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Expression of *Escherichia coli* AppA2 phytase in four yeast systems

Seonho Lee, Taewan Kim, Chad H. Stahl & Xin Gen Lei*
Department of Animal Science, Cornell University, Ithaca, NY, 14853-4801, USA
**Author for correspondence (Fax: +1-607-255-9829; E-mail: XL20@cornell.edu)*

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Abstract

To develop an effective fermentation system for producing *Escherichia coli* phytase AppA2, we expressed the enzyme in three inducible yeast systems: *Saccharomyces cerevisiae* (pYES2), *Schizosaccharomyces pombe* (pDS472a), and *Pichia pastoris* (pPICZαA), and one constitutive system: *P. pastoris* (pGAPZαA). All four systems produced an extracellular functional AppA2 phytase with apparent molecular masses ranging from 51.5 to 56 kDa. During 8-day batch fermentation in shaking flasks, the inducible *Pichia* system produced the highest activity (272 units ml^{-1} medium), whereas the *Schizo. pombe* system produced the lowest activity (2.8 units ml^{-1}). The AppA2 phytase expressed in *Schizo. pombe* had 60–75% lower K_m for sodium phytate and 28% higher heat-stability at 65 °C than that expressed in other three systems. However, all four recombinant AppA2 phytases had pH optimum at 3.5 and temperature optimum at 55 °C and similar efficacy in hydrolyzing phytate–phosphate from soybean meal.

Introduction

Phytate–phosphate in plant-derived foods and feeds is poorly available to humans and simple-stomached animals, such as swine and poultry. Their ingested phytate–phosphate is largely excreted which causes nutritional deficiencies and environmental pollution. As phytases are phosphohydrolases that initiate dephosphorylation of phytate complexes, these enzymes can be used to solve these nutritional and environmental problems associated with phytate. Compared with fungal phytases, *Escherichia coli* phytase has several favorable characteristics: its acidic pH optimum is similar to the physiological pH of stomach; it is resistant to pepsin, and it has higher catalytic efficiency for phytic acid than other phytases (Lei & Stahl 2001). Our laboratory has previously isolated a phytase-producing *E. coli* strain from the pig colon and cloned an *appA2* DNA fragment that encodes a protein of 433 amino acids with three putative *N*-glycosylation sites. We have successfully expressed the *appA2* gene in an inducible (pPICZαA) *Pichia pastoris* system (Rodriguez *et al.* 1999) and the resulting extracellular phytase is highly effective in releasing phytate–phosphate and in con–soy diets for young pigs and chickens (Augspurger *et al.* 2003). Since the yield and properties of any given protein or enzyme are significantly affected by the expression capability of hosts (Müller *et al.* 1998) and their post-translational modifications, such as glycosylation (Stahl *et al.* 2003), a number of expression hosts and systems have been tested for improving phytase function and production (Han *et al.* 1999, Rodriguez *et al.* 1999, 2000a,b, Miksch *et al.* 2002, Stahl *et al.* 2003).

Although *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have been used to produce *Aspergillus niger* PhyA (Han *et al.* 1999) and other enzymes (Müller *et al.* 1998), it is unclear if these systems could express *E. coli* AppA2 phytase as a functional extracellular protein. In addition, the constitutive expression system (pGAPZαA) of *P. pastoris* is relatively new and bears several advantages over the inducible *Pichia* system (Cereghino & Cregg 2000). Therefore, our objective was to compare the expression efficiency

and biochemical characteristics of *E. coli* AppA2 phytase in these three yeast systems with that in the inducible *Pichia* system.

Materials and methods

Strains, plasmids, constructs

The list of yeast strains and plasmids used in this study is shown in Table 1. To facilitate cloning, the phytase gene *(appA2)* from *E. coli* was amplified by PCR using a forward primer (E2) containing an *Eco*RI site and a reverse primer (K2) containing a *Kpn*I site (Rodriguez *et al.* 1999). All primers used for PCR are summarized in Table 2.

The first plasmid, pPAα2, was designed to express *appA2* gene in *Pichia pastoris* with the pPICZαA (Invitrogen, Carlsbad, CA). The second plasmid, pGAα2, was produced by ligating pGAPZαA (Invitrogen) with the coding region of the *appA2* amplified from pPAα2 after the restriction digestion of pPAα2 and pGAPZαA with *Eco*RI and *Kpn*I. The third plasmid, pYAα2, was to express *appA2* gene in *Saccharomyces cerevisiae* and was prepared by ligating pYES2 (Invitrogen) with the coding region of the *appA2* gene and α factor, the signal peptide in pGAPZαA (Invitrogen). The α factor signal peptide and the *appA2* coding region were amplified from pGAα2 by PCR using a forward primer (A2) containing a *Kpn*I site and a reverse primer (X2) containing a *Xba*I site. The PCR fragment was inserted into pYES2 at the *Kpn*I and *Xba*I sites to produce pYAα2. The fourth plasmid, pDAα2, was to express *appA2* gene in *Schizosaccharomyces pombe* by ligating pDS472a (ATCC 87615) with the coding region of the *appA2* gene and α factor. The latter (the *appA2* gene and α factor) was amplified from pGAα2 by PCR using a forward primer (SP1) containing *Bgl*II site and a reverse primer (SP2) containing a *Not*I site, and was inserted into pDS472a at *Bgl*II and *Not*I sites. All PCR fragments were cloned into pGEMT vector (Promega, Madison, WI) and transformed into *E. coli* JM 109 cells according to the manufacturer's instructions. After selective restriction digestions, the appropriate inserts and the expression vectors were gel purified, ligated, and transformed into *E. coli* JM 109 cells by electroporation (2.5 kV, 129 Ω, 5 ms, ECM 600 Electro Cell Manipulator, Genetronics, BTX Instrument Division, San Diego, CA). The positive colonies were then grown to prepare DNA for the following yeast transformations.

Table 1. Cloning and expression plasmids.

Plasmids	Description	Host strains	Source or reference
pDAα2	pDS472a + α factor[a] + appA2	*Schizosaccharomyces pombe* (ATCC 38436)	ATCC (Forsburg & Sherman 1997)
pYAα2	pYES2 + α factor[a] + appA2	*Saccharomyces cerevisiae* INVSc1	Invitrogen
pGAα2	pGAPZαA + appA2	*Pichia pastoris* X33	Invitrogen
pPAα2	pPICZαA + appA2	*Pichia pastoris* X33	Invitrogen

[a]Signal peptide of alpha factor from *Saccharomyces cerevisiae*.

Table 2. PCR primers used in this study.

Primer pair	Sequence (5′–3′)
E2–K2	GGA ATT CGC TCA GAG TGA GCC GGA
	GGG GTA CCT TAC AAA CTG CAC G
A2–X2	GCA CGG TAC CAT GAG ATT TCC TTC AAT T
	GGT CTA GAT TAC AAA CTG CAC G
SP1–SP2	GCG GAA GAT CTA TGA GAT TTC CTT CAA
	ATA AGA ATG CGG CCG CTT ACA AAC TGC ACG

Transformation, induction, and sample preparation

The transformation, culture, and induction of plasmid pPAα2 for the expression of *appA2* gene in *P. pastoris* strain X33 (Invitrogen) were conducted as described previously (Rodriguez *et al.* 1999). Transformation of pGAα2 into *P. pastoris* strain X33 (Invitrogen) was performed with 10 μg linearized plasmid using restriction enzyme *Bsp*HI, according to the manufacturer's instructions (Invitrogen). Positive colonies were selected from YPD (1% yeast extract, 2% peptone, 2% glucose) agar plates containing 100, 300 or 500 μg zeocin ml^{-1}. Selected transformants were grown in YPD medium at 30 °C for various time points to collect supernatant for analysis. Transformations of plasmids pYAα2 into *Sacc. cerevisiae* INVSc1I (Invitrogen) and pDAα2 into *Schizo. pombe* (ATCC 38436) were carried out by electroporation (1.5 kV, 129 Ω, 4.9 ms, ECM 600 Electro Cell Manipulator, Genetronics, BTX Instrument Division). The transformed cells were plated in synthetic minimal defined agar medium containing 2% (w/v) glucose and lacking uracil (SC-U) to screen for positive transformants. After isolated positive colonies were initially grown overnight at 30 °C in SC-U broth, the whole culture was centrifuged at $1500 \times g$, 4 °C for 10 min. The cell pellets were re-suspended to obtain an OD_{600} of 0.4 in YPG medium (1% yeast extract, 2% peptone, and 2% galactose) for the *Sacc. cerevisiae* transformants and EMM (or EMMP) media (Moreno *et al.* 1991) lacking thiamine for the *Schizo. pombe* transformants to induce expression for various time periods. For all analyses described below, the cultures were first centrifuged at $3000 \times g$ and 4 °C and then the supernatants were concentrated through a 30 kDa cut-off ultrafiltration membrane (Millipore, Bedford, MA) in a stirred cell at 4 °C. Samples were washed (desalted) through the same membrane with 10 mM Tris/HCl, pH 7.4.

Western analysis

Samples of expressed phytases were subjected to SDS-PAGE (Laemmli 1970). The separated proteins were transferred onto a Protran nitrocellulose membrane with a Mini Trans-Blot cell (Bio-Rad Laboratories). A purified rabbit antibody, diluted 1:1000 prior to application, was used as the primary antibody (Stahl *et al.* 2003). A goat anti-rabbit immunoglobulin G-horseradish peroxidase system (Bio-Rad Laboratories) was used for the final colorimetric detection. The proteins were deglycosylated by incubation with endoglycosidase H_f (Endo H_f, New England Biolabs, Beverly, MA) according to the manufacturer's instructions. The reaction mixture was subjected to Western blot analyses as described above.

Phytase activity and properties

Phytase activity was determined using 0.5 ml sample containing 0.02 to 0.2 unit (U) phytase. The sample was diluted with 0.2 M glycine HCl, pH 3.5, incubated at 37 °C for 5 min, and then 0.5 ml 1% sodium phytate (in 0.2 M glycine HCl, pH 3.5) (Sigma) was added to start the reaction. After the hydrolysis was carried out at 37 °C for 15 min, 1 ml 15% (w/v) trichloroacetic acid was added to stop it. The release of phosphate from sodium phytate in the reaction was determined colorimetrically using sulfuric acid, ammonium molybdate and ascorbic acid solutions (Rodriguez *et al.* 2000a). Blanks were run by incubating the enzyme samples with trichloroacetic acid for 15 min before adding the substrate. One U of phytase activity was defined as that releases 1 μmol inorganic phosphate from sodium phytate per minute at pH 3.5 and 37 °C. Optimal pH, optimal temperature, thermostability of the expressed phytases were determined as described in the legend of Figure 3. Kinetics of the expressed phytases was determined as described in Table 3.

Phytate–phosphate hydrolysis

To compare the effectiveness of the expressed phytases in hydrolyzing phytate–phosphate in natural food, 1 g soybean meal was incubated with 250 mU enzyme in 5 ml 0.2 M glycine/HCl, pH 3.5, at 37 °C for 1 h (Han *et al.* 1999). The released phosphate in the supernatant was determined as previously described (Chen *et al.* 1956).

Statistical analysis

Data were analyzed using SAS (release 8.1, SAS Institute, Cary, NC), and significance level was set at $p < 0.05$.

Table 3. Comparison of kinetic parameters for hydrolysis of sodium phytate by AppA2 phytases produced by different yeast expression host systems*.

Expression host	pH 3.5		pH 5.5	
	V_m (mU ml^{-1})	K_m (mM)	V_m (mU ml^{-1})	K_m (mM)
Schizosaccharomyces pombe	108 ± 0.9[a]	0.3 ± 0.1[a]	83 ± 1.2[a]	1.5 ± 0.1[a]
Saccharomyces cerevisiae	128 ± 3.9[b]	1.2 ± 0.1[c]	70 ± 3.2[a]	2.1 ± 0.2[b]
Pichia pastoris (pGAPZαA)	118 ± 3[ab]	0.8 ± 0.1[b]	68 ± 3.8[a]	1.8 ± 0.2[ab]
Pichia pastoris (pPICZαA)	118 ± 3.5[ab]	0.7 ± 0.1[b]	61 ± 1.4[a]	1.8 ± 0.01[ab]

*To determine K_m of the expressed phytases, the partially purified enzyme samples (100 mU, diluted in 0.2 M glycine/HCl, pH 3.5 or 0.2 M pH 5.5) were incubated with 10 different concentrations of sodium phytate ranging from 0.1 to 10-fold of the estimated K_m (Bisswanger 2002). The substrate was dissolved in 0.2 M glycine/HCl, pH 3.5 or in 0.2 M citrate buffer, pH 5.5, and the reaction was carried at 37 °C and sampled at 0, 1, 2, 3, 5, and 10 min. Initial velocities were calculated from linear phases of the hydrolysis curve and plotted against inorganic phosphate concentration. Linear transformation was achieved using Lineweaver–Burk plot and Hanes–Woolf plot to estimate the K_m values (Bisswanger 2002). Values without sharing a common letter within the same column are different ($p < 0.05$).

Results

Functional expression of AppA2

All four yeast systems produced functional, extracellular recombinant AppA2 proteins. Figure 1 shows the time-dependent changes in the cell growth and accumulation of phytase activity in the culture. The phytase activity paralleled the cell mass. While phytase activity in the inducible *Pichia* system continued to increase to 272 U ml^{-1} (equivalent to 0.63 mg of phytase protein ml^{-1}) for 8 days of fermentation, cell growth and phytase activity accumulation reached a plateau in the other three systems after only 2 days. The plateau phytase activity was 92, 6.7 and 2.8 U ml^{-1} (0.21, 0.015 and 0.006 mg of phytase protein ml^{-1}) for the constitutive *Pichia*, *Sacc. cerevisiae*, and *Schizo. pombe* systems, respectively. All recombinant AppA2 proteins showed similar molecular mass by Western blot analysis (Figure 2). There were several levels of glycosylation, with apparent molecular mass ranging from 51.5 to 56 kDa. Deglycosylation of those recombinant enzymes resulted in a single band with an apparent molecular size of 46.3 kDa.

Biochemical characterization of the expressed AppA2

All four recombinant AppA2 demonstrated a pH optimum of 3.5 and similar pH profiles under the conditions of the present study (Figure 3a). Likewise, the expressed AppA2 enzymes from the four systems shared the same temperature optimum (55 °C) and similar activities from 25 to 85 °C (Figure 3b). Only slight losses of phytase activity were observed after heating the expressed phytases for 10 min at 25–55 °C, while there was a sharp decrease in activity between 55 and 75 °C treatments (Figure 3c). At 55 °C, the thermostability of phytases expressed in *Sacc. cerevisiae* was 6–9% lower ($p < 0.05$) than that of the other three systems. At 65 °C, the thermostability of the phytase expressed in *Schizo. pombe* was 28% higher ($p < 0.05$) than those of the other three systems.

Phytate affinity and hydrolysis of the expressed AppA2

At both levels of pH, AppA2 expressed in *Schizo. pombe* and *Sacc. cerevisiae* had the lowest and highest K_m values for sodium phytate, respectively, among the four enzymes (Table 3). At pH 3.5, the K_m values were similar between the two *Pichia* yeast systems, but there was significant difference ($p < 0.05$) between any two of the three hosts. At pH 5.5, all systems had higher K_m values than at pH 3.5, and there was a significant difference between *Schizo. pombe and Sacc. cerevisiae*. However, *in vitro* hydrolysis of phytate–phosphate in soybean meal showed similar effectiveness for all the four enzymes. The total amounts of phosphate released from soybean meal within 1 h were 18.4 ± 0.7, 16.9 ± 0.8, 16.6 ± 0.4 and 17.2 ± 0.5 μmol g^{-1} soybean meal by the phytase expressed in *Schizo. pombe,*

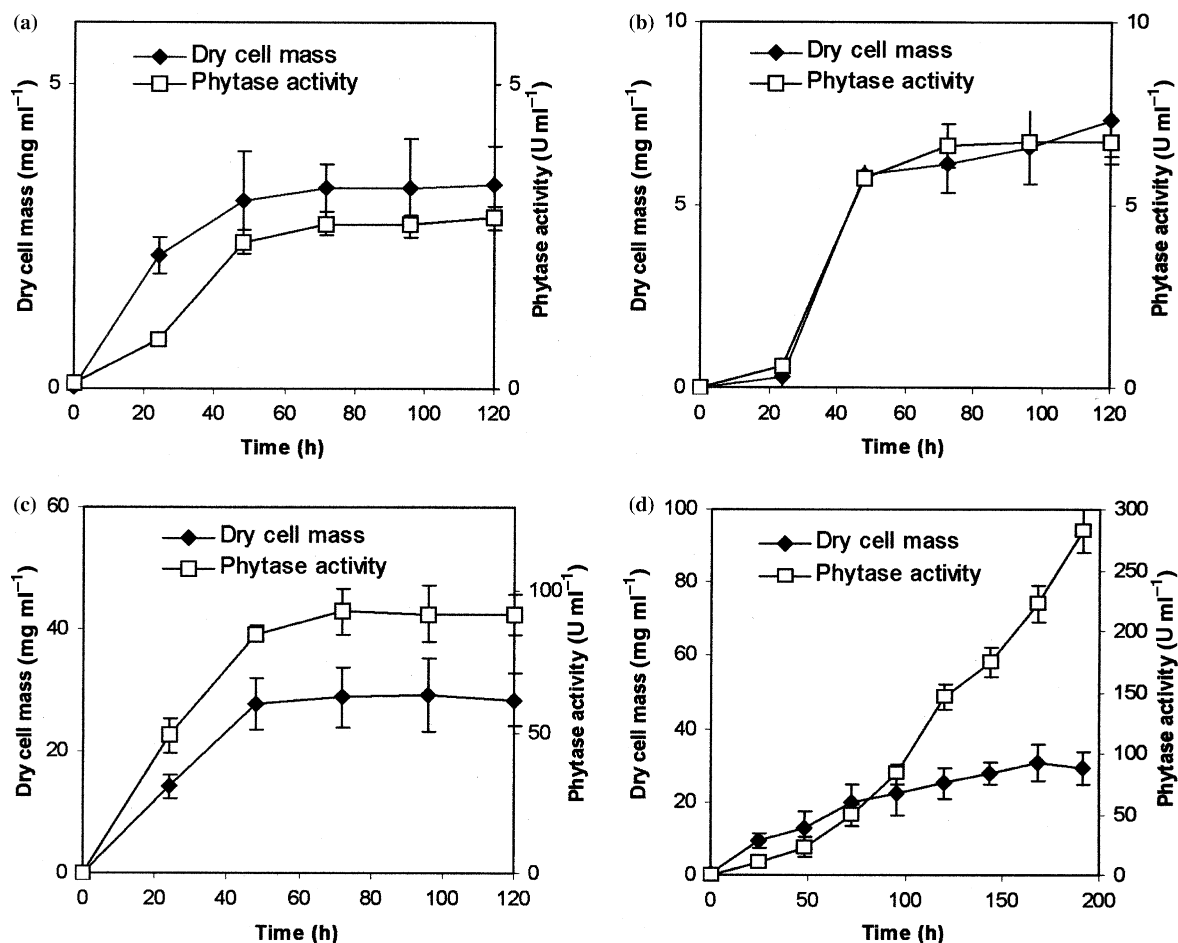

Fig. 1. Time course of cell mass (○) of the *appA2* transformants and the expressed phytase activity (●) in the media for (a) *Schizo. pombe*, (b) *Sacc. cerevisiae,* (c) *Pichia pastoris*–pGAPZαA system, and (d) *Pichia pastoris*–pPICZαA system. The transformants were grown in EMM media lacking thiamine for the *Schizo. pombe*, YPG for *Sacc. cerevisiae*, YPD for *P. pastoris*–pGAPZαA system, and BMMY for *P. pastoris*–pPICZαA system at 30 °C for various time points. Phytase activity was determined at 37 °C with 0.2 M sodium citrate buffer, pH 5.5. Results are expressed as the means ± standard errors of the means from three independent experiments.

Sacc. cerevisiae, constitutive *Pichia*, and inducible *Pichia* systems, respectively.

Discussion

As extracellular production is the preferred mode of industrial enzyme fermentation (Sreekrishna *et al.* 1997, Miksch *et al.* 2002), we have compared the extracellular expression of *E. coli appA2* in four yeast systems in the present study. Our results have demonstrated that *Sacc. cerevisiae* and *Schizo. pombe* are able to produce functional AppA2 extracellularly. In both systems, the α factor signal peptide from *Sacc. cerevisiae* functioned well in leading the secretion of the

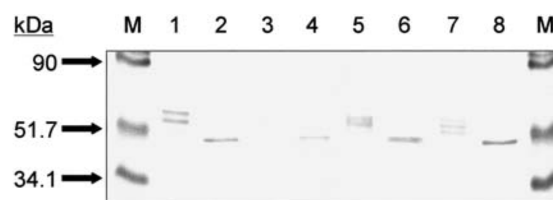

Fig. 2. Western blot analysis of the recombinant AppA2 phytases expressed in *Schizo. pombe* (SP), *Sacc. cerevisiae* (SC), *P. pastoris*–pGAPZαA system (PG), and *P. pastoris*–pPICZαA system (PP) before and after deglycosylation, using a primary antibody raised against purified *E. coli* AppA phytase. Lane M: pre-stained markers; lane 1: SP; lane 2: deglycosylated SP; lane 3: SC; lane 4: deglycosylated SC; lane 5: PG; lane 6: deglycosylated PG; lane 7: PP; and lane 8: deglycosylated PP. Numbers on the left are molecular masses in kDa.

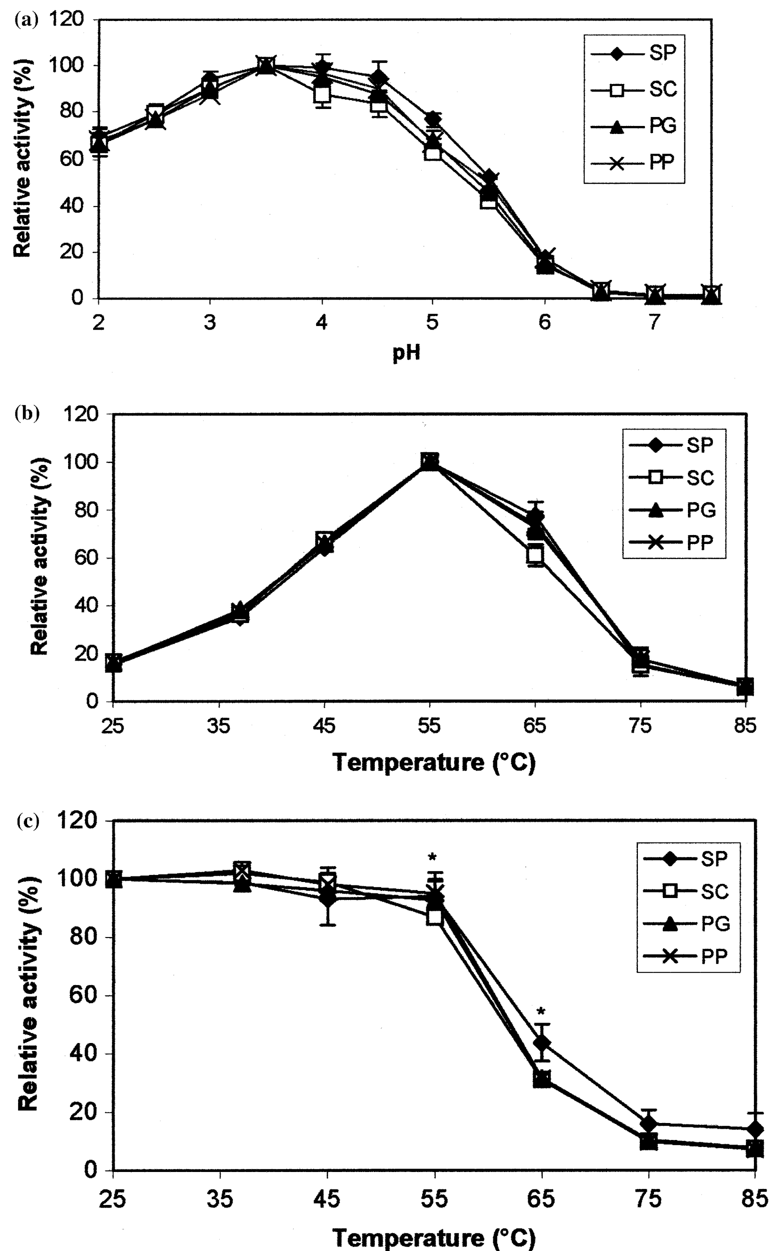

Fig. 3. Enymatic properties of the recombinant AppA2 phytases expressed in *Schizo. pombe* (SP), *Sacc. cerevisiae* (SC), *P. pastoris*–pGAPZαA system (PG), and *P. pastoris*–pPICZαA system (PP). (a) Optimal pH: the optimal pH of the expressed phytases was determined at 37 °C with two different buffers (final concentration: 0.2 M): glycine/HCl for pH 2.5, 3 and 3.5; sodium citrate for pH 4, 4.5, 5, 5.5, 6 and 6.5. Sodium phytate (1%, w/v) was dissolved in each of the designated buffers (2×). Phytase samples were diluted to 0.1 U ml^{-1} in de-ionized water. The reaction was started by mixing an equal volume (0.5 ml) of diluted enzyme and substrate for each assayed pH. Activity at the optimal pH was expressed as 100%. (b) Optimal temperature: the optimal temperature of the expressed phytases was determined at 25–85 °C with phytase samples diluted to 0.1 U ml^{-1} in 0.2 M glycin/HCl, pH 3.5. Activity at the optimal temperature was expressed as 100%. (c) Thermostability: phytase samples were diluted to 0.2 U ml^{-1} in 0.2 M glycin HCl, pH 3.5, and incubated for 10 min at 25, 37, 45, 55, 65, 75, and 85 °C. The samples were cooled on ice for 30 min and their remaining phytase activities were expressed as the percentage of the initial activity (100%). Asterisk indicates significant ($p < 0.05$) differences from other groups at the same temperature point.

expressed AppA2 into the culture media. However, the phytase activity yields in these two systems were much lower than those produced in the *Pichia* systems. The inducible *Pichia* system with pPICZαA vector produced the highest phytase activity among the four yeast systems. This expression system uses the alcohol oxidase I (AOX I) promoter that is induced by methanol and repressed by glucose, glycerol, and ethanol (Minning *et al.* 2001). Comparatively, the constitutive *Pichia* system produced lower plateau phytase activity than the inducible system. However, the system with the glyceraldehyde-3-phosphate dehydrogenase (GAP) gene promoter does not require the change of culture media for methanol induction during fermentation (Cereghino & Cregg 2000). In addition, the system uses relatively low cost YPD medium and does not consume high levels of oxygen (Han *et al.* 1999). With ease of manipulation, rapid growth, and relatively high production yield, recombinant strains *of Pichia* with pGAPZαA vector may offer considerable advantages for phytase fermentation.

The four recombinant enzymes showed similar molecular mass, glycosylation, pH optimum and profile, temperature optimum and profile, and phytate–phosphate hydrolysis efficiency. These enzymes also exhibited similar pH stability at 37 °C (data not shown). The lack of significant impact of the expression systems on phytase property offers flexibility for the selection of phytase fermentation systems. Compared with that reported previously (Rodriguez *et al.* 1999), the most effective pH range of the AppA2 expressed in the inducible *Pichia* system shifted somewhat broad up to 4–4.5. This change might be mainly due to modification of the assay conditions. Despite this, other properties of the enzyme, including molecular mass, glycosylation, kinetics, and phytate–phosphate hydrolysis, were similar between the past (Rodriguez *et al.* 1999) and the present study.

It is interesting to note the differences in K_m for sodium phytate and thermostability between the AppA2 expressed in *Schizo. pombe* and in other systems. At pH 3.5, AppA2 phytase from *Schizo. pombe* had a much lower K_m for sodium phytate, implying a stronger substrate affinity than those from other expression systems. These differences in K_m might be due to post-translational modification of phytases by the expression hosts (Ingham *et al.* 1995, Rudd *et al.* 1995, Wang *et al.* 1996, Wyss *et al.* 1999, Rodriguez *et al.* 2000a). As we have not determined the fidelity of processing of the *N*-terminal secretion signals in these four systems, we do not know if any of this variation (Lin *et al* . 2004) contributed to the K_m differences. As the enzyme from *Schizo. pombe* did not release a significantly greater amount of phosphate from phytate in soybean meal than those of other three enzymes, it might be due to the relatively high phytate concentration of soybean meal in the hydrolysis mixture and that not all phytate in soybean meal is in the form of sodium phytate. Although deglycosylation of AppA2 expressed in the four different systems resulted in a single protein band with a molecular mass of 46.3 kDa (Miksch *et al.* 2002, Stahl *et al.* 2003), the phytase produced by *Schizo. pombe* was more thermostable at 65 °C than the enzymes expressed in other systems. Previous studies have shown the positive effect of elevated glycosylation on the thermostability of *Aspergillus niger* PhyA phytase (Han *et al.* 1999). Other studies have shown that additional glycosylation of enzymes facilitate folding and increase stability (Haraguchi *et al.* 1995, Imperiali & Rickert 1995). However, there is no significant difference in thermostability, protein folding, or specific activity among phytases expressed in *A. niger*, *Hansenula polymorpha,* and *Sacc. cerevisiae* (Wyss *et al.* 1999). In contrast, increased levels of glycosylation have been shown to reduce thermostability of phytase (Fierobe *et al.* 1997). Previous research in our laboratory has created an *E. coli* AppA mutant with improved thermostability, but without elevated glycosylation compared with the wild-type enzyme (Rodriguez *et al.* 2000b). Thus, effects of glycosylation on phytase thermostability cannot be simply generalized. The observed improvement in thermostability of AppA2 expressed *in Schizo. pombe* might be a specific interaction between the enzyme and the expression host (Stahl *et al.* 2000b). In addition, caution should be given in comparing thermostability of different phytases with relatively low purity as many factors may interfere with the assay (Rodriguez *et al.* 2000b).

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