JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Overexpression of *Escherichia coli* Phytase in *Pichia pastoris* and Its Biochemical Properties

Hsueh-Ming Tai,[†,‡] Li-Jung Yin,[§] Wei-Chuan Chen,[∥] and Shann-Tzong Jiang*[,†,∥]

[†]Department of Food and Nutrition, Providence University, Number 200, Section 7, Taiwan Boulevard, Salu, Taichung 43301, Taiwan

[‡]Nugen Bioscience (Taiwan) Company, Limited, Number 29, 40th Road, Taichung Industry Park, Taichung 40768, Taiwan

[§]Department of Seafood Science, National Kaohsiung Marine University, Number 142, Hai-Chuan Road, Nan-Tzu, Kaohsiung 81143, Taiwan

[∥]Department of Food Science, National Taiwan Ocean University, Number 2, Beining Road, Keelung 20224, Taiwan

Supporting Information

ABSTRACT: To obtain a *Pichia pastoris* mutant with an *Escherichia coli* phytase gene, which was synthesized according to *P. pastoris* codon preference, a mature phytase cDNA of *E. coli* being altered according to the codons usage preference of *P. pastoris* was artificially synthesized and cloned into an expression vector of pGAPZαC. The final extracellular phytase activity was 112.5 U/mL after 72 h of cultivation. The phytase, with a molecular mass of 46 kDa, was purified to electrophoretical homogeneity after Ni Sepharose 6 Fast Flow chromatography. The yield, purification fold, and specific activity were 63.97%, 26.17, and 1.57 kU/mg, respectively. It had an optimal pH and temperature of 4.0−6.0 and 50 °C, respectively, and was stable at pH 3.0−8.0 and 25−40 °C. The purified recombinant phytase was resistant to trypsin, highly inhibited by Cu^{2+}, Zn^{2+}, Hg^{2+}, Fe^{2+}, Fe^{3+}, phenylmethylsulfonyl fluoride, and *N*-tosyl-L-lysine chloromethyl ketone, but activated by Mg^{2+}, Ca^{2+}, Sr^{2+}, Ba^{2+}, glutathione, ethylenediaminetetraacetic acid, and *N*-ethylmaleimide. It revealed higher affinity to calcium phytate than to other phosphate conjugates.

KEYWORDS: *Phytase, Escherichia coli, Pichia pastoris, cloning, expression*

INTRODUCTION

Phytic acid, a major component of plant seeds, is about 1−3% in many cereals and oilseeds and amounts to 60−90% of total phosphorus.[1] Although phytates are very important in many physiological functions, especially in seed germination, they are considered solely as antinutrients because of the binding with starch and protein and their strong chelating ability with divalent minerals, such as Ca^{2+}, Mg^{2+}, Zn^{2+}, and Fe^{2+}.[2] Conversely, as a strong chelator of iron and zinc, phytate in plant foods can actually serve as an antioxidant to reduce free-radical formation mediated by these metals.[2] The formation of insoluble mineral−phytate complexes at physiological pH values is considered to be the major reason for poor mineral bioavailability, because these complexes are hardly absorbed in the human gastrointestinal tract.[3] Because many minerals are involved in the activation of intra- and extracellular enzymes, a deficiency of any one of these essential minerals might result in severe metabolic disorders and compromise the health of the organism. Some mineral deficiencies are common in developing countries, but mineral sub-deficiencies are also found in developed countries.[4]

Effective reduction of phytic acid can be obtained via the action of both enzymatic and non-enzymatic degradation.[3] Enzymatic degradation can be performed by the addition of either the isolated form of wild-type or recombinant exogenous phytic-acid-degrading enzymes from various sources of fungi and bacteria. Phytase can reduce the antinutritional factor of phytic acid and also eutrophication, caused by the excretion of undigested phytic acid by monogastrics because of the lack of adequate levels of phytase in their digestive tracts.[3] Phytases from several plants and microbial species have been purified and characterized.[5] Most phytases in animal digestive tracts are from the secretion by the gastrointestinal tract, production by microorganisms inside tracts, and foods or feeds with the addition of microbial phytases. However, microbial phytases are usually sensitive to pH and heat, which consequently greatly lowered their activities during processing or passing through the gastrointestinal tract.[6]

Non-enzymatic hydrolysis during food processing or by physical removal of phytate-rich parts from plant seeds is also frequently employed to reduce the phytate levels in the final foods. Phytases (*myo*-inositol hexakisphosphate 3- or 6-phosphohydrolases; EC 3.1.3.8 or EC 3.1.3.26) are phosphatases that can catalyze the sequential hydrolysis of *myo*-inositol hexakisphosphate or phytic acid (InsP6) to lower the formation of phosphorylated *myo*-inositol derivatives and inorganic phosphate.[5] Biotechnological engineering to optimize phytase catalytic features is considered as a promising strategy for efficient reduction of phytate. Enhancement of thermal tolerance and an increase in specific activity are two important issues for not only animal feed but also food processing

Received: February 5, 2013
Revised: June 4, 2013
Accepted: June 5, 2013
Published: June 5, 2013

dx.doi.org/10.1021/jf401853b | J. Agric. Food Chem. 2013, 61, 6007−6015

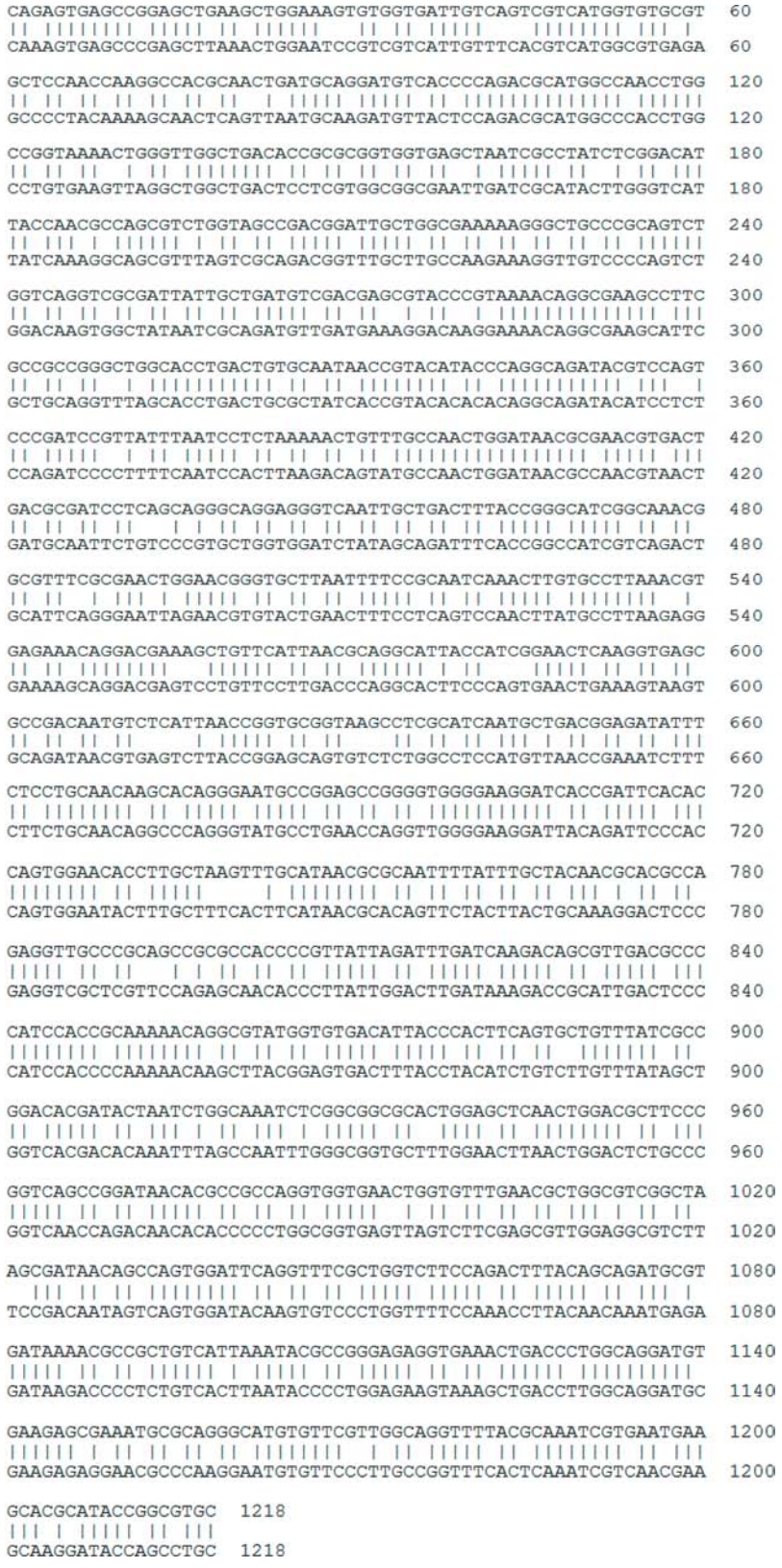

Figure 1. Comparison of nucleotide sequences between the optimized and original phytase genes (upper sequences, original phytase genes; lower sequences, optimized gene sequences).

applications of phytases. Various strategies have been used to obtain the phytases with higher thermal stability, such as shifting in the temperature optimum of the *Escherichia coli* phytase from 55 to 65 °C achieved by expression in *Pichia pastoris* after introducing three glycosylation sites in the amino acid sequence by site-directed mutagenesis.[7]

Nevertheless, there are still limited sources of phytase that is suitable for all food applications. Thus, screening for ideal

phytase-producing bacteria with more favorable properties and biotechnological engineering to optimize the catalytic and stability features of phytase are still attracting researchers. Some of the criteria of ideal phytase are the capability of remaining highly active during food processing or preparation. Moreover, it is a favorable property of phytase to have high phytate-degrading capability even at room temperature, withstanding of acceptable temperatures, such as in the digestive tract of animals or humans, and a high activity over a broad pH range.[3]

Monogastric animals, such as pig, poultry, fish, and also humans, lack phytase to hydrolyze phytate.[8] Supplemental dietary phytase is needed for them to use phosphorus and other minerals bound in phytates. Although currently phytases are used mainly as animal feed additives in diets of monogastric animals, there is great potential for use in the processing and manufacturing of food for human consumption.[9] Phytases used in animal feed can reduce/avoid the supplementation of inorganic phosphate,[8] which consequently lead to the lowering of the feed costs as well as the pollution caused by fecal phosphorus excretion of animals.[10]

Phytase activity, thermal stability, pH stability, degradation of different phytate forms, and production yields need to be improved to make these phytases possible for industrial application.[8] However, there is no phytase that can fulfill all of the mentioned requirements.[8] Site-directed mutagenesis has been used to improve the specific activity or thermal stability of phytases from *Aspergillus fumigatus*[11] and *E. coli*[7] and to change the pH profile of *Aspergillus niger* phytase.[12] Fungal and *E. coli* phytases are acidic histidine phytases, which can degrade phytate at low pH in the stomach where phytate exists in metal-free form and results in *myo*-inositol monophosphate.[13] It has been reported that the *E. coli* phytase was more resistant to pepsin activity than fungal phytases.[14] Two types of phytases are known as 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3.1.3.26).[5] The phytase from *E. coli* belongs to the 6-phytase type.[15] Augspurger et al.[8] reported that a bacterial phytase derived from *E. coli* liberated more P in broilers than two recombinant fungal phytases based on increases in tibia ash relative to inorganic P supplementation.

Chen et al.[16] modified appropriately the medium composition and fermentation strategy of *E. coli* phytase gene *appA* expressed in *P. pastoris* KM71-61 strain to enhance the phytase activity level from 118 to 204 U/mL at the flask scale.

This study was to obtain *P. pastoris* mutant with *E. coli* phytase gene, which was synthesized without altering the amino acid sequence according to *P. pastoris* codon preference. It was overexpressed, and the biochemical properties of the purified recombinant *E. coli* phytase were determined.

MATERIALS AND METHODS

Microorganisms and Plasmids. *E. coli* BL21(DE3) (Stratagene, La Jolla, CA) was used as the donor of DNA. *E. coli* TOP10F′ (Invitrogen, Carlsbad, CA) was used for the construction of the *E. coli* genomic library and for DNA manipulations. The plasmid pUC57 cloning vector (GenScript, Piscataway, NJ) was used for DNA fragment cloning. The DNA encoding phytase with His tag was ligated into pGAPZαC (Invitrogen) expression vector. *P. pastoris* SMD1168H (Invitrogen) was used for the expression host of phytase.

Design and Synthesis of Codon-Optimized Phytase Gene. The usage biases of codon in *E. coli* and *P. pastoris* are significantly different. To further improve the expression level of recombinant phytase in *P. pastoris*, the optimized phytase gene was designed in favor of *P. pastoris* expression by artificial gene synthesis.[17,18] The synthesized 1218 base pair (bp) phytase gene showed 74% homology with the wild-type phytase gene (Figure 1). This synthetic phytase sequence, in which 319 nucleotides were changed, was designed to be preferential for *P. pastoris* by the online program DNAWorks. However, the encoding amino acid sequences of both wild-type and synthetic genes were exactly the same. The GC content decreased from 54.5 to 49.4%, closer to the actual GC content of other high expression genes in *P. pastoris*.[19] Moreover, to avoid premature termination, the AT-rich codons were eliminated and changed according to the favor codons usage of *P. pastoris* expression.

PCR Synthesis and Construction of Phytase Expression pGAPZαC Plasmid. The phytase gene of *E. coli* BL21(DE3) (GenBank accession number CP001509.3), with an additional His-tag sequence at the 3′ end, was optimized using codon usage preference[17] and synthesized by the polymerase chain reaction (PCR) method. The standard techniques of recombinant DNA protocols were performed according to the studies by Ausubel et al.[20] and Sambrook et al.[21] A 1.29 kb DNA fragment was amplified. The PCR product was first cloned into pUC57 vector (GenScript) and then transformed into *E. coli* TOP10F′ competent cells. The nucleotide sequence of resulting products was confirmed using primers by sequencing. After Midi preparation, the correct plasmid was digested with *Xho*I and *Xba*I and finally ligated into pGAPZαC vector (Novagen).

Transformation and Selection in *P. pastoris* Host. The pGAPZαC plasmid, ligated with phytase, was digested with *Bsp*HI in the GAP promoter region to linearize the vector and then transformed into *P. pastoris* SMD1168H. The colonies were selected by plating on YPDS agar plates (BD Biosciences, Clontech) containing 1500 μg/mL Zeocin. The yeast colony integrated by recombinant pGAPZαC-phytase DNA with correct in-frame coding sequence into the *Pichia* genome was used for the protein expression.

Cultivation of *P. pastoris* SMD1168H and Production of Phytase. The *P. pastoris* SMD1168H strain integrated with recombinant pGAPZαC-phytase DNA was cultivated in YPDS broth (BD Biosciences, Clontech) at 30 °C with shaking at 250 rpm overnight. The resulting culture was inoculated into a medium containing 1% yeast extract, 2% peptone, 2% dextrose, and 100 μg/mL Zeocin and incubated at 30 °C. The phytase activity, pH, and viable cell counts (CFU/mL) were monitored during 5 days of incubation. After incubation, it was centrifuged at 13000*g* and 4 °C for 30 min. The supernatant was used for further purification.

Determination of Enzyme Activity. Phytase activity was determined according to the study by Zou et al.[22] After 30 min of centrifugation at 13000*g* and 4 °C and being filtered through a 0.45 μm sterilized membrane (Gelman Sciences, Ann Arbor, MI) to remove cells, the phytase sample (0.25 mL) or collected phytase samples during the purification process were incubated with 1 mL of 2 mM sodium phytate (dissolved in 100 mmol/L sodium acetate at pH 5.5). After the hydrolysis, 1 mL of 15% (w/v) trichloroacetic acid (TCA) was added to stop the reaction. The release of inorganic phosphate from sodium phytate was determined colorimetrically by adding ammonium molybdate, sulfuric acid, and ferrous sulfate solution. After 10 min of reaction, the absorbance at 700 nm was measured. A total of 1 unit of activity (U) was defined as the amount of enzyme that can liberate 1 μmol of inorganic phosphorus within 1 min of reaction at 37 °C.[22]

Purification of Phytase. The broth was centrifuged at 13000*g* and 4 °C for 30 min and then filtered through a 0.45 μm sterilized membrane (Gelman Sciences, Ann Arbor, MI) to remove cells. After the sample was concentrated by Amicon ultrafiltration (cutoff of 10 kDa, Amicon Division, W. R. Grace and Co., Beverly, MA), the concentrated sample was loaded onto a Ni Sepharose 6 Fast Flow column (2.6 × 4.7 cm) pre-equilibrated with a buffer containing 20 mM sodium acetate and 10 mM imidazole (pH 5.5). After washing with the same buffer, the bound proteins were eluted with an elution buffer containing 20 mM sodium acetate and 200 mM imidazole (pH 5.5). The absorbance of eluents at 280 nm was measured, and the phytase activity in each fractions was determined according to the study by Zou et al.[22]

Determination of the Protein Concentration. Protein concentrations were determined by the dye binding method[23] using bovine serum albumin (BSA) as the standard.

Deglycosylation. Deglycosylation was performed using Enzymatic Protein Deglycosylation Kit (Sigma-Aldrich, St. Louis, MO) according to the protocols of the manufacturer. Briefly, 100 μg of recombinant phytase were dissolved in 30 μL of distilled water. A total of 10 mL of reaction buffer and 2.5 mL of denaturation solution were added to the phytase solution and mixed gently. The mixture was heated at 100 °C for 5 min and then cooled to room temperature. About 2.5 mL of Triton X-100 solution was added to the resulting sample. The phytase solution was then deglycosylated by mixing with 1 mL each of the PNGase F, *O*-glycosidase, and α-(2,3,6,8,9)-neuraminidase solutions and incubated at 37 °C for 3 h. The deglycosylated protein was analyzed by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE).

SDS−PAGE. SDS−PAGE analysis was performed with a 10% polyacrylamide gel according to Laemmli.[24] After electrophoretic running, gels were stained with Coomassie Blue R-250 and destained according to the study by Neuhoff et al.[25] The low-molecular-weight calibration kit (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) was used as markers (phosphorylase β subunit, 97.0 kDa; BSA, 66.0 kDa; ovalbumin, 45.0 kDa; carbonic anhydrase, 30.0 kDa; soybean inhibitor, 20.1 kDa; and α-lactalbumin, 14.0 kDa).

Optimal pH and Temperature of the Purified Phytase. The optimum pH was determined by measuring the activity at various pH values (100 mM glycine-HCl buffer, pH 1.0, 2.0, 3.0, and 4.0; 100 mM sodium acetate buffer, pH 5.0 and 6.0; and 100 mM Tris-HCl buffer, pH 7.0, 8.0, and 9.0) at 37 °C, while the optimal temperature was measured by incubating the reaction mixture at different temperatures (25−90 °C) for 30 min using 1.5 mM sodium phytate as the substrate.[22] The maximal phytase activities at optimal pH and temperature were defined as 100%. About 0.225 nmol of the purified recombinant phytase was used in the determination of optimal pH and temperature.

pH and Thermal Stability of the Purified Phytase. The pH stability was determined by measuring the residual phytase activity after 30 min of incubation at 37 °C and different pH values (100 mM glycine-HCl, pH 1.0−4.0; 100 mM sodium acetate, pH 4.0−6.0; and 100 mM Tris-HCl, pH 6.0−9.0) according to the study by Zou et al.[22]

The thermal stability was determined by measuring the residual phytase activity after 5 and 30 min of incubation in 20 mM sodium acetate buffer (pH 5.5) at temperatures from 25 to 95 °C according to the study by Zou et al.[22]

Effects of Metal Ions, Inhibitors, or Reducing Agent. To investigate the effect of metal ions, purified recombinant phytase in 20 mM sodium acetate buffer (pH 5.5) with 5 and 10 mM Ag^{+}, K^{+}, Li^{+}, Na^{+}, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, and Fe^{3+} was incubated at 37 °C. After 30 min of incubation, the residual phytase activity was measured according to the study by Zou et al.[22]

To investigate different inhibitor effects, the purified phytase in 20 mM sodium acetate buffer (pH 5.5) with 1−5 mM ethylenediaminetetraacetic acid (EDTA), ethyleneglycoltetraacetic acid (EGTA), *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (*p*CMB), and phenylmethylsulfonyl fluoride (PMSF) or reducing agents, such as dithiothreitol (DTT), glutathione (GSH), and β-mercaptoethanol (β-Me), was incubated at 37 °C for 30 min. The residual enzyme activity was measured according to the study by Zou et al.[22]

Substrate Specificity and Kinetic Analysis. To determine the substrate specificity, purified phytase in 20 mM sodium acetate (pH 5.5) was incubated with 1.5 mM *p*-nitrophenyl phosphate (*p*NPP), sodium−phytate (with nine sodium, Sigma-Aldrich, Inc.), calcium−phytate (with six sodium, Sigma-Aldrich, Inc.), fructose 1,6-bisphosphate (F-1,6-PP), and glucose 6-phosphate (G-6-P) at 37 °C for 30 min. The activity was measured according to the study by Zou et al.[22] The kinetic parameters of K_M and V_{max} were determined by incubating the phytase with various substrate concentrations (0.1−20 mM) in 20 mM sodium acetate buffer (pH 5.5) at 37 °C and calculated by the Michaelis−Menten equation. k_{cat} was determined with the equation: $V_{max} = k_{cat}[E]$, where $[E]$ is the enzyme concentration. The ratio of k_{cat}/K_M was used to evaluate the catalytic efficiency of recombinant phytase.

Pepsin and Trypsin Tolerance Tests. Pepsin in 80 mM glycine-HCl (pH 2.0) and trypsin in 80 mM $NH_4H_2CO_3$ (pH 7.5) were used to evaluate the proteolytic effects on phytase. Purified recombinant phytase was incubated with various amounts of pepsin or trypsin at ratios (protease/phytase) ranging from 0.002, 0.005, 0.01, 0.02, and 0.1 at 37 °C for 2 h. The residual activity was measured by the method described above.[22]

Genetic Stability of the Recombinant *P. pastoris*. To investigate the stability of inheritance and expression level of the recombinant phytase genes in *P. pastoris*, the mutant colonies were selected on a YPDS [yeast, peptone, and dextrose (YPD) with sorbitol] agar plate containing 1500 μg/mL Zeocin. They were transferred to 10 mL of YPD broth and incubated with shaking (250 rpm) at 30 °C. After 24 h of incubation, 1% of culture was inoculated into 250 mL of YPD broth with/without 100 μg/mL Zeocin. This procedure was repeated 10 times. Phytase activities and gene expression levels were determined after 10 generations according to the studies by Zaghloul et al.[26] and Xiong et al.[27] [plasmid stability (%) = (CFU of YPD broth with Zeocin/CFU of YPD broth without Zeocin) × 100%].

Statistical Analysis. One-way analysis of variance (ANOVA) was used to determine the significance of differences within treatments using the Statistical Analysis System (SAS/STAT, Release 8.0; Cary, NC). For each treatment, three replicates were measured and the mean values were calculated. Values were considered to be significantly different when $p < 0.05$.

RESULTS AND DISCUSSION

Construction of Phytase-pGAPZαC Expression Vector. To ensure that the phytase DNA fragment was in the correct reading frame, the PCR-amplified fragment was first cloned into pUC57 cloning vector for screening and sequencing. The vector containing the correct in-frame phytase DNA sequence was used to construct the phytase expression vector. The DNA for *E. coli* phytase was then ligated with pGAPZαC expression vector in *Xho*I and *Xba*I restriction enzyme sites and introduced in-frame downstream of the α factor in the pGAPZαC vector. The pGAPZαC vector is a powerful expression vector, which uses the GAP promoter to constitutively express recombinant proteins in *P. pastoris*. It was then transformed into *E. coli* TOP10F′ cell and grew in a low-salt Lauria-Bertani (LB) plate with 25 μg/mL Zeocin. The colony PCR-amplified DNA products indicated that the phytase-pGAPZαC expression vector had transformed to *E. coli* TOP10F′ cell (see Supplemental Figure 1 of the Supporting Information). The plasmid of the transformant was also digested with *Xho*I and *Xba*I to check the 1.3 kb of phytase and 3.1 kb of pGAPZαC expression vector (see Supplemental Figure 2 of the Supporting Information). After the phytase-pGAPZαC plasmid had been transformed into *P. pastoris* SMD1168H expression host, the expression vector was integrated into the genomic DNA because of the existence of the GAP promoter sequence. PCR analysis of transformed *P. pastoris* colonies ensured that they contained the phytase-pGAPZαC plasmid (see Supplemental Figure 3 of the Supporting Information).

Expression of the Recombinant Phytase in *P. pastoris*. A high level of the recombinant phytase was expressed under the control of the GAP promoter and secreted into broth by α-factor pre-prosequence during shaking cultivation. After 36 h of incubation, the *P. pastoris* transformant was grown to stationary phase, and the total count was approximately 9.1 log CFU/mL after 48 h of cultivation. The highest level of phytase activity

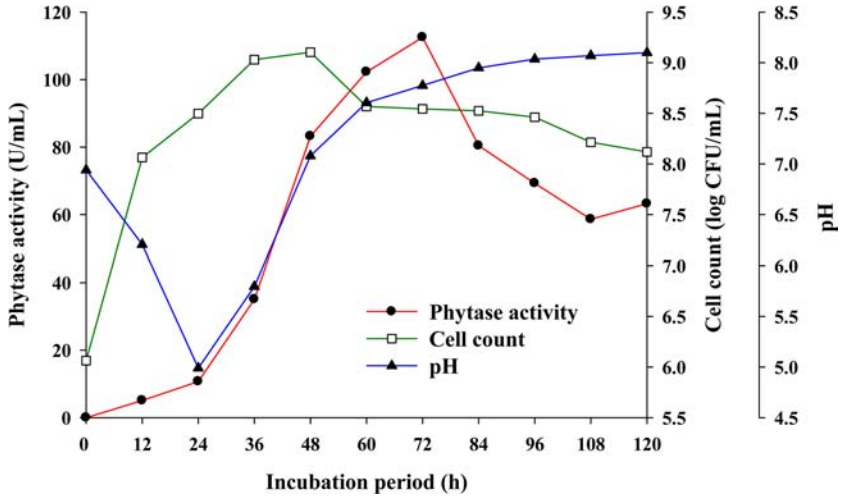

Figure 2. Changes in phytase activity, pH, and cell counts of broth during incubation of *P. pastoris* SMD1168H transformant at 30 °C in YPD medium. A total of 1 unit of activity (U) was defined as the amount of enzyme that can liberate 1 μmol of inorganic phosphorus from sodium phytate within 1 min of reaction at 37 °C and pH 5.5.

(112.5 U/mL) was observed after 72 h of cultivation, while the pH increased to 7.7 (Figure 2). No significant increase in phytase activity was observed during prolonged cultivation. The alkaline pH might not be good for *P. pastoris* growth. This phenomenon could be confirmed by the stability of the purified recombinant phytase, which was unstable in the alkaline environment (pH > 8.0; Figure 6). Therefore, control of pH at <8.0 during the fermentation may be of necessity. It was clear that the pGAPZαC expression vector with the α-factor leader-sequence-constituted GAP promoter could produce recombinant phytase. These data indicated that *P. pastoris* could be employed to express the optimized phytase from *E. coli*. The yield obtained from the *P. pastoris* SMD1168H transformant was higher than those of optimized *phyC* gene encoding neutral phytase expressed in *P. pastoris* (18.5 U/mL)[28] and sequence-optimized rLlAlp2 of alkaline phytase production in *P. pastoris* (0.2 U/mL).[29] The yield was also higher than that of previous studies of *Eupenicillium parvum* (BCC17694, 2.7 U/mL).[30] The yield obtained from the *P. pastoris* SMD1168H transformant was almost similar to that from the r-AppA gene encoding phytase expressed in *P. pastoris* (117 U/mL); however, it was higher than those from mutants A131N/V134N/D207N/S211N (35 ± 4 U/mL) and A131N/V134N/C200N/D207N/S211N (57 ± 8 U/mL).[7]

Recombinant phytase, with a molecular weight of 55 kDa, was purified to electrophoretic homogeneity using Ni Sepharose 6 Fast Flow affinity chromatography (Figure 3). As we know, when expressed in *P. pastoris*, the recombinant proteins are usually glycosylated. Accordingly, the diffused protein band occurred during the migration of glycosylated phytase in SDS−PAGE (lane 2 in Figure 3). However, the sharp single band was clearly observed after the purified phytase was deglycosylated (lane 3 in Figure 3). The specific activity and recovery were 1.57 kU/mg and 63.97%, respectively (Table 1). The specific phytase activity of the recombinant phytase in this study (1570 U/mg) was much higher than those from *P. pastoris* mutants with r-AppA (41 U/mg), A131N/V134N/D207N/S211N (32 U/mg), and A131N/V134N/C200N/D207N/S211N (51 U/mg),[7] which was reflected on the low K_M of the recombinant phytase in this study (see later part of Table 5). The deglycosylated phytase, after being treated with

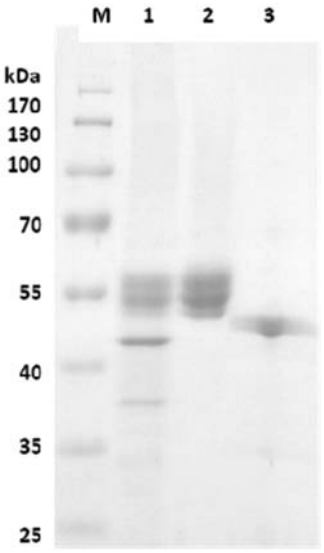

Figure 3. Profiles of SDS−PAGE of recombinant phytase purified by affinity chromatography on the Ni Sepharose 6 Fast Flow column. M, prestained SDS−PAGE marker; lane 1, crude enzyme; lane 2, purified enzyme; and lane 3, deglycosylated enzyme.

glycosidase, displayed a molecular weight of 46 kDa, calculated from SDS−PAGE and amino acid composition (Figure 3). These results indicated that the recombinant phytase was glycosylated protein and expressed successfully in the *P. pastoris* expression system.

Effects of the Temperature and pH. The optimal temperature of phytase from the *P. pastoris* transformant was 50 °C, which was similar to those of previous reports of *Aspergillus japonicus* BCC18313 (TR86), *A. niger* BCC18081 (TR170), and *E. parvum* BCC17694.[30,31] The thermal stability of the recombinant protein was investigated by incubating at 25−95 °C for 5 and 30 min. The results showed that the protein was stable at temperatures of 25−40 °C. Almost 80% activity of the purified recombinant phytase was left after 30 min of incubation at 35 °C, and more than 90% activity remained after 5 min of incubation at 40 °C (Figure 4). From the previous studies, the phytases from *E. parvum* (BCC17694)

dx.doi.org/10.1021/jf401853b | J. Agric. Food Chem. 2013, 61, 6007−6015

Table 1. Summary of the Purification of Phytase from the *P. pastoris* SMD1168H Transformant

procedures	total protein (mg)	total activity (kU)	specific activity (kU/mg)	recovery (%)	purification (fold)
crude enzymes	179.14 ± 3.04	10.02 ± 0.07	0.06	100.00	1.00
ultrafiltration	58.23 ± 1.31	8.33 ± 0.03	0.14	83.13	2.33
nickel affinity column	4.09 ± 0.80	6.41 ± 0.15	1.57	63.97	26.17

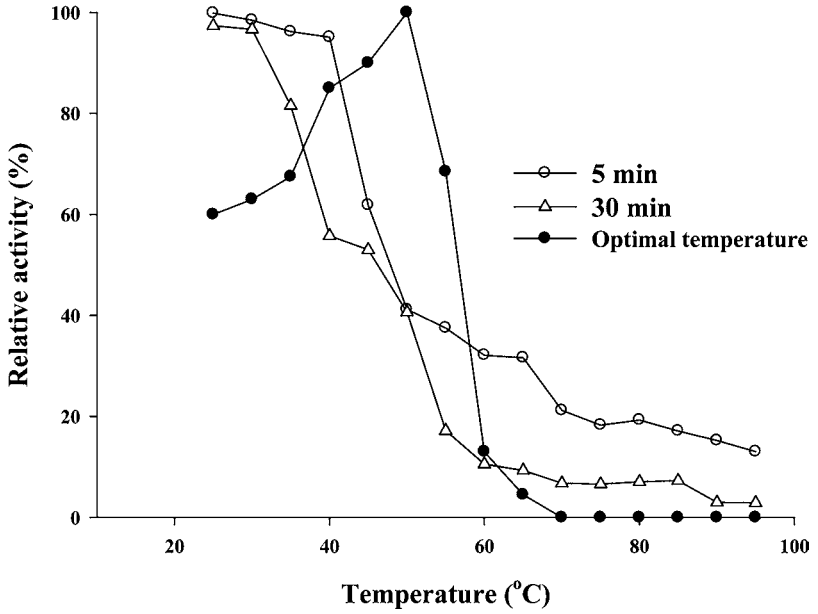

Figure 4. Temperature optimum and thermal stability of the recombinant phytase from *P. pastoris* [temperature optimum, closed symbols; thermal stability, open symbols (○, after 5 min of incubation; △, after 30 min of incubation, at various temperatures)].

had 40% activity left after 30 min of incubation at 40 °C, while those from *A. japonicus* BCC18313 (TR86) and *A. niger* BCC18081 (TR170) had 60% activity retained after 30 min of incubation at 80 °C.[30,31]

The optimal pH range was relatively broad, from 4.0 to 6.0. It was similar to that from *Neosartorya spinosa* BCC 41923.[32] However, it was stable at a broad pH ranging from 3.0 to 8.0 (Figure 5), which was similar to that of *E. coli* ATCC 33965 phytase.[33] This wide-range pH stability will be a high benefit to the industrial application for this enzyme.

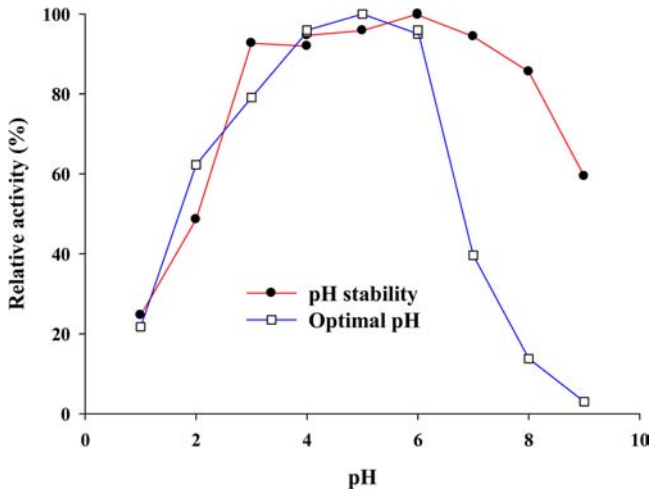

Figure 5. Effect of pH on recombinant *E. coli* phytase from the *P. pastoris* SMD1168H transformant.

Effects of Metal Ions, Inhibitors, or Reducing Agent. The purified phytase was moderately inhibited by Mn^{2+}, Co^{2+}, Ni^{2+}, and Al^{3+}, but highly inhibited by Cu^{2+}, Zn^{2+}, Fe^{2+}, Hg^{2+}, and Fe^{3+}. It was activated by Ca^{2+}, Mg^{2+}, Sr^{2+}, and Ba^{2+} (Table 2). From the previous studies, the phytase from *Selenomonas*

Table 2. Effect of Metal Ions on Recombinant *E. coli* Phytase from the *P. pastoris* SMD1168H Transformant

	relative activity (%)	
metal ions[a]	10 mM	5 mM
none[b]	100.00	100.00
Ba^{2+}	113.61 ± 0.61[c]	116.60 ± 0.55[c]
Sr^{2+}	105.29 ± 1.02[c]	102.08 ± 3.02
Ca^{2+}	104.46 ± 1.76	103.00 ± 0.99
Mg^{2+}	104.40 ± 0.45	100.42 ± 0.36
K^{+}	88.21 ± 0.94[c]	97.56 ± 1.53
Na^{+}	89.79 ± 1.75[c]	96.70 ± 1.66[c]
Li^{+}	79.99 ± 0.37[c]	93.01 ± 1.42[c]
Mn^{2+}	75.16 ± 2.05[c]	76.76 ± 0.47[c]
Co^{2+}	62.07 ± 1.05[c]	69.74 ± 1.98[c]
Ni^{2+}	46.30 ± 0.80[c]	45.73 ± 0.10[c]
Al^{3+}	31.70 ± 0.30[c]	48.53 ± 0.41[c]
Fe^{2+}	13.26 ± 0.59[c]	19.30 ± 0.27[c]
Cu^{2+}	1.87 ± 0.37[c]	0.00[c]
Fe^{3+}	0.03 ± 0.34[c]	0.00[c]
Zn^{2+}	2.16 ± 0.08[c]	6.16 ± 0.47[c]
Hg^{2+}	1.14 ± 0.45[c]	0.00[c]

[a]The counter ion of all metals was chloride. [b]Without metal ion added (as 100%). [c]Values in the same column differ significantly from that without metal added ($p < 0.05$).

ruminantium JY35 was inhibited by Zn^{2+}, Cu^{2+}, Fe^{2+}, Fe^{3+}, and Hg^{2+},[34] while those from *A. japonicas* BCC18313 and *A. niger* BCC18081 were inhibited by Zn^{2+} and Cu^{2+}.[31] The inhibitory effect of metal ions of Fe^{2+}, Fe^{3+}, Zn^{2+}, and Cu^{2+} might be due to the binding to the enzyme and, subsequently, forming poor soluble metal−phytate complexes. The purified enzyme was strongly inhibited by inhibitors, such as PMSF and *N*-tosyl-L-lysine chloromethyl ketone (TLCK), and was partially inhibited by DTT and *p*CMB (Table 3). Both PMSF and TLCK are inhibitors of the serine protease family. These serine protease inhibitors can modify the serine residues on the protein molecules. In this experiment, the activity of recombinant phytase could be inhibited by PMSF and TLCK, indicating the importance of the serine residue for the enzyme catalytic activity of phytase. EDTA did not inhibit phytase activity, indicating that it is metal-independent. This phenomenon is similar to most phytases, except for alkaline phytases, which require Ca^{2+}.[35] Substrate specificity revealed that the purified recombinant phytase had high affinity to the sodium and calcium phytates. However, it could hardly hydrolyze other phosphorylated compounds of pNPP, F-1, 6-PP, and G-6-P (Table 4). It is quite similar to that from *Bacillus* sp. DS11, which also did not hydrolyze pNPP, ATP, ADP, AMP, β-glycerophosphate, sodium pyrophosphate, and α-naphthylphosphate.[36]

Table 3. Effect of Chemicals on Recombinant *E. coli* Phytase from the *P. pastoris* SMD1168H Transformant

	relative activity (%)		
chemicals	5 mM	2 mM	1 mM
none[a]	100.00	100.00	100.00
DTT	46.58 ± 0.70[b]	47.98 ± 0.76[b]	76.60 ± 4.00[b]
GSH	106.29 ± 0.41[b]	104.03 ± 1.06[b]	107.15 ± 4.93[b]
β-ME	71.68 ± 2.69[b]	97.28 ± 2.16	103.26 ± 2.37
EGTA	100.1 ± 2.38	103.76 ± 5.41	100.98 ± 1.36
EDTA	144.3 ± 3.95[b]	121.08 ± 2.90[b]	104.29 ± 3.74
*p*CMB	55.0 ± 2.23[b]	73.70 ± 1.46[b]	85.92 ± 1.14[b]
PMSF	0.9 ± 0.07[b]	2.05 ± 0.13[b]	11.53 ± 0.99[b]
TLCK	2.4 ± 0.09[b]	1.63 ± 0.11[b]	4.19 ± 0.18[b]
IAA	97.6 ± 1.67	102.44 ± 0.76[b]	101.23 ± 2.60

[a]The activity of that without chemicals added was defined as 100%. [b]Values in the same column differ significantly from that without chemicals added ($p < 0.05$).

Table 4. Effect of Various Substrates on Recombinant *E. coli* Phytase from the *P. pastoris* SMD1168H Transformant

substrate	phytase activity (U/mL)
phytate Na	341.1 (100.0%)
phytate Ca	175.4 (51.4%)
*p*NPP	1.3 (0.4%)
F-1,6-PP	0.00 (0.0%)
G-6-P	0.00 (0.0%)

Kinetic Parameters of the Purified Recombinant Phytase. The kinetic parameters for the recombinant phytase were investigated with Na−phytate and Ca−phytate as substrates (Table 5). According to K_M, the recombinant phytase had higher affinity to Na−phytate than Ca−phytate. K_M of recombinant phytase from this study (0.26 ± 0.01 mM on Na−phytate and 0.70 ± 0.03 mM on Ca−phytate) was much lower than that of r-AppA (3.66 ± 0.44 mM) and mutants A131N/V134N/D207N/S211N (7.87 ± 0.84 mM), C200N/D207N/S211N (1.86 ± 0.35 mM), and A131N/V134N/C200N/D207N/S211N (3.18 ± 0.39 mM).[7] It suggests that the recombinant phytase obtained in this study had much higher affinity to the substrate compared to those from *P. pastoris* mutants with r-AppA, A131N/V134N/D207N/S211N, and A131N/V134N/C200N/D207N/S211N.[7] It was also similar to those from *A. fumigatus* WY-2 (114 μM) and *Aspergillus* thermostable phytases, r-PhyA86 and r-PhyA170 (0.14−0.16 mM).[31] However, both K_M values for Ca−phytate and Na−phytate were still higher than those for *A. fumigatus* phytase (*phyA*, 30 μM) and *Aspergillus ficuum* phytase (40 μM), which were expressed in *P. pastoris*.[37] Among the substrates (Na−phytate and Ca−phytate), the highest V_{max} was 8.20 mmol/min against calcium−phytate. In this study, the k_{cat}/K_M value of Na−phytate (891.92 mM^{-1} s^{-1}) was higher than that of Ca−phytate (863.43 mM^{-1} s^{-1}). The kinetic efficiency of an enzyme was validated by the k_{cat}/K_M values for a given substrate, which indicated that the substrate of Na−phytate has high affinity for recombinant phytase. The k_{cat}/K_M values for Ca−phytate (863.43 mM^{-1} s^{-1}) and Na−phytate (891.92 mM^{-1} s^{-1}) are slightly lower than that from the study by Golovan et al.[33] (1340 mM^{-1} s^{-1}, in which authors concluded that this is the highest value for the phytate-degrading enzyme).

Pepsin and Trypsin Tolerance Tests. When the purified phytase was incubated with pepsin at a protease/phytase (w/w) ratio of 0.002, about 70% of phytase activity was lost. However, more than 65% of activity was left when it was incubated with trypsin at the protease/phytase (w/w) ratios from 0.002 to 0.02 (Figure 6). It was interesting that the purified recombinant phytase revealed remarkable stability to proteolytic digestion with trypsin (Figure 6). A very high percentage of phytase activity remained undigested even after 2 h of incubation with a high ratio of 0.1 of protein/trypsin at 37 °C. These phenomena indicated that the resistance to pepsin and trypsin was different with the previous reports.[30,38]

Genetic Stability. According to the data obtained, the transformant of *P. pastoris* still maintained 99% of the plasmid stability even after 10 generations of expression. No significant difference in phytase activity was observed when the transformant was cultivated in YPD or YPD with Zeocin (Figure 7). This phenomenon indicated that the plasmids were highly stable during the incubation of the *P. pastoris* SMD1168H transformant at 30 °C in YPD medium. The genetic stability of the recombinant protein in this study was similar to that of *A. niger* SK-57 *phyA* gene with MF4I signal peptide expressed in *P. pastoris*, which had 98 and 97% of phytase yield and activity, respectively.[39]

Table 5. Kinetic Parameters of the Purified Recombinant Phytase

substrate	K_M (mM)	V_{max} (mmol/min)	k_{cat} (s^{-1})	k_{cat}/K_M (mM^{-1} s^{-1})
Na−phytate	0.26 ± 0.01	3.13 ± 0.11	231.90 ± 3.07	891.92 ± 7.89
Ca−phytate	0.70 ± 0.03	8.20 ± 0.19	604.40 ± 4.15	863.43 ± 3.26

dx.doi.org/10.1021/jf401853b | J. Agric. Food Chem. 2013, 61, 6007−6015

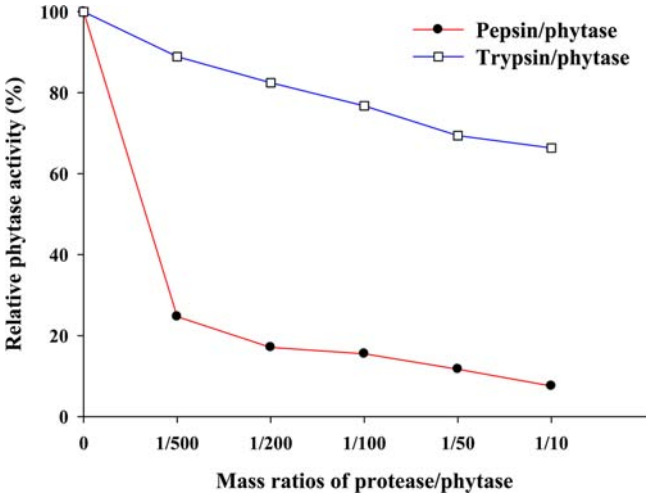

Figure 6. Residual activity of the recombinant *E. coli* phytase from the *P. pastoris* SMD1168H transformant after digestion with pepsin or trypsin at different protease/phytase (w/w) ratios.

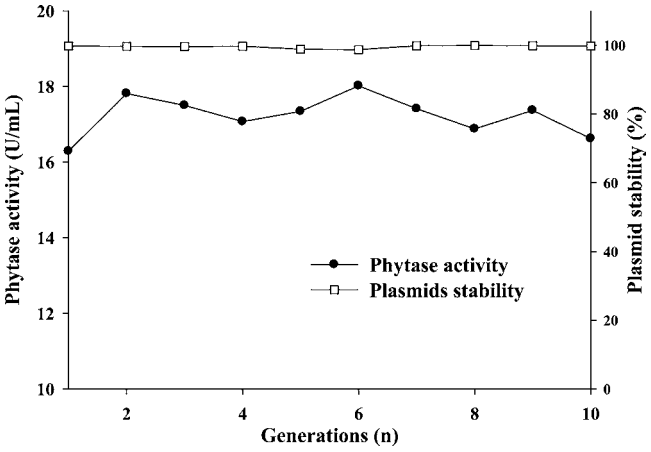

Figure 7. Changes in phytase activity and plasmid stability of broth during incubation of the *P. pastoris* SMD1168H transformant at 30 °C in YPD medium [plasmid stability (%) = (CFU of YPD broth with Zeocin/CFU of YPD broth without Zeocin) × 100%].

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Agarose gel analysis of the PCR-amplified DNA products from *E. coli* TOP10F′ phytase-pGAPZαC transformed colony (Supplemental Figure 1), restriction enzyme digestion analysis of the phytase-pGAPZαC expression vector (Supplemental Figure 2), and agarose gel analysis of the PCR-amplified DNA products from *P. pastoris* SMD1168H phytase-pGAPZαC transformed colony (Supplemental Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +886-4-26328001, ext. 15375. Fax: +886-943207241. E-mail: stjiang@pu.edu.tw.

Notes

The authors declare no competing financial interest.

■ REFERENCES

(1) Graf, E. Applications of phytic acid. *J. Am. Oil Chem. Soc.* **1983**, *60*, 1861−1867.

(2) Lei, X. G.; Porres, J. M. Phytase enzymology, applications, and biotechnology. *Biotechnol. Lett.* **2003**, *25*, 1787−1794.

(3) Greiner, R.; Farouk, A.; Carlsson, N.; Konietzny, U. *myo*-Inositol phosphate isomers generated by the action of a phytase from a Malaysian wastewater bacterium. *Protein J.* **2007**, *26*, 577−584.

(4) Lopez, H.; Leenhardt, F.; Coudray, C.; Remesy, C. Minerals and phytic acid interaction: Is it a real problem for human nutrition? *Int. J. Food Sci. Technol.* **2002**, *37*, 727−739.

(5) Haros, M.; Bielecka, M.; Honke, J.; Sanz, Y. *myo*-Inositol hexakisphosphate degradation by *Bifidobacterium infantis* ATCC 15697. *Int. J. Food Microbiol.* **2007**, *117*, 76−84.

(6) Sebastian, S.; Touchburn, S. P.; Chavez, E. R.; Lague, P. C. Efficacy of supplemental microbial phytase at different dietary calcium levels on growth performance and mineral utilization of broiler chickens. *Poult. Sci.* **1996**, *75*, 1516−1523.

(7) Rodriguez, E.; Wood, Z.; Karplus, P.; Lei, X. Site-directed mutagenesis improves catalytic efficiency and thermostability of *Escherichia coli* pH 2.5 acid phosphatase/phytase expressed in *Pichia pastoris*. *Arch. Biochem. Biophys.* **2000**, *382*, 105−112.

(8) Augspurger, N. R.; Webel, D. M.; Lei, X. G.; Baker, D. H. Efficacy of an *E. coli* phytase expressed in yeast for releasing phytatebound phosphorus in young chicks and pigs. *J. Anim. Sci.* **2003**, *81*, 474−483.

(9) Jorquera, M.; Martinez, O.; Maruyama, F.; Marschiner, P.; Mora, M. Current and future biotechnology applications of bacterial phytases and phytase-producing bacteria. *Microb. Environ.* **2008**, *23*, 182−191.

(10) Vohra, A.; Satyanarayana, T. A cost-effective cane molasses medium for enhanced cell-bound phytase production by *Pichia anomala*. *J. Appl. Microbiol.* **2004**, *97*, 471−476.

(11) Tomschy, A.; Tessier, M.; Wyss, M.; Brugger, R.; Broger, C.; Schnoebelen, L.; van Loon, A. P.; Pasamontes, L. Optimization of the catalytic properties of *Aspergillus fumigatus* phytase based on the three-dimensional structure. *Protein Sci.* **2000**, *9*, 1304−1311.

(12) Mullaney, E.; Daly, C.; Kim, T.; Porres, J.; Lei, X.; Sethumadhavan, K. Sitedirected mutagenesis of *Aspergillus niger* NRRL 3135 phytase at residue 300 to enhance catalysis at pH 4.0. *Biochem. Biophys. Res. Commun.* **2002**, *297*, 1016−1020.

(13) Lassen, S. F.; Breinholt, J.; Ostergaard, P. R.; Brugger, R.; Bischoff, A.; Wyss, M. Expression, gene cloning, and characterization of five novel phytase from four Basidomycete fungi: *Peniophora lycii*, *Agrocybe pediades*, a *Ceriporia* sp., and *Trametes pubescens*. *Appl. Environ. Microbiol.* **2001**, *67*, 470−407.

(14) Rodriguez, E.; Wood, Z. A.; Karplus, P. A.; Lei, X. G. Site-directed mutagenesis improves catalytic efficiency and thermostability of *Escherichia coli* pH 2.5 acid phosphatase/phytase expressed in *Pichia pastoris*. *Arch. Biochem. Biophys.* **2000**, *382*, 105−112.

(15) Onyango, E.; Bedford, M.; Adeola, O. The yeast production system in which *Escherichia coli* phytase is expressed may affect growth performance, bone ash, and nutrient use in broiler chicks. *Poult. Sci.* **2004**, *83*, 421−427.

(16) Chen, C. C.; Wu, P. H.; Huang, C. T.; Cheng, K. J. A *Pichia pastoris* fermentation strategy for enhancing the heterologous expression of an *Escherichia coli* phytase. *Enzyme Microb. Technol.* **2004**, *35*, 315−320.

(17) Sharp, P.; Tuohy, T.; Mosurski, K. Codon usage in yeast: Cluster analysis clearly differentiates highly and lowly expression genes. *Nucleic Acids Res.* **1986**, *14*, 5125−5143.

(18) Zhao, X.; Huo, K. K.; Li, Y. Y. Synonymous codon usage in *Pichia pastoris*. *Shengwu Gongcheng Xuebao* **2000**, *16*, 308−311.

(19) Huang, H.; Yang, P.; Luo, H.; Tang, H.; Shao, N.; Yuan, T.; Wang, Y.; Bai, Y.; Yao, B. High-level expression of a truncated 1,3−1,4-β-D-glucanase from *Fibrobacter succinogenes* in *Pichia pastoris* by optimization of codons and fermentation. *Appl. Microbiol. Biotechnol.* **2008**, *78*, 95−103.

(20) Ausubel, F. M.; Brent, R.; Kingston, R. E.; Moore, D. D.; Seidman, J. G.; Smith, J. A.; Struhl, K. *Current Protocols in Molecular Biology*; Greene Publishing Associates, Inc.: New York, 1993.

(21) Sambrook, J.; Fritsch, E. F.; Maniatis, T. In *Molecular Cloning: A Laboratory Manual*; Nolan, C., Ed.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.

(22) Zou, L. K.; Wang, H. N.; Pan, X.; Tian, G. B.; Xie, Z. W.; Wu, Q.; CHen, H.; Yang, Z. R. Expression, purification and characterization of a *phyA*^{m}*–phyCs* fusion phytase. *J. Zhejiang Univ., Sci., B* **2008**, *9*, 536–545.
(23) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(24) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.
(25) Neuhoff, V.; Arold, N.; Taube, D.; Ehrhardt, W. Improved staining of proteins in polyacrylaminde gel including isoelectric focusing gels with clear background at nanogram sensitivity using coomassie brilliant blue G-250 and R-250. *Electrophoresis* **1988**, *9*, 255–262.
(26) Zaghloul, T.; Abdelaziz, A.; Mostafa, M. High level of expression and stability of the cloned alkaline protease (aprA) gene in *Bacillus subtilis*. *Enzym. Microbiol. Technol.* **1994**, *16*, 534–537.
(27) Xiong, A.; Yao, Q.; Peng, R.; Zhang, Z.; Xu, F.; Liu, J.; Han, P.; Chen, J. High level expression of a synthetic gene encoding *Peniophora lycii* phytase in methylotrophic yeast *Pichia pastoris*. *Appl. Microbiol. Biotechnol.* **2006**, *72*, 1039–1047.
(28) Zou, L. K.; Wang, H. N.; Pan, X.; Xie, T.; Wu, Q.; Xie, Z. W.; Zhou, W. R. Design and expression of a synthetic phyC gene encoding the neutral phytase in *Pichia pastoris*. *Acta Biochim. Biophys. Sin.* **2006**, *38*, 803–811.
(29) Yang, M.; Johnson, S. C.; Murthy, P. P. Enhancement of alkaline phytase production in *Pichia pastoris*: Influence of gene dosage, sequence optimization and expression temperature. *Protein Expression Purif.* **2012**, *84*, 247–254.
(30) Fugthong, A.; Boonyapakron, K.; Sornlek, W.; Tanapongpipat, S.; Eurwilaichitr, L.; Pootanakit, K. Biochemical characterization and in vitro digestibility assay of *Eupenicillium parvum* (BCC17694) phytase expressed in *Pichia pastoris*. *Protein Expression Purif.* **2010**, *70*, 60–67.
(31) Promdonkoy, P.; Tang, K.; Sornlake, W.; Harnpicharnchai, P.; Kobayashi, R. S.; Ruanglek, V.; Upathanpreecha, T.; Vesaratchavest, M.; Eurwilaichitr, L.; Tanapongpipat, S. Expression and characterization of *Aspergillus* thermostable phytases in *Pichia pastoris*. *FEMS Microbiol. Lett.* **2009**, *290*, 18–24.
(32) Pandee, P.; Summpunn, P.; Wiyakrutta, S.; Isarangkul, D.; Meevootisom, V. A thermostable phytase from *Neosartorya spinosa* BCC 41923 and its expression in *Pichia pastoris*. *J. Microbiol.* **2011**, *49*, 257–264.
(33) Golovan, S.; Wang, G.; Zhang, J.; Forsberg, C. Characterization and overproduction of the *Escherichia coli* appA encoded bifuntional enzyme that exhibits both phytase and acid phosphatase activity. *Can. J. Microbiol.* **2000**, *46*, 59–71.
(34) Yanke, L. J.; Selinger, L.; Cheng, K. Phytase activity of *Selenomonas ruminantium*: A preliminary characterization. *Lett. Appl. Microbiol.* **1999**, *29*, 20–25.
(35) Oh, B. C.; Choi, W. C.; Park, S.; Kim, Y. O.; Oh, T. K. Biochemical properties and substrate specificities of alkaline and histidine acid phytases. *Appl. Microbiol. Biotechnol.* **2004**, *63*, 362–372.
(36) Kim, Y. O.; Lee, J. K.; Kim, H. K.; Yu, J. H.; Oh, T. K. Cloning of the thermostable phytase gene (phy) from *Bacillus* sp. DS11 and its overexpression in *Escherichia coli*. *FEMS Microbiol. Lett.* **1998**, *162*, 185–191.
(37) Ullah, A.; Sethumadhavan, K.; Lei, X.; Mullaney, E. Biochemical characterization of cloned *Aspergillus fumigates* phytase (phyA). *Biochem. Biophy. Res. Commun.* **2000**, *275*, 279–285.
(38) Rodriguez, E.; Mullaney, E. J.; Lei, X. G. Expression of the *Aspergillus fumigatus* phytase gene in *Pichia pastoris* and characterization of the recombinant enzyme. *Biochem. Biophys. Res. Commun.* **2000**, *268*, 373–378.
(39) Xiong, A. S.; Yao, Q. H.; Peng, R. H.; Han, P. L.; Cheng, Z. M.; Li, Y. High level expression of a recombinant acid phytase gene in *Pichia pastoris*. *J. Appl. Microbiol.* **2005**, *98*, 418–428.