Expression of an *Aspergillus niger* Phytase (*phyA*) in *Escherichia coli*

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The gene (*phyA*) for the *Aspergillus niger* phytase with optima at pH 5.5 and 2.2 was expressed in *Escherichia coli* under the control of the T7*lac* promoter. A 56 kDa fusion protein comprised of phytase linked to an S-Tag leader peptide accumulated in inclusion bodies at 30 °C. The yield of unglycosylated recombinant phytase purified from 50 mL cultures by anion exchange chromatography of solubilized inclusion body protein was 10 mg. The refolded enzyme had an activity of 1.5 μmol mg^{-1} min^{-1} at 37 °C, but most of the protein was in the form of inactive aggregates. Recombinant phytase displayed a single pH optimum at pH 5.1, was irreversibly denatured at pH 2.0 and was not active above 55 °C. As with *A. niger* phytase, the initial breakdown product observed was inositol 1,2,4,5,6-pentakis(phosphate). K_m values for the hydrolysis of inositol hexakis(phosphate) and *p*-nitrophenylphosphate were 96 μM and 2.0 mM, respectively, at pH 4.5.

Keywords: *Phytase; phosphatase; phytic acid; inositol phosphates; Aspergillus niger*

Domesticated fowl and swine consume feed made from corn and soybeans that contains appreciable amounts of phytic acid [*myo*-inositol hexakis(dihydrogen phosphate)]. Since monogastric animals do not digest phytic acid very well, this results in two problems. First, to compensate for the low availability of phytic acid phosphorus, inorganic phosphate has to be supplemented to the diets. Second, the high levels of phytate in the fecal waste can pollute surface water and are a primary cause of algal blooms in freshwater environments.

In recognition that enzymatic hydrolysis of the phytic acid will simultaneously resolve both of the above problems, several approaches have been investigated. Fermentation of the feed as a pretreatment may be effective (Han and Wilfred, 1988; Nair and Duvnjuk, 1990), but it is inconvenient. Genetically engineering the seeds to contain high levels of phytase is effective (Pen et al., 1993), but it may have limited application. Incorporation of crude phytase preparations as an ingredient of the feed has been successfully implemented (Simons et al., 1990) and is also the focus of our research group.

Several phosphatases have been identified for potential utilization in feeds. Two phytases, *phyA* and *phyB*, from *Aspergillus niger* have been cloned (Ehrlich et al., 1993; Piddington et al., 1993; van Hartingsveldt et al., 1993) that have primary pH optima for the hydrolysis of phytic acid at pH 5.5 and 2.5, respectively. However, both enzymes are unstable above 65 °C (Ullah and Cummins, 1987; Ullah and Gibson, 1987), and loss of activity during feed pelleting can be a problem (Simons et al., 1990). A prokaryotic model of *phyA* could benefit efforts to improve the physiochemical characteristics of the enzyme via crystallographic analysis and site-directed mutagenesis. The expression and properties of *phyA* cloned in *Escherichia coli* are the subject of this paper.

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MATERIALS AND METHODS

Materials. A 2.7 kb *Sph*I fragment containing the *phyA* gene (GenBank Accession No. M94550) from an *A. niger* SRRC 256 EMBL3 genomic DNA library was cloned into the *Sph*I site of plasmid pBR322 and designated pMD4.21. Primers were synthesized by National Biosciences, Inc. (Plymouth, MN). The pET-29a(+) translation vector, S-Tag kits, and *E. coli* strains NovaBlue and BL21(DE3)pLysS were from Novagen, Inc. (Madison, WI). Terrific broth, Luria broth base, dodecasodium phytate, and *Aspergillus ficuum (niger)* phytase were purchased from Sigma Chemical Co. (St. Louis, MO).

Construction of Expression Vector. Polymerase chain reaction was used to amplify the *phyA* gene from pMD4.21, delete the signal sequence, and incorporate restriction sites. No introns interrupt the region coding for the mature enzyme. Primer 1, which added an *Eco*RI site to the 5′ end, was 5′-TCCGAATTCCTGGCAGTCCCCGCCTCGAGA-3′. Primer 2, which added a *Hin*dIII site to the complementary strand of the 3′ end, was 5′-CGCAAGCTTAGCTAAGCAAAACACTC-CGCC-3′. Amplification yielded the expected 1.3 kb product, which was digested with *Eco*RI and *Hin*dIII and ligated into the multiple cloning site of pET-29a(+) to give pET-29a(+)−*phyA* (Figure 1). The recombinant plasmid was used to transform *E. coli* NovaBlue and subcloned into the expression strain *E. coli* BL21(DE3)pLysS.

Expression and Purification of Recombinant Phytase. Fifty milliliters of Terrific broth containing 30 μg/mL kanamycin and 34 μg/mL chloramphenicol was inoculated with *E. coli* BL21(DE3)pLysSpET-29a(+)−*phyA* and shaken at 220 rpm and 30 °C. After 3 h (when the absorbance at 600 nm was approximately 0.6), the culture was induced with 500 μL of 100 mM isopropyl β-D-thiogalactopyranoside. Cells were shaken for an additional 3 h and harvested by centrifugation for 5 min at 5000*g* and 4 °C. The supernatant was decanted, and the cells were resuspended in 5 mL of 2 mM EDTA/50 mM Tris, pH 8.0. Cells were frozen briefly at −80 °C to disrupt the inner membrane and allow T7 lysozyme made from a gene carried by pLysS to reach the peptidoglycan. After the cells thawed, 500 μL of 1% Triton X-100 was added, and the cells were incubated for 15 min at 30 °C. The lysed cells were sonicated on ice for 20 s and centrifuged for 15 min at 12000*g* and 4 °C. The pellet was washed by sonication with 5 mL of 0.1% Triton X-100, 2 mM EDTA, and 50 mM Tris, pH 8.0, and centrifuged as before. After the supernatant was discarded, the pellet was dissolved by vortexing in 5 mL of 8 M urea, 100 mM β-mercaptoethanol, and 50 mM Tris, pH 8.0. The 5 mL of solubilized material was combined with 5 mL of 4 M

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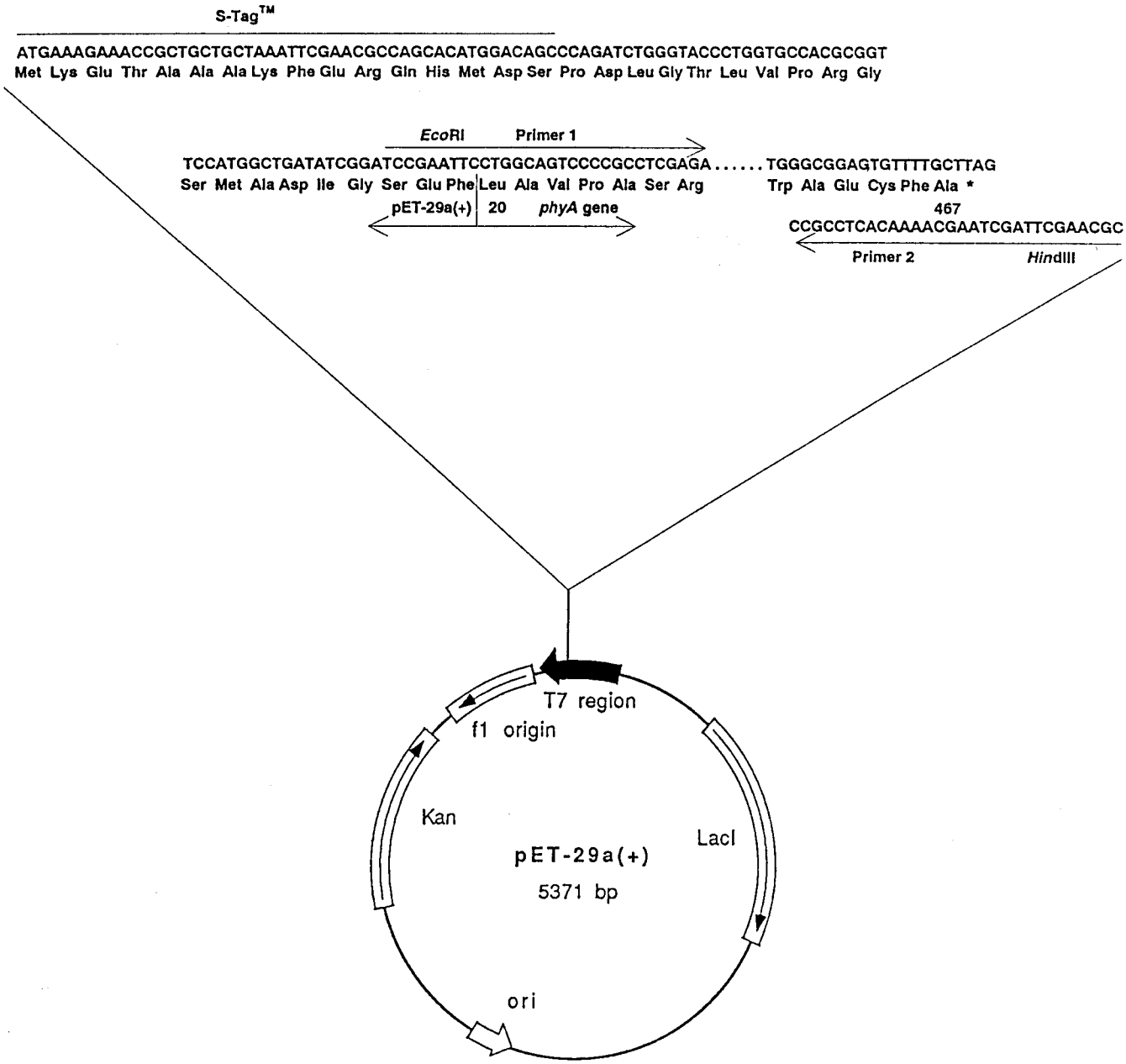

Figure 1. Construction of vector pET-29a(+)−*phyA*. PCR-amplified *phyA* sequence corresponding to amino acid residues 20−467 was ligated into the multiple cloning site of restriction digested pET-29a(+).

urea and loaded on a 1.5 × 3.0 cm Toyopearl DEAE 650M column equilibrated with 6 M urea, 5 mM β-mercaptoethanol, 0.1 M phenylmethanesulfonyl fluoride, and 20 mM HEPES, pH 7.0, at 1 mL/min. Twenty 5-mL fractions were collected in a gradient of 0−0.3 M KCl in the same buffer, and aliquots from each fraction were assayed for protein and phytase activity. The peak fractions of protein and activity coincided and were combined for an average yield of 10 mg of recombinant protein/50 mL of culture. The purified phytase was refolded by dialyzing 20 mL of enzyme diluted to 100 μg/mL with 20 mM Tris, 50 mM KCl, and 5 mM β-mercaptoethanol, pH 7.0, against 1 L of the same buffer containing 4, 2, 1, and 0 M urea for 2, 16, 3, and 3 h, respectively, at 4 °C.

S-Tag Assay and Purification. Recombinant phytase was assayed according to the protocol supplied with the S-Tag assay kit. In this method the S-Tag peptide of the fusion protein binds to the ribonuclease S-protein to form active ribonuclease, which is assayed spectrophotometrically with the substrate poly(C). The S-Tag purification kit contained S-protein agarose to bind the S-Tag fusion protein, biotinylated thrombin to cleave the recombinant protein from the S-Tag and streptavidin agarose to precipitate the biotinylated thrombin after cleavage.

Enzyme Assays. Two micrograms or less of recombinant phytase was incubated in 200 μL of 100 mM sodium acetate and 1 mM sodium phytate, pH 4.5, for 30 min at 37 °C. The reaction was stopped by adding 800 μL of freshly prepared acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1), and inorganic phosphate was determined by measuring the absorbance at 355 nm (Heinonen and Lahti, 1981). Acid phosphatase activity was determined in 200 μL of 20 mM *p*-nitrophenyl phosphate and 100 mM sodium acetate, pH 4.5, incubated for 30 min at 37 °C. The reaction was stopped by addition of 0.8 mL of 1 N NaOH, and *p*-nitrophenol was determined by measuring the absorbance at 405 nm. In experiments to determine kinetic properties, 0.01% Triton X-100 was included in the incubation buffers.

Ion Chromatography. An HPLC procedure for gradient ion chromatography of inositol phosphates was described previously (Phillippy and Bland, 1988).

Electrophoresis and Protein Determination. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed on 10% gels in a Mini-Protean II (Bio-Rad Laboratories, Hercules, CA) (Laemmli, 1970), while nondenaturing 7.5% polyacrylamide gels were stained for acid phosphatase activity with α-naphthyl phosphate and Fast Blue

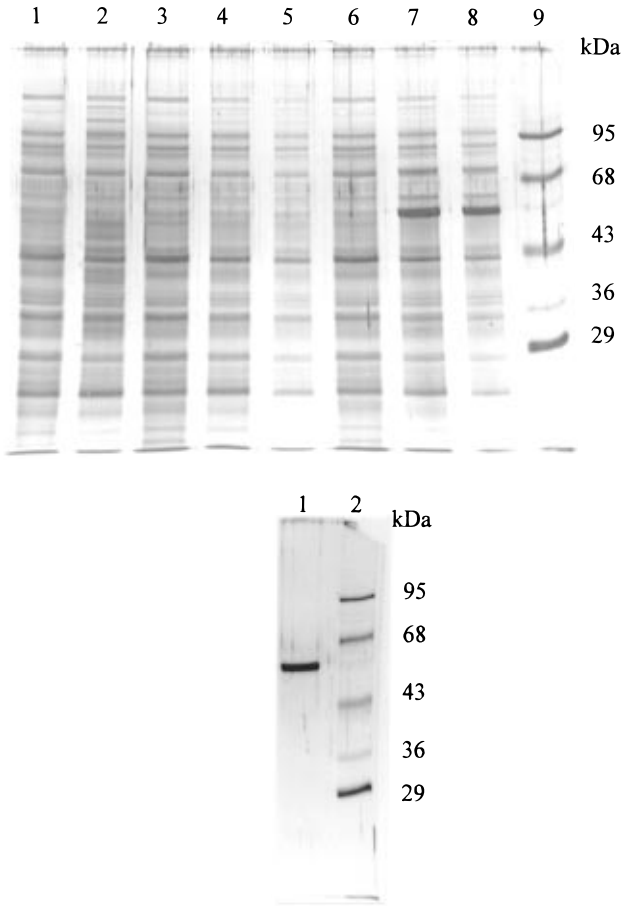

Figure 2. (a, upper lanes) SDS−PAGE of total cell extracts. Cells were grown in Luria broth supplemented with 34 μg/mL chloramphenicol (all cells) and 30 μg/mL kanamycin [cells containing pET-29a(+) or pET-29a(+)−*phyA*) at 30 °C and induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 1 mM at 3 h]: (lanes 1 and 2) *E. coli* BL21-(DE3)pLysS; (lanes 3−5) *E. coli* BL21(DE3)pLysSpET-29a(+); (lanes 6−8) *E. coli* BL21(DE3)pLysSpET-29a(+)−*phyA*; (lanes 1, 3, and 6) 3 h cultures; (lanes 2, 4, 5, 7, and 8) 5 h cultures (after 2 h of induction). Standards in lane 9 were phosphorylase B (95 000), bovine serum albumin (68 000), ovalbumin (43 000), lactate dehydrogenase (36 000), and carbonic anhydrase (29 000). (b, lower lanes) SDS−PAGE of recombinant phytase. Lane 1 was 0.5 μg of phytase purified by Toyopearl DEAE 650M chromatography as described under Materials and Methods. Standards in lane 2 were the same as in (a).

RR (Gabard and Jones, 1986). Protein concentration was determined using Coomassie Blue with ovalbumin as the standard (Bradford, 1976).

Protein Sequencing. N-terminal analysis was performed by Commonwealth Biotechnologies, Inc. (Richmond, VA).

RESULTS

Expression of Recombinant Phytase in *E. coli*. S-Tag is a 15 amino acid leader peptide fused to recombinant proteins made from pET-29 translation vectors. It binds to the ribonuclease S-protein with a high-affinity K_d of 10^{-9} M and can be used for quantitative assays, Western blotting, and affinity purification. When *E. coli* BL21(DE3)pLysSpET-29a(+)−*phyA* was grown at 37 °C, there was no difference in the protein electrophoretic patterns of total cell extracts before and after induction with IPTG, although small amounts of S-Tag were detected (results not shown).

In contrast, when the cells were grown at 30 °C, much more S-Tag was detected and an intense 56 kDa band was visible specifically in the induced extract from cells containing the recombinant plasmid (Figure 2a).

To determine the distribution of recombinant phytase between soluble and insoluble fractions, cells were lysed and centrifuged at 12000*g*. Upon electrophoresis the 56 kDa protein was observed only in the insoluble fraction. The expression of recombinant proteins in *E. coli* often occurs in the form of insoluble inclusion bodies, and a switch to soluble protein can sometimes be attained by lowering the growth temperature (Schein, 1989). However, when the recombinant cells were grown and induced at 13 °C, the 56 kDa band was absent from both the soluble and insoluble fractions (results not shown).

The recombinant phytase was solubilized in 8 M urea and refolded by dialysis. When recombinant phytase was folded at 4.5 mg/mL prior to purification on a Toyopearl DEAE 650M column, most of the activity eluted prior to the bulk of the 56 kDa protein, which may not have refolded properly. Conversely, fractions containing the recombinant protein purified prior to refolding at 25 μg/mL coincided with enzyme activity and appeared to be pure (Figure 2b). The activity of the refolded enzyme increased with the number of dialysis steps and with dilution of the protein. The maximum activity obtained was 1.5 μmol min^{-1} mg^{-1} at 37 °C. For characterization experiments a four-step dialysis over 24 h and dilution to a protein concentration of 100 μg/mL were found to be the most convenient.

To verify that the phytase activity was from the recombinant protein rather than from an intrinsic *E. coli* phytase, control BL21(DE3)pLysS cells were transformed with pET-29a(+). Inclusion bodies were prepared and the protein was refolded in a two-step dialysis without DEAE purification. Phytase activity was associated with insoluble protein from *phyA* cells but not with insoluble protein from the control cells. However, since the control had negligible insoluble protein compared to the *phyA* cells, additional attempts were made to show that the phytase activity was specifically associated with the S-Tag−*phyA* fusion protein. When purified folded phytase was incubated with S-protein agarose from the S-Tag purification kit, all of the recombinant protein appeared to bind to the agarose but most of the phytase activity remained in solution. Assuming the S-Tag was inaccessible when the fusion protein was folded into an enzymatically active conformation, binding in the unfolded state was required. Efforts to purify the enzyme with S-protein agarose in 2 M urea repeatedly failed to give sufficient protein to detect reproducible activity, even though improved yields were achieved by increasing the concentration of thrombin used to cleave phytase from the S-Tag and by increasing the cleavage time (results not shown). Part of the problem may have resulted from the fact that the unfolded recombinant protein was unstable in urea and lost most of its ability to refold into an active enzyme after 16 h on a rotator in 2 M urea at 22 °C.

Although purified phytase could not be recovered from the S-protein agarose in sufficient amounts to measure activity, the S-protein agarose could be used to precipitate the recombinant protein. When unfolded inclusion body protein was precipitated with S-protein agarose, most of the activity and protein were depleted from solution (Table 1). In a control incubated without S-protein agarose, most of the protein and activity were recovered.

Characterization of the Recombinant Phytase. N-terminal analysis of the recombinant phytase yielded the sequence Met-Lys-Glu-Thr-Ala, which corresponds to the initial methionine plus the first four residues of the S-Tag leader peptide. Due to the numerous stop

Table 1. Binding of Recombinant Phytase Activity by S-Protein Agarose[a]

	unbound material remaining in solution[b]		
agarose	protein, μg	activity, nmol/min	specific activity, nmol mg^{-1} min^{-1}
none (control)	98 ± 13	15.1 ± 1.9	155 ± 6
S-protein	26 ± 3	2.9 ± 0.1	114 ± 15

[a] One hundred and five micrograms of inclusion body protein was incubated in 390 μL of 2 M urea, 150 mM NaCl, 0.1% Triton X-100, and 20 mM Tris, pH 7.5, plus or minus 200 μL of S-protein agarose for 30 min on a rotator at 22 °C. Following centrifugation of the mixtures for 10 min at 1000*g*, the supernatant solutions were dialyzed sequentially against two 1 L solutions of 20 mM Tris, 50 mM KCl, and 100 mM β-mercaptoethanol, pH 7.0, containing 1 and 0 M urea, respectively, for 2.5 h each. [b] Data represent the means ± SD (n = 4).

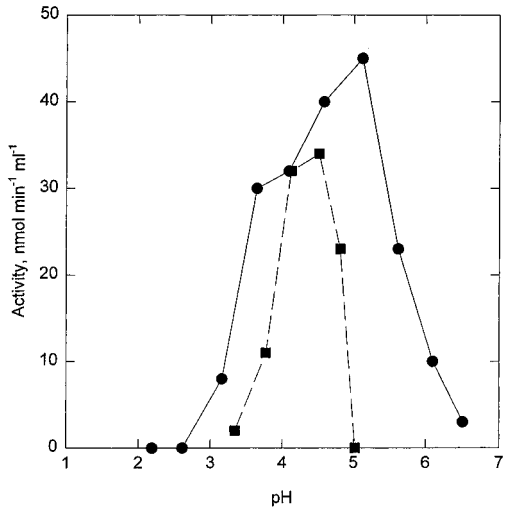

Figure 3. Effect of pH on enzyme activity. Two hundred microliters of 0.01% Triton X-100 containing 20 mM each of glycine, formic acid, acetic acid, and 2-(*N*-morpholino)ethanesulfonic acid buffers was incubated with 1 μg of recombinant phytase and 1 mM sodium phytate (●) or 0.5 μg of recombinant phytase and 20 mM *p*-nitrophenyl phosphate (■) at the indicated pH for 30 min at 37 °C.

codons in the two incorrect reading frames, the anticipated size of the recombinant protein was proof of the phytase amino acid sequence. Cleavage of the S-Tag with thrombin resulted in the expected decrease in size from 56 to 52 kDa of most of the protein but was accompanied by a 28% loss of activity. The S-Tag purification kit can be used to remove thrombin and the S-Tag and provide a purified recombinant protein. However, since the recombinant phytase fusion protein was already pure and displayed maximum activity, this procedure was not needed.

Since the above results with DEAE chromatography and S-Tag purification were indicative of improper folding, recombinant phytase was analyzed by electrophoresis on nondenaturing 7.5% polyacrylamide gels and stained with α-naphthyl phosphate and Fast Blue RR for activity or silver stain for protein. A single activity band was detected midway down the gel, while nearly all of the protein was aggregated near the top (results not shown).

The effect of pH on the activity of recombinant phytase was examined using phytic acid and *p*-nitrophenyl phosphate as substrates. Optimum activity was observed at pH 5.1 for the former and at pH 4.5 for the latter (Figure 3). Since *A. niger* phytase has optima at both pH 5.5 and 2.2 (Ullah and Gibson, 1987), the stability of the recombinant phytase at low pH was determined. In contrast to *A. niger* phytase, the recombinant enzyme was completely and irreversibly denatured by transient exposure to pH 2.0 (Figure 4). Lineweaver−Burk plots revealed K_m values of 96 μM and 2.0 mM for phytic acid and *p*-nitrophenyl phosphate, respectively, at pH 4.5 (Table 2). The temperature optimum for phytate hydrolysis was 43−50 °C (Figure 5), and the activation energy was calculated to be 6.5 kcal/mol.

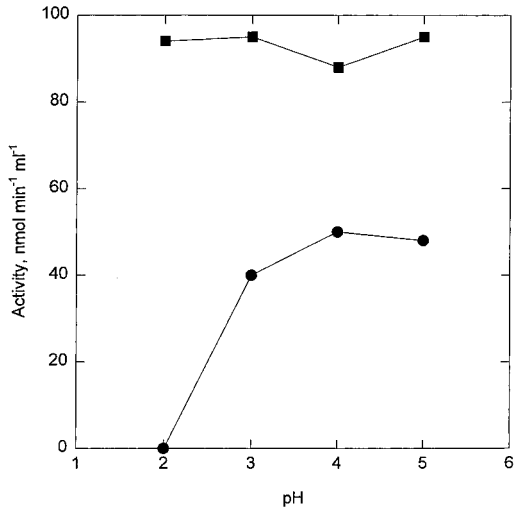

Figure 4. Effect of pH on enzyme stability. One milliliter aliquots containing 100 μg of refolded phytase (●) or *A. ficuum (niger)* phytase (■) were dialyzed at 23 °C for 2 h against 30 mM glycine, 30 mM formic acid, and 30 mM acetic acid, pH 2, 3, 4, or 5, followed by 2 h against 100 mM sodium acetate, pH 4.5. Activity was determined as described under Materials and Methods.

Table 2. Kinetic Properties of Microbial Phytases

	A. niger phyA[a]	refolded phytase[b]	*E. coli* P2[c]
K_m for phytic acid	40 μM	96 μM	130 μM
K_m for *p*-nitrophenyl phosphate	265 μM	2.0 mM	7.8 mM
k_p for phytic acid	1.30×10^4 min^{-1}	79 min^{-1}	3.73×10^5 min^{-1}
k_p for *p*-nitrophenyl phosphate	6.24×10^3 min^{-1}	38 min^{-1}	2.94×10^4 min^{-1}

[a] Assays were performed at 55 °C and pH 5.0 (Ullah and Cummins, 1987). [b] Assays were performed at 37 °C and pH 4.5 (this paper). [c] Assays were performed at 35 °C and pH 4.5 (Greiner et al., 1993).

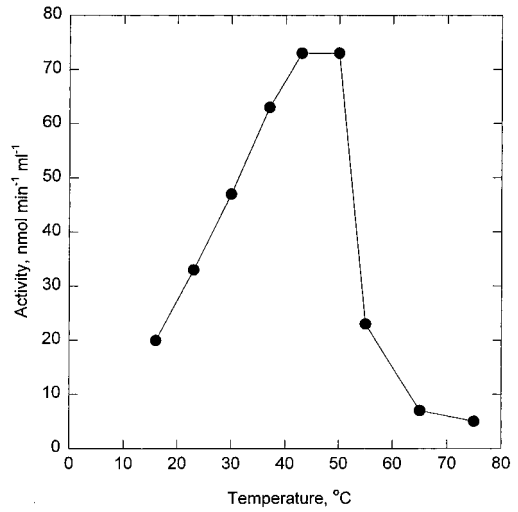

Figure 5. Effect of temperature on enzyme activity. One microgram of recombinant phytase was incubated with 200 μL of 0.01% Triton X-100, 100 mM sodium acetate, and 1 mM sodium phytate, pH 4.5, for 30 min at the indicated temperature.

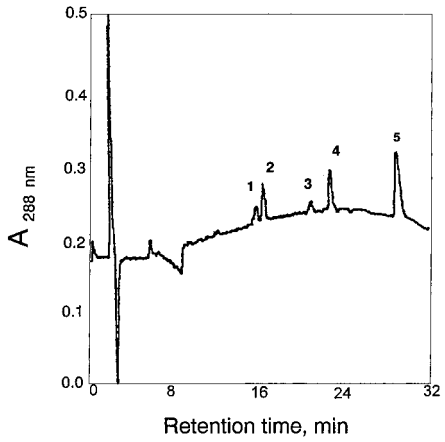

Figure 6. Identification of inositol phosphate breakdown products. Four micrograms of recombinant phytase was incubated in 200 μL of 100 mM sodium acetate and 1 mM sodium phytate, pH 4.5, for 30 min at 37 °C. The reaction was stopped with 200 μL of 1.2% HCl, and 200 μL of the mixture was analyzed by gradient ion chromatography (Phillippy and Bland, 1988). Peak identifications were as follows: 1, Ins-(1,2,3,4)P_4; 2, Ins(1,2,5,6)P_4 and Ins(2,3,4,5)P_4; 3, Ins(1,2,3,4,5)-P_5; 4, Ins(1,2,4,5,6)P_5; 5, InsP_6.

To identify the inositol phosphate products of the reaction, 200 μL solutions of 1 mM phytic acid in 100 mM sodium acetate, pH 4.5, were hydrolyzed until the initial breakdown products were evident (Figure 6). Upon analysis by gradient ion chromatography, the predominant InsP_5 peak corresponded to Ins(1,2,4,5,6)-P_5, while a smaller Ins(1,2,3,4,5)P_5 peak was also detected. The major InsP_4 appeared to be Ins(1,2,5,6)-P_4, with Ins(1,2,3,4)P_4 present in lesser amounts.

DISCUSSION

A. niger phyA has a molecular mass of approximately 80 000, of which only 48 800 is protein (van Hartingsveldt et al., 1993). The remaining 39% of the molecule is essentially carbohydrate. Nevertheless, the unglycosylated version of the enzyme, synthesized in *E. coli*, retained catalytic activity. The refolded recombinant phytase had an activity of 1.5 μmol min^{-1} mg^{-1} at 37 °C compared to 120 μmol min^{-1}mg^{-1} at 55 °C for the fungal enzyme. Most of the kinetic properties of the enzymes were similar. The major difference was that the recombinant phytase had an uninterrupted pH activity curve with an optimum at pH 5.1, whereas *A. niger* phytase had a primary peak at pH 5.5 and a secondary one at pH 2.2 (Ullah and Gibson, 1987). Although the calculated turnover number, k_p, of recombinant phytase was low, probably due to imperfect refolding and/or a lack of post-translational modification, both the recombinant and *A. niger* phytases had approximately twice as much activity for phytic acid as for *p*-nitrophenyl phosphate (Table 2).

There were three indications that the low activity can be explained by a small proportion of highly active phytase in the presence of a majority of improperly refolded and/or aggregated enzyme. First, refolded recombinant phytase activity eluted from a DEAE column before the bulk of the protein, in contrast to unfolded phytase in the presence of 6 M urea, which exhibited coincident activity and protein peaks. Second, most of the recombinant protein was retained near the top of a 7.5% nondenaturing polyacrylamide gel, whereas a single band midway down the gel was detected with an activity stain. Third, in the presence of 2 M urea unfolded protein and activity bound to S-protein agarose, whereas most of the refolded recombinant protein bound to the S-protein resin while the phytase activity remained in solution. Apparently the S-Tag was inaccessible when the fusion protein was folded into an enzymatically active conformation. Aggregation is a recognized problem when normally glycosylated eukaryotic proteins are expressed in *E. coli* (Schein, 1989). When maize phytase was expressed in *E. coli*, no activity was detected despite the fact that the original enzyme was unglycosylated (Maugenest et al., 1997). In contrast, a partially glycosylated form of *A. niger* phytase expressed in tobacco leaves showed no reduction in specific activity (Verwoerd et al., 1995).

Recombinant phytase was stable to 55 °C compared to 65 °C for the *A. niger* enzyme. The reported effects of glycosylation on the thermostability of enzymes have been variable. Glycosylation had no effect on human milk bile salt-activated lipase (Downs et al., 1994) but did increase the thermostability of several hybrids of *Bacillus* (1,3−1,4)-β-glucanase, although in one hybrid a decrease was observed (Melgaard and Svendsen, 1994). While it is likely that lack of glycosylation was primarily responsible for the decreases in thermostability and pH stability of recombinant phytase, other changes in post-translational modification cannot be discounted.

The predominant initial product of the reaction was identified as Ins(1,2,4,5,6)P_5, which is the same isomer produced by *A. niger* phytase (Irving and Cosgrove, 1972). In contrast, the *E. coli* phytase purified by Greiner et al. (1993) formed mostly Ins(1,2,3,4,5)P_5. Although Ins(1,2,4,5,6)P_5 accumulated initially when InsP_6 was hydrolyzed by the recombinant phytase, smaller amounts of Ins(1,2,3,4,5)P_5 were also detected. This pattern is the same as that of the glycosylated fungal enzyme (Irving and Cosgrove, 1972). In contrast to the microbial phytases, InsP_6 is hydrolyzed by wheat phytase to predominantly Ins(1,2,3,5,6)P_5 (Phillippy, 1989).

At this time several phytases are prospective candidates for feed additive use. Like the *A. niger* enzymes, *E. coli* phytase lacks thermostability and has an optimum of only 55 °C (Greiner et al., 1993). Recently, an extracellular phytase with optimal activity and stability at pH 7.5 was characterized from a soil isolate of *Enterobacter* (Yoon et al., 1996). However, activity at low pH appears to be essential for an optimally effective dietary phytase (Sandberg et al., 1996). *A. niger* phytase was expressed in *E. coli* to obtain a model to genetically engineer for improved physiochemical properties. The recombinant phytase was active but had to be solubilized in urea and refolded by dialysis. Cloning the enzyme in another system such as a fusion protein with thioredoxin (LaVallie et al., 1993) or noninduced expression in a host lacking an F′ episome (Sakamoto et al., 1996) might yield a soluble active enzyme if it is not toxic to the cells. A newly successful effort to crystallize the deglycosylated fungal phytase has provided a subtrate binding model that will be helpful for mutagenesis experiments (Kostrewa et al., 1997). We are currently investigating the properties of the more thermostable phytase from *Aspergillus terreus* (Yamada et al., 1968; Mitchell et al., 1997). Expression of phytase in a thermophile to evaluate the potential benefits of thermoprotectants such as di-*myo*-inositol phosphate (Martins and Santos, 1995) that could be retained in the crude phytase preparations may also be worth considering.

ABBREVIATIONS USED

*phy*A, gene encoding phytase A; phosphorylated *myo*-inositols are designated according to the general formula Ins(X)P_Y, where X represents positions of the phosphates and Y is the number of phosphates (the D configuration of enantiomers is assumed).

NOTE ADDED IN PROOF

A new report describes a fungal phytase with good thermostability at temperatures up to 100 °C (Paramontes, L.; Haiker, M.; Wyss, M.; Tessier, M.; van Loon, A. P. G. M. Gene cloning, purification, and characterization of a heat-stable phytase from the fungus *Aspergillus fumigatus*. *Appl. Environ. Microbiol.* **1997**, *63*, 1696−1700).

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