The first is upstream of the postulated TATA box (see below) and the following amino acids are not predicted to form an effective signal sequence for protein secretion (von Heijne, 1983). The other two possible initiation codons are close together and each is followed by a putative signal peptide. Since use of the last ATG in expression plasmids resulted in efficient phytase secretion (data not shown), we propose that the third ATG is the start codon. The coding sequence of \textit{phyA} thus encodes a protein of 466 aa and is interrupted...
D. B. MITCHELL and VAL bereits TSGTPLGPRGK intron reported thus far in fungi (Gwynne 2048, 2208-2210, 2226-2228 expected splice consensus sites presented in Fig. 3).

The complete sequence of the 3995 bp SalI fragment containing phyA from M. thermophila (Fig. 1) is presented in Fig. 3. There are four possible initiation codons in the correct reading frame (positions 2046–2048, 2208–2210, 2226–2228 and 2235–2237). The second ATG is postulated as the initiation codon; it is followed by a putative signal sequence (von Heijne, 1983). The predicted coding sequence is 1000 bp long and is interrupted by a single intron of 48 bp. The intron contains the expected splice consensus sites (5' GTRNGY, YAG 3') and a putative internal lariat sequence, RCTRAC, that would be followed by unusually short signal sequences (see above) as does the putative internal lariat sequence.

The A. terreus phyA gene is the same length as the smallest intron reported thus far in fungi (Gwynne et al., 1987).

**Isolation and characterization of the phyA gene of M. thermophila**

The complete sequence of the 3995 bp SalI fragment containing phyA from M. thermophila (Fig. 1) is presented in Fig. 3. There are four possible initiation codons in the correct reading frame (positions 2046–2048, 2208–2210, 2226–2228 and 2235–2237). The second ATG is postulated as the initiation codon; it is followed by a putative signal sequence (von Heijne, 1983). The first precedes the putative TATA box (see below) and is not followed by a predicted signal sequence, and the third and fourth ATG codons do not show a good consensus for translation-initiation and would be followed by unusually short signal sequences (von Heijne, 1983). The predicted coding sequence is thus 487 aa long and is interrupted by a single intron of 57 bp. The intron splice sites conform to the consensus sequences (see above) as does the putative internal lariat sequence.

The A. terreus and M. thermophila PhyA proteins show higher homology to the A. niger phytase than to acid phosphatases

The amino acid compositions of the putative phytases of A. terreus (von Heijne, 1983) and M. thermophila were compared with those of the A. niger PhyA phytase and several known acid phosphatases (Fig. 4). The M. thermophila and A. terreus strain 9A-1 proteins showed 48% and 60% identity, respectively, to the PhyA of A. niger. In contrast, only 21–28% identity was seen when
the putative PhyA proteins were compared with three known acid phosphatases, including the A. niger pH 2.5-optimum acid phosphatase. Thus, the two sequences are more similar to the A. niger PhyA phytase than to other histidine acid phosphatases. Direct comparison of the amino acid sequences of the A. terreus strain 9A-1 and M. thermophila 5) encoding the phyA gene. The sequence of the putative phyA gene. The sequence of the PCR fragment used to isolate the complete genomic region of M. thermophila is shown in capitals; the consensus intron splice sites and intron internal elements are underlined.

Fig. 3. DNA sequence of genomic region of M. thermophila encoding the phyA gene. The DNA sequence of the PCR fragment used to isolate the complete phyA gene is shown with double underlining. The DNA sequence of the putative intron is shown in capitals; the consensus intron splice sites and intron internal elements are underlined. A potential TATA box (italics) and the purine-rich region upstream of the start codon are underlined.

Phytase subfamily of histidine acid phosphatases

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Fig. 3. DNA sequence of genomic region of M. thermophila encoding the phyA gene. The DNA sequence of the PCR fragment used to isolate the complete phyA gene is shown with double underlining. The DNA sequence of the putative intron is shown in capitals; the consensus intron splice sites and intron internal elements are underlined. A potential TATA box (italics) and the purine-rich region upstream of the start codon are underlined.
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Fig. 4. The phytases from A. terreus strain 9A-1 and M. thermophila show higher homology to the A. niger phytase than to other histidine acid phosphatases. The amino acid sequences of the phytases (phyA) of A. terreus strain 9A-1 (At) and M. thermophila (Mt) were compared with A. niger (An) PhyA phytase and the pH 2.5-optimum acid phosphatase (AP) of A. niger and the acid phosphatases (AP) of Saccharomyces cerevisiae (SCV) and rat prostate (Rat P). The A. niger pH 2.5-optimum acid phosphatase (Piddington et al., 1993) is 99% identical to the A. niger PhyB protein (Ehrlich et al., 1993) and thus, both enzymes may represent the same protein.

Both the A. terreus and M. thermophila PhyA proteins have phytase activity

The enzyme activity profiles of both proteins were determined and compared with those of the A. niger phytase and the A. niger pH 2.5-optimum acid phosphatase. In each case culture supernatants from A. niger transformants overexpressing one of the four proteins were used and the pH dependence of the enzyme activities against phytic acid and 4-nitrophenyl phosphate was determined. The A. terreus strain 9A-1 protein showed enzymic activity between pH 2.5 and 7.5 with phytic acid as substrate, with maximal activity at pH 5.5 (Fig. 6a). The M. thermophila protein showed enzymic activity against phytic acid between pH 3-5 and 8.5, while the highest activity was reached between pH 5.5 and 6.0 (Fig. 6b). Both proteins also accept 4-nitrophenyl phosphate, which is commonly used to determine acid phosphatase activity, as substrate. Interestingly, the pH optima for 4-nitrophenyl phosphate were clearly shifted to more acidic pH values. For

Fig. 5. Alignment of the predicted amino acid sequences of the PhyA phytases of A. niger (Anig), A. terreus strain 9A-1 (Aterr9a1) and M. thermophila (Mthermo). Identical residues are shown in black boxes. Dots indicate gaps introduced to optimize the alignment.
We isolated and characterized the genes encoding phytases from two different fungi, A. terreus strain 9A-1 and M. thermophila. Thus far, the only phytase gene to be isolated and characterized is that from A. niger (Piddington et al., 1993; van Hartingsveldt et al., 1993). The A. terreus and M. thermophila phytases, the A. niger phytase and acid phosphatases such as the pH 2.5-optimum acid phosphatase of A. niger (Piddington et al., 1993; Ehrlich et al., 1993) all belong to the histidine acid phosphatase family. However, based on protein sequence homology and enzyme activity profiles, the A. terreus, M. thermophila and A. niger PhyA proteins form a separate subclass of this family. The substrate specificities and pH dependence of the enzyme activities of the A. terreus, M. thermophila and A. niger PhyA proteins differ clearly from those of the A. niger pH 2.5-optimum acid phosphatase. All three PhyA proteins clearly prefer phytic acid as substrate and have drastically different pH optima than that of the pH 2.5-optimum acid phosphatase. A. terreus, M. thermophila and A. niger PhyA proteins showed 47 to 60% aa sequence identity, and the sequence homologies were evenly distributed along the entire lengths of the proteins. Other acid phosphatases show a much lower degree of amino acid identity when compared to each other (18 to 35%) or to the three phytases (21 to 29%). The A. niger pH 2.5-optimum acid phosphatase (Piddington et al., 1993), shows only 25 to 28% identity compared to the three phytases. A more detailed analysis of the characteristics of this phytase subfamily and the residues relevant for catalytic activity is currently ongoing.

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