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

David B. Mitchell,† Kurt Vogel, Bernd J. Weimann, Luis Pasamontes and Adolphus P. G. M. van Loon

Author for correspondence: Adolphus P. G. M. van Loon. Tel: +41 61 688 7027. Fax: +41 61 688 1645.
e-mail: Adolphus.van_Loon@Roche.com

Biotechnology Section, Vitamins and Fine Chemicals Division, F. Hoffmann-La Roche AG, CH-4070 Basel, Switzerland

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 phytate 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...
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 the *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, 0.1 g KCl, 0.1 g MgSO₄·7H₂O, 0.4 mg CuSO₄·5H₂O, 8 mg ZnSO₄·7H₂O, 0.04 mg Na₂B₄O₇·10H₂O and 0.8 mg MnSO₄·H₂O. When appropriate, 0.14 g KH₂PO₄·11 and 0.68 g K₂HPO₄·4 were added. For phytase growth assays on plates, the plates were made up with agarose to decrease the background level of phosphate. The following fungi showed background level of phosphate. The following fungi showed background level of phosphate. The following fungi showed background level of phosphate. The following fungi showed background level of phosphate. The following fungi showed background level of phosphate.

**Background of the method.** The method consisted of PCR amplification of DNA from the fungi. The fungi were grown in a shaking incubator at 27°C for 5 days. The DNA was then prepared and purified by phenol/chloroform extraction. The DNA was then tested for hybridization with the original PCR fragments. The DNA from the positive *M. thermophila clone was used to digest with *Sal*I and the insert ligated into pBluescript II SK(+) (Stratagene). The λ DNA of the positive *M. thermophila* clone was used to digest with *Sal*I and the insert ligated into pBluescript 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 *Nco*I 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 *Nco*I site at the ATG initiation codon. This resulted in an exchange of the second amino acid from Thr to Ala.

**Transformation of the plasmids.** The plasmids were transformed into *S. cerevisiae* using *CaCl₂* transformation. The expression cassettes were then isolated as *Nco*I-KpnI fragments and cloned into the corresponding sites of a pUC19 plasmid. The expression cassettes were then isolated as *Nco*I-KpnI fragments and cloned into the corresponding sites of a pUC19 plasmid. The expression cassettes were then isolated as *Nco*I-KpnI fragments and cloned into the corresponding sites of a pUC19 plasmid. The expression cassettes were then isolated as *Nco*I-KpnI fragments and cloned into the corresponding sites of a pUC19 plasmid. The expression cassettes were then isolated as *Nco*I-KpnI fragments and cloned into the corresponding sites of a pUC19 plasmid.
are pPAT1 (containing *A. terreus* strain 9A-1 *phyA*), pMT1 (containing *M. thermophila* *phyA*), pPAN1 (containing *A. niger* *phyA*) and pAPAN1 (containing *A. niger* *aph*).

**Transformation of Aspergillus and screening of transformants.** *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 MgSO4, 10 mM HEPES/HCl pH 5, 0.001% nicotinamide, 0.26 g arginine, 10 g glucose, 0.04 mg Na2B4O7, 0.4 mg CuSO4, 0.8 mg FeSO4 and 0.8 mg MnSO4, 0.8 mg Na2MoO4 and 8 mg ZnSO4. After growth for 16 to 24 h at 30 °C, the mycelium was overlaid with 1 ml of a solution containing 0.6 M H2SO4, 0.5% (NH4)6Mo7O24 and 2% ascorbic acid and incubated for 30 s to 20 min at 50 °C. Transformants showing the most intense blue colour and, thus, the highest inorganic phosphate release were chosen for isolation of individual transformants.

**Determination of enzyme activity.** YPD (1% yeast extract, 1% bactopeptone and 2% glucose) medium was inoculated with 104 spores ml−1 from individual transformants, each of which overproduces one of the four proteins. The shake flask cultures were incubated at 30 °C and either used as preculture (overnight culture, diluted 1:100) to inoculate fermenter cultures for *A. niger* transformants containing an additional *phyA* gene, or grown for 3 d for the *A. niger* cells expressing the *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 KH2PO4, 2 g K2SO4, 0.5 g MgSO4, 7H2O, 0.05 g MnSO4, 4H2O, 0.05 g FeSO4, 0.03 g ZnCl2 and 0.02 g CaCl2. 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 *et al.* (1993). ‘Acid phosphatase’ activity was determined in a 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 *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 *A. terreus* strain 9A-1 and *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 *Aspergillus* strains tested exhibited phytase activity, while of the other fungi screened about 50% exhibited phytase activity (not shown). *M. thermophila* and *A. terreus* strain 9A-1 (Yamada *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 *A. terreus* strain 9A-1 (146 bp; double-underlined in Fig. 2) and *M. thermophila* (179 bp; double-underlined in Fig. 3) were determined. Each fragment encoded part of a protein having higher homology to the *A. niger* phytase (van Hartingsveldt *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 *M. thermophila* and *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 *phyA* gene of *A. terreus* strain 9A-1**

The nucleotide sequence of the 2327 bp *XbaI-PstI* fragment (Fig. 1) of *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 *phyA* thus encodes a protein of 466 aa and is interrupted...
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 intron splice sites and intron internal elements are underlined. A possible TATA box is shown with underlined italics.

**Fig. 2.** DNA sequence of genomic region of *A. terreus* strain 9A-1 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 PCR fragment used to isolate the complete genomic region 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 possible TATA box is shown with underlined italics.

by a single intron of 48 bp. The intron contains the expected splice consensus sequence (5' GTRNGY, YAG 3') and a putative internal lariat sequence, RCTRAC (Unkles, 1992) (Fig. 2, underlined). The intron in 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* strain 9A-1 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 proteins with the A. niger phytase (Fig. 5) showed that the homology is distributed evenly along the length of the proteins. Interestingly, the M. thermophila protein contains three inserts, of 3, 11 and 13 aa, respectively, and has two deletions, of 8 and 2 aa, respectively, when compared to

**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 *A. niger* DNA sequence of the putative *phyA* gene is shown with single underlining. The *TATA box* and the purine-rich region upstream of the start codon are underlined.
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 \( S. \ cerevisiae \) (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](image-url)  
**Fig. 5.** Alignment of the predicted amino acid sequences of the PhyA phytases of \( A. \ niger \) (Anig), \( A. \ terreus \) strain 9A-1 (Ater9a1) and \( M. \ thermophila \) (Mthermo). Identical residues are shown in black boxes. Dots indicate gaps introduced to optimize the alignment.
DISCUSSION

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.

ACKNOWLEDGEMENTS

We thank Maria Huecas, Rachel Manzetti, Anke Middendorf, Roland Remy and Denis Hug for excellent technical assistance. We also thank Professor T. Kodama for kindly providing the *A. terreus* strain 9A1, Dr F. Buxton for the *A. niger* strain NW205.

REFERENCES


Received 3 June 1996; revised 20 August 1996; accepted 2 September 1996.