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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 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

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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 phytate (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 $MgSO_4.7H_2O$, 0·4 mg $CuSO_4$, 0·8 mg $FeSO_4.7H_2O$, 0·8 mg $Na_2MoO_4.2H_2O$, 8 mg $ZnSO_4.7H_2O$, 0·04 mg $Na_2B_4O_7.10H_2O$ and 0·8 mg $MnSO_4.H_2O$. When appropriate, 1·4 g KH_2PO_4 l^{-1} and 0·68 g K_2HPO_4 l^{-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 phytase activity: *Absidia sporophora-variabilis*, ATCC 36019; *Acrophialophora levis*, ATCC 48380; *Aspergillus fischeri*, Roche isolate; *Aspergillus flavipus*, DSM 874; *Aspergillus giganticus*, CBS 101.64; *Aspergillus ochraceus*, ATCC 1008; *Aspergillus petrakii*, ATCC 12337; *Aspergillus sclerotiorum*, CBS 549.65; *Aspergillus sojae*, CBS 126.59; *Aspergillus ustus*, Roche isolate; *Calcarisporiella thermophila*, ATCC 22718; *Chaetomium rectopilium*, ATCC 22431; *Chaetomium thermophilum*, ATCC 58420; *Corynascus thermophilus*, ATCC 22066; *Humicola sp.*, ATCC 60849; *Malbranchea cinnamomea*, ATCC 42825; *Mycelia sterlia*, ATCC 20350; *Myceliophthora thermophila*, ATCC 48102; *Myrococcum thermophilum*, ATCC 22112; *Rhizomucor miehei*, ATCC 22064; *Rhizomucor pusillus*, ATCC 22074; *Sporotrichum cellulophilum*, ATCC 20494; *Sporotrichum thermophile*, ATCC 22482; *Scytalidium indonesiacum*, ATCC 46858; *Talaromyces thermophilus*, ATCC 20186; *Thermomucor indicae-seudaticae*, ATCC 28404; and *Thielavia albomyces*, ATCC 28084 (ATCC, American Type Culture Collection; CBS, Centraal Bureau voor Schimmelcultures; DSM, Deutsche Sammlung von Mikroorganismen).

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 MDMCSFD 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, $T_{m\,max}$ 60 °C, $T_{m\,min}$ 52 °C); primer B, 5′ TT(AG) CC(AG) GC(AG) CC(GA) TGN CC(GA) TA 3′ (antisense, degeneracy = 128, $T_{m\,max}$ 70 °C, $T_{m\,min}$ 58 °C).

Isolation and characterization of PCR fragments. PCR was performed using a GeneAmp kit (Perkin-Elmer Cetus) according to the manufacturer's instructions. The reactions contained the degenerate primers at a final concentration of 10 μM. All components of the reaction, with the exception of the *Taq* polymerase, were incubated at 95 °C for 10 min, 50 °C for 10 min and then the sample was placed on ice. *Taq* polymerase was added and 35 cycles of PCR performed according to the cycle profile: 95 °C for 60 s, 50 °C for 90 s, 72 °C for 120 s. PCR products were excised from low melting point 1·5% agarose gel and purified from a NACS column (Life Technologies) essentially according to the manufacturer's protocol. The fragment was polyadenylated (5 min reaction time) and cloned into the p123T vector (Mitchell *et al.*, 1992). DNA sequence analysis was performed on Qiagen-purified dsDNA using the dideoxy method and the Pharmacia T_7 kit according to the protocol supplied by the manufacturer.

Construction of λ libraries and isolation of phytase genes. A genomic library in Lambda FIX II (Stratagene) of *A. terreus* strain 9A-1 DNA digested with *Bam*HI was constructed according to the manufacturer's protocols. Similarly, a genomic library of *M. thermophila* DNA digested with *Bgl*II was constructed. The λ 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 λ DNA was then prepared and again tested for hybridization with the original PCR fragments. The DNA from 9A1λ17/9A1λ22 was digested with *Pst*I or *Bgl*II/*Xba*I and ligated into pBluescript II SK(+) (Stratagene). The λ DNA of the positive *M. thermophila* clone was digested 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 (CCATGG) site at the ATG initiation codon. This resulted in an exchange of the second amino acid from Thr to Ala. The 1782 bp *Nco*I–*Sal*I fragment was cloned between the 2·3 kb *Kpn*I–*Nco*I fragment of the *gpdA* (glyceraldehyde-3-phosphate dehydrogenase) promoter from *A. nidulans* (Punt *et al.*, 1988, 1990) and the 710 bp *Bam*HI–*Xba*I fragment of the *trpC* terminator (Mullaney *et al.*, 1985) in pUC19. Similarly, the 1956 bp *Nco*I–*Eco*RI fragment of the *A. terreus* strain 9A-1 gene, the 1512 bp *Nco*I–*Hin*dIII fragment of the *A. niger phyA* gene and the 1623 bp *Cla*I–*Sma*I fragment of the *A. niger aph* gene were cloned between the *gpdA* promoter and the *trpC* terminator. The *Cla*I site in the *aph* gene directly preceding the ATG start codon (ATCGATATG) 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* phytase 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-optimum acid phosphatase (Piddington *et al.*, 1993). The expression cassettes were then isolated as *Kpn*I–*Xba*I fragments and cloned into the corresponding sites of a pUC19 derivative containing the ~2·3 kb *Cla*I–*Sph*I fragment of the *Neurospora crassa pyr4* gene (Oakley *et al.*, 1987) cloned into the *Nar*I site of pUC19. The *pyr4* gene is used as a selection marker for transformation of *A. niger*. The resulting plasmids

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 $MgSO_4$, 10 mM HEPES/HCl pH 5, 0·001% nicotinamide, 0·26 g arginine, 10 g glucose, 0·04 mg $Na_2B_4O_7$, 0·4 mg $CuSO_4$, 0·8 mg $FeSO_4$ and 0·8 mg $MnSO_4$, 0·8 mg Na_2MoO_4 and 8 mg $ZnSO_4$. After growth for 16 to 24 h at 30 °C, the mycelium was overlaid with 1 ml of a solution containing 0·6 M H_2SO_4, 0·5% $(NH_4)_6Mo_7O_{24}$ 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 10^6 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 KH_2PO_4, 2 g K_2SO_4, 0·5 g $MgSO_4.7H_2O$, 0·05 g $MnSO_4.4H_2O$, 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 *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

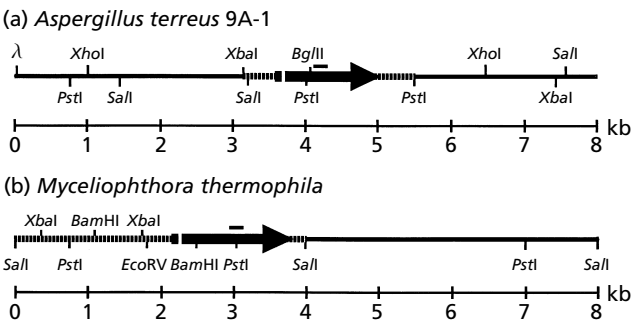

Fig. 1. Genomic map of the *phyA* genes of *A. terreus* strain 9A-1 (a) and *M. thermophila* (b). The PCR fragments (small bars above the restriction maps) obtained using the degenerate PCR primers were used to construct genomic maps of the regions containing the phytase genes. Arrows indicate the coding sequences for the *phyA* genes, the white gap in each arrow shows the single intron present within each of the coding sequences and the striped regions delineate the 5′- and 3′-flanking regions of the *phyA* genes of which the DNA sequence is presented (see Figs 2 and 3). The λ indicates the border of the λ insert in the case of the *A. terreus* strain 9A-1 clone. The restriction maps of the corresponding regions in chromosomal DNA and in the λ clones were identical.

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 *Xba*I–*Pst*I 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

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tctagaacaataacaggtactccctaggtacccgaaggaccttgtggaaaatgtatggaggtggacacggcaccaaccaccacccgcgatggcgcacgtg   100
gtgccctaacccctttgctccctcaggatggaatccatgtcgactctttaccctcaccatcgcctggatgaaacctccccgctaagctcacgacgatcgct   200
atttccgaccgatttgaccgtcatggtggagggctgattcggtcgatgctcctgccttcatttcggagttcggagacatgaaaggct tatatgaggacgt   300
cccaggtcggggacgaaatccgccctgggctgtgctccttcgtcggaaacatctgctgtccgtgatggctaccatgggctttcttgccattgtgctctcc   400
                                                                    M  G  F  L  A  I  V  L  S                9
gtcgccttgctctttagaagGTATGCACCCCTCTACGTCCAATTCTCTGGGCACTGACAACGGCGCAGcacatcgggcaccccgttgggcccccggggca   500
V  A  L  L  F  R  S                                                 T  S  G  T  P  L  G  P  R  G  K     27
aacatagcgactgcaactcagtcgatcacggctatcaatgctttcctgaactctctcataaaatggggactctacgcgccctacttctccctccaggacga   600
  H  S  D  C  N  S  V  D  H  G  Y  Q  C  F  P  E  L  S  H  K  W  G  L  Y  A  P  Y  F  S  L  Q  D  E     60
gtctccgtttcctctggacgtcccagaggactgtcacatcaccttcgtgcaggtgctggcccgccacggcgcgcggagcccaacccatagcaagaccaag   700
 S  P  F  P  L  D  V  P  E  D  C  H  I  T  F  V  Q  V  L  A  R  H  G  A  R  S  P  T  H  S  K  T  K     93
gcgtacgcggcgaccattgcggccatccagaagagtgccactgcgtttccgggcaaatacgcgttcctgcagtcatataactactccttggactctgagg   800
A  Y  A  A  T  I  A  A  I  Q  K  S  A  T  A  F  P  G  K  Y  A  F  L  Q  S  Y  N  Y  S  L  D  S  E  E   127
agctgactcccttcgggcggaaccagctgcgagatctgggcgcccagttctacgagcgctacaacgccctcacccgacacatcaaccccttcgtccgcgc   900
  L  T  P  F  G  R  N  Q  L  R  D  L  G  A  Q  F  Y  E  R  Y  N  A  L  T  R  H  I  N  P  F  V  R  A   160
caccgatgcatcccgcgtccacgaatccgccgagaagttcgtcgagggcttccaaaccgctcgacaggacgatcatcacgccaatccccaccagccttcg  1000
 T  D  A  S  R  V  H  E  S  A  E  K  F  V  E  G  F  Q  T  A  R  Q  D  D  H  H  A  N  P  H  Q  P  S    193
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P  R  V  D  V  A  I  P  E  G  S  A  Y  N  N  T  L  E  H  S  L  C  T  A  F  E  S  S  T  V  G  D  D  A   227
cggtcgccaacttcaccgccgtgttcgcgccggcgatcgcccagcgcctggaggccgatcttcccggcgtgcagctgtccaccgacgacgtggtcaacct  1200
  V  A  N  F  T  A  V  F  A  P  A  I  A  Q  R  L  E  A  D  L  P  G  V  Q  L  S  T  D  D  V  V  N  L   260
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 M  A  M  C  P  F  E  T  V  S  L  T  D  D  A  H  T  L  S  P  F  C  D  L  F  T  A  T  E  W  T  Q  Y    293
aactacctgctctcgctggacaagtactacggctacggcgggggcaatccgctgggtccggtgcaggggtcggctgggcgaacgagctgatggcgcggc  1400
N  Y  L  L  S  L  D  K  Y  Y  G  Y  G  G  G  N  P  L  G  P  V  Q  G  V  G  W  A  N  E  L  M  A  R  L   327
taacgcgcgcccccgtgcacgaccacacctgcgtcaacaacaccctcgacgcgagtccggccaccttcccgctgaacgccaccctctacgccgacttctc  1500
  T  R  A  P  V  H  D  H  T  C  V  N  N  T  L  D  A  S  P  A  T  F  P  L  N  A  T  L  Y  A  D  F  S   360
ccacgacagcaacctggtgtcgatcttctgggcgctgggcctgtacaacggcaccgcgccgctgtcgcagacctccgtcgagagcgtctcccagacggac  1600
 H  D  S  N  L  V  S  I  F  W  A  L  G  L  Y  N  G  T  A  P  L  S  Q  T  S  V  E  S  V  S  Q  T  D    393
gggtacgccgccgcctggacggtgccgttcgccgctcgcgcgtacgtcgagatgatgcagtgtcgcgccgagaaggagccgctggtgcgcgtgctggtca  1700
G  Y  A  A  A  W  T  V  P  F  A  A  R  A  Y  V  E  M  M  Q  C  R  A  E  K  E  P  L  V  R  V  L  V  N   427
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  D  R  V  M  P  L  H  G  C  P  T  D  K  L  G  R  C  K  R  D  A  F  V  A  G  L  S  F  A  Q  A  G  G   460
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 N  W  A  D  C  F                                                                                      466
gcccgtcaactgccttgcgccatccacctctcaccctggacgcaaccgagcggtctaccctgcacacggcttccaccgcgacgcgcacggataaggcgct  2000
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gccacaatcgtcgagagccatctgcag
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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 possible TATA box is shown with underlined italics.

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 (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 *Sal*I 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

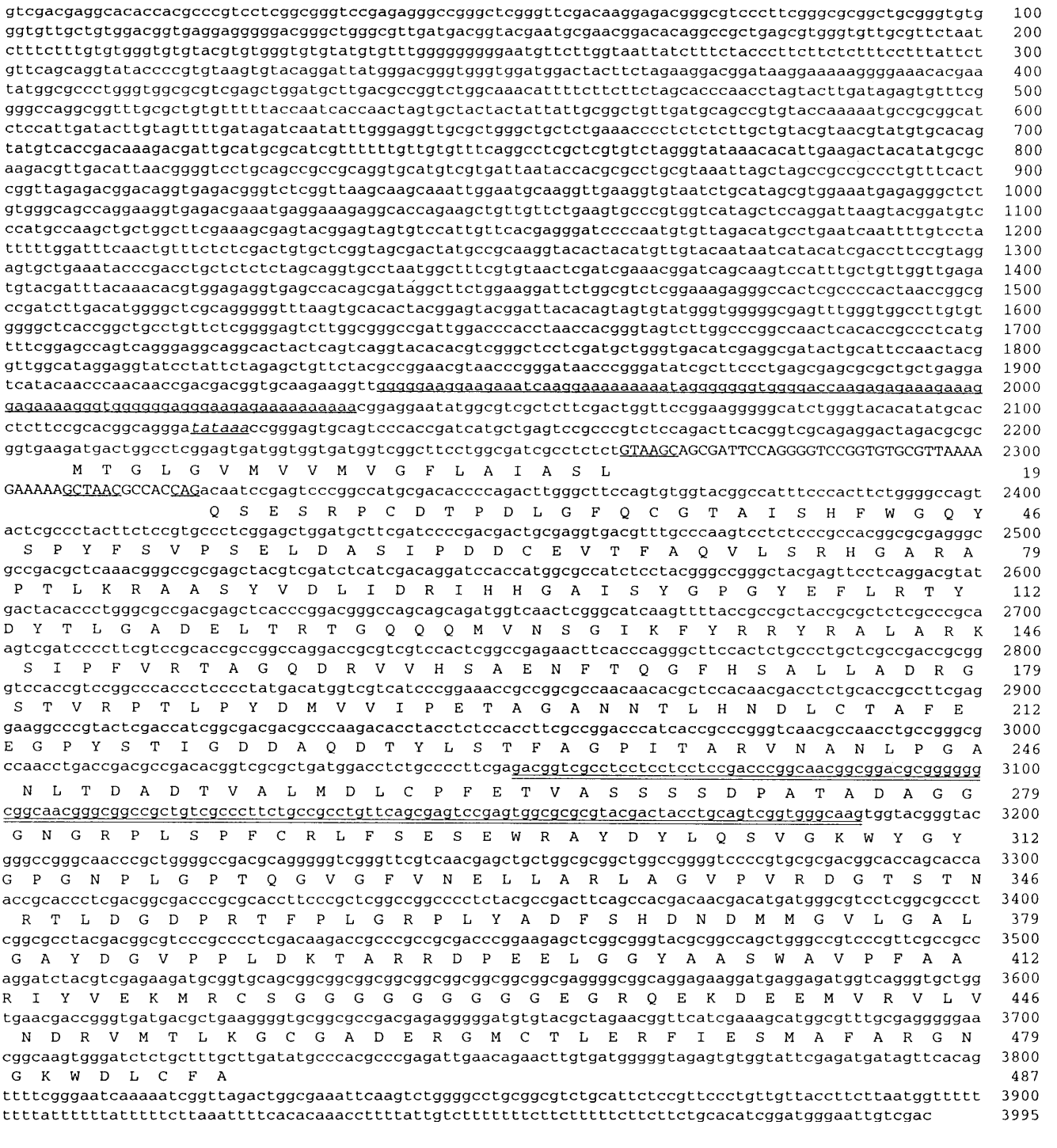

Fig. 3. DNA sequence of genomic region of *M. thermophila* encoding the *phyA* gene. The DNA sequence of the PCR fragment used to isolate the complete *phyA* gene is shown with double underlining. The DNA sequence of the putative intron is shown in capitals; the consensus intron splice sites and intron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

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 cloned 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

	An phyA	At phyA	Mt phyA	An AP	SCV AP	Rat P AP
An phyA		60	48	28	27	23
At phyA	72		47	26	28	21
Mt phyA	65	62		25	29	24
An AP	50	47	47		35	21
SCV AP	48	48	48	57		18
Rat P AP	45	44	49	44	42	

Identity (%)

Similarity (%)

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.

the two other proteins. The third insertion has a highly unusual aa sequence: 10 glycines in 13 aa. Thus, based on protein sequence analysis, both proteins are predicted to be phytases.

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

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Anig     ...MGVSAVLLPLYLLSGVTSGLAVPASRNQSSCDTVDQGYQCFSETSHLWGQYAPFFSL  57
Aterr9al ...MGFLAIVLSVALLFRSTSGTPLGPRGKHSDCNSVDHGYQCFPELSHKWGLYAPYFSL  57
Mthermo  MTGLGVMVVMVGFLAIASLQSES.........RPCDTPDLGFQCGTAISHFWGQYSPYFSV  52

Anig     ANESVISPEVPAGCRVTFAQVLSRHGARYPTDSKGKKYSALIEEIQQNATTFDGKYAFLK  117
Aterr9al QDESPFPLDVPEDCHITFVQVLARHGARSPTHSKTKAYAATIAAIQKSATAFPGKYAFLQ  117
Mthermo  PSE..LDASIPDDCEVTFAQVLSRHGARAPTLKRAASYVDLIDRIHHGAISYGPGYEFLR  110

Anig     TYNYSLGADDLTPFGEQELVNSGIKFYQRYESLTRNIVPFIRSSGSSRVIASGKKFIEGF  177
Aterr9al SYNYSLDSEELTPFGRNQLRDLGAQFYERYNALTRHINPFVRATDASRVHESAEKFVEGF  177
Mthermo  TYDYTLGADELTRTGQQQMVNSGIKFYRRYRALARKSIPFVRTAGQDRVVHSAENFTQGF  170

Anig     QSTKLKDPRAQPGQSSPKIDVVISEASSSNNTLDPGTCTVFED...SELADTVEANFTAT  234
Aterr9al QTARQDDHHANPHQPSPRVDVAIPEGSAYNNTLEHSLCTAFES...STVGDDAVANFTAV  234
Mthermo  HSALLADRGSTVRPTLPYDMVVIPETAGANNTLHNDLCTAFEEGPYSTIGDDAQDTYLST  230

Anig     FVPSIRQRLENDLSGVTLTDTEVTYLMDMCSFDTISTS..........TVDTKLSPFCD  283
Aterr9al FAPAIAQRLEADLPGVQLSTDDVVNLMAMCPFETVSLT..........DDAHTLSPFCD  283
Mthermo  FAGPITARVNANLPGANLTDADTVALMDLCPFETVASSSSDPATADAGGGNGRPLSPFCR  290

Anig     LFTHDEWINYDYLQSLKKYYGHGAGNPLGPTQGVGYANELIARLTHSPVHDDTSSNHTLD  343
Aterr9al LFTATEWTQYNYLLSLDKYYGYGGGNPLGPVQGVGWANELMARLTRAPVHDHTCVNNTLD  343
Mthermo  LFSESEWRAYDYLQSVGKWYGYGPGNPLGPTQGVGFVNELLARLAGVPVRDGTSTNRTLD  350

Anig     SSPATFPLNSTLYADFSHDNGIISILFALGLYNGTKPLSTTTVENITQTDGFSSAWTVPF  403
Aterr9al ASPATFPLNATLYADFSHDSNLVSIFWALGLYNGTAPLSQTSVESVSQTDGYAAAWTVPF  403
Mthermo  GDPRTFPLGRPLYADFSHDNDMMGVLGALGAYDGVPPLDKTARRDPEELGGYAASWAVPF  410

Anig     ASRLYVEMMQC.............QAEQEPLVRVLVNDRVVPLHGCPVDALGRCTRDSFV  450
Aterr9al AARAYVEMMQC.............RAEKEPLVRVLVNDRVMPLHGCPTDKLGRCKRDAFV  450
Mthermo  AARIYVEKMRCSGGGGGGGGGEGRQEKDEEMVRVLVNDRVMTLKGCGADERGMCTLERFI  470

Anig     RGLSFARSGGDWAECFA  467
Aterr9al AGLSFAQAGGNWADCF.  466
Mthermo  ESMAFARGNGKWDLCFA  487
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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.

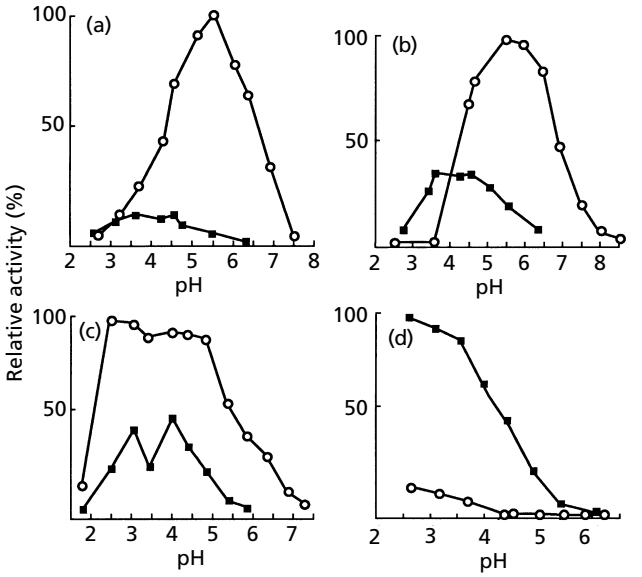

Fig. 6. Enzymic activity of the *A. terreus* strain 9A-1 (a), *M. thermophila* (b) and *A. niger* (c) PhyA phytases and of the *A. niger* pH 2·5-optimum acid phosphatase (d). Each of the four proteins was overexpressed in recombinant *A. niger* cells. Culture supernatants were used to determine the pH optima for each of the enzymes using phytic acid (○) and 4-nitrophenyl phosphate (■) as substrates. The release of inorganic phosphate was measured and for each protein the relative activities for both substrates at different pH values are shown. The growth conditions (excess inorganic phosphate) were such that expression of the endogenous *A. niger* acid phosphatase and phytase was repressed and did not contribute to the measured enzyme activities (see Methods). Representative results are shown. In the transformed *A. niger* strains used here, maximal enzyme activities for the *A. terreus* PhyA protein against phytic acid and 4-nitrophenyl phosphate were 22 and 2 U ml^{-1}, respectively; for the *M. thermophila* PhyA protein 8·5 and 3 U ml^{-1}, respectively; for the *A. niger* PhyA protein 4 and 2 U ml^{-1}, respectively, and for the *A. niger* acid phosphatase 18 and 125 U ml^{-1}, respectively. One unit of phytase or acid phosphatase activity equals release of 1 μmol P_i min^{-1} at 37 °C.

the *A. terreus* strain 9A-1 phytase, a broad maximum between pH 3·0 and pH 4·5 was seen (Fig. 6a). The *M. thermophila* protein showed maximal activity against 4-nitrophenyl phosphate between pH 3·5 and 4·5 (Fig. 6b). Both proteins therefore showed a clear preference for phytic acid as substrate, similar to the *A. niger* PhyA protein (Fig. 6c), and their substrate specificity and pH profiles clearly differed from those of the *A. niger* pH 2·5-optimum acid phosphatase (Fig. 6d). Thus, both the *A. terreus* strain 9A-1 and *M. thermophila* PhyA proteins are phytases.

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.

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