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Gene Cloning, Purification, and Characterization of a Heat-Stable Phytase from the Fungus *Aspergillus fumigatus*

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The finding of heat-stable enzymes or the engineering of moderately thermostable enzymes into more stable ones by random or site-directed mutagenesis has become a main priority of modern biotechnology. We report here for the first time a heat-stable phytase able to withstand temperatures up to 100°C over a period of 20 min, with a loss of only 10% of the initial enzymatic activity. The gene (*phyA*) encoding this heat-stable enzyme has been cloned from *Aspergillus fumigatus* and overexpressed in *Aspergillus niger*. The enzyme showed high activity with 4-nitrophenyl phosphate at a pH range of 3 to 5 and with phytic acid at a pH range of 2.5 to 7.5.

In recent years, considerable efforts have been made to improve the nutritive value of animal feedstuffs through supplementation with exogenous enzymes. The currently used feed enzymes can be divided into two main groups: the hemicellulases, including β-glucanase and xylanases able to degrade nonstarch polysaccharides, and the phytases.

Phytases (EC 3.1.3.8) belong to the family of histidine acid phosphatases and catalyze the hydrolysis of phytate, the major storage form of phosphate for plant seeds (9), into inorganic phosphate, inositol, and inositol mono- to pentaphosphates. Although a large amount of phosphate is present in feed in the form of phytate phosphorus, monogastric animals, like pigs and poultry, lack the ability to use this form of phosphate. As a consequence, phytate is excreted in the manure, causing environmental problems in areas of intensive livestock production (2). Furthermore, in order to compensate for the limited supply of readily available phosphorus, inorganic phosphate usually has to be included in the feed. The concept of adding phytases to the feed of monogastric animals to reduce the amount of phosphate excreted in the manure and to circumvent supplementation of the feed with inorganic phosphate has been described (13).

A major drawback to the wide use of phytases and of feed enzymes in general is the constraint of thermal stability (65 to 95°C) required for these enzymes to withstand inactivation during the feed-pelleting and/or expansion processes. Therefore, the availability of heat-resistant enzymes would circumvent the aforementioned problem. Currently available industrial phytases all originate from *Aspergillus niger*, but these enzymes have a low intrinsic resistance to heat inactivation (6). As a consequence, costly formulations are required to limit activity loss in these phytases during the feed-pelleting process.

We report the cloning of the phytase-encoding gene from the fungus *Aspergillus fumigatus*, known to secrete phytase (5). The enzyme was overexpressed in *A. niger* and purified to homogeneity, to study heat stability and the effect of pH on its activity. Furthermore, circular dichroism measurements were done to compare the secondary structure of the protein before and after exposure to high temperatures.

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MATERIALS AND METHODS

Organisms and growth conditions. *A. fumigatus* (ATCC 34625) was grown in potato dextrose broth (Difco Laboratories, Detroit, Mich.) at 28°C. Transformed *Escherichia coli* (TG-1) was grown in Luria broth at 37°C with 100 μg of ampicillin/ml.

Genomic DNA. Fungal mycelium was obtained by incubating potato dextrose medium inoculated with spores at a high density (10^6 per ml) and grown overnight with shaking (200 rpm) at 28°C. Up to 2 g of mycelium, obtained by filtration through a Wero-Lene N tissue (Wernli AG, Rothrist, Switzerland), was used for the isolation of genomic DNA. The mycelium was ground to a fine powder in liquid nitrogen, and the solution was immediately mixed with 10 ml of extraction buffer (200 mM Tris-HCl, 250 mM NaCl, 25 mM EDTA, 0.5% sodium dodecyl sulfate [SDS] [pH 8.5]). Phenol (7 ml) was added, and the sample was gently mixed, followed by addition of 3 ml of chloroform and further mixing. The sample was then centrifuged for 15 min at 20,000 × *g*, and the aqueous phase was recovered. After addition of RNase A to a final concentration of 2.5 μg/ml, the sample was incubated for 15 min at 37°C. The mixture was extracted once with 1 volume of chloroform, and the aqueous phase was recovered after centrifugation for 10 min at 10,000 × *g*. The DNA was precipitated by addition of 0.54 volume of isopropanol and incubation for 1 h at room temperature. The DNA was recovered by spooling and was resuspended in water. Proteinase K (50 μg/ml) was added to the DNA, followed by incubation for 2 h at 37°C. The sample was then extracted twice with phenol-chloroform, followed by ethanol precipitation. The pelleted DNA was resuspended in water.

DNA amplification. A DNA fragment of the coding region of the *A. fumigatus* phytase gene was obtained by "touchdown" PCR (4). The PCR was performed with a Gene Amp kit (Perkin-Elmer, Norwalk, Conn.), according to the manufacturer's instructions, with the degenerate primers A and B, which are derived from regions that are conserved between the *A. niger* phytase and other histidine acid phosphatases (10): primer A, 5′-ATGGA(C/T)ATGTG(C/T)TC(N)TT(C/T)GA-3′; primer B, 5′-TT(A/G)CC(A/G)GC(A/G)CC(G/A)TG(N)CC(A/G)TA-3′. The final concentration in the reaction mixture was 0.2 mM for each primer. The amplification cycles were as follows. Genomic DNA was denatured for 3 min at 95°C, followed by two cycles for 1 min each at each of the following annealing temperatures: 60, 59, 58, 57, 56, 55, 54, 53, 52, and 51°C. Prior to annealing, the incubation temperature was increased to 95°C for 1 min, and after annealing, elongation was performed at 72°C for 30 s. Cycles 21 to 35 were as follows: denaturation for 1 min at 95°C, annealing for 1 min at 50°C, and elongation for 30 s at 72°C. As a template, 1 μg of the genomic *A. fumigatus* DNA was used in a total reaction volume of 50 μl. An aliquot of the reaction mixture was analyzed on a 1.5% agarose gel. A PCR product of about 150 bp was detected, excised from the agarose, and isolated by centrifugation as described by Heery et al. (7). The fragment was subsequently cloned into pUC18 with the Sure-Clone ligation kit (Pharmacia Biotech AB, Uppsala, Sweden) according to the manufacturer's instructions and was sequenced. The sequence of the amplified DNA fragment (PCRAfu) and its location within the *A. fumigatus* phytase gene are shown in Fig. 1.

Screening of the genomic library. The genomic *A. fumigatus* (National Institutes of Health stock 5233) Lambda FIX II library was obtained from Stratagene (La Jolla, Calif.). The sizes of the cloned fragments generated by partial *Sau*3AI digestion of genomic DNA ranged from 9 to 22 kb. The library was screened according to the manufacturer's instructions, with the DNA fragment, PCRAfu, as a probe. A total of 5.3×10^6 plaques were screened, resulting in 115 hybridizing plaques. Two plaques were subjected to a second round of purification.

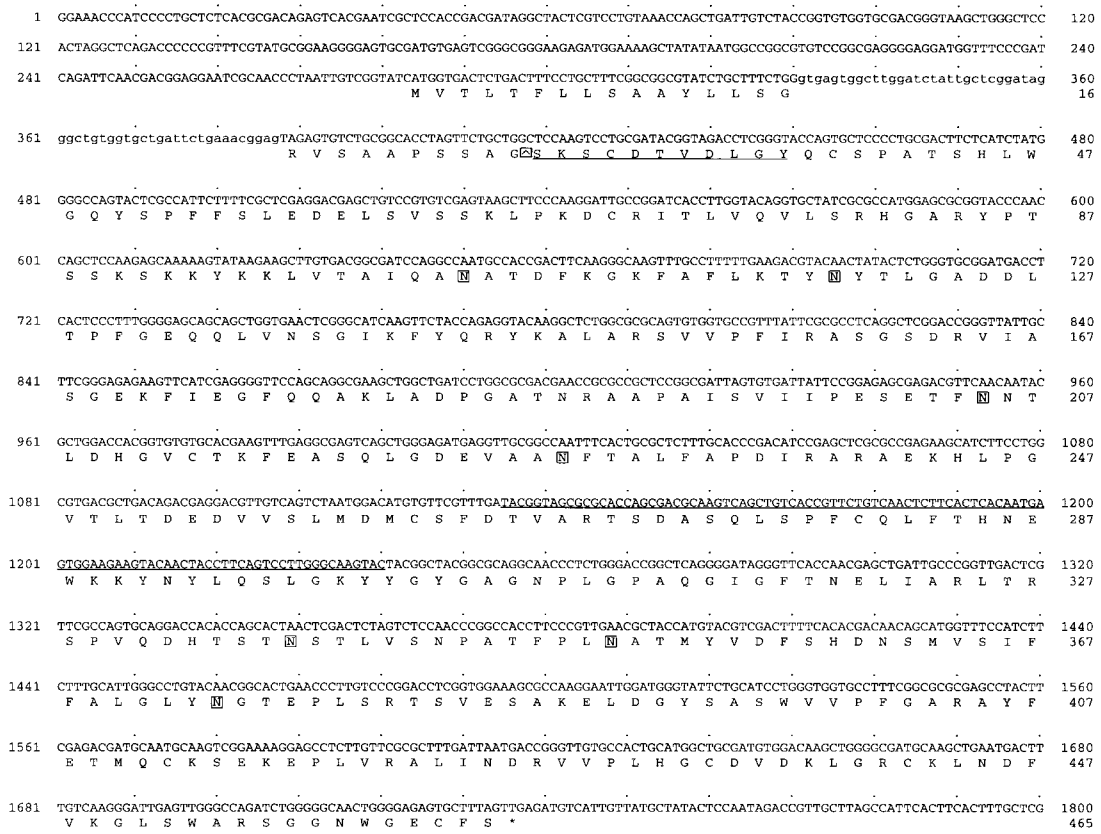

FIG. 1. Partial sequence of the 6-kb *Bam*HI fragment, including the complete phytase-encoding gene (*phyA*) of *A. fumigatus*. The intron is indicated by lowercase letters. The proposed intron donor and acceptor sites are in accordance with the GT-AG rule of Breathnach et al. (1). The signal peptide comprises the first 26 amino acids, and the cleavage site for the signal peptidase is marked (▽). The underlined amino acids were determined by N-terminal sequencing of the mature protein. The sequence of the PCR fragment PCRAfu obtained with primers A and B on genomic DNA from *A. fumigatus* is underlined. Potential N glycosylation sites are boxed.

Bacteriophage DNA from the two candidates was isolated and digested with *Not*I. The Lambda clone having the largest insert (approximately 15 kb) was further mapped by restriction analysis and genomic Southern blotting. A 6-kb *Bam*HI fragment giving a strong signal with the aforementioned probe was isolated, and the sequence encoding the putative phytase gene was determined. Figure 1 shows 1,571 nucleotides (nt) of the insert carrying the complete phytase gene, *phyA*.

DNA sequencing. The sequence was determined by the dideoxy chain termination technique (16) with the Sequenase kit, version 1.0 (United States Biochemicals, Cleveland, Ohio). Both strands were sequenced completely, and the sequence was analyzed with the GCG sequence analysis software package, version 8.0 provided by Genetics Computer Group, Inc. (Madison, Wis.) (3).

Construction of the expression plasmid for *A. niger*. The *phyA* gene of *A. fumigatus* was isolated with the Expand long-range kit (Boehringer, Mannheim, Germany), according to the supplier's instructions, with primer 39 (5′-TAT ATC ATG ATT ACT CTG ACT TTC CTG CTT TCG-3′) and primer 40 (5′-TAT ATA GAT ATC TCA ACT AAA GCA CTC TCC-3′). The reaction mixture included 10 pmol of each primer and 200 ng of template DNA (the plasmid carrying the 6-kb *Bam*HI insert). Ten rounds of amplification were done with the following cycles: 95°C for 1 min, 56°C for 1 min, and 72°C for 90 s. The PCR-amplified *A. fumigatus phyA* gene had a new *Bsp*HI site at the ATG start codon, introduced with primer 39, which resulted in the change of the second amino acid from a valine to an isoleucine. Furthermore, an *Eco*RV site was created with primer 40, downstream from the TGA termination codon. The amplified fragment was digested with *Bsp*HI and *Eco*RV and ligated into the *Nco*I site downstream of the glucoamylase promoter of *A. niger* (*glaA*) and the *Eco*RV site upstream of the *Aspergillus nidulans* tryptophan C terminator (*trpC*) (12). The resulting expression plasmid, pAfum, had in addition the orotidine-5′-phosphate decarboxylase gene (*pyr-4*) of *Neurospora crassa* as a selection marker (Fig. 2) (18).

Transformation and screening of *A. niger* transformants. Transformation of *A. niger* NW205 (Pyr^- Arg^- Nic^-) was done basically as described previously (15), with minor modifications (10). Transformants were grown at 37°C in medium that contained (per liter) 70 g of maltodextrin (Sugro, Basel, Switzerland), 12.5 g of yeast extract, 25 g of casein-hydrolysate, 2 g of KH_2PO_4, 2 g of K_2SO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.03 g of $ZnCl_2$, 0.02 g of $CaCl_2$, 0.05 g of $MnSO_4 \cdot 4H_2O$, and 0.05 g of $FeSO_4$ adjusted to pH 5.6. The screening of the transformants for phytase activity was also performed as described by Mitchell et al. (10).

Purification of *A. fumigatus* phytase. *A. fumigatus* phytase was overexpressed in *A. niger* NW205. The culture broth was centrifuged to remove the cells and concentrated approximately 50-fold by ultrafiltration in Amicon 8400 cells (PM30 membranes) and ULTRAFREE-15 centrifugal filter devices (Biomax-30K; Millipore, Bedford, Mass.). The concentrate, with an enzymatic activity of 350 U/ml, was desalted in aliquots of 1.5 ml on a Fast-Desalting HR 10/10

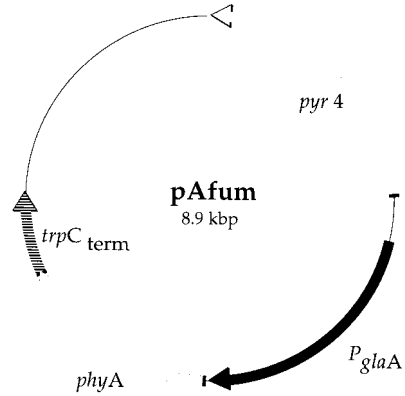

FIG. 2. Expression plasmid containing the *phyA* gene of *A. fumigatus* used to transform *A. niger*. *pyr-4*, orotidine-5′-phosphate decarboxylase gene of *N. crassa*; P_{glaA}, glucoamylase promoter of *A. niger*; *phyA*, *A. fumigatus* phytase gene; $trpC_{term}$, *A. nidulans* tryptophan C terminator.

column (Pharmacia Biotech), with 10 mM sodium acetate, pH 5.0, serving as the elution buffer. The eluate (3 ml) was loaded directly onto a 1.7-ml Poros HS/M cation-exchange column (PerSeptive Biosystems, Paul Bucher, Basel, Switzerland), from which the proteins were eluted by a step gradient from 0 to 1 M NaCl. Deglycosylation of purified *A. fumigatus* phytase was done by incubating the protein with a mixture of recombinant *N*-glycosidase F and endoglycosidase F1 (both fusion proteins with glutathione *S*-transferase; a kind gift from Fiona Grüninger, Roche, Basel, Switzerland). Deglycosylation was stopped by addition of fourfold-concentrated SDS-polyacrylamide gel electrophoresis (PAGE) sample buffer and incubation for 3 min at 95°C. Proteins were analyzed by SDS-PAGE with 8 to 16% Tris-glycine gradient gels (Novex, San Diego, Calif.) and stained with Coomassie blue.

Enzymatic assays. Enzymatic activity measurements with phytic acid at different pHs were done by diluting the purified enzyme (2.4 mg/ml) 1:200 in 10 mM sodium acetate, pH 5.0, and adding an equal volume of substrate solution containing 1% phytate (dodecasodium salt of phytic acid [$C_6H_6O_{24}P_6Na_{12}$]; Sigma, St. Louis, Mo.) in one of the following buffers: 0.4 M glycine-HCl between pH 2.0 and 3.0, 0.4 M sodium acetate between pH 3.5 and 5.5, 0.4 M imidazole-HCl between pH 6.0 and 6.5, and 0.4 M Tris-HCl between pH 7.0 and 9.0. After incubation of the sample (0.5 ml) for 15 min at 37°C, the reaction was stopped by addition of an equal volume of 15% trichloroacetic acid. Free inorganic phosphate was measured at 820 nm after 0.1 ml of the aforementioned sample was mixed with 0.9 ml of H_2O and 1 ml of a solution containing 0.6 M H_2SO_4, 2% ascorbic acid, and 0.5% ammonium molybdate, followed by incubation for 20 min at 50°C.

The enzymatic activity against 4-nitrophenyl phosphate was determined by incubating 0.25 ml of a 1:1,000 dilution of the purified *A. fumigatus* phytase (2.4 mg/ml) in 10 mM sodium acetate, pH 5.0, with an equal volume of 0.64% 4-nitrophenyl phosphate disodium (Merck 6850) dissolved in the following buffers: 0.4 M glycine-HCl between pH 2.0 and 3.0, 0.4 M sodium acetate between pH 3.5 and 5.5, 0.4 M imidazole-HCl between pH 6.0 and 6.5, and 0.4 M Tris-HCl between pH 7.0 and 9.0. After 15 min of incubation at 37°C, the reaction was terminated by adding an equal volume of 15% trichloroacetic acid. Free inorganic phosphate was measured at 820 nm as outlined above.

For short-term-thermostability assays, the purified enzymes (0.2 mg/ml) were diluted 1:100 in 10 mM sodium acetate, pH 5, and incubated for 20 min at each of the following temperatures: 30, 40, 45, 50, 55, 60, 70, 80, 90, and 100°C. The samples were placed on ice for 30 min, and the enzymatic activity with phytic acid was measured at pH 5.0 as outlined above.

For long-term-thermostability assays with the *A. fumigatus* phytase, the enzyme (0.2 mg/ml) was incubated at 60, 90, and 100°C for 20, 40, 60, and 120 min before being tested as described above.

N-terminal amino acid sequence analysis. Automated Edman degradation was done on an Applied Biosystems 494A sequencer (Perkin-Elmer) with on-line microbore phenylthiohydantoin detection.

CD spectroscopy. *A. fumigatus* and *Aspergillus terreus* 9A1 phytase used for the circular dichroism (CD) measurements had protein concentrations of 0.37 and 0.39 mg/ml, respectively. Samples were incubated for 20 min at either 30 or 90°C, followed by 1 h on ice. Far-UV CD spectra were recorded at 30°C on a model CD 6 dichrograph (Jobin Yvon, Longjumeau Cedex, France) from 190 to 260 nm with 1-nm increments. The baseline, obtained with solvent alone, was subtracted from the sample spectra.

Nucleotide sequence accession number. The *A. fumigatus* sequence shown in Fig. 1 has been deposited in the GenBank database under accession no. U59804.

RESULTS

Isolation and characterization of the phytase gene from *A. fumigatus*. In order to generate a specific probe hybridizing to the *phyA* gene of *A. fumigatus*, we used the "touchdown" PCR approach described by Mitchell et al. (10). The amplified fragment, PCRAfu, shown to encode part of a protein having homology to the phytase of *A. niger* (10, 19), was then used to isolate the complete *A. fumigatus* gene. Figure 1 shows 1,750 nt of the insert carrying the complete phytase gene. One single intron of 56 nt is found close to the 5′ end of the gene, in accordance with results reported for other phytase genes (10, 14, 19). The *phyA* gene of *A. fumigatus* encodes an enzyme of 465 amino acids with 66, 61, and 48% identity to the phytases of *A. niger*, *A. terreus* 9A1, and *Myceliophthora thermophila*, respectively (10, 19). With 29% sequence identity, the pH 2.5 acid phosphatase (*phyB*) of *A. niger* (14) had a higher proportion of identical amino acids than all the other members of the histidine acid phosphatase family (20). A putative TATA box (TATAA) is found 83 bp upstream of the initiation codon, but no CAAT box could be identified (Fig. 1). Analysis of the codon usage of the *phyA* gene showed a strong bias for G and C at the third position (77%, excluding the Trp and Met codons). This high frequency has been correlated with high protein expression levels in other *Aspergillus* species (8).

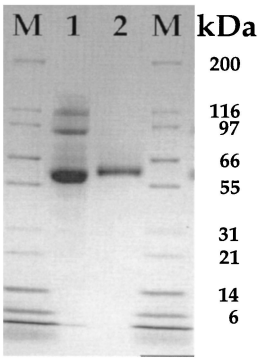

FIG. 3. SDS-PAGE analysis of the *A. fumigatus* phytase in the culture broth (lane 1) and after purification (lane 2). Lanes M, M_r standard (Mark 12; Novex) comprising myosin (M_r, 200,000), β-galactosidase (M_r, 116,300), phosphorylase *b* (M_r, 97,400), albumin (M_r, 66,300), glutamic dehydrogenase (M_r, 55,400), lactate dehydrogenase (M_r, 36,500), carbonic anhydrase (M_r, 31,000), trypsin inhibitor (M_r, 21,500), lysozyme (M_r, 14,400), and aprotinin (M_r, 6,000).

Purification and characterization of the phytase from *A. fumigatus*. Analysis of the protein sequence of the *A. fumigatus* phytase revealed a theoretical pI that was considerably higher than the pI values for the other phytases whose primary structures are currently known (7.3 versus 4.5 to 5.5). Since this pI also seemed to be more basic than those of all the other proteins present in the culture broth, purification of the phytase could be achieved essentially by a one-step procedure. When subjected to cation-exchange chromatography at pH 5.0, the *A. fumigatus* phytase was eluted as a single peak at 500 mM NaCl in the step gradient. The possibility that the purified *A. fumigatus* phytase was contaminated with *A. niger* NW205 phytase can be excluded on the basis of both the much lower expression of the latter and the completely different behaviors of the phytases during purification (not shown).

Analysis of the purified enzyme by SDS-PAGE revealed a protein with an apparent M_r of approximately 60,000 (Fig. 3). N-terminal sequencing showed that the first 26 amino acids represent the signal sequence necessary for proper secretion. This sequence is 6 amino acids longer than the one predicted according to the von Heijne rules (21). The mature enzyme therefore consists of 439 amino acids, with a theoretical M_r of 48,270. The observed difference between theoretical and apparent M_r points towards protein glycosylation, a notion that is supported by the presence of seven potential glycosylation sites of the NXT type, as shown in Fig. 1, as well as by a shift in protein size observed by SDS-PAGE after deglycosylation of the enzyme (not shown).

To prove that the cloned gene represents a phytase, rather than a pH 2.5 acid phosphatase, we determined the activity of the purified *A. fumigatus* enzyme at different pHs with phytic acid and 4-nitrophenyl phosphate as substrates. With phytic acid as the substrate, the enzyme showed activity between pH 2.5 and 8.0, with optima at pH 4 and 6.0 to 6.5 and with maximum activity at the latter (Fig. 4). With 4-nitrophenyl phosphate as the substrate, enzymatic activity occurred at pH values that were lower than those for phytic acid. The highest level of activity was measured at pH 5, and roughly 60% of the activity occurred between pH 3.0 and 3.5 (Fig. 4).

Investigation of the resistance of *A. fumigatus* phytase to heat inactivation revealed that even after 20 min of exposure to

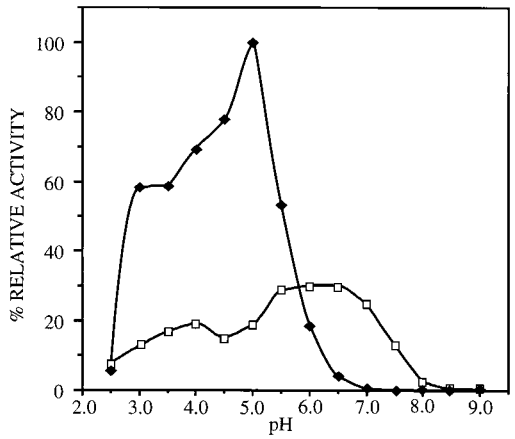

FIG. 4. pH dependence of the enzymatic activity at 37°C of *A. fumigatus* phytase with phytic acid (□) or 4-nitrophenyl phosphate (◆) as the substrate. The enzyme (2.4 mg/ml) was diluted for the assays 1:200 and 1:1,000, respectively. The maximal activity with 4-nitrophenyl phosphate was defined as 100% relative activity and was 3.3 times higher than the maximal activity for phytic acid. The percent error of the activity measurements was below 2%.

100°C, the enzyme still retained 90% of its initial activity (Fig. 5A). In contrast, the phytases from *A. terreus* 9A1 (Fig. 5A) and *M. thermophila* (Fig. 5A) lost 50% of their initial activity within 20 min of exposure to 50 and 55°C, respectively. Further assays with *A. fumigatus* phytase showed that even after prolonged incubation for 120 min at 90°C, the enzyme still retained at least 70% of its initial activity (Fig. 5B). Incubation at 100°C resulted in an almost linear loss of enzymatic activity over a period of 120 min (Fig. 5B). However, after 2 h at 100°C, 20% of the initial activity had still been retained. This linear loss of activity over time may be explained by the chemical deterioration of amino acids such as Cys and Met or of critical covalent links in proteins such as amide bonds (11). Nevertheless, the half-life of the enzymatic activity at 100°C is close to 70 min, which is remarkable considering that the reported half-lives of stabilized proteases are similar at a much lower temperature (76°C) (11).

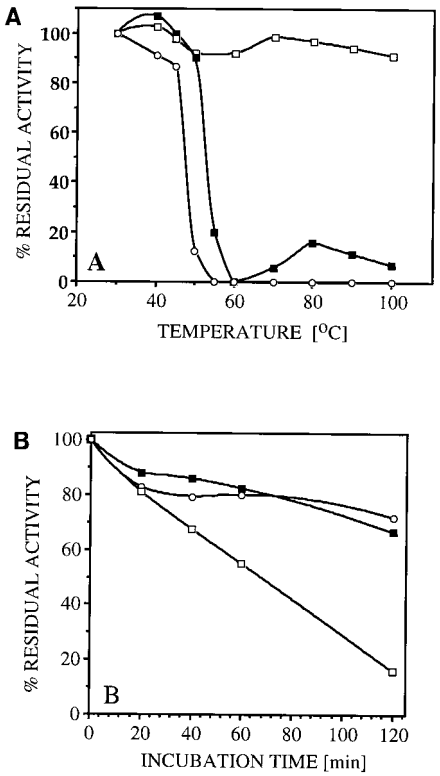

FIG. 5. (A) Residual enzymatic activity of the phytases from *A. fumigatus* (□), *A. terreus* 9A1 (○), and *M. thermophila* (■) after exposure for 20 min to the indicated temperatures. (B) Residual enzymatic activity of the purified *A. fumigatus* phytase after exposure for different periods to 60°C (○), 90°C (■), and 100°C (□).

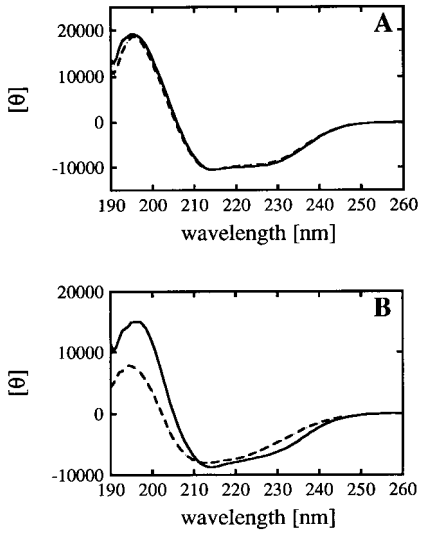

FIG. 6. Effect of exposure to high temperatures on the CD spectra of the phytases of *A. fumigatus* (A) and *A. terreus* 9A1 (B). The enzymes were incubated first for 20 min at 30°C (——) or 90°C (– – – –) and then for 1 h on ice. Residual molar ellipticities (in degrees · centimeter^2 · decimole^{-1}) are plotted against wavelength.

Finally, incubation of the enzyme for 20 min at 90°C had no discernible effect on the secondary structure of the *A. fumigatus* phytase (Fig. 6A), while considerable changes were seen for the phytase of *A. terreus* 9A1 when analyzed by CD spectroscopy (Fig. 6B).

DISCUSSION

The substrate specificity and the pH profile of the *phyA* enzyme of *A. fumigatus* resemble those described for the phytases of *A. niger* T213, *A. terreus* 9A1, *M. thermophila*, and *Aspergillus ficuum* but clearly differ from those of the acid phosphatase of *A. niger*, which has optimum activity at pH 2.5 (10, 17). These results confirm that we have cloned a phytase. Furthermore, the *A. fumigatus phyA* enzyme catalyzes phytic acid hydrolysis over the broadest pH range (2.5 to 8) of all the aforementioned phytases. The pH dependence of the activity with 4-nitrophenyl phosphate is very similar to that of the *A. niger* T213 *phyA* enzyme, with maximal activity found at pH 3.0 and 5.0.

Most remarkable and not described so far for any other phytase or acid phosphatase is the resistance of the enzyme to high temperatures. Incubation at 90 or even 100°C for 20 min resulted in only a minor loss of activity (10%). Even after exposure to 90°C for 120 min, 70% of the initial activity remained.

From the far-UV CD spectra (Fig. 6), it is clear that no overall changes in the secondary-structure elements of the *A.*

fumigatus phytase occurred after prolonged incubation at 90°C. Two possible explanations are (i) that the *A. fumigatus* phytase displays increased resistance to heat inactivation, similar to enzymes from hyperthermophilic organisms, and (ii) that this enzyme has the ability to refold properly after denaturation. It is the goal of ongoing experiments to understand in more detail the ability of the *A. fumigatus* enzyme to withstand high temperatures.

The strategy of cloning phytases from thermophilic fungi in order to obtain enzymes with increased resistance to heat inactivation was successful in the present study and led to the identification and cloning of a phytase with an extraordinary intrinsic capacity for heat resistance. This phytase is the first to withstand temperatures normally reached during the feed-pelleting process without a significant loss of activity. Furthermore, the broad pH range over which enzymatic activity occurs makes this phytase a promising candidate for commercial applications.

ACKNOWLEDGMENTS

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