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# Biophysical Characterization of Fungal Phytases (*myo*-Inositol Hexakisphosphate Phosphohydrolases): Molecular Size, Glycosylation Pattern, and Engineering of Proteolytic Resistance

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**Phytases (*myo*-inositol hexakisphosphate phosphohydrolases) are found naturally in plants and microorganisms, particularly fungi. Interest in these enzymes has been stimulated by the fact that phytase supplements increase the availability of phosphorus in pig and poultry feed and thereby reduce environmental pollution due to excess phosphate excretion in areas where there is intensive livestock production. The wild-type phytases from six different fungi, *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus*, *Emericella nidulans*, *Myceliophthora thermophila*, and *Talaromyces thermophilus*, were overexpressed in either filamentous fungi or yeasts and purified, and their biophysical properties were compared with those of a phytase from *Escherichia coli*. All of the phytases examined are monomeric proteins. While *E. coli* phytase is a nonglycosylated enzyme, the glycosylation patterns of the fungal phytases proved to be highly variable, differing for individual phytases, for a given phytase produced in different expression systems, and for individual batches of a given phytase produced in a particular expression system. Whereas the extents of glycosylation were moderate when the fungal phytases were expressed in filamentous fungi, they were excessive when the phytases were expressed in yeasts. However, the different extents of glycosylation had no effect on the specific activity, the thermostability, or the refolding properties of individual phytases. When expressed in *A. niger*, several fungal phytases were susceptible to limited proteolysis by proteases present in the culture supernatant. N-terminal sequencing of the fragments revealed that cleavage invariably occurred at exposed loops on the surface of the molecule. Site-directed mutagenesis of *A. fumigatus* and *E. nidulans* phytases at the cleavage sites yielded mutants that were considerably more resistant to proteolytic attack. Therefore, engineering of exposed surface loops may be a strategy for improving phytase stability during feed processing and in the digestive tract.**

Phytic acid (*myo*-inositol hexakisphosphate) is the major storage form of phosphorus in plants. In the context of human and animal nutrition, the following two aspects of phytic acid are critically important (18, 29): (i) monogastric animals have only low levels of phytate-degrading enzymes in their digestive tracts, and since phytic acid itself is not resorbed, feed for pigs and poultry commonly is supplemented with inorganic phosphate in order to meet the phosphorus requirements of these animals; and (ii) phytic acid is an antinutrient factor, since it forms complexes with proteins and a variety of metal ions and therefore decreases the dietary availability of these nutrients.

Because of these problems, there is considerable interest in phytate-degrading enzymes. The phytases (*myo*-inositol hexakisphosphate 3- and 6-phosphohydrolases; EC 3.1.3.8 and 3.1.3.26) are a subfamily of the histidine acid phosphatases (14) and are found naturally in plants and microorganisms, particularly fungi. As a class, the phytases have been rather poorly characterized biochemically (for a recent review see reference 29). In order to more clearly define this class of enzymes, several phytases of fungal origin have been cloned and overexpressed by workers in our company (14–16). In this paper we describe the purification of these fungal phytases, the structural and biophysical properties of these enzymes, and the results of a comparison with a prokaryotic phytase from *Escherichia coli*.

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## MATERIALS AND METHODS

**Expression in *Aspergillus niger*.** The DNA fragments encoding the *A. niger* CB (30), *Aspergillus terreus* 9A1 (GenBank accession no. U59805), *Aspergillus fumigatus* (GenBank accession no. U59804), *Emericella nidulans* (GenBank accession no. U59803), and *Myceliophthora thermophila* (GenBank accession no. U59806) phytases were ligated as 5′ *Nco*I or *Bsp*HI (introduced sites at the ATG start codon) 3′ blunt-ended fragments downstream of the *glaA* promoter into the *Nco*I-*Eco*RV site of the expression vector, as described by Mitchell et al. (14) and Pasamontes et al. (15). Transformation of *A. niger* NW205 (*ura*$^-$ *arg*$^-$ *nic*$^-$; kindly provided by F. Buxton) and screening for phytase-producing transformants were also done as described previously (15).

The phytase of *A. niger* (Natuphos; GenBank accession no. Z16414) was a commercial preparation obtained from BASF (Ludwigshafen, Germany) and was purified to homogeneity by anion-exchange chromatography.

**Expression in *Saccharomyces cerevisiae*.** The phytase genes were cloned into pScer-Ro11, a 2μ-based vector harboring a shortened version of the *gap*(FL) promoter and the *pho5* terminator (12), as well as the *ura3* gene as a selection marker. The intronless phytase genes of *A. fumigatus*, *A. niger* CB, and *A. terreus* CBS were cloned as *Eco*RI-*Eco*RV fragments downstream of the *gap*(FL) promoter in the *Eco*RI-*Bam*HI blunt-ended expression cassette. The gene for *E. nidulans* phytase was cloned as an *Eco*RI fragment into the corresponding site of pScer-Ro11. *S. cerevisiae* YMR4 (*ura*$^-$ *his*$^-$ *leu*$^-$ *pho3*$^-$ *pho5*$^-$; kindly provided by M. Riederer) was used for transformation. Individual transformants were

grown initially for 1 to 2 days in minimal medium. Phytase production was tested after subsequent culture for 2 to 3 days in YPD medium.

**Expression in *Hansenula polymorpha*.** The phytase genes (intronless) of *A. terreus* CBS (GenBank accession no. U60412), *A. fumigatus*, and *Talaromyces thermophilus* (GenBank accession no. U59802) were cloned as *Eco*RI fragments into the corresponding site of the *H. polymorpha* expression vector pFP (4) downstream of the formate dehydrogenase (FMD) promoter (9). The resulting plasmids were transformed into *H. polymorpha* RB11 (*ura*$^-$). About 300 to 400 transformants of each construct were inoculated into minimal medium (YNB containing 2% glucose). After several passages under selective pressure to force multiple integration of the expression plasmids into the genome of *H. polymorpha*, single stable clones were tested for phytase activity.

In order to obtain acceptable expression levels, the first 35 N-terminal amino acids of the *T. thermophilus* phytase were replaced by the amino acid sequence MGVFVVLLSIATLFGSTSGTALGPRGNHSKSCDTA$_{35}$ (the underlined amino acids originate from *A. terreus* CBS phytase and contain the signal sequence). Amino acids K$_{30}$SCDTA$_{35}$ were unrelated residues resulting from the cloning strategy used to replace the N terminus. Computer modelling of the chimeric *T. thermophilus* phytase suggested that modification of the first 16 amino acids of the mature protein was not likely to have an effect on the biochemical properties of the enzyme.

**Protein purification.** Independent of the expression system used, the culture broths (typically 500 to 1,000 ml) were centrifuged to remove the cells and were concentrated by ultrafiltration with Amicon 8400 cells (PM30 membranes; Grace AG, Wallisellen, Switzerland) and ultrafree-15 centrifugal filter devices (Biomax-30K; Millipore, Bedford, Mass.). The concentrates (typically 1.5 to 5 ml) were desalted with either Fast Desalting HR 10/10 or Sephadex G-25 Superfine columns (Pharmacia Biotech, Dübendorf, Switzerland); 10 mM sodium acetate (pH 5.0) was used as the elution buffer. The desalted *A. fumigatus* samples were directly loaded onto a 1.7-ml Poros HS/M cation-exchange chromatography column (PerSeptive Biosystems, Framingham, Mass.). When the other phytases were expressed in *A. niger* or *H. polymorpha*, they were loaded onto a 1.7-ml Poros HQ/M anion-exchange chromatography column. During both anion-exchange and cation-exchange chromatography, phytase was eluted in pure form by using an optimized sodium chloride gradient.

All of the phytases expressed in *S. cerevisiae* (except *A. fumigatus* phytase) were brought to 2 M $(NH_4)_2SO_4$ after desalting and were loaded onto a 1-ml Butyl Sepharose 4 Fast Flow hydrophobic interaction chromatography column (Pharmacia Biotech). The enzymes were eluted with a linear 2 to 0 M $(NH_4)_2SO_4$ gradient in 10 mM sodium acetate (pH 5.0). The phytases eluted in the breakthrough and were concentrated and loaded onto a 120-ml Sephacryl S-300 gel permeation chromatography column (Pharmacia Biotech). They eluted as symmetrical peaks and were determined to be pure by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

***E. coli* phytase.** In order to clone the *appA* gene of *E. coli*, total DNA from *E. coli* M15 (25) was prepared as described by Davis et al. (3). A 500-ng portion of this DNA was used in a PCR performed with the following primers designed by using the *appA* sequence published by Dassa et al. (2) (GenBank accession no. M58708): appA I (5′-AAACATAT*TCATGA*AAGCGATCTTAATCCCA-3′), including an *Rca*I site (sequence in italics); and appA II (5′-ATATA*GGATCC* CAAACTGACAGCCGGTTATGCG-3′), including a *Bam*HI site (sequence in italics). The PCR was performed as described by the manufacturer (Expand High Fidelity PCR kit; Boehringer, Mannheim, Germany) by using a hybridization temperature of 55°C. The resulting PCR product was separated from the primers by using a PCR purification kit obtained from Qiagen (Hilden, Germany), was digested with *Bam*HI and *Rca*I, and was purified by agarose gel electrophoresis and a subsequent gel elution step (Qiaex II; Qiagen). The *appA* gene was ligated into the *Bam*HI and *Nco*I sites of the pBluescript II SK vector (Stratagene, La Jolla, Calif.). A DNA sequence analysis of the gene revealed two differences when it was compared to the sequence deposited by Dassa et al. (2). One of these differences was a change from A to G at position 620. This difference also affected the amino acid sequence of the *appA* gene product; the glycine at position 207 was replaced by aspartic acid. The second sequence difference was at position 984 (change from G to A) and was a silent mutation. The *appA* gene was transferred into the pQE60 expression vector (Qiagen) containing a C-terminal 6xHis tag and a short linker sequence (Gly-Ser-Arg-Ser-His-His-His-His-His-His) and was transformed into *E. coli* BL21 (Stratagene).

A 500-ml portion of Luria-Bertani medium containing 200 μg of ampicillin per ml and 30 μg of kanamycin per ml was inoculated with 8 ml of an overnight culture of strain BL21 harboring the *appA* expression plasmid. When the culture reached an optical density at 600 nm of 1.0, the cells were induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and incubated for an additional 5 h at 37°C with vigorous shaking. The cells were harvested by centrifugation at 4000 × *g* for 20 min, resuspended in 30 ml of sonication buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 20 mM imidazole, 1 mg of lysozyme per ml). The suspension was incubated on ice for 15 min before the cells were disrupted by sonication (Vibra Cell 72408; Bioblock Scientific, Illkirch, France) at 60% power by using 2.5-s bursts, 2.5-s cooling periods, and a net sonication time of 3 min. The supernatant was clarified by centrifugation at 10,000 × *g* for 15 min, mixed with 8 ml of $Ni^{2+}$-agarose (50% resin suspension), equilibrated with sonication buffer, and incubated for 1 h on ice. Then the mixture was poured into a column and washed with 85 ml of sonication buffer. Phytase was eluted with 40 ml of 20 mM sodium acetate buffer (pH 4.5) containing 300 mM NaCl.

**SDS-PAGE and IEF.** SDS-PAGE was performed on 8 to 16% Tris–glycine gradient gels, and isoelectric focusing (IEF) was performed on IEF pH 3 to 7 or pH 3 to 10 gels (Novex, San Diego, Calif.). The gels were stained with colloidal Coomassie blue (Novex) or were semi-dry-blotted onto Immobilon P$^{SQ}$ (polyvinylidene difluoride) membranes (Millipore) and then stained with amido black (naphthol blue black).

**Gel permeation chromatography.** The molecular sizes of the purified proteins were determined at room temperature by gel filtration performed with a calibrated Superdex 200 column (fast-protein liquid chromatography; Pharmacia Biotech). The elution buffer normally contained 50 mM sodium phosphate, 150 mM NaCl, 0.2 mM $Na_2EDTA$, 2 mM 2-mercaptoethanol, and 1 mM sodium azide (pH 7.2). In order to determine the effect of phosphate on the molecular size of *A. fumigatus* phytase, gel permeation chromatography was also performed with an elution buffer containing 100 mM sodium acetate (pH 5.0). The gel filtration column was calibrated for both elution buffers with high- and low-molecular-weight kits obtained from Pharmacia Biotech, which contained thyroglobulin ($M_r$, 749,000; Stokes radius, 85.0 Å), ferritin ($M_r$, 421,000; Stokes radius, 61.0 Å), catalase ($M_r$, 211,000; Stokes radius, 52.2 Å), aldolase ($M_r$, 163,000; Stokes radius, 48.1 Å), bovine serum albumin ($M_r$, 71,700; Stokes radius, 35.5 Å), ovalbumin ($M_r$, 45,700; Stokes radius, 30.5 Å), chymotrypsinogen A ($M_r$, 20,200; Stokes radius, 20.9 Å), and RNase A ($M_r$, 15,700; Stokes radius, 16.4 Å).

**Analytical ultracentrifugation.** Analytical ultracentrifugation was performed in 10 mM sodium acetate (pH 5.0) by using a model Optima XL-A ultracentrifuge (Beckman, Palo Alto, Calif.) equipped with a type An-60 Ti rotor and cells having standard double-sector Epon aluminum-filled centerpieces. The data were analyzed with the program DISCREEQ of Schuck (20), which is based on an iterative Marquardt procedure. The specific volume of a protein depends not only on the amino acid sequence but also on the extent and type of glycosylation. Since glycosylation of fungal phytases most likely is the high-mannose type of glycosylation, all sugars were assumed to be mannose. This assumption was a simplification but introduced only a small error into the $M_r$ calculation.

**Mass spectrometry.** The peptides of interest obtained from trypsin digestion of *A. niger* phytase (Fig. 1) were analyzed by electrospray mass spectrometry. All analyses were performed in the positive ion mode with a triple quadrupole instrument (model API III; SCIEX, Concord, Ontario, Canada). Scans between *m*/*z* 400 and *m*/*z* 1600 were recorded with 0.2-average-mass-unit steps. *A. fumigatus* phytase was analyzed with a PerSeptive Biosystems Voyager Elite mass spectrometer equipped with a reflectron and delayed extraction. The ion acceleration voltage was 20 kV, and the results of 100 to 200 scans were averaged.

**N-terminal sequencing and calculation of theoretical $M_r$ and pI values.** Automated Edman degradation of purified phytases was performed with a model ABD 494HT sequencer (Perkin-Elmer, Foster City, Calif.) with on-line microbore phenylthiohydantoin-amino acid detection. Theoretical $M_r$ and pI values were calculated from the amino acid sequences of the native proteins (without a signal sequence) with the programs EditSeq and Protean from the Lasergene software package of DNASTAR Inc. (Madison, Wis.).

**Proteolytic susceptibility of *A. fumigatus* phytase mutants.** Purified *A. fumigatus* wild-type phytase and the *A. fumigatus* phytase mutants S126N and R125L/S126N were pretreated for 20 min at 90°C in order to inactivate potentially interfering traces of protease and then were renatured in the cold. Subsequently, they were incubated at 50°C at a concentration of 175 μg/ml in 10 mM sodium acetate (pH 5.0) with a 1:400-diluted 3-day culture supernatant of *A. niger* NW205 containing proteolytic activity. After 0, 20, 40, 60, 90, and 120 min of incubation, aliquots were subjected to SDS-PAGE or phytase activity was measured at 37°C. In control experiments, *A. fumigatus* wild-type phytase was incubated under the same conditions without NW205 culture supernatant or with NW205 culture supernatant that had been pretreated for 20 min at 90°C in order to inactivate the protease(s).

**Identification of the glycosylation sites of *A. niger* phytase.** *A. niger* phytase was S-carboxymethylated and digested with trypsin in 100 mM ammonium bicarbonate buffer (pH 8.0) at a phytase-to-protease ratio of 50:1 (wt/wt) for 18 h at 37°C. The reaction was stopped by adding 10% trifluoroacetic acid. The proteolytic fragments were separated at room temperature by reversed-phase high-performance liquid chromatography (HPLC) by using a $C_{18}$ column (250 by 2.1 mm; Vydac, Hesperia, Calif.) and a linear 0 to 95% acetonitrile gradient in 0.1% trifluoroacetic acid (Fig. 1). All of the peptides were collected and analyzed by electrospray mass spectrometry. In addition, fractions 9, 17, 21, 27 to 30, 35, 37, and 43 to 45 (Fig. 1) were analyzed by Edman degradation. Assignments of glycosylated Asn residues were made on the basis of the absence of the phenylthiohydantoin derivative of Asn in the corresponding Edman cycles and by comparison of the peptide sequences with the predicted sequence of *A. niger* phytase.

**Phytase activity measurements.** Phytase activity was measured in an assay mixture containing 0.5% (~5 mM) phytic acid and 200 mM sodium acetate (pH 5.0). After 15 min of incubation at 37°C (or at temperatures between 37 and 90°C), the reaction was stopped by adding an equal volume of 15% trichloroacetic acid. The liberated phosphate ions were quantified by mixing 100 μl of the assay mixture with 900 μl of $H_2O$ and 1 ml of 0.6 M $H_2SO_4$–2% ascorbic acid–0.5% ammonium molybdate. After 20 min of incubation at 50°C, absorbance at 820 nm was measured. Standard solutions of potassium phosphate were

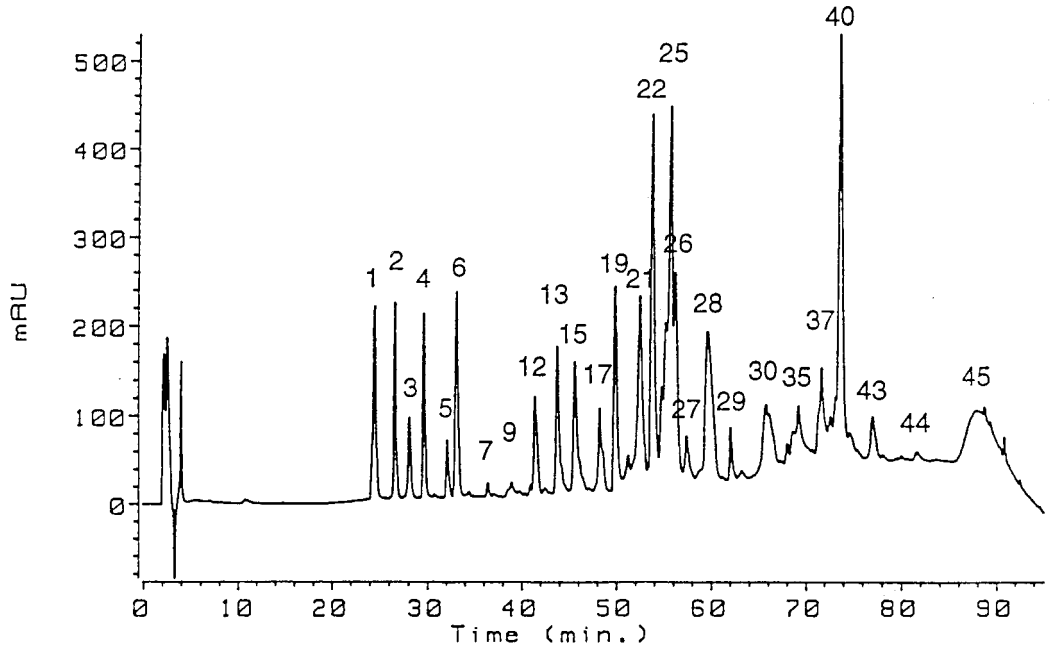


FIG. 1. Separation of *A. niger* phytase fragments by reversed-phase HPLC after carboxymethylation and trypsin digestion of the protein. For experimental details see the text. AU, arbitrary units.

used as a reference. One unit of phytase activity was defined as the amount of activity that liberates 1 μmol of phosphate per min at 37°C.

## RESULTS

**Purification of the phytases.** Phytase genes from different fungal species were expressed in three different host organisms. *A. fumigatus* phytase is the only fungal phytase having an isoelectric point greater than 7.0 (Table 1). Since the pI of this phytase is considerably higher than the pIs of all of the extracellular proteins produced by the different expression strains, purification of *A. fumigatus* phytase was essentially a one-step procedure. As Fig. 2 shows, *A. fumigatus* phytase eluted as a symmetrical peak from a cation-exchange chromatography column. Probably because of acidic glycosylation, the pI of *A. fumigatus* phytase expressed in *H. polymorpha* was less than 4.0 (Fig. 3A). Nevertheless, cation-exchange chromatography could still be used for purification of this protein, although the capacity was much lower (data not shown).

The other fungal phytases expressed in *A. niger* or *H. polymorpha* had pI values that were less than 5.5 and, accordingly, could be purified by anion-exchange chromatography (data not shown). Although the high level of glycosylation associated with expression in *S. cerevisiae* had no effect on the pI of *A. niger* CB, *A. terreus* CBS, or *E. nidulans* phytase (data not shown), all attempts to bind these proteins to anion-exchange chromatography columns failed. Consequently, we used an alternative approach, in which these proteins were subjected to hydrophobic interaction chromatography followed by preparative gel permeation chromatography, which yielded homogeneous products (data not shown).

**N-terminal sequences.** In no instance did the N terminus of the mature fungal phytase that was determined experimentally (Table 2) correspond to the N terminus that was predicted on the basis of the von Heijne rules (26, 27). This finding is surprising and contrasts with observations made with other acid phosphatases (data not shown). Whether this deviation from the theoretical results is an inherent property of phytases or whether the initial processing conformed to the von Heijne rules but was followed by additional proteolytic cleavage could not be determined in this study. However, the latter possibility was supported by the finding that the *A. niger* NRRL 3135 (24), *A. niger* CB, *A. terreus* CBS, and *E. nidulans* phytases had multiple N-terminal sequences (Table 2). In the case of *E. coli* phytase, our sequence data agree with previously published N-terminal sequence data for the protein (2, 6). However, the amount of amino acid recovered in the Edman cycles was small compared with the amount of purified protein applied, suggesting that a large proportion of *E. coli* phytase is N-terminally blocked.

**Biophysical properties of fungal phytases are not affected by different extents of glycosylation.** *A. fumigatus* phytase was expressed in three different expression systems, *A. niger*, *H. polymorpha*, and *S. cerevisiae*. In all of these expression systems, the protein was glycosylated, although to different extents (Fig. 3B). While glycosylation was moderate in *A. niger*, it was excessive and highly variable in *H. polymorpha* and *S. cerevisiae*, as indicated by the broad bands spanning $M_r$s ranging from 75,000 to 160,000. Different extents of glycosylation were observed even with different batches of *A. fumigatus* phytase expressed in the same expression system (Table 3).

When the protein was expressed in *A. niger* and *S. cerevisiae*, the different extents of glycosylation either had no or only a minor effect on the pI of the protein, suggesting that glycosylation was neutral (Fig. 3A). On the other hand, the pI of *A. fumigatus* phytase expressed in *H. polymorpha* was less than 4.0. Since deglycosylation with endoglycosidase F1 resulted in a pI of >8.0 (data not shown), the latter finding must have been due to acidic glycosylation. Remarkably, the different extents and patterns of glycosylation had no significant effect on the specific activity of the enzyme (31).

Virtually identical observations were made with *A. niger* CB, *A. terreus* CBS, and *E. nidulans* phytases which were also produced in different expression systems (data not shown).

Different extents of glycosylation may have an impact on the

TABLE 1. Isoelectric points of fungal and *E. coli* phytases

| Source of phytase | pI calculated by using amino acid sequence of mature protein | pI determined experimentally by IEF | *n* |
|---|---|---|---|
| *A. niger* (Natuphos) | 4.78 | 5.06 ± 0.01 (m)[b] | 4 |
| | | 5.24 ± 0.01 | 4 |
| | | 4.92 ± 0.01 | 4 |
| | | 4.80 ± 0.01 | 4 |
| *A. niger* CB | 4.79 (27)[a], 4.85 (24, 25) | 5.21 ± 0.01 | 4 |
| *A. terreus* 9A1 | 5.08 | 4.97 ± 0.02 (double band) | 3 |
| | | 4.93 ± 0.01 | 3 |
| *A. terreus* CBS | 5.50 (20), 5.38 (26) | —[c] | |
| *A. fumigatus* | 7.28 | 8.56 ± 0.02 (m) | 6 |
| | | 8.12 ± 0.02 | 6 |
| *E. nidulans* | 5.27 (23, 26) | 5.29 ± 0.01 (m) | 3 |
| | | 5.39 ± 0.01 | 3 |
| | | 5.14 ± 0.01 | 3 |
| *M. thermophila* | 4.95 | 4.83 ± 0.01 (quadruplet) | 6 |
| | | 4.73 ± 0.01 | 6 |
| | | 4.63 ± 0.01 | 6 |
| | | 4.55 ± 0.01 | 6 |
| *T. thermophilus* | 5.23 | — | |
| *E. coli* | 7.01[d] | 7.52 ± 0.01 | 3 |
| | | 7.37 ± 0.02 (m) | 3 |

[a] N-terminal residue of the mature protein species (see Table 2) having the respective calculated pI value.
[b] (m), major band.
[c] —, There was a broad smear below pI 4.0 due to acidic glycosylation when the phytase was expressed in *H. polymorpha*; all of the other fungal phytases were expressed in *A. niger*.
[d] The rather high pI of *E. coli* phytase was probably due to the $His_6$ tag at the C terminus of the protein. Without this $His_6$ tag, the predicted pI was 6.35.

structure, stability, and function of proteins (11, 19, 28). In order to determine whether the extent of glycosylation had an effect on phytase stability, the activities of *A. fumigatus* phytase expressed in *A. niger*, *H. polymorpha*, or *S. cerevisiae* (Fig. 4A) and the activities of *A. niger* CB phytase expressed in *A. niger* or *S. cerevisiae* (Fig. 4B) were measured at a range of temperatures between 37 and 90°C. Evidently, the different extents of glycosylation had no effect on the thermostabilities of these two phytases. In addition, *A. niger* CB phytase expressed in either *A. niger* or *S. cerevisiae* was incubated for 20 min at temperatures between 37 and 90°C and then was incubated for 1 h at 4°C in order to allow (partial) refolding of the heat-denatured protein. Subsequent activity measurements at 37°C (Fig. 4C) revealed that the different extents of glycosylation had no effect on the refolding properties.

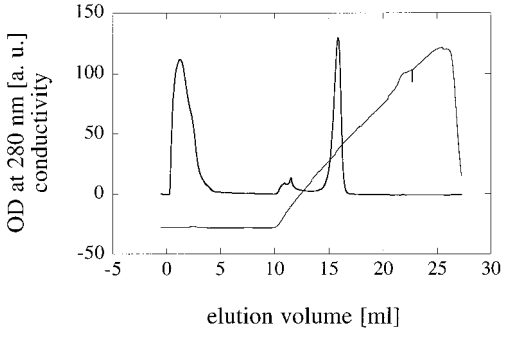


FIG. 2. Purification of *A. fumigatus* phytase by cation-exchange chromatography. An aliquot of a concentrated *A. niger* culture supernatant containing *A. fumigatus* phytase was loaded onto a 1.7-ml Poros HS/M cation-exchange chromatography column and eluted with a linear sodium chloride gradient. *A. fumigatus* phytase eluted as a symmetrical peak at an elution volume of approximately 15.5 ml. OD, optical density; a.u., arbitrary units.

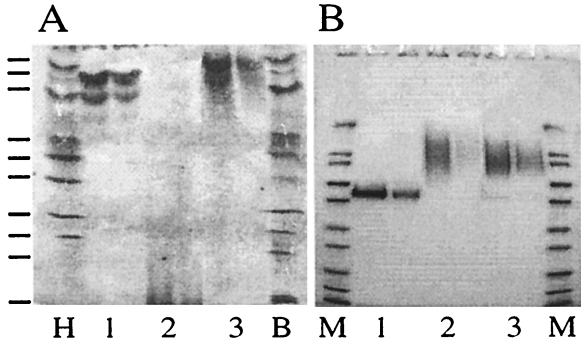


FIG. 3. Extent of glycosylation and its effect on the isoelectric point of *A. fumigatus* phytase in different expression systems. (A) IEF pH 3 to 10 gel. (B) SDS-PAGE gel. Purified *A. fumigatus* phytase was expressed in *A. niger* (lanes 1), *H. polymorpha* (lanes 2), or *S. cerevisiae* (lanes 3) (two lanes with different protein concentrations were used for each expression system). Lane H, high-pI kit (Pharmacia Biotech); lane B, broad-pI kit (Pharmacia Biotech) (from top to bottom, pI 8.65, 8.45, 8.15, 7.35, 6.85, 6.55, 5.85, 5.20, 4.55, and 3.50); lanes M, Mark 12 molecular weight standard (Novex) containing myosin ($M_r$, 200,000), β-galactosidase (116,300), phosphorylase *b* (97,400), albumin (66,300), glutamic dehydrogenase (55,400), lactate dehydrogenase (36,500), carbonic anhydrase (31,000), trypsin inhibitor (21,500), lysozyme (14,400), and aprotinin (6,000).

TABLE 2. N-terminal sequences of fungal and *E. coli* phytases

| Source of phytase | Sequence[b] | Position of first residue |
|---|---|---|
| *A. niger* (Natuphos) | XQSSXDTVDQGYQXF | 27 (19)[c] |
| *A. niger* CB | XQSTXDTV | 27 (19) |
| | SRXQSTX | 25 |
| | ASRXQST | 24 |
| *A. terreus* 9A1 | SDXNSVDHGY | 29 (19) |
| *A. terreus* CBS[a] | TALGPXGX | 20 (19) |
| | GXHSDXTSVD | 26 |
| *A. fumigatus* | SKSXDTVDLGY | 27 (21) |
| *E. nidulans* | VVQNHSX | 23 (20) |
| | NHSXNTA | 26 |
| *M. thermophila* | SESRP | 21 (18) |
| *T. thermophilus*[a] | TALGPRGXHSK(S)XDTAX(G)(G)XQ | 20 (19) |
| *E. coli* | QSE(P)ELKL(E) | 23 (23) |

[a] These proteins were expressed in *H. polymorpha*; all of the other fungal phytases were expressed in *A. niger*.
[b] Some of the Xs represent Asn residues that were probably glycosylated.
[c] The number in parentheses indicates the first residue of the mature protein predicted by using the von Heijne rules.

**Fungal phytases are monomeric proteins.** The molecular sizes of the phytases were determined by SDS-PAGE, analytical ultracentrifugation, gel permeation chromatography, and mass spectrometry and were calculated theoretically by using the amino acid sequences of the mature proteins (Table 3). While SDS-PAGE, mass spectrometry, and amino acid sequence determinations provided the $M_r$ of the protomers, analytical ultracentrifugation and gel permeation chromatography provided values for the native proteins. The results obtained by the different methods agreed closely, and the data showed that all of the phytases investigated are monomeric proteins. There was no indication of higher oligomeric forms of phytase in any experiment. This interpretation is consistent with the results of an analysis of the three-dimensional structure, which also showed that the *A. niger* phytase is a monomeric protein (13).

The difference between the $M_r$ determined by SDS-PAGE and the $M_r$ calculated by using the amino acid sequence is an indication of the extent of glycosylation of a protein. In the examples listed above, the extent of glycosylation ranged from 20 to 65% of the total $M_r$, but it may be even higher. While mass spectrometry of *E. coli* phytase was straightforward, a broad, diffuse peak was obtained for *A. fumigatus* phytase. This finding is consistent with the difficulties encountered with mass spectrometry of highly glycosylated proteins.

For all of the glycosylated phytases, gel permeation chromatography consistently gave a higher $M_r$ than expected. The difference between the values determined by gel filtration and SDS-PAGE (or analytical ultracentrifugation) increased with the extent of glycosylation, which was calculated from the difference between the $M_r$ obtained by SDS-PAGE (or analytical ultracentrifugation) and the $M_r$ obtained by amino acid sequence analysis (Fig. 5). Therefore, the higher $M_r$s obtained by gel filtration are most likely artifacts due to glycosylation. Similar observations have previously been made for Sephadex columns, in which glycoproteins also eluted earlier than expected (1).

**Isoelectric points.** The isoelectric points of the different phytases were calculated by using the amino acid sequences of the mature proteins (without signal sequences) and were determined experimentally by IEF (Table 1). In general, there was a good correlation between the calculated and observed pI values, and the maximum difference was 1.28 pH units. This suggests that glycosylation in most instances either had no effect or had only a minor effect on the pI of the protein. The only exceptions were phytases expressed in *H. polymorpha*, in which a pronounced shift to acidic pI values and considerable pI heterogeneity were observed (Fig. 3A). As Table 1 shows, *A. fumigatus* phytase is peculiar in that it has a much higher pI than all of the other fungal phytases. Since the pI of this phytase also is higher than the pI values of most extracellular proteins produced by the expression strains used, *A. fumigatus* phytase is particularly suited to easy and efficient purification on an industrial scale. Because of the differences in pI values between individual phytases or for a single phytase expressed in different production hosts, different strategies had to be used for protein purification (see above).

**Site-directed mutagenesis of surface-exposed cleavage sites decreases the proteolytic susceptibility of fungal phytases.** When expressed in *A. niger* and stored as concentrated culture supernatants at 4°C, the phytases from *A. fumigatus*, *E. nidulans*, *A. terreus* 9A1, and *M. thermophila* had a tendency to undergo proteolytic degradation (data not shown). The results of N-terminal sequencing of the fragments ($V_{153}$VPFIRASGS for *A. fumigatus* phytase, $R_{187}$ATPVVNV and $A_{188}$TPVVNV for

TABLE 3. Molecular sizes of fungal and *E. coli* phytases as determined experimentally or by using the amino acid sequences of the mature proteins

| Source of phytase | $M_r$ as determined by: | | | Gel filtration analysis | | | $M_r$ as determined by mass spectrometry |
|---|---|---|---|---|---|---|---|
| | Sequence analysis | SDS-PAGE | Analytical ultracentrifugation | $M_r$ | Stokes radius (Å) | $n$[c] | |
| *A. niger* (Natuphos) | 48, 423 | 66,360 ± 2,440 (5)[b] | 64,890 | 82,360 ± 1,710 | 39.1 ± 0.3 | 4 | ND[c] |
| *A. niger* CB | 48,658-48,973 | 74,530 ± 2,800 (5) | 71,020 | 103,030 ± 1,840 | 42.3 ± 0.3 | 4 | ND |
| *A. terreus* 9A1 | 48,189 | 60,550 ± 1,340 (8) | 70,380 | 79,900 ± 910 | 38.7 ± 0.2 | 3 | ND |
| *A. terreus* CBS[a] | 48,570-49,166 | 82,110 ± 720 (3) | 78,030 | 115,100 ± 300 | 43.8 ± 0.1 | 3 | ND |
| *A. fumigatus* batch 1 | 48,276 | 72,360 ± 520 (5) | 70,740 | 90,500 ± 1,630 | 40.5 ± 0.3 | 4 | |
| *A. fumigatus* batch 2 | | 60,770 ± 2,150 (9) | | | | | 62,500-62,800 |
| *E. nidulans* batch 1 | 49,034-49,360 | 66,430 ± 2,300 (5) | 67,400 | 77,850 ± 600 | 38.4 ± 0.1 | 3 | ND |
| *E. nidulans* batch 2 | | | | 86,930 ± 1330 | 39.9 ± 0.2 | 3 | |
| *M. thermophila* | 50,524 | 62,890 ± 1,210 (4) | 66,150 | 73,800 ± 200 | 37.6 ± 0.1 | 3 | ND |
| *T. thermophilus*[a] | 49,775 | 128,400 ± 1,700 (3) | 137,140 | ND | ND | | ND |
| *E. coli* | 45,846 | 47,270 ± 60 (3) | 47,060 | 39,190 ± 200 | 28.7 ± 0.1 | 3 | 45,833 |

[a] These proteins were expressed in *H. polymorpha*; all of the other fungal phytases were expressed in *A. niger*.
[b] The numbers in parentheses are the numbers of preparations examined.
[c] ND, not determined.

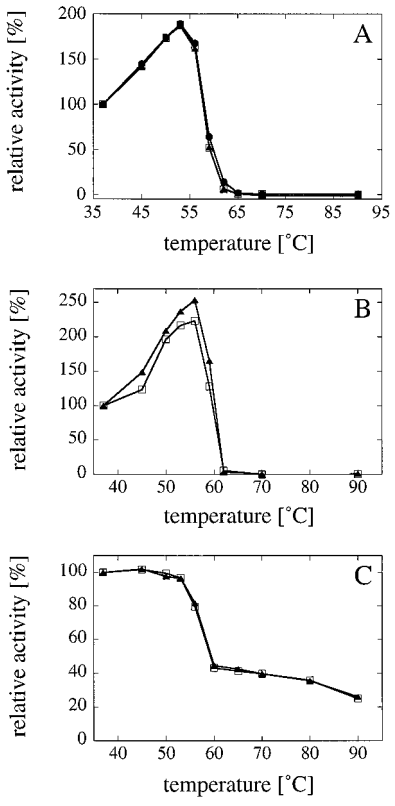


FIG. 4. Different extents of glycosylation do not affect the thermostability and refolding of phytase. The enzymatic activity of *A. fumigatus* phytase (A) expressed in *A. niger* (□), *H. polymorpha* (●), or *S. cerevisiae* (▲) and the enzymatic activity of *A. niger* CB phytase (B) expressed in *A. niger* (□) or *S. cerevisiae* (▲) were measured at a series of temperatures between 37 and 90°C. (C) *A. niger* CB phytase expressed in *A. niger* (□) or *S. cerevisiae* (▲) was incubated for 20 min at 37, 45, 50, 53, 56, 60, 65, 70, 80, or 90°C and then for 1 h at 4°C. Subsequently, phytase activity was measured at 37°C. It is evident that the different extents of glycosylation had no or only minor effects on the thermostability and refolding properties of the phytases.

*E. nidulans* phytase, $XP_{192}$SPRVDVAI for *A. terreus* 9A1 phytase, and $G_{282}$RPLSPFXR for *M. thermophila* phytase) suggested that cleavage occurred between amino acids S-152 and V-153, K-186 and R-187 or R-187 and A-188, H-190 and Q-191, and N-281 and G-282, respectively. A comparison with the three-dimensional structure of *A. niger* phytase (13) and homology modelling of the other phytases (23a) revealed that all of the cleavage sites occur within surface-exposed loop structures or turns and are therefore accessible to proteases. While limited proteolysis of the *A. fumigatus* phytase between amino acids 152 and 153 was associated with pronounced or even complete inactivation of the enzyme (Fig. 6B), cleavage of the *E. nidulans* phytase between amino acids 186 and 187 or between amino acids 187 and 188 seemingly had no effect on the specific activity (data not shown). The two proteolytic fragments of both phytases remained associated with each other, as shown by gel permeation chromatography, as well as by copurification (data not shown). Site-directed mutagenesis at the protease-sensitive sites of *A. fumigatus* phytase (S152N and R151L/S152N) (Fig. 6B) and *E. nidulans* phytase (K186G/R187Q) (data not shown) yielded mutant proteins whose susceptibility to proteolysis was reduced considerably. On the other hand, the R151L and S152N mutations had no effect on the specific activity of *A. fumigatus* phytase, which was 20.3, 20.5, and 21.2 U/mg after heat pretreatment for the wild-type, the R151L/S152N, and the S152N enzymes, respectively.

In contrast to expression in *A. niger*, problems of proteolytic degradation were not encountered when the phytases were expressed in *H. polymorpha* (data not shown).

**Identification of the glycosylation sites of *A. niger* phytase.** *A. niger* phytase has 10 potential sites for N-linked glycosylation of the Asn-X-Ser/Thr type; these sites occur at residues 27, 59, 105, 120, 207, 230, 339, 352, 376, and 388, all of which are exposed on the surface of the molecule, as determined by an examination of the three-dimensional structure (13). In order to identify which of these residues are actually glycosylated in the mature protein, *A. niger* phytase was reduced, carboxymethylated, and digested with trypsin. The proteolytic fragments were separated by reversed-phase HPLC (Fig. 1) and analyzed by electrospray mass spectrometry and Edman sequencing. Asn residues 27 (four peptides analyzed), 105 (one peptide analyzed), 207 (two peptides analyzed), 230 (two peptides analyzed), 339 (one peptide analyzed), and 376 (three peptides analyzed) were glycosylated in all of the peptides analyzed. In contrast, Asn-59 and Asn-120 were both glycosylated in only one of the two peptides examined. No glycosylation was observed for Asn-352 and Asn-388 in the one and three peptides analyzed, respectively. The incomplete glycosylation of Asn residues 59 and 120 may be one of the reasons for the $M_r$ heterogeneity of *A. niger* phytase.

## DISCUSSION

Depending on the application, an enzyme in which there is commercial interest should fulfill a series of predefined quality criteria. In the case of phytase, which is added to animal feed to increase the availability of phosphorus, these criteria include high specific activity, broad substrate specificity, a broad pH optimum, and good stability during storage, feed pelleting, and passage through the digestive tract. Thermostability is a particularly important issue since feed pelleting is commonly performed at temperatures between 65 and 95°C. In order to obtain information which can be used for engineering improved phytases, the range of phytase properties that occur in nature was investigated in this study by biochemically and

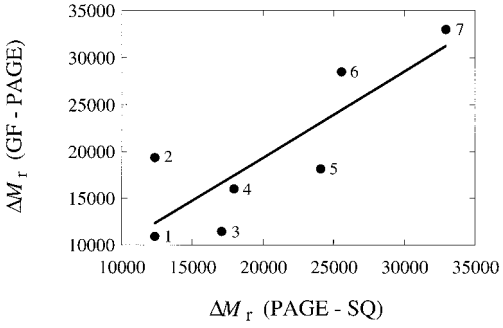


FIG. 5. Gel filtration artifacts due to protein glycosylation. Gel permeation chromatography of the glycosylated phytases resulted in higher $M_r$s than expected on the basis of SDS-PAGE, mass spectrometry, or analytical ultracentrifugation data. The overestimation, as expressed by the difference between the $M_r$s obtained by gel filtration and SDS-PAGE ($y$ axis), increased with the extent of protein glycosylation, as expressed by the difference between the $M_r$s obtained by SDS-PAGE and amino acid sequence analysis ($x$ axis). The regression line has the equation $y = 904.1 + 0.9203x$ and an $R$ value of 0.837. The data were obtained from Table 3. Data point 1, *M. thermophila* phytase; data point 2, *A. terreus* 9A1 phytase; data point 3, *E. nidulans* phytase; data point 4, *A. niger* phytase (Natuphos); data point 5, *A. fumigatus* phytase; data point 6, *A. niger* CB phytase; data point 7, *A. terreus* CBS phytase. GF, gel filtration; SQ, sequence analysis.

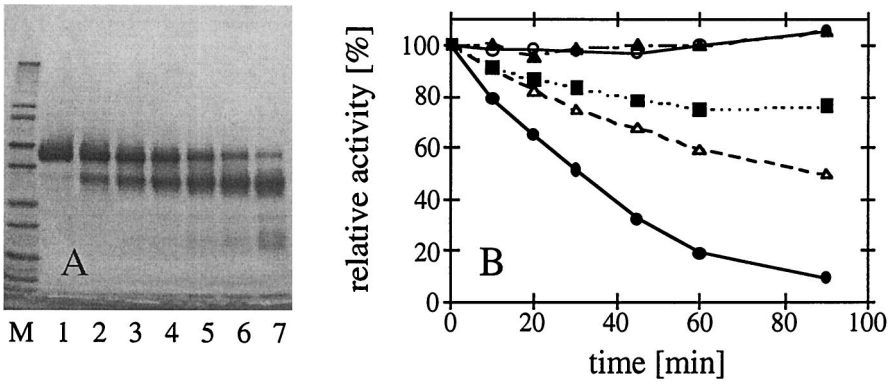


FIG. 6. Proteolytic susceptibility of *A. fumigatus* phytase expressed in *A. niger*. (A) Purified *A. fumigatus* phytase was incubated at 50°C with a diluted *A. niger* NW205 culture supernatant containing proteolytic activity. After 0, 10, 20, 30, 45, 60, and 90 min of incubation, aliquots were subjected to SDS-PAGE (lanes 1 to 7, respectively). Lane M contained the Mark 12 molecular weight standard (see the legend to Fig. 3). (B) *A. fumigatus* wild-type phytase (●), *A. fumigatus* S126N (■), and *A. fumigatus* R125L/S126N (△) were incubated with diluted NW205 culture supernatant at 50°C for 0, 10, 20, 30, 45, 60, and 90 min, and then phytase activity was measured at 37°C. As controls, *A. fumigatus* wild-type phytase was incubated under the same conditions without NW205 culture supernatant (○) or with NW205 culture supernatant that had been pretreated for 20 min at 90°C in order to inactivate the protease(s) (▲). The decreased rate of inactivation of the mutants was paralleled by much slower accumulation of degradation products on SDS-PAGE gels (data not shown).

biophysically characterizing different wild-type phytases, most of which were fungal phytases.

A comparison of the molecular sizes obtained by analytical ultracentrifugation, gel permeation chromatography, mass spectrometry, SDS-PAGE, and amino acid sequence analyses (Table 3), as well as by X-ray crystallography (13), clearly showed that all of the fungal phytases considered as well as *E. coli* phytase are monomeric proteins. This conclusion is consistent with the findings of Ullah and coworkers for fungal and soybean seed phytases (5; for a review see reference 29), of Greiner et al. (6, 7) for *E. coli* and *Klebsiella terrigena* phytases, and of Shimizu (22) for *Bacillus subtilis* phytase. On the other hand, the phytases of *A. terreus*, *Aspergillus oryzae*, *Schwanniomyces castellii*, and maize roots were reported to be homohexameric, heterotetrameric or dimeric proteins (10, 21, 23, 32). The proposed hexameric structure of *A. terreus* phytase is particularly surprising, since in our study there was no indication of oligomeric forms of *A. terreus* phytases isolated from two different strains. Since inorganic phosphate might act as an effector molecule for phytases and the phosphate normally present in the gel filtration buffer may therefore have caused dissociation of phytase oligomers into monomers, gel permeation chromatography was also performed in the absence of inorganic phosphate. Again, there was no indication of higher oligomeric forms (data not shown).

In the four conflicting examples mentioned above, the molecular sizes of the phytases were determined by gel filtration on Sephadex G-200, Sepharose S-200, and Sephacryl HR S300 (10, 21, 32) or by native PAGE (23). As shown previously (1) and in this study (Fig. 5), gel filtration of glycosylated proteins results in overestimates of the molecular sizes, and the error increases with the extent of glycosylation. Calculation of the $M_r$ from gel filtration experiments is based on the assumption that the proteins have a compact globular shape. As indicated by Andrews (1), glycoproteins may "have more expanded structures [. . . ], which may well be due to a greater hydration in solution of carbohydrate chains as compared with polypeptide chains." In conclusion, gel permeation chromatography and possibly also native PAGE should not be considered reliable techniques for determining the molecular sizes of fungal phytases and of (highly) glycosylated proteins in general. Most, if not all, phytases of fungi, bacteria, and plants are monomeric proteins, and conflicting conclusions should be regarded with caution at least until there is more convincing evidence that oligomeric forms occur.

We found that glycosylation of the phytases was highly variable. It differed not only among the three expression systems used (Fig. 2) but also among different batches of a phytase produced in the same expression system (Table 3). As shown by the analysis of the glycosylation pattern of *A. niger* phytase (Fig. 1), some of the heterogeneity is due to the fact that 2 of the 10 potential N-glycosylation sites, Asn-59 and Asn-120, are nonstoichiometrically modified. Remarkably, different extents of glycosylation, such as those obtained when a phytase was produced in different expression systems (Fig. 3), had no effect on phytase thermostability and refolding properties (Fig. 4). The importance of glycosylation for phytase structure and function is further questioned by the fact that only two potential N-glycosylation sites, Asn-207 and Asn-339, are conserved in all of the fungal phytases whose primary structures are known (16).

Glycosylation may have a number of effects on the properties of an enzyme. First, it may have an impact on the stability of a protein, or it may influence the catalytic properties. Second, in case of acidic carbohydrate modification, it may influence the pI of a protein (Fig. 3) and thereby change the behavior of the protein during purification. And third, by diverting metabolic energy, it may lower the level of expression of a phytase. With regard to basic research, it is noteworthy that heterogeneous glycosylation may impede crystallization of a protein and therefore determination of its three-dimensional structure. Strategies for circumventing this problem by deglycosylating glycoproteins, particularly phytase, with recombinant fusion protein glycosidases have recently been described by Grueninger-Leitch et al. (8).

All of the fungal, bacterial, and plant phytases investigated so far have acidic pI values (5–7, 10, 22, 23; for a review see reference 29). The difference between the pI values for *E. coli* phytase determined previously (about 6) (6) and in the present investigation (7.4 to 7.5) is most probably due to the $His_6$ tag attached to the C terminus in our preparation. In any case, *A. fumigatus* phytase, which has a pI of 8.1 to 8.6, is by far the most basic wild-type phytase. Since the great majority of contaminating proteins secreted by the expression systems into the culture supernatants have acidic pI values, this property makes

*A. fumigatus* phytase a particularly well-suited enzyme for straightforward purification on an industrial scale (Fig. 2).

Finally, some of the phytases in some of the expression systems which we used showed a tendency to slowly undergo limited proteolysis. Identification of the cleavage sites by N-terminal sequencing of the fragments revealed that cleavage invariably occurred at exposed loops on the surface of the molecule. Site-directed mutagenesis of *A. fumigatus* phytase (Fig. 6) and *E. nidulans* phytase (data not shown) at these cleavage sites in fact reduced the susceptibility to proteases. Engineering of surface-exposed loops may therefore be a promising strategy for improving the resistance of phytases to proteases present in the culture supernatant or in the digestive tract.

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