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Cloning, Sequencing, and Expression of an *Escherichia coli* Acid Phosphatase/Phytase Gene (*appA2*) Isolated from Pig Colon

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Bacterial strains were isolated from the pig colon to screen for phytase and acid phosphatase activities. Among 93 colonies, Colony 88 had the highest activities for both enzymes and was identified as an *Escherichia coli* strain. Using primers derived from the *E. coli* pH 2.5 acid phosphatase *appA* sequence (Dassa *et al.* (1990), *J. Bacteriol.* 172, 5497–5500), we cloned a 1482 bp DNA fragment from the isolate. In spite of 95% homology between the sequenced gene and the *appA*, 7 amino acids were different in their deduced polypeptides. To characterize the properties and functions of the encoded protein, we expressed the coding region of the isolated DNA fragment and *appA* in *Pichia pastoris*, respectively, as r-appA2 and r-appA. The recombinant protein r-appA2, like r-appA and the r-phyA phytase expressed in *Aspergillus niger*, was able to hydrolyze phosphorus from sodium phytate and *p*-nitrophenyl phosphate. However, there were distinct differences in their pH profiles, K_m and V_{max} for the substrates, specific activities of the purified enzymes, and abilities to release phytate phosphorus in soybean meal. In conclusion, the DNA fragment isolated from *E. coli* in pig colon seems to encode for a new acid phosphatase/phytase and is designated as *E. coli appA2*.

***Key Words: E. coli;* acid phosphatase; phytase; pigs; cloning; heterologous expression; *Pichia pastoris; Aspergillus niger* (*phyA*).**

Phytases catalyze the hydrolysis of phytate (*myo*-inositol hexakisphosphate), thereby releasing inorganic phosphate (1, 2). These enzymes are of interest for biotechnological applications, in particular for improving dietary phytate-phosphorus utilization by simple-stomached animals such as swine and poultry. Because these species, as well as humans, have little endogenous phytase activity, most of the ingested phytate is excreted. Supplemental phytase to diets for pigs effectively improves the bioavailability of phytate-phosphorus, thus decreasing phosphorus pollution (2-6). Although plant seeds such as wheat, bean, rice, corn, and maize have some phytase activity, microorganisms may be a more feasible source of the enzyme (7, 8). Phytase genes have been isolated from *Aspergillus niger* (9, 10), *Emericella nidulans* and *Talaromyces thermophilus* (11), *A. fumigatus* (12), and *A. terreus* and *Myceliophthora thermophila* (13). Phytases and acid phosphatases have also been identified from bacteria such as *Escherichia coli* (14-18), *Aerobacter aerogenes* (19), and *Bacillus* sp. (21, 22). Two phytases have been purified from *E. coli* (18), but the corresponding genes are unknown. In the present study, we isolated an *E. coli* strain from pig colon that produced relatively high phytase and acid phosphatase activities. A DNA fragment was amplified by PCR from the strain, cloned, sequenced, and expressed in *Pichia pastoris*. Compared with the recombinant enzymes of the *E. coli* pH 2.5 acid phosphatase (r-appA) and the *A. niger* phytase (r-phyA), this expressed protein was classified as a new acid phosphatase/phytase and the gene was designated as *E. coli appA2*.

MATERIALS AND METHODS

Isolation and identification of phytase producing bacterium colonies. Colon contents were obtained from crossbreed Hampshire-Yorkshire-Duroc pigs (13 weeks of age) raised under confinement at Cornell University Swine Farm. These pigs were fed a practical corn-soybean meal diet. Immediately after the pigs were killed, the content of colon was removed by aseptic procedures and kept in anaerobic, sterile plastic bags. A 10-g sample was diluted with 190

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Abbreviations used: *appA, Escherichia coli* pH 2.5 acid phosphatase gene; *appA2, Escherichia coli* acid phosphatase/phytase gene (newly isolated); BMGY, buffered glycerol-complex medium; BMMY, buffered methanol-complex medium; DEAE, diethylaminoethyl; dNTPs, deoxynucleotides; LB, Luria-Bertani medium; PCR, polymerase chain reaction; *phyA, Aspergillus niger* phytase gene; pNPP, *p*-nitrophenyl phosphate; r-appA, recombinant protein produced by *appA* in *Pichia pastoris;* r-appA2, recombinant protein produced by *appA2* in *Pichia pastoris;* r-phyA, recombinant protein produced by *phyA* in *Aspergillus niger;* SDS, sodium dodecyl sulfate; YPD, yeast extract peptone dextrose.

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ml of an anaerobic rumen fluid glucose medium in a 250 ml rubber-stoppered Erlenmeyer flask. The mixture was shaken vigorously for 3 min under a CO_2 atmosphere. Serial successive dilutions were made accordingly.

Diluted samples were cultured at 37°C for 3 days in a modified rumen fluid-glucose-cellobiose-Agar medium containing insoluble calcium phytate (22, 23). Colonies with a clear zone were tested as a potential producer of intra and extracellular phytase activity. Phytase activity was measured using sodium phytate as a substrate (9). One phytase unit (PU) was defined as the activity that releases one μmole of inorganic phosphorus from sodium phytate per minute at 37°C. Acid phosphatase activity was assayed using *p*-nitrophenyl phosphate (*p*-NPP) as a substrate according to the manufacturer instructions (Sigma, St Louis, MO). Identification of the selected colony was conducted in the Diagnostic Laboratory of Cornell Veterinary College (Ithaca, NY). Morphological and physiological characteristics of the isolated colony were determined by standard procedures.

DNA amplification and sequencing. Because the colony that produced the highest acid phosphatase and phytase activities was identified as an *E. coli* strain, we used primers derived from the DNA sequence of *E. coli* pH 2.5 acid phosphatase gene (*appA*, GeneBank Accession number 145283) (17) to isolate the gene. Primers Pf1 [forward: 1-22]: 5′-TAAGGAGCAGAAACAATGTGGT-3′, E2 [forward: 254-264]: 5′-GGAATTCCAGAGTGAGCCGGA-3′ and K2 [reverse: 1468-1491]: 5′-GGGGTACCTTACAAACTGCACG-3′ were synthesized at the Cornell University Oligonucleotide Synthesis Facility. The whole sequence and the coding region were amplified using [Pf1-K2] and [E2-K2] primers, respectively. The PCR reaction mixture (100 μL) contained 500 ng of genomic DNA as template, 100 pmole of each primer, 5 U of Ampli*Taq* DNA polymerase (Perkin Elmer, Norwalk, CT), 10 mM Tris-HCl pH 8.3, 50 mM KCl, 12.5 mM MgCl2, and 200 μM each dNTPs (Promega, Madison, WI). The reaction was performed by the GeneAmp PCR system 2400 (Perkin Elmer). The thermal program included 1 cycle at 94°C (3 min), 30 cycles of [94°C (0.8 min), 54°C (1 min) and 72°C (2 min)] and 1 cycle at 72°C (10 min). Amplified PCR products were resolved by 1% low melting agarose (Gibco BRL, Grand Island, NY) gel electrophoresis. A gel slice containing the expected size band was excised and DNA was eluted with GENECLEAN II kit (Bio101, Vista, CA). The PCR products were sequenced at the Cornell University DNA Service Facility using Taq Cycle automated sequencing with Dye Deoxy terminators (Applied Biosystems, Forster City, CA). Sequencing experiments were performed five times and the deduced amino sequence was aligned with other acid phosphatases and phytases using the Multi-align Program CLUSTAL BLAST (24). The two identified PCR fragments [Pf1-K2] and [E2-K2] were described, respectively, as *appA2′* and *appA2* in the following text. For comparative purposes, the *appA* gene was amplified from *E. coli* BL21(DE3) using the primers [E2-K2]. The PCR reactions and the resulting fragments were processed as described above.

Subcloning and construction of expression vectors. The PCR products [E2-K2] and [Pf1-K2] were cloned into pGEM T-easy vector (Promega) according to the manufacturer instructions and transformed into TOP10F′ to screen for positive colonies. The isolated *appA2* and *appA* fragments were inserted into the pPICZαA (Invitrogen, San Diego, CA) at the *Eco*RI and *Kpn*I sites, as described by the manufacturer instruction. The constructs were transformed into TOP10F′ cells which were plated on LB medium containing 25 μg zeocin/ml. The positive colonies were then grown to prepare DNA for transformation.

Yeast transformation and expression. *Pichia pastoris* strain X33 (Invitrogen) were grown in YPD medium and prepared for transformation, according to the manufacturer instructions. Two μg of plasmid DNA was linearized using *Bgl*II and then transformed into *Pichia* by electroporation. After incubation for 3 h at 30°C in 1 M sorbitol without agitation, cells were plated in YPD-zeocin agar medium to screen integration of the transformed gene into the 5'AOX1 region of the host chromosomal DNA. After 2 days, transformants were incubated in minimal media with glycerol (GMGY medium) for 24 h. After the culture reached a density of about 2.5×10^8 cells/ml ($OD_{600} = 5$), the cells were spun down (3500g, 5 min) and then suspended in 0.5% methanol medium (GMMY) to induce the phytase gene expression.

RNA quantification. Total RNA was extracted from the *appA2* transformants at different times after induction. The RNA was separated in 1% formaldehyde-agarose gel, transferred onto Hybond N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by capillary blotting and UV cross-linked for 2 min. The membrane was then pre-hybridized for 4 h at 42°C. The probe was the *appA2* [E2-K2] PCR fragment, and was labeled with [α-^{32}P]-dCTP (DuPont, Boston, MA.) using Ready-To-Go TM DNA Labeling Beads (Amersham Pharmacia Biotech). The membrane was hybridized with the probe overnight at 42°C, and washed three times for 20 min at 25°C and twice at 50°C in 2X SSC (0.15 M NaCl, 0.015 M sodium citrate), 1% sodium dodecyl sulfate (SDS), and finally twice at 50°C in 0.2X SSC, 0.1% SDS. The autoradiogram was produced by exposing the membrane to an intensifying screen of BAS-III FUJI Imaging plate (Fuji, Japan) for 10 h and quantified using a Bio-Imaging Analyzer (Kohshin Graphic Systems, Fuji, Japan). Results were normalized by the relative levels of 18S rRNA.

Purification of the expressed enzymes. All operations were carried out at 4°C. Both expressed r-appA and r-appA2 enzymes, and the r-phyA phytase expressed in *A. niger* (kindly provided by BASF, Mt Olive, NJ), were suspended in 50 mM Tris-HCl, pH 7 with 25% saturation of ammonium sulfate. The suspension was then centrifuged at 25,000g for 20 min. The supernatant was mixed with 75% saturated ammonium sulfate under agitation for 12 h, and the mixture was centrifuged at 25,000g for 20 min. The pellet was then suspended in 10 ml 25 mM Tris-HCl, pH 7 and dialyzed overnight against the same buffer. The dialyzed sample was loaded onto a DEAE-Sepharose column (Sigma) equilibrated with 25 mM Tris-HCl, pH 7. After the column was washed with 200 ml of the same buffer, the bound phytase was eluted with 1 M NaCl in 25 mM Tris-HCl, pH 7. Three fractions exhibiting the highest phytase and acid phosphatase activities were pooled and dialyzed against 25 mM Tris-HCl, pH 7.5 overnight for the following studies.

Electrophoretic analysis. Protein concentration was measured by the Lowry's method (25). Non-denaturing gel electrophoresis and SDS-PAGE (15%) were performed as described by Laemmli (26). Proteins in SDS-PAGE were stained with Coomassie brillant blue R-250. Acid phosphatase or phytase activity in bands of the non-denaturing gel was detected as described previously (10). After electrophoresis, the gel was incubated for 20 min at 25°C in 0.2% α-naphtylphosphate (or sodium phytate), 0.1% Fast Garnet GBC salts, 1 mM $CaCl_2$, and 0.5 M Tris-HCl buffer pH 7.0.

Deglycosylation of the enzymes. Deglycosylation of r-appA2 was done using 0.3 IU of endoglycosidase H*f* (Endo H_f) for 4 h at 37°C according to the manufacturer instructions (New England Biolabs, Beverly, MA). The deglycosylated proteins were analyzed in a 15% SDS-PAGE as described above.

Enzyme properties and hydrolysis of phytate phosphorus in soybean meal. Phytase activity at different pH was determined at 37°C, using three different buffers. The temperature optimum for each enzyme was determined at its optimal pH. The K_m and V_{max} values for r-appA2 and r-appA were determined at the optimal pH of each enzyme and 37°C. Hydrolysis of phytate phosphorus by r-appA2 was compared with that of r-appA and r-phyA. Different amounts of the purified enzymes were incubated with 1 g soybean meal in a 5 mL buffer (10 mM HCl or 0.2 M citrate) at their respective optimal pH (2.5 for r-appA, 3.5 for r-appA2, and 5.5 for r-phyA) at 37°C for 2 h. The released inorganic phosphorus was determined as previously described (27). Thermostabilities of these three enzymes were com-

pared. Each of the enzymes (2 mg/ml) was diluted 1:200 in 0.2 M sodium citrate, pH 5.5, and incubated for 20 min at 25, 37, 55, 65, 80 and 100°C. The samples were placed on ice for 30 min and the remaining phytase activity was measured at 37°C.

Statistical test employed. The Mann-Whitney U-test was used for all the statistical evaluations (28).

RESULTS

Bacterial colony screening and identification. A total of 93 colonies were isolated. Over 70 colonies had intracellular phytase activity less than 500 U/g protein, and 6 colonies had activities greater than 1,000 U/g protein. Colony 88 demonstrated the highest phytase activity (2,927 U/g protein), with an acid phosphatase activity (1,391 U/g protein). Thus, it was chosen for further experiments. The production of phytase and acid phosphatase activities by the colony was greater in Sweet E than LB broth and greater at anaerobic than aerobic conditions (data not shown). Subsequently, the colony was identified as a gram negative *E. coli.* This was confirmed, in particular, by the substrate fermentation profile.

Cloning and sequencing of the pig E. coli appA2 gene. A 1482 bp (whole) and a 1241 bp (coding region) fragments were amplified from the genomic DNA of Colony 88 (Fig. 1). Except for the *E. coli appA* gene and the *Bacillus* phytase gene, no significant sequence homologies were found in the GenPro databank (version 61), GeneBank or EMBL databases using BLAST program. The whole nucleotide sequence had 47 and 95% homology with the *Bacillus* sp. DS11 phytase gene (GeneBank accession number 3150039) and *E. coli appA*, respectively. In spite of such a high nucleotide sequence homology, there were distinct differences between *appA* and *appA2* and their encoding polypeptides. First, seven amino acids were different in the deduced peptide sequences: one in the signal peptide, L4F; six in the coding region, S102P, P195S, S197L, K202N, K298M, and T299A. Second, the 73 bp untranslated region, located between the lead sequence and coding region, was shorter by 6 bp than that of *appA*. However, the three putative N-glycosylation sites were still located in the coding region at the same positions. The DNA fragment was sequenced for five times to verify these differences. Compared with *phyA*, *appA2* had only a 19% of amino acid sequence homology. The sequence has been transmitted to GeneBank data library with the accession number 250016.

Expression of appA2 in Pichia pastoris. A total of 42 transformants were analyzed for phytase and acid phosphatase activities at various intervals. Three days after methanol induction, 13 transformants produced phytase activity from 18 to 114 U/mL of medium and acid phosphatase activity from 7 to 42 U/mL. Meanwhile, 22 *appA* transformants expressed phytase activity from 25 to 130 U/mL and acid phosphatase activity from 59 to 85 U/mL. The *appA2* transformant that demonstrated the highest activities was used in the expression time course (Fig. 2) and other studies. The *appA2* mRNA level reached the peak at 4 h (Figs. 2 and 3), remained high until 12 h, and thereafter declined significantly. No *appA2* mRNA signal was detected in the control cells (data not shown). Both the extracellular phytase and acid phosphatase activities produced by the transformant increased sharply between 0 and 24 h (Fig. 2). Thereafter, the acid phosphatase activity remained nearly unchanged while phytase activity increased much less over time than that at the earlier phase.

Characterization of the purified enzymes. The specific phytase activity of the purified r-appA2, r-appA, and r-phyA enzymes was 28.9, 30.7, and 19.8 U/mg protein, respectively. The purified r-appA2 demonstrated a higher affinity for sodium phytate than pNNP, as shown by the K_m and V_{max} values (Fig. 2, Table 1). When sodium phytate was used as the substrate, the pH curve was significantly different among the three enzymes. The pH optimum was between 2.5 and 3.5 for r-appA2, 2.5 for r-appA, and 2.5 and 5.5 for r-phyA phytase (Fig. 4). However, the two *E. coli* enzymes demonstrated the same pH optimum (2.5) for the substrate pNNP (data not shown). In addition, both r-appA and r-appA2 had the same temperature optimum (55°C) which was slightly lower than that of r-phyA (data not shown). These two enzymes also had very similar thermostabilities of phytase activity which were slightly higher between 37 and 60°C and lower between 65 and 100°C than that of *r-phyA* (data not shown). The acid phosphatase activity of r-appA2 that remained after different temperature treatments was shown in the non-denaturing gel, as a unique band of 71 kDa (Fig. 5). The activity was largely or completely lost at 65 or 80°C, but somehow recovered partially at 100°C. When the three purified recombinant enzymes were incubated with soybean meal, r-appA2 protein released significantly more phosphorus from phytate than the other two enzymes (Fig. 6).

Effects of deglycosylation on enzyme properties. After the three purified enzymes were treated with β-mercaptoethanol and Endo H_f, more than 90% of their activities for both sodium phytate and pNNP were lost (data not shown). But, Endo H_f alone had no significant effect on their catalytic activities. Deglycosylation of r-appA2 resulted in a single band with an apparent M*r* of 46.3 kDa from three distinguished bands for the glycosylated forms with apparent M*r* of 50.5, 53 and 56 kDa (Fig. 7). This gave a range of glycosylation for r-appA2 between 8.3 and 17.3%.

DISCUSSION

In the present study, we have isolated a phytase-producing *E. coli* strain from the pig colon content.

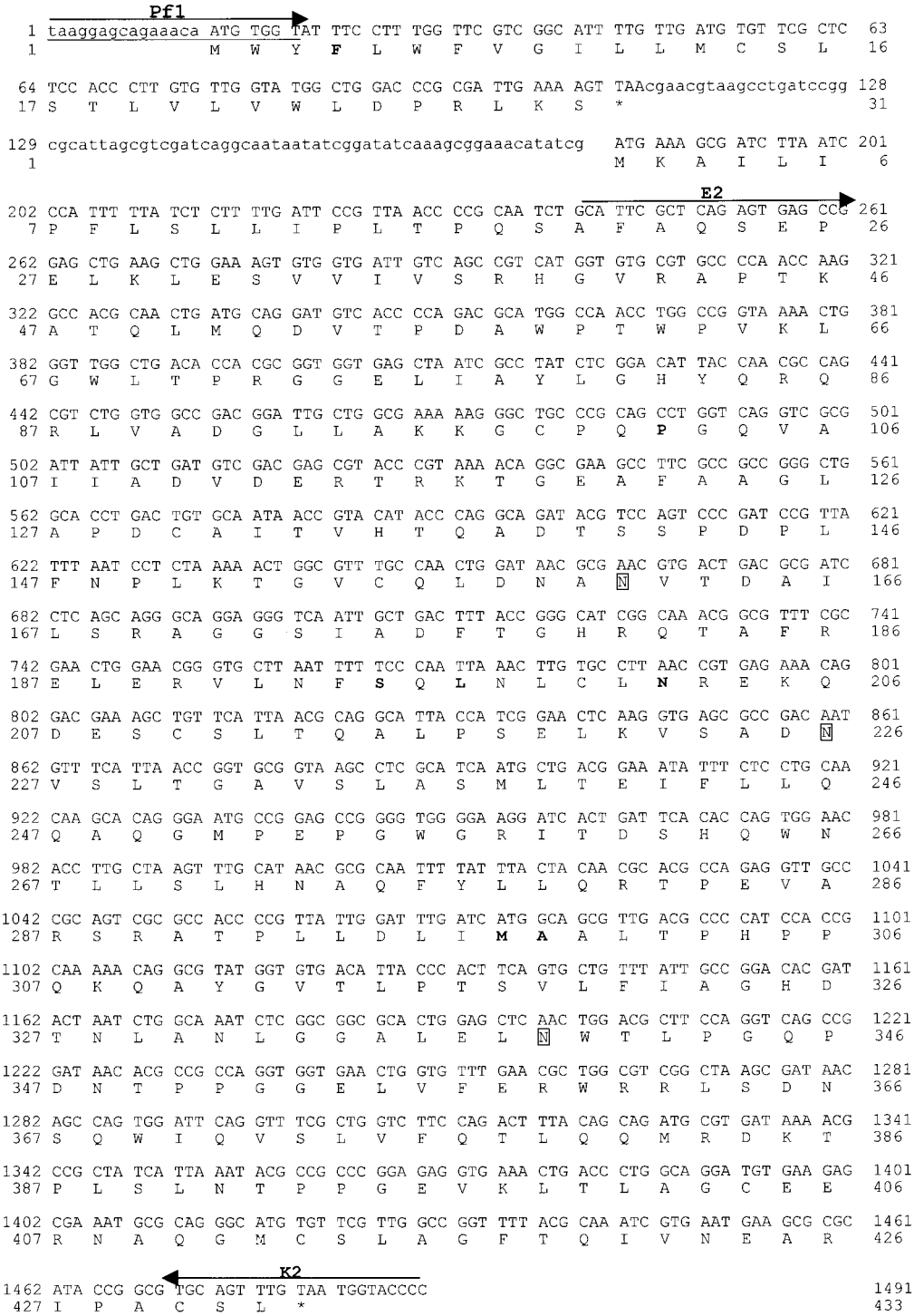

FIG. 1. Nucleotide sequence of the *appA2* gene and its deduced amino acid sequence. The untranslated region is indicated by lowercase letters. The underlined sequences are the primers used to amplify *appA2′* (Pf1: 1-22, and K2: 1468-1490), *appA2* (E2: 243-252, and K2: 1468-1490). Potential N-glycosylation sites are boxed. The sequence of *appA2* has been transmitted to Genebank data library with accession number 250016.

Using primers based on the *E. coli* pH 2.5 acid phosphatase gene (*appA*) described by Dassa et al. (17), we have amplified a 1487 bp DNA fragment from the genomic DNA of the strain. This fragment, designated as *appA2*, encodes a protein of 433 amino acids with 3 putative N-glycosylation sites. The deduced peptide

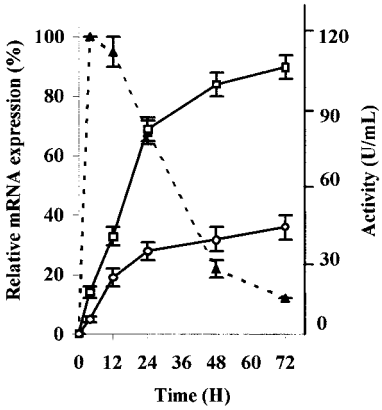

FIG. 2. Time course of extracellular phytase (□) and acid phosphatase (○) activities, and *appA2* mRNA expression (▲) in *Pichia pastoris* transformed with *appA2* after induction. Results are expressed as the mean ± SEM from three experiments.

contains both the N-terminal motif (RHGXRXP, position: 38-44) and the C-terminal motif (HD, position: 325-326), characteristic for histidine acid phosphatases (2). In addition, there is a lead sequence of 30 amino acids and an untranslated region of 73 bp. Among the available sequence databases, only the *E. coli appA* pH 2.5 acid phosphatase and the *Bacillus sp.* DS11 phytase genes share some homology with *appA2* (95% and 47% in nucleotide sequence, respectively). In spite of the high homology between *appA* and *appA2*, there are distinct differences between these two genes and their respective proteins. First, seven amino acids differ between the two deduced polypeptide sequences: one within the signal peptide and six in the coding region. Second, the 73 bp untranslated region between the lead sequence and the coding region was shorter by 6 bp than that of *appA*. All those differences have been confirmed by five repetitive nucleotide sequencing analysis.

More importantly, when these two genes are transformed into the same host, *Pichia pastoris*, the expressed proteins r-appA and r-appA2 show differently

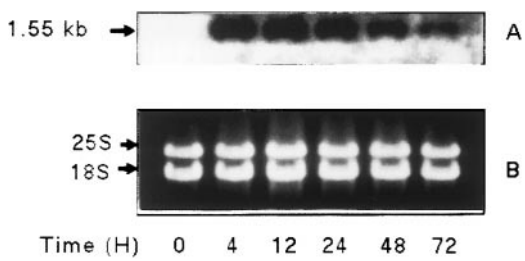

FIG. 3. Northern blot analysis of *appA2* mRNA expression in *Pichia pastoris* transformed with *appA2* after induction (Panel A). Hybridization was realized using [α-32P] labeled *appA2* as a probe. Twenty μg of total RNA was loaded per lane. Panel B represents the equal RNA loading visualized by the yeast rRNA under UV.

TABLE 1

Kinetic Parameters of the Purifed r-appA and r-appA2 Expressed in *Pichia pastoris*

	r-appA	r-appA2
K_m, mM		
Sodium phytate	1.03	0.66
p-NPP	2.26	1.43
V_{max}, μmole min^{-1} mg^{-1}		
Sodium phytate	89	117
p-NPP	310	340

biochemical characteristics. Although both exhibit the same pH optimum of 2.5 for pNNP, r-appA2 has a broad pH optimum between 2.5 and 3.5 while r-appA had it at 2.5 for sodium phytate. Compared with r-appA, the r-appA2 has a higher affinity for both substrates, as shown by the lower K_m and higher V_{max} values, and releases more phosphorus from phytate in soybean meal *in vitro*. Thus, the catalytic function of r-appA2, towards phosphorus hydrolysis from phytate or phosphate, seems to be better than that of r-appA. Apparently, the six amino acid exchanges in the polypeptide may not be not just a polymorphism of the enzyme, but rather responsible for the observed kinetic differences. Thus, it seems reasonable to state that the *appA2* is a different gene from *appA*, although a more defined structural analysis is needed to elucidate the relationship between specific amino acid exchanges and functional alterations of these two enzymes. It will be necessary to produce the crystal of both enzymes first for future structural studies (29).

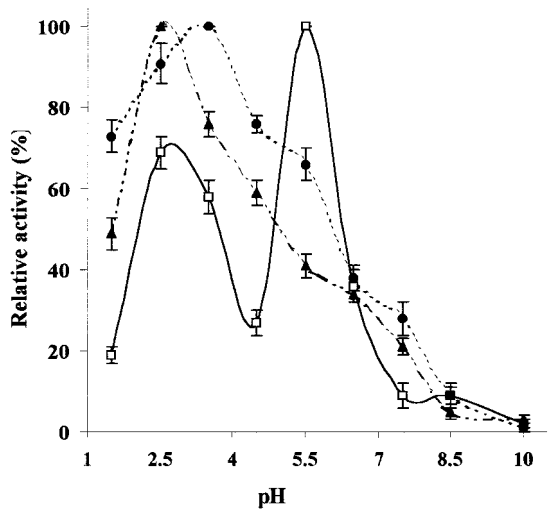

FIG. 4. pH dependence of the enzymatic activity at 37°C of the purified r-appA2 (●), r-appA (▲), and r-phyA (□) with sodium phytate as the substrate. Buffers: pH 1.5-4.5, 0.2M glycine-HCl; pH 5.5-7.5, 0.2 M citrate; pH 8.5-11, 0.2 M Tris-HCl. Results are expressed as the mean ± SEM from three experiments.

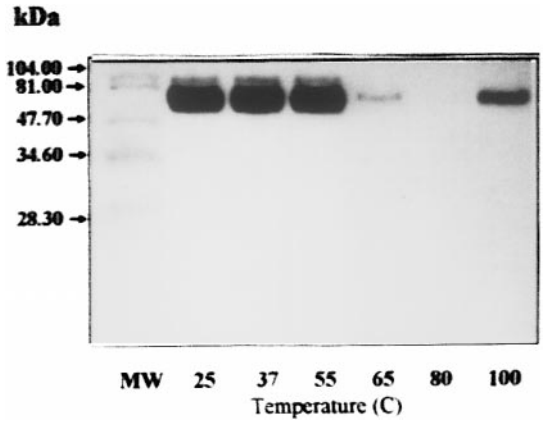

FIG. 5. Non-denaturing gel (15%) electrophoresis analysis of the remaining acid phosphatase activity of r-appA2 after incubated at different temperatures for 20 min. After the heat treatment, the samples were put on ice for 5 min before being loaded onto the gel (200 μg protein/lane).

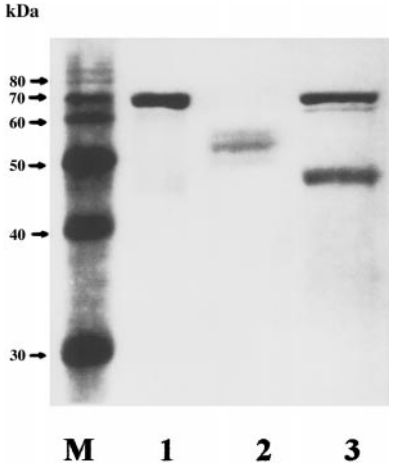

FIG. 7. SDS-polyacrylamide gel electrophoresis analysis of r-appA2 before and after deglycosylation by Endo H_f. Lane M: standard 10 kDa from Gibco; Lane 1: Endo H_f (M*r* = 70 kDa); Lane 2: r-appA2 with 3 levels of glycosylation (M*r* = 50.5, 53, and 56 kDa); Lane 3: r-appA2 deglycosylated by Endo H_f (M*r* = 46.3 kDa).

Previously, several *E. coli* enzymes have been reported to hydrolyze pNNP or sodium phytate (16-20). Greiner et al. (18) characterized two *E. coli* phytases (P1 and P2). They found that the purified *E. coli* phytase P2 shares a great identity with the *E. coli* pH 2.5 acid phosphatase in the N-terminal sequence, chemical properties, and kinetics. Thus, they suggested that these two enzymes might be the same protein and the *E. coli* pH 2.5 acid phosphatase should better be regarded as a phytase. Indeed, we have demonstrated herein that both r-appA acid phosphatase and r-appA2 are not only able to hydrolyze phytate in the pure chemical form or in the natural food, but also have a higher affinity for sodium phytate than pNNP. Therefore, these two enzymes could be classified as phytases.

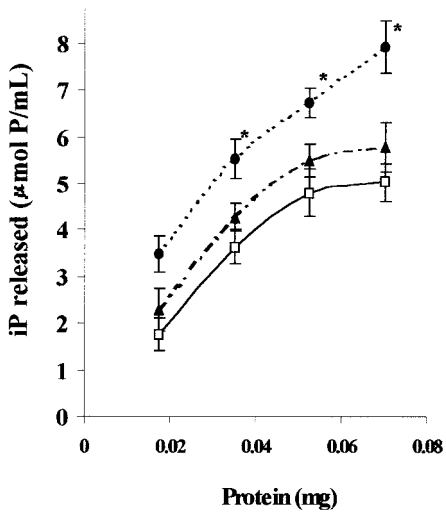

FIG. 6. Hydrolysis of phytate phosphorus in soybean meal by different amounts (100, 300, 600, and 900 PU) of purified r-appA2 (●), r-appA (▲), and r-phyA (□) enzymes. * indicates significant differences ($P < 0.05$) between r-appA2 and other two enzymes. Results are expressed as the mean ± SEM from three experiments.

Compared with the purified phytase P2 (18), r-appA2 has the same optimum temperature (55°C) and similar molecular mass after deglycosylation (46.3 kDa). Based on the SDS-PAGE and non-denaturing gel analyses, the protein is also monomeric. However, r-appA2 has a more acidic pH optimum (2.5 to 3.5 vs. 4.5 for P2) and contains 8 to 14% of sugar moieties because of the N-glycosylation in *Pichia*. Deglycosylation of r-appA2 with Endo H_f reduces the molecular size but has a minimal effect on its activity. In contrast, when the protein is incubated with β-mercaptoethanol and Endo H_f, the phytase and acid phosphatase activities of r-appA2 are considerably reduced. This indicates that disulfide bonds are required for its phytase activity as previously shown for the *A. ficuum* phytase (30).

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