Phytase Expressed by pIAβ8 and pGAPZαA Vectors and Analysis of its Biochemical Characters

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Abstract: Phytase and phytase gene from *Aspergillus ficuum* (*A. ficuum*) were used in this study. The results showed that phytase activity reached the peak of 0.17 U/g after 4 d incubation in solid medium for *A. ficuum*; the optimum pH and temperature of phytase were 2.5 and 50 °C, respectively. A 1.4-kb DNA containing the coding region of phytase gene was isolated and inserted into the expression vectors of pIAβ8 and pGAPZαA, which were transformed into *E. coli* (Top 10). The maximal phytase activities in the supernatant and cells were 2.31 and 9.04 U/ml for the *E. coli* with pIAβ8, 8.04 and 2.93 U/ml for the *E. coli* with pGAPZαA, respectively. It was concluded that the recombinant of pIAβ8-phytase could express intracellular phytase, while the recombinant of pGAPZαA-phytase could express extracellular phytase. The molecular weight of phytase protein was 54.61 kDa.

Key Words: Phytase, vectors, gene expression, biochemical characters.

INTRODUCTION

Many species of plants contain appreciable amounts of phytate [1]. Phytate is the major storage form of phosphorus and accounts for more than 80% of the total phosphorus in cereals and legumes [2]. Monogastric animals such as pig, poultry and fish, are not able to metabolize phytic acid or phytate. Phytic acid also acts as an anti-nutritional agent in feed by chelating other metal ions and nutrients needed by the animals [3]. Phytase could hydrolyze phytate phosphorus and release the phosphorus to be available for the animals. Although some feedstuffs contain natural phytase, it is not enough to hydrolyze phytic acid effectively. Because the microorganisms can produce phytase with low cost, the microbial phytase preparations become more and more important for its commercial application. There have been some reports of microbial phytase from a variety of microbial species, such as *A. ficuum, A. niger, A. fumigatus, A. terrus, A. nidulans, Myceliophthora thermophila, Talaromyces thermophila, Bacillus subtilis* and *E. coli,* etc [4-7]. Through analysis of phytase characters of the above microorganisms, the phytase from *A. ficuum* was found to be corresponding to the environment of animal's stomach, which was considered to be more effective for releasing phosphorous phytate in animal's stomach. Even though phytase gene transformation has been done in many microorganisms [5-7], the study on effective and applicable phytase gene expression still needs further exploration.

Our objectives were to determine the biochemical characters of phytase from *A. ficuum*, to compare the ability of phytase gene expression in *E. coli* by pIAβ8 and pGAPZαA vectors, and to choose the optimum vector for further researches.

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MATERIALS AND METHODS

Phytase Activity and Properties

A. ficuum (purchased from Institute of Microbiology, Chinese Academy of Sciences, Beijing, P. R. China) was incubated at 30 °C for 4 d in five 500 ml cone bottles with solid medium. The solid medium consisted of 90 g wheat bran and 10 g soybean meal added with 0.3 g $(NH_4)_2SO_4$ and 0.3 g NaCl which was dissolved in 120 ml deioned water, and then added into the solid medium. The medium was autoclaved at 121 °C for 15 min. Five grams were taken daily to estimate phytase activity. After 5th d incubation, the left culture was added with physiological saline (1:3), filtrated with four-fold gauze. The filtrate was centrifuged at 1500×g for 15 min. Phytase in the supernatant was extracted and purified by salting-out process, dialysis, laminar analysis. The purified phytase was used for thermostability and pH determination. The thermostability of phytase was estimated according to the following protocol: 4 ml were heated for 15 min at the following temperature levels: 30, 40, 50, 60, 70, 80, 90 and 100 °C. The tubes were put into ice immediately after 15 min reaction. The supernatant was adjusted to six different pH levels by HCl or NaOH additions (pH 1.0 2.0, 3.5, 5.0, 5.5, 6.5). The corresponding 1% sodium phytates at the different pH levels were also prepared respectively for estimating phytase activity. The specific activity of enzyme at the different pH and temperature levels was calculated as the following protocol: 0.5 ml sample was pre-warmed at 37 °C water bath for 5 min, mixed with 0.5 ml 1% sodium phytate (Dissolved in 0.2 M sodium citrate-citric acid buffer, pH 5.5), incubated at 37 °C for 15 min and then added with 1 ml of 15% trichloroacetic acid (TCA) to stop

1874-0707/09

the reaction. The blank samples were prepared by adding 0.5 ml sample and 1.0 ml TCA, and then mixed, 15 min later 0.5 ml sodium phytate was added. The absorbance against that of sample blank at 820 nm was measured. One phytase unit was defined as the activity that released 1 μmol of inorganic phosphorous from sodium phytate per minute [8].

Extract Genomic DNA from *A. ficuum*

A. ficuum was incubated at 30 ^{o}C for 3 d in cone bottles with PDA medium (6 g solvable starch, 2 g yeast extract, 5 g peptone, 20 g glucose, 2 g KH_2PO_4, 0.3 g $MgSO_4$, the volume was adjusted to 1 L with deioned water.). The extracted protocol is as follows: The culture medium was filtrated with sterilized four-fold gauze and filter paper, the biomass was put into mortar and grinded in liquid nitrogen sufficiently, and then the powder was kept in refrigerator at -20 ^{o}C. Weighed 50 mg sample, putted it into a 1.5 ml centrifugal tube and resuspended in 500 μl extraction solution (250 mM NaCl, 25 mM EDTA, 200 mM Tris-HCl, pH 8.5). Turned up and down for 10 min. Added 500 μl balanced phenol (the extraction solution and phenol (1:1) was mixed and kept standing for 5-10 min, and then the supernatant was discarded) and incubated for 5 min, and added 150 μl chloroform and incubated for another 5 min, centrifuged at 12000×g for 30 min at 4 ^{o}C. Added 25 μl buffer (20 mg/ml RNAse, 15 mM NaCl, 10 mM Tris-HCl, pH 7.5) in the supernatant and kept at 37 ^{o}C for 10 min. Added the same volume of chloroform and incubated for 5 min, centrifuged for 10 min. Added double volume isopropanol in the supernatant and kept at 4 ^{o}C for 1 h, centrifuged at 12000×g for 10 min. After removal of the aqueous phase, 1 ml of 70% ethanol was added, the sample was held at 4 ^{o}C for 1 h, centrifuged at 12000×g for 10 min. Removed the upper phase and resuspended in 100 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). At last, the genomic DNA was examined by agarose gel electrophoresis.

The Phytase Gene Cloning

Phytase gene (1.4 kb) used in the recombinant of pIAβ8-phyA was amplified from genomic DNA of *A. ficuum* by PCR with the following primers [9]. An *Xba*Ⅰsite at 5' end and a *Kpn*Ⅰsite at 3' end were added to the primers, respectively.

Upstream: 5'-ATGTCTAGACTGGCAGTCCCCGCCTCG AGA-3';

Downstream: 5'-CTAGGTACCCTAAGCAAAACACTCC GCCCAATC-3'.

The phytase gene used in the recombinant of pGAPZαA-phyA was amplified by PCR with the following primers. A *Kpn* Ⅰ site at 5' end and an *Xba* Ⅰ site at 3' end were added to the primers, respectively.

Upstream: 5'-ATGGGTACCCTGGCAGTCCCCGCCTCG AGA-3';

Downstream: 5'-CTATCTAGACTAAGCAAAACACTCC GCCCAATC-3'.

The PCR reaction mixtures contained 250 ng of genomic DNA as template, 100 pmole of each primer, 5 U of Ampli-Taq DNA polymerase, 12.5 Mm $MgCl_2$, 200 μmole each dNTP and the volume was adjusted to 50 μl with de-ionized water. The reaction was performed using the GeneAmp PCR system 2400 (Perkin Elmer, Norwalk, CT. USA). The thermal program consisted of 1 cycle at 94 ^{o}C (3 min), 30 cycles of 94 ^{o}C for 1 min, 60 ^{o}C for 1 min, and 72 ^{o}C for 2 min, 1 cycle of 72 ^{o}C for 10 min and stored at 4 ^{o}C. PCR products were purified with the Qiaquick PCR purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and were then introduced into *E. coli* using the pGEM-T vector (Promega, Madison, Wis. USA). The plasmids containing a unique insert of the appropriate size were purified by the QIAprep spin miniprep kit and were subjected to DNA sequence analysis. One microgram of purified plasmid was used for sequence analysis of the cloned DNA fragments.

Shuffle Vector Cassettes

The recombinant pGEM-T vectors was partially digested with *Xba* Ⅰ and *Kpn* Ⅰ to isolate phytase gene and then purified with the kits as above. The plasmids (pIAβ8 and pGAPZαA) were also partially digested with *Xba* Ⅰ and *Kpn* Ⅰ and purified with the kits, respectively. Ligase was used to construct the recombinant of pIAβ8 and pGAPZαA vectors. The recombinant shuffle vectors were inserted into *E. coli* by chemical method [10]. The antibiotics for selecting the positive colonies were ampicillin for pIAβ8 and zeocin for pGAPZαA, respectively.

Incubation and Phytase Activity Assays

Picked five colonies and incubated in LB medium for *E. coli* with the recombinant pIAβ8 containing 100 μg/ml ampicillin, and low salt LB (Peptone 10 g, yeast extract 5 g, NaCl 10 g, the volume was adjusted to 1 L with deioned water, pH 7.0) medium for *E. coli* with the recombinant pGAPZαA containing 100 μg/ml Zeocin at 37^{o}C for 5 d, respectively. Four ml of incubating bacterial liquid were taken daily to be centrifuged at 12000×g for 2 min. The supernatant was used for determining the extracellular phytase activity, and the cell was grinded by liquid nitrogen for determining the intercellular phytase activity. After grinding, 4 ml normal saline were added and mixed sufficiently. Another 4 ml incubating bacterial liquid were taken daily to be centrifuged for getting the cell precipitations, the cells were dried at 65 ^{o}C for 24 h to determine cell dry weight. The phytase activity assays were performed as the above protocol.

SDS-PAGE

To detect natural phytase protein from *A. ficuum* and to confirm the phytase expression in the transformed *E. coli*, the purified phytase from *A. ficuum* and the phytase from the supernatant and cells of the recombinant *E. coli* with pIAβ8-phyA and pGAPZαA-phyA vectors were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) for analysis.

Statistic Analysis

Experimental data were expressed as the means and standard errors. The data were analyzed using the ANOVA procedures of Statistical Analysis Systems Institute. Duncan's multiple range test was used to compare treatment means. Difference were considered statistically significant at $P<0.05$.

RESULTS

Enzymatic Properties of Phytase from *A. ficuum*

Phytase activity of *A. ficuum* slowly increased in the first 3 d solid incubation, reached the peak (0.17 U/g, P<0.05) at the 4th d, and then quickly decreased to 0.04 U/g (Fig. **1**). Fig. (**2**) showed that phytase activity increased with temperature increasing below 50 °C, and decreased beyond 50 °C (P<0.05). Fig. (**3**) indicated the maximal phytase activity was at pH 2.0 (P<0.05), slipped down at pH 3.5, and then increased to the second peak at pH 5.0 (P>0.05).

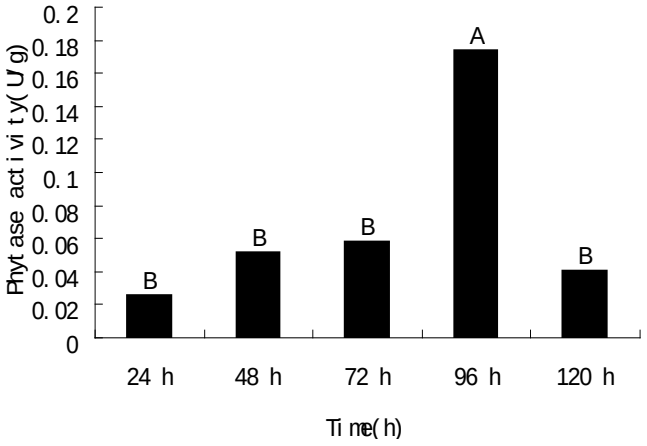

Fig. (1). Phytase activity changing with the incubating time for *A. ficuum*. Means (n=4) lacking a common letter differ (P<0.05).

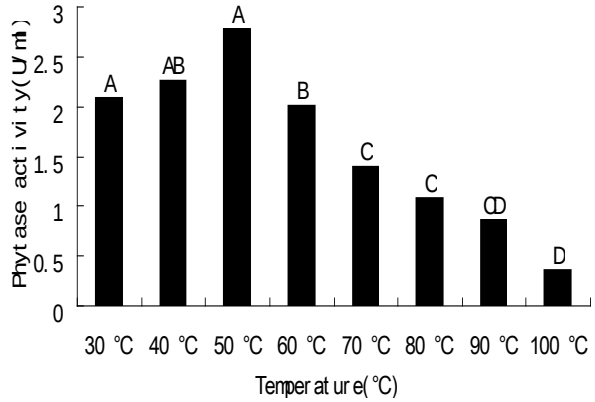

Fig. (2). The thermostabiliy of the phytase from *A. ficuum*. Means (n=5) lacking a common letter differ (P<0.05).

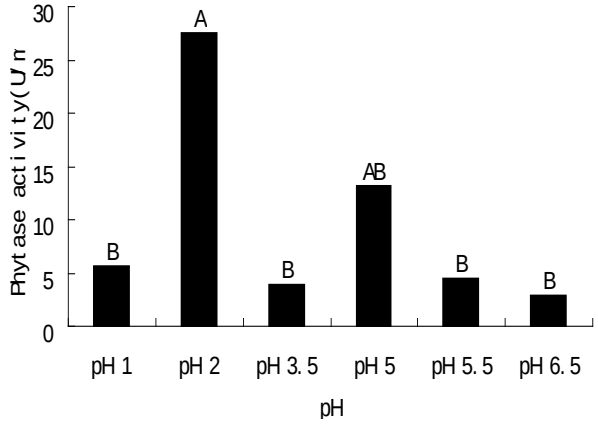

Fig. (3). The phytase activity at the different pH levels for *A. ficuum*. Means (n=5) lacking a common letter differ (P<0.05).

Phytase Gene Amplification and Construction of the Expression Vector

A 1.4 Kb phytase gene was amplified by PCR with the designed two pairs of primers. Then the pGEM-T vector and 1.4-kb DNA were ligated. The recombinants were screened with antibiotics and blue/ white blot test for positive clones, from which vectors were extracted. The nucleotide sequence of phytase gene was analyzed, and it has been deposited in the GenBank nucleotide sequence database (Ref. EF206311). The homologies of the gene nucleotide sequences and deduced amino sequences between the amplified gene products and the target sequence of phytase were 99.5% and 99.6%, respectively (Ref. AY013315).

The recombinants of pGAPZαA-phyA and pIAβ8-phyA were digested by *Xba* Ⅰ and *Kpn*, then the products were subject to electrophoresis. The result clearly showed 2 bands for pIAβ8-phyA (7.9 and 1.4 kb) and 2 bands for pGAPZαA-phyA (3.1 and 1.4 kb). Phytase genes (1.4 kb) were amplified from the incubating *E. coli* (used as template) transformed with pIAβ8-phyA and pGAPZαA-phyA, which indicated that the expected vectors were successfully expressed in *E. coli*.

Analysis of Expressed Phytase Activity

Fig. (**4**) showed that the phytase activity expressed by pIAβ8-phyA in the supernatant were significantly lower than that in bacterial cells (P<0.05); while the phytase activity expressed by pGAPZαA-phyA in the supernatant were much higher than that in bacterial cells (P<0.05). Fig. (**5**) indicated that phyatse activities in the dry cells of the transformed *E. coli* with pIAβ8-phyA and pGAPZαA-phyA had no significant difference (P>0.05). The natural purified phytase protein from *A. ficuum* and the phytase proteins in the cells and supernatant of the transformed *E. coli* were analyzed with SDS-PAGE. The bands of phytase proteins (54.61 kDa) were showed in Fig. (**6**), which indicated that phytase proteins could be produced and expressed by pIAβ8 and pGAPZαA in *E. coli*.

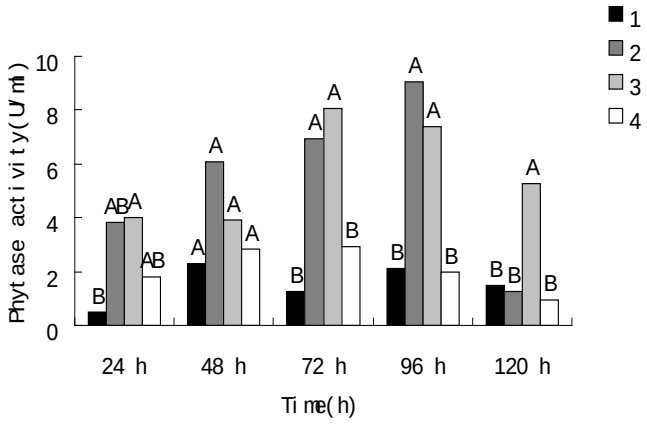

Fig. (4). Comparison of phyatse activities in the supernatant and cells of the transformed *E. coli* with pGAPZαA-phyA and pIAβ8-phyA. 1, supernatant of pIAβ8-phyA; 2, cells of pIAβ8-phyA; 3, supernatant of pGAPZαA-phyA; 4, cells of pGAPZαA-phyA. Means (n=4) lacking a common letter differ (P<0.05). After the cells were grinded in liquid nitrogen, normal saline was added to the cells to return to the original volume for comparing phytase activity at the same unit.

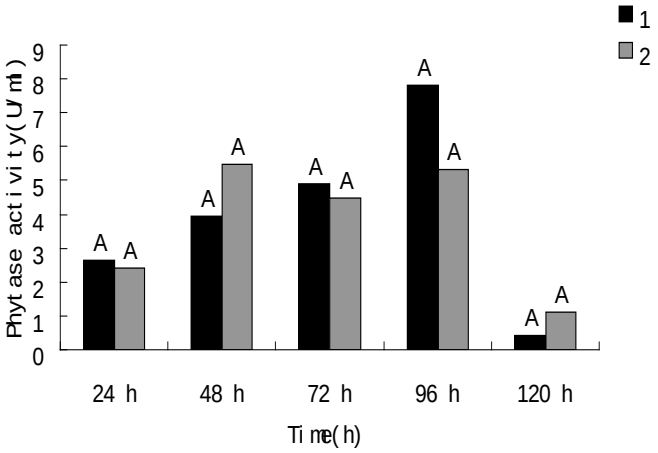

Fig. (5). Phyatse activities in the dry cells of the transformed *E. coli* with pIAβ8-phyA and pGAPZαA-phyA. 1, for pIAβ8-phyA; 2, for pGAPZαA-phyA. Means (n=4) lacking a common letter differ (P<0.05).

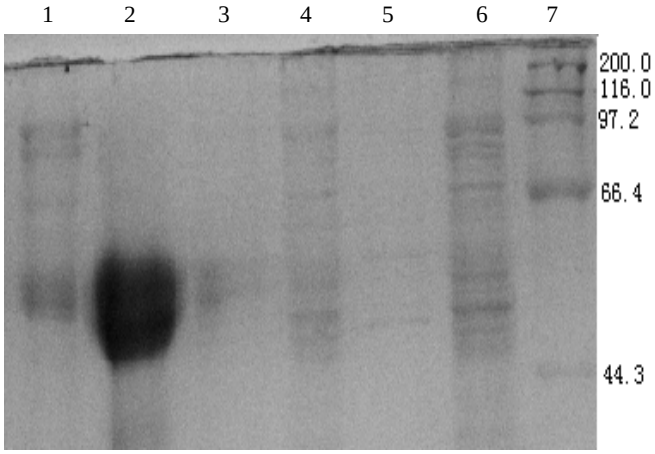

Fig. (6). Polyacrylamide gel electrophoresis of phytase protein stained with Coomassie Brilliant Blue. Lane 1 is the purified phytase from *A. ficuum* in this study, lane 2 is the commercial phytase of *A. ficuum*, lane 3 and 4 are the phytase proteins in the supernatant and cells of the *E. coli* with the recombinant pGAPZαA-phyA, lane 5 and 6 are the phytase proteins in the supernatant and cells of the recombinant pIAβ8-phyA, and lane 7 is the protein marker.

DISCUSSION

The highest phytase activity was obtained after incubation for 4 d because *A. ficuum* kept growing within 4 d, and then began to degenerate due to the insufficient nutrients. The physical and enzymatic characteristics of the purified phytase from *A. ficuum* in this study is similar to the phytase from *A. ficuum* NRRL3135 described by Simons [11]. Fig. (**3**) indicates that there was little phytase activity left at pH 1 and 6.5, the reason is that the protein structure of phytase is easily changed under the strong acidic or basic conditions to make phytase lose its activity. The optimum pH point of phytase from *A. ficuum* is in the pH range (pH 2.0-3.0) of stomach of the non-ruminant animals [12], so it can begin to hydrolyze phytate early when it is used in animal feed. This pH character of phytase makes it have potential commercial function. Every kind of enzyme has its own optimum temperature to show the highest enzyme activity due to its different structure and composition of protein. The responses of phytase activity to temperature in this study are similar to the former results to some extents. The earlier researches showed that the wild-type phytase retained 40% (50% in this study) of its activity after it was heated at 68°C for 10min [13], and the expressed phytase activity in soybean cells decreased largely when the temperature was above 63°C [14].

The molecular weights of phytase from fungi were 48.5 kDa without glycosylation [15], and 85-100 kDa with glycosalation [13]. It is the glycosylation that makes molecular weight of phytase bigger [16]. The molecular weight of the fungal phytase in this study (54.61 kDa) is within the range of the published results. It had the same molecular mass as that of the commercial phytase as determined by SDS-PAGE (Fig. **6**).

The phytase gene has been cloned successfully, and the selected clones were sequenced that have significant homology to each other as well as the target sequence of phytase. The deduced amino acid sequences have homology to those of phytase listed in the databases, which conserve the sequence in the active site of phytase [17]. By the comparison of the expressed phytase activity between pGAPZαA-phyA and pIAβ8-phyA in the supernatant of culture and the cells, it could be concluded that the recombinant of pGAPZαA-phyA and pIAβ8-phyA could secrete extracellular and intracellular phytase, respectively.

ACKNOWLEDGMENTS

This program was funded by National Natural Science Foundation of China (No. 30571346). Thanks to Biomedal Company of Spain and Invitrogen Company of USA to supply pIAβ8 and pGAPZαA plasmids kindly for this research.

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Received: September 17, 2008 Revised: January 13, 2009 Accepted: January 26, 2009