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Overexpression and Characterization of appA Phytase Expressed by Recombinant Baculovirus-Infected Silkworm

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Abstract An *Escherichia coli* strain with high phytase activity was screened from pig excreta. The phytase gene, *appA*, was amplified by PCR technique. To obtain large amounts of appA phytase, the *appA* gene was subcloned into the baculovirus transfer vector pVL1393 under the control of the *Polyhedrin* promoter. The recombinant baculovirus harboring the *appA* gene was obtained after co-transfection and screening. The early 5th instar larvae of silkworm were infected with the recombinant virus. Using this system, the appA phytase was overproduced up to 7,710 U per ml hemolymph. SDS-PAGE analysis revealed the baculovirus-derived appA phytase to be approximately 47 kDa in size. The optimal temperature and pH of the expressed phytase were 60°C and pH 4.5, respectively. The enzymatic activity was increased by the presence of 1 mM Ca^{2+}, 1 mM Mn^{2+}, or 0.02% Triton X-100.

Key words: *Escherichia coli*, appA, phytase, baculovirus, silkworm

Phytic acid (*myo*-inositol hexaphosphate) is the major storage form of phosphorus and accounts for more than 80% of the total phosphorus in cereals and legumes, which are the principal components of animal feeds [16]. Phytases (*myo*-inositol-hexakisphosphate phosphohydrolase) are a subclass of histidine acid phosphatase enzymes, which can efficiently cleave phytate into *myo*-inositol and inorganic phosphate [25]. Monogastric animals such as pigs and poultry virtually lack phytase activity in their digestive tracts; consequently, feed is commonly supplemented with inorganic phosphate to satisfy the phosphorus requirements. Furthermore, phytic acid is an antinutrient factor, since it can chelate proteins and a variety of metal ions and therefore depress utilization of these nutrients. The undigested phytate also results in phosphorus pollution [13].

The *Escherichia coli* phytase, appA, is an alternative enzyme with the highest catalytic efficiency for phytate among all the known phytases [13, 24]. The appA phytase also has an acidic pH optimum close to the physiological pH range of the stomach of pigs and chickens. It was demonstrated that appA phytase displayed higher affinity to sodium phytate and greater resistance to pepsin than the commercially available *A. niger* phyA [18]. The appA phytase was originally defined as a pH 2.5 acid phosphatase [3]. Greiner *et al.* [9] purified phytase from *E. coli*, but they did not present the DNA sequence of the gene to show if it was identical to the *appA* gene.

Although phytases are found naturally in microorganisms, the content of native phytase is too low. To overcome this major limiting factor, several different heterologous systems have been studied for expression of appA phytase, such as *E. coli* [7, 11] and yeast [17, 19, 21]. In transgenic mice models, the appA phytase was endogenously expressed in the parotid salivary glands and reduced fecal phosphorus by 11% [6]. Recombinant baculoviruses are widely used to express heterologous genes in cultured insect cells and insect larvae. For large-scale applications, the baculovirus expression vector system (BEVS) is particularly advantageous. But whether BEVS is suitable for producing recombinant appA phytase still remains unknown. In the present report, we described the overexpression of appA phytase by BEVS in silkworm larvae.

Materials and Methods

Enzymes and Chemicals

Restriction endonucleases, T4 DNA ligase, Platinum *pfx* polymerase, and lipofectin kit were purchased from Invitrogen (U.S.A.). DNA purification kit was obtained from Promega

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Corporation (U.S.A.). Proteinase K was purchased from Merck. Sodium phytate was from Sigma.

Virus, Cell Lines, and Silkworm Larvae

The parental baculovirus BmBacPAK6 was generously provided by Professor XF Wu (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences) (Chinese patent: 1242428). The Bm-5 cells were propagated at 27°C in TC-100 insect medium, supplemented with 10% heat-inactivated (56°C, 30 min) fetal bovine serum (Invitrogen). The hyperexpression variety of silkworm (JY1) was maintained in our lab. The silkworm larvae were reared at the temperature of 25–26°C and the humidity of 70%–90%. The fresh mulberry leaves, with good enough quality to meet the preference of silkworm, were fed 3 times a day throughout this investigation [12].

Screening of *Escherichia coli* Strains with High Phytase Activity

Newly excreted feces were collected from sows that were locally fed on silages instead of compound feed. After dilution with sterilized water, samples were spread on LB plates. Then, 120 colonies were picked up and cultured, and the harvested cells were disrupted by sonication for phytase activity assay. Genomic DNA of the strain with the highest phytase activity was extracted [20].

Construction of Plasmids

Two primers were designed according to the *appA* gene reported by Dassa *et al.* [4] (GenBank Accession No: M58708) to amplify the *appA* gene from the genomic DNA of the screened *E. coli* by PCR (PTC-100^{TC}, MJ Research Inc.). 5'-GAGGATCCACGATGAACGATCTT-AATCCCAT-3' (Forward), 5'-TTGAATTCATTACAAAC-TGCACGCCGGTAT-3' (Reverse) (the *Eco*RI and *Bam*HI sites are shown in underlines), respectively. PCR was carried out using high fidelity DNA polymerase (Platinum *pfx*) according to the manufacturer's instructions. The PCR product was separated on agarose gel and purified by a DNA purification kit. After digestion with *Eco*RI and *Bam*HI, the fragment was inserted into pGEM-3Z digested by the same enzymes. The recombinant plasmid was sequenced to confirm that no insertion or deletion mutation had occurred during the PCR. The confirmed *appA* gene was excised from pGEM3Z-appA by *Eco*RI and *Bam*HI digestion and subcloned into the baculovirus transfer vector pVL1393 to generate the end plasmid pVL-appA.

Generation of Recombinant Baculovirus

BmBacPAK6, a genetically modified *Bombyx mori* nucleopolyhedrovirus (BmNPV), similar to *Autographa californica* multinucleocapsid nucleopolyhedrovirus (AcMNPV) BacPAK6 [5], was employed as the parental virus in this study. After the BmBacPAK6 viral DNA was digested with *Bsu*36I, the linearized viral DNA and the transfer plasmid DNA containing the *appA* gene were co-transfected into Bm-5 cells, mediated by Lipofectin following the manufacturer's protocol. After incubation at 27°C for 4–5 d, the co-transfection supernatant was subjected to plaque assays to screen individual viral plaques. PCR amplification was conducted to confirm whether the *appA* gene had incorporated into the viral genome. Primers were designed as follows: Sense, 5'-ACTGTTTTCGTAACAGT-TTTGTAA-3' and appA Reverse, 5'-TTGAATTCATTACA-AACTGCACGCCGGTAT-3'. Another 2 rounds of purification were performed, and the pure recombinant virus was used to generate high titer viral stocks for protein expression.

Expression of appA in Silkworm Larvae

Early fifth-instar silkworm larvae (JY1 variety) were used for expression studies. Each larva was injected with 1×10^5 PFU recombinant baculovirus and reared for 4–5 days until the hemolymph was collected by puncturing the abdominal legs. The hemolymph samples were either ready to use or stored at −20°C until analyzed.

SDS-PAGE Analysis

The properly diluted hemolymph samples were separated on 10% SDS-PAGE gel (with 5% stacking gel) as described by Sambrook *et al.* [20]. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250.

appA Phytase Activity Assay

For the phytase activity assays, 100 μl diluted sample was added to 900 μl 4 mM sodium phytate in 0.1 M sodium acetate buffer (pH 4.5). The reaction was incubated for 30 min at 37°C, and then stopped by the addition of 1 ml 10% (w/v) trichloroacetic acid. Inorganic phosphate was measured by the addition of an equal volume of freshly prepared phosphomolybdate color reagent containing 7.3% $FeSO_4{\cdot}7H_2O$ (w/v), 1% $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ (w/v), and 3.2% H_2SO_4 (v/v). Following a 5 min color development, absorbance was measured at 750 nm with a Shimazu UV-260 spectrophotometer. One unit (U) of phytase was defined as the amount of enzyme required to liberate 1 μmol inorganic phosphate per min under the given assay conditions. All activity assays were carried out in triplicate.

Determination of Temperature, pH Optima, and Thermostability

To determine the temperature optimum of expressed appA, phytase activity assays were carried out over a temperature range of 10 to 90°C in 0.1 M sodium acetate at pH 4.5. To determine the optimal pH value of the appA phytase, activity assays were performed at 37°C in different buffers with pH varied from 1.2 to 9.0. The four different buffers were as follows: 0.25 M Gly-HCl (pH 1.2–3.5), 0.25 M NaAc-HAc (pH 4–6), 0.25 M Tris-HCl (pH 6–7), and

0.1 M Tris-HCl (pH 6–9). To assess the thermostability of the baculovirus-derived appA phytase, samples were dissolved properly in 0.1 M sodium acetate (pH 4.5). After incubation for 20 min in a water bath at 40, 45, 55, 60, 65, 70, 75, 80, and 90°C, the enzyme samples were allowed to renature for 30 min at room temperature. Each sample was diluted 1:80 in 0.1 M sodium acetate buffer (pH 4.5) before standard activity assays were carried out.

Modulation of Phytase Activity by Ions or Triton X-100
Activity assays were performed in the presence of several ions at the concentration of 1 mM or Triton X-100 at the concentration of 0.02%, 0.1%, 0.5%, and 1.5%, respectively. Except the presence of ions or Triton X-100 in the reaction system, the appA phytase activity assays were carried out under the standard assay conditions mentioned above.

RESULTS

Cloning of the *appA* Gene
Upto 120 *E. coli* colonies were tested for the presence of phytase activity and colony 13 exhibited the highest phytase activity. Using genomic DNA of colony 13 as a template, the *appA* gene was amplified by PCR. The predicted 1.3 kb PCR fragment was separated and cloned into the plasmid pGEM-3Z. DNA sequencing of the cloned *appA* gene showed an open reading frame of 1,299 bp (GenBank accession No. AF537219). The *appA* gene was 99.8% identical with the sequence reported by Dassa *et al.* [4] and only two nucleotides were different. One of these differences was a change from G to A at position 721. This transition also affected the amino acid sequence in which the Glu was replaced by Lys. The second sequence difference was at position 835 (changing from G to A) and was a silent mutation. The deduced amino acid sequence of the appA phytase was composed of 432 amino acids with a molecular mass of 47.06 kDa, predicted by DNAStar. It also contained the active-site motif RHGXRXP (the X could be any amino acid), which was shared by other known phytases and acid phosphatases except phyC [10, 23]. Three potential glycosylation sites were found in the deduced amino acid sequence.

Isolation of Recombinant Baculovirus
The baculoviral transfer plasmid pVL-appA was co-transfected with linearized BmBacPAK6 DNA into Bm-5 cells. The linearized BmBacPAK6 lacked an essential gene for viral propagation, and co-transfecting linearized BmBacPAK6 with a baculoviral transfer vector rescued the virus. More than 90% virus was recombined and further represented in plaque assay. Recombinant virus was also confirmed by PCR. The forward primer was designed according to the -40 nucleotide with respect to the initial translational site of the *Polyhedrin* gene. Fragments of expected size were all amplified from genomic DNA of three independent isolates. After another 2 rounds of purification, the pure virus clone was obtained.

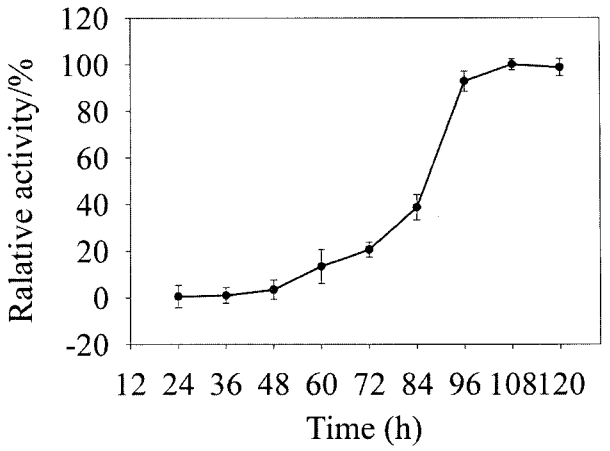

Fig. 1. The time course of appA phytase expression in silkworm. Larval hemolymph was collected on ice every 12 h from 24 h post-infection. The amount of expressed appA phytase was represented by the relative activity at the corresponding time.

High Expression Level in Silkworm Larvae
Hemolymph of silkworm larvae was harvested on ice every 12 h from 24 h post-infection (h.p.i.). Although a small

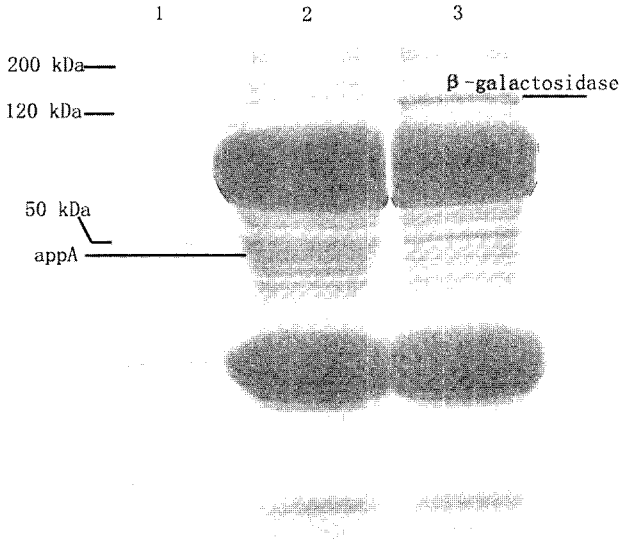

Fig. 2. SDS-PAGE analysis of the BEVS-derived appA phytase. Lane 1, 10 kDa standard protein ladder; lane 2, the silkworm hemolymph with expressed appA phytase; lane 3, hemolymph of silkworm infected with BmBacPAK6.

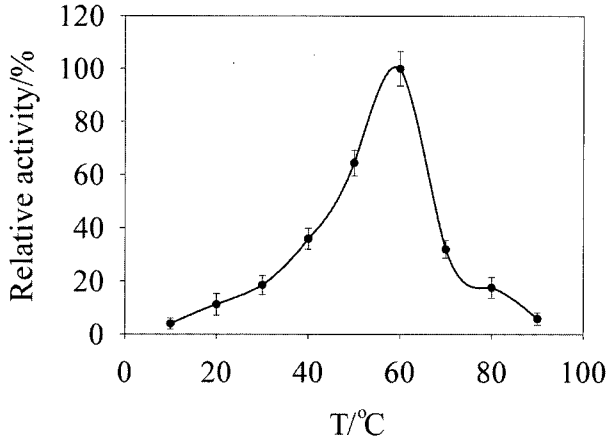

Fig. 3. The temperature profile of the expressed appA phytase.
The enzymatic activity of appA phytase expressed in silkworm was measured at a series of temperatures between 10 and 90°C. The optimal temperature for enzymatic reaction was 60°C.

quantity of appA phytases were expressed before 72 h, the accumulation of recombinant product was dramatically increased during the late phase of infection (Fig. 1). Up to 7,710 U of the appA phytase activity was detected in 1 ml hemolymph, which was harvested at 108 h.p.i. As indicated in Fig. 2, the appA phytase was a 47 kDa band, with a remarkable increment of protein amount represented on the gel. The hemolymphs of BmBacPAK6-infected silkworms that expressed β-galactosidase were collected and subjected to SDS-PAGE analysis as control. The molecular mass of expressed appA phytase was in good accordance with prediction.

Enzymatic Characterization of appA Phytase

The temperature optimum and pH optimum of appA phytase were recorded at 60°C and pH 4.5, respectively

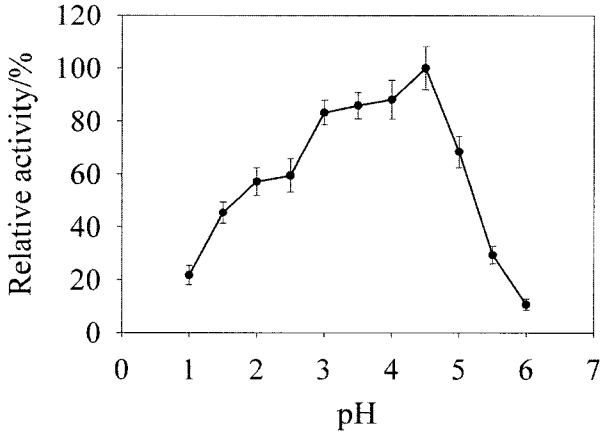

Fig. 4. The pH profile of the BEVS-derived appA phytase.
The enzymatic reactions were carried out at different pH values from 1.2 to 9.0. Representative results are shown. The optimal pH for enzymatic reaction was 4.5.

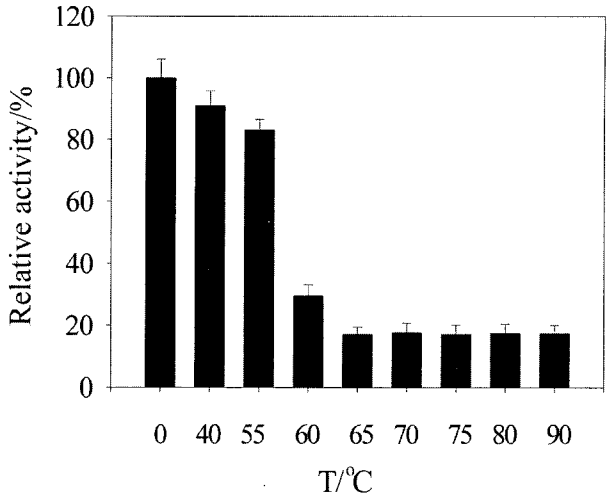

Fig. 5. Thermostability analysis of the expressed appA phytase.
Samples were incubated for 20 min at temperatures ranging over 40–90°C. The enzyme samples were allowed to renature for 30 min at room temperature and then stored at 0°C until standard activity assays were performed. Samples incubated at 0°C were carried out as the control.

(Fig. 3; Fig. 4). More than 60% phytase activity remained in a pH ranged from 2 to 5, which suggested that the expressed appA might function well in the digestive tracts of monogastric animals. There was a sharp drop in the appA phytase activity from pH 5 to 6 (Fig. 4). In a thermostability assay, about 30% activity remained when the enzyme was pre-incubated at 60°C for 20 min. The phytase activity was low and constant at temperatures from 65°C to 90°C (Fig. 5). The appA phytase activity was increased 27% in the presence of 1 mM Mg^{2+}, while 76% remained in the presence of 1 mM Zn^{2+} (Fig. 6). In some cases, phytase activity could be strongly enhanced by Triton X-100 [7, 8]. In this report, Triton X-100 at a concentration of 0.02% enhanced catalytic activity by 18% (Fig. 6).

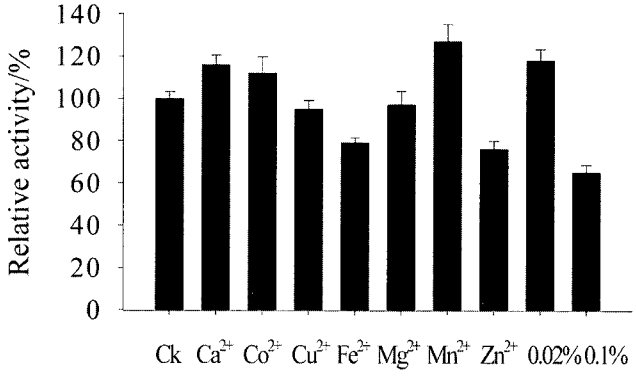

Fig. 6. The modulation effects of various ions or Triton X-100 on the activity of the BEVS-derived appA phytase.
All ions were at a concentration of 1 mM; 0.02% and 0.1% indicate the concentrations of Triton X-100 (v/v). Enzymatic activity of the non-additive samples was performed as the control.

DISCUSSION

Phytase, as a feed supplement to poultry and swine diets, is a substance of growing interest, and it has created a potential $500 million market [1]. Although several groups have expressed appA in other heterologous systems, using insect BEVS, to our knowledge, these have not been reported. The most distinguishing feature of the silkworm-BEVS is its potential to express foreign genes with high levels. We demonstrated here that it is an alternative approach to express large-scale appA phytase in silkworm. There are three potential N-glycosylation sites in the appA phytase. The enzyme expressed in *Pichia pastori* was over-glycosylated [19, 21]. In this report, by the indication of the molecular mass, there was no glycosylation presented in the expression product. It implies that less influence would occur in biochemical characters with respect to the native enzyme.

Two of the most common isolates used in foreign gene expression are AcMNPV and BmNPV. Although both the AcMNPV and BmNPV systems provide high-level expression of foreign genes using larval hosts [14, 15], the silkworm larvae have several additional advantages, such as being easy to rear and manipulate, short life cycle as well as nonpathogenicity to any vertebrate host, which also means few treatment are needed to produce appA phytase as a feed supplement.

The phytase derived from *Aspergillus niger* is currently used in the livestock industry. The *A. niger* phytase, phyA2, was expressed by BEVS using silkworm hosts and the expression level was 177-235 U/ml in hemolymph [22]. According to the previous studies, the phytase activities of purified appA and phyA2 were 3,615 U/mg [7] and 150 U/mg [26], respectively. In the present investigation, the expression level of the *E. coli* phytase appA was up to 7,710 U/ml in silkworm hemolymph. To some extent, the high level of activity was attributed by the highest catalytic efficiency of appA phytase.

More than 60% of phytase activity remained at a pH ranged from 2 to 5, which suggested that the expressed appA might function well in the digestive tracts of monogastric animals. The appA phytase expressed by *Schizosaccharomyces pombe* was also demonstrated to be not genotoxic or to have any *in vivo* toxicity to monogastric animals [2]. Due to the favorable enzymatic characteristics of appA phytase and its highest catalytic ability among the phytases known to date, baculovirus-derived appA phytase seems to be a valuable candidate for the application of feed supplement.

Acknowledgments

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