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Properties of beta-propeller phytase expressed in transgenic tobacco

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Abstract

Phytases are enzymes that liberate inorganic phosphates from phytate. In a previous study, a beta-propeller phytase (*168phyA)* from *Bacillus subtilis* was introduced into transgenic tobacco, which resulted in certain phenotypic changes. In the study described herein, the recombinant phytase (t168phyA) was purified from transgenic tobacco to near homogeneity by a three-step purification scheme. The biochemical properties and kinetic parameters of t168phyA were compared with those of its counterpart from *B. subtilis.* t168phyA was glycosylated, and it showed a 4 kDa increase in molecular size in SDS–PAGE (44 kDa vs. 40 kDa). Although its thermostability remained unchanged, its temperature optimum shifted from 60 °C to 45–50 °C and its pH optimum shifted from pH 5.5 to 6.0. Kinetic data showed that the t168phyA had a lower K_{cat}, but a higher K_m than the native enzyme. Despite these changes, t168phyA remained catalytically active and has a specific activity of 2.3 U/mg protein. These results verify the activity of recombinant *Bacillus* phytase that is expressed in plants.

Keywords: *Bacillus*; Beta-propeller phytase; Tobacco; Phytate; Phytic acid

Phytate is the main form of phosphorus storage in plant seeds. It is an anti-nutrient for monogastric animals, a major source of phosphorus pollution in animal manure [1], and a major form of organic phosphorus in soil [2]. Phytases are enzymes that hydrolyze phytate to produce lower inositol phosphates and inorganic orthophosphate. Based on their structures and catalytic mechanisms, phytases are classified into four families: histidine acid phosphatases (HAPs),[1] purple acid phosphatases (PAPs), beta-propeller phytases (BPPs) [3], and a novel phytase from *Selenomonas ruminantium* [4].

HAPs, which share an active site motif, RHGXRXP [5], have been found in fungi [6,7], bacteria [8], and plants [9]. The most extensively studied HAP is *phyA* from *Aspergillus niger* [6]. It has been used as a feed additive to help monogastric animals to utilize dietary phytate and to reduce the fecal excretion of phosphorus [10]. Extensive studies have been conducted on the expression of *phyA* in transgenic plants, including tobacco [11–14], alfalfa [15], canola [16], and *Arabidopsis thaliana* [17]. PAPs are non-specific phosphomonoesterases with dinuclear Fe(III)–Me(II) centers (where the Me can be Fe, Zn, or Mn) at their active sites. They show broad substrate specificity and have acidic pH optima. Most of the characterized PAPs do not exhibit phytase activity, with only two exceptions: the *Gmphy* from soybean [18] and a wheat PAP [19]. These are the only two known PAPs that show significant activity against phytic acid.

To date, all reported BPPs have been of *Bacillus* origins. The first *Bacillus* phytase was discovered by Powar et al. [20] in *Bacillus subtilis.* Later, phytase from *B. subtilis* (natto) N-77 [21], *B. subtilis* strain VTT E-68013 [22], *Bacillus amyloliquefaciens* DS11 [23], *Bacillus* sp. KHU-10 [24], *B. subtilis 168*, and *Bacillus licheniformis* [25] were also characterized. BPPs have structures of a six-bladed propeller, with each blade made up of

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[1] *Abbreviations used:* HAPs, histidine acid phosphatases; PAPs, purple acid phosphatases; BPPs, beta-propeller phytases; PMSF, phenylmethylsulfonyl fluoride.

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beta-sheets [26]. They require Ca^{2+} ions for their stability and activity, and are very specific toward phytic acid [27,28]. Unlike HAPs and PAPs, which have acidic pH optima, BPPs have high activity over the neutral pH range and also exhibit high thermostability [23–25]. These properties make BPP an interesting candidate for transgenic plant studies because the pH value of plant cells and plant growth environments range from slightly acidic to neutral. In our laboratory, transgenic tobacco lines that expressed *168phyA* from *B. subtilis 168* intracellularly [29] and extracellularly [30] have been generated. The intracellular expression lines showed decreases in seed phytate content, increases in numbers of flowers and fruit, improved growth performance with phosphate starvation, and the "small seed syndrome" [29], and the transgenic lines that secreted phytase were able to assimilate exogenous phytate [30]. However, the biochemical and kinetic properties of the *Bacillus* phytase that is expressed in tobacco have not been determined. In the study described herein, the recombinant phytase from transgenic tobacco was purified, characterized, and compared with its bacterial counterpart.

Materials and methods

Materials

All enzymes used for molecular biology were obtained from Roche Diagnostics (Hong Kong, China) or Promega (Hong Kong, China), and all other chemicals were purchased from Sigma (St. Louis, USA).

Purification of recombinant phytase from tobacco line A3SP-4

Transgenic tobacco (*Nicotiana tabacum*) line A3SP-4 was used as the plant source [30]. The leaves were first ground in liquid nitrogen into a fine powder. Then, total soluble protein was extracted with chilled 100 mM Tris–HCl buffer, pH 7.0, supplemented with 0.1 mM $CaCl_2$ and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein extract was then precipitated with ethanol at −20 °C for 2 h. The precipitates were redissolved into and dialyzed against 20 mM Tris–HCl, pH 8.5, with 1 mM $CaCl_2$. The dialyzed sample was loaded into a Hi-trap Q column (FPLC System, Pharmacia), which was equilibrated with 20 mM Tris–HCl, pH 8.5, with 1 mM $CaCl_2$. The protein was eluted with a linear salt gradient from 0 to 0.5 M NaCl. The active fractions were pooled and then loaded into a Superose 12 HR 10/30 column (FPLC System, Pharmacia) that was equilibrated with 100 mM Tris–maleate, pH 7.0, with 1 mM $CaCl_2$ and 150 mM NaCl. A flow rate at 0.5 ml per minute was maintained for 60 min. 0.5 ml fractions were collected and assayed for both phytase activity and protein content.

Gel electrophoresis and Western blot analysis

The purified samples were fractionated by SDS–PAGE and visualized by silver stain [31]. To confirm the identity of the proteins, Western blotting was conducted using a rabbit polyclonal antiserum raised against native *Bacillus* phytase [30].

Phytase activity assay

Phytase activity assays were carried out in 100 μl 100 mM Tris–maleate, pH 7.0, 1 mM $CaCl_2$ at 37 °C for 30 min using 1 mM sodium phytate (Sigma Cat. No. P3168) as substrate. The reaction was stopped by adding 100 μl cold 4% (v/v) TCA. The liberated inorganic orthophosphates were quantified spectrophotometrically by the molybdate-blue reaction [32]. One unit (U) of phytase activity was defined as the amount of enzyme that was required to produce 1 μmol of phosphate per minute under the described assay conditions. The kinetic constants were determined using 0.2, 0.4, 0.6, 0.8, and 1.0 mM sodium phytate as substrate and calculated from the Lineweaver–Burk plots of the data.

Protein assay

The protein concentration of the samples was determined by the standard Bradford protein assay using the Protein Assay Dye Reagent Concentrate (Bio-Rad, Hong Kong), according to the manufacturer's protocol.

Immunoblot detection for glycoproteins

The Immun-Blot Kit for glycoprotein (Bio-Rad, Hong Kong) was used to determine whether the purified phytase from tobacco was glycosylated. The samples were first subjected to SDS–PAGE and then blotted onto a Hybond C Extra nitrocellulose membrane. After being washed with 1× PBS twice for 5 min, the membrane was immersed in the dark in reagent A (21.4 mg periodate in 10 ml of 100 mM sodium acetate/EDTA buffer) for 20 min with agitation. The membrane was then washed three times with 1× PBS before immersing in the biotinylation solution, which was prepared by adding 2 μl reagent B (hydrazide in dimethylformamide) to 10 ml sodium acetate/EDTA solution. After incubating for 60 min at room temperature, the membrane was washed three times with 1× TBS and then immersed in the blocking solution (1% (w/v) BSA in TBS supplemented with 0.5% (v/v) Tween 20) for 1 h. After washing three times with TBS, streptavidin–alkaline phosphatase conjugate was applied to the membrane for 1 h. Finally, after washing with TBS, the development solution (BCIP/NBT) was added for color detection. A negative control that omitted periodate in reagent A was also conducted.