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| Title | Properties of beta-propeller phytase expressed in transgenic tobacco |
| Author(s) | Chan, WL; Lung, SC; Lim, BL |
| Citation | Protein Expression And Purification, 2006, v. 46 n. 1, p. 100-106 |
| Issued Date | 2006 |
| URL | http://hdl.handle.net/10722/54343 |
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Title: Properties of beta-propeller phytase expressed in transgenic tobacco

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Number of text pages: 20

Number of tables: 3

Number of figures: 6

Abstract

Phytases are enzymes that liberate inorganic phosphates from phytate. In a previous study, a beta-propeller phytase (*I68phyA*) 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 *Bacillus subtilis*. t168phyA was glycosylated, and it showed a 4kDa increase in molecular size in SDS-PAGE (44kDa vs. 40kDa). 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 kcat, but a higher Km 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.

Key words: *Bacillus*, Beta-propeller phytase, tobacco, phytate, phytic acid

Introduction

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), purple acid phosphatases (PAPs), beta-propeller phytases (BPPs) [3], and a novel phytase from *Selenomonas ruminantium* [4].

HAPs, which share an active site motif, RHGXRX [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 of the expression of *phyA* in transgenic plants, including tobacco [11-14], alfalfa [15], canola [16], and *Arabidopsis thaliana* [17]. PAPs are nonspecific 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. in 1982 in *Bacillus subtilis* [20]. Later, phytase from *Bacillus subtilis* (natto) N-77 [21], *Bacillus subtilis* strain VTT E-68013 [22], *Bacillus*

amyloliquefaciens DS11 [23], *Bacillus* sp. KHU-10 [24], *Bacillus subtilis* 168, and *Bacillus licheniformis* [25] were also characterized. BPPs have structures of a six-bladed propeller, with each blade made up of 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 *Bacillus 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 has 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 100mM Tris-HCl buffer, pH 7.0 supplemented with 0.1 mM CaCl₂ and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein extract was then precipitated with ethanol at -20°C for 2 hours. The precipitates were redissolved into and dialyzed against 20mM Tris-HCl pH8.5 with 1mM CaCl₂. 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 CaCl₂. 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 100mM Tris-Maleate pH 7.0 with 1 mM CaCl₂ and 150 mM NaCl. A flow rate at 0.5 ml per minute was maintained for 60 minutes. 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 μ l 100 mM Tris-Maleate pH 7.0, 1 mM CaCl₂ at 37°C for 30 minutes using 1 mM sodium phytate (Sigma cat. no. P3168) as substrate. The reaction was stopped by adding 100 μ l 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 μ mol 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 1X PBS twice for 5 minutes, the membrane was immersed in the dark in reagent A (21.4 mg periodate in 10ml 100 mM sodium acetate/EDTA buffer) for 20 minutes with agitation. The membrane was then washed three times with 1X PBS before immersed in the biotinylation solution, which was prepared by adding 2 μ l reagent B

(hydrazide in dimethylformamide) to 10 ml sodium acetate/EDTA solution. After being incubated for 60 minutes at room temperature, the membrane was washed three times with 1X TBS and then immersed in the blocking solution (1% (w/v) BSA in TBS supplemented with 0.5% (v/v) Tween 20) for 1 hour. After washing three times with TBS, streptavidin-alkaline phosphatase conjugate was applied to the membrane for 1 hour. 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.

Results

Expression level of t168phyA in tobacco leaves

168phyA from *Bacillus subtilis 168* was expressed in tobacco leaves. Table 1 summarizes the phytase activity in the leaves of the transgenic tobacco A3SP-4. No phytase activity was detected in the control plants. Therefore, all of the detected phytase activity was contributed by the phytase transgene. The phytase that was expressed in tobacco was named t168phyA, while that expressed in *Bacillus subtilis* was referred to as b168phyA for easy discrimination.

Purification of t168phyA from transgenic tobacco

The purification was achieved by ethanol precipitation, anion exchange chromatography, and gel filtration. For the anion exchange chromatography, the target protein was eluted from the column with 40-80 mM sodium chloride. The results of the purification are shown in Table 2. The purified phytase after the gel filtration gave a specific activity of 2.3 U/mg of protein. The overall yield was 5.2% with a 287 times increase in specific activity. The percentage of t168phyA in total soluble protein was determined based on the specific activity of t168phyA, and was found to be 0.4% (w/w) of total soluble protein.

Purity, Molecular Weight and Glycosylation

In SDS-PAGE (Fig. 1A), the t168phyA had an apparent molecular size of ~44kDa, and the b168phyA was ~40kDa. This showed that the t168phyA was approximately 10% larger in molecular size than the b168phyA under denaturing. The identities of the t168phyA and b168phyA were verified by a specific antiserum against native *Bacillus* phytase (Fig. 1B).

The results of glycoprotein detection (Fig. 2) showed that only t168phyA could be oxidized by periodate and give positive results, whereas no band was observed from b168phyA and the non-oxidized controls. The results indicated that t168phyA was glycosylated, and this was one of the reasons for its size increment. Three potential N-glycozylation sites, Asn 96, Asn 129, and Asn 256, were present in the coding sequence. One or more of these sites might have been glycosylated when the enzyme was expressed in tobacco.

Biochemical characterization of t168phyA from transgenic tobacco

pH optima As shown in figure 3, b168phyA had highest activity at pH 5.5-6.0, and t168phyA gave considerably higher activity at pH 6.0. Moreover, the shapes of the pH profiles were different. b168phyA performed well over a broad pH range as it maintained over 80% activity from pH 5.5-8.5 and retained over 60% activity at pH 9.0. However the pH profile of t168phyA was dumbbell shaped, with the highest activity at pH 6.0 before gradually decreasing in activity from pH 6.5-8.5. No activity was detected at pH 9.0.

Temperature optima t168phyA showed a downward shift in temperature optima when compared to b168phyA (Fig. 4). t168phyA had the highest activity at temperatures of 45-50°C, and b168phyA gave highest activity at 60°C. Moreover, t168phyA showed no activity at temperatures higher than 65°C, but b168phyA remained active up to 80°C. Although t168phyA lost most of its activity at high temperatures, t168phyA exhibited 75-85% of its activity at 37-40°C, at which temperatures b168phyA exhibited only 50% of its optimal activity.

Thermostability The thermostability of t168phyA was very similar to that of b168phyA

(Fig. 5). In the presence of 1 mM CaCl₂ at pH 7.0, both retained most of their activity after 10 minutes of incubation at 50°C. However, incubation at 60°C or above resulted in 80% loss of activity.

Kinetic parameters The turnover number of t168phyA (107 min⁻¹) was smaller than that of b168phyA (310 min⁻¹) and the K_m for phytate was almost double for t168phyA (0.340 mM) than that of b168phyA (0.192 mM). Therefore the kinetic perfection of t168phyA was only one-fifth of b168phyA (314 mM⁻¹ min⁻¹ vs. 1614 mM⁻¹ min⁻¹).

Discussion

A beta-propeller phytase, *168phyA* from *Bacillus subtilis 168* was introduced to and expressed in tobacco [29, 30]. The enzyme was extracted from tobacco leaves and purified through ethanol precipitation, anion-exchange chromatography, and gel filtration chromatography. To date, there are five publications on the expression of histidine acid phosphatases (HAP) in transgenic tobacco [11-14, 29], three of which have characterized the HAP that is expressed in transgenic plants. Verwoerd [11] recorded a very high accumulation level of HAP in tobacco leaves, accounting for 8.8-14.4% of the total soluble protein from extracts of 7-week old leaves. Ullah [12] reported a lower accumulation of HAP in tobacco than in Verwoerd's study, but the accumulation level was still quite high and reached 6.7% of the total soluble protein from the leaves of three week old tobacco plants. Zhang [13] recorded the highest accumulation level of HAP in transgenic plants, which accounted for 17.6% of the total soluble protein in 8-week old leaves. In this study, the BPP phytase only accumulated up to 0.4% of the total soluble protein from three week old tobacco leaves. The differences in accumulation levels between the fungal HAP phytase (*phyA*) and beta-propeller phytase could have several possible reasons. First, their mRNA transcription levels may be different. Second, the codon usage of *Bacillus* sp. is different from that of tobacco. According to the codon usage database (www.kazusa.or.jp/codon), the *168phyA* gene utilizes several codons that are not preferred by tobacco e.g. the codon frequency of "CCG" (Proline) in *168phyA* is 15.7, whereas that in tobacco is 4.9; and the codon frequency of "AGC" (Serine) in *168phyA* is 18.3, whereas that in tobacco is 9.9. These examples illustrate that there is substantial codon bias between *168phyA* and the tobacco genes, which might have lowered the translation efficiency of *168phyA*. In contrast, Verwoerd [11] claimed that the difference in codon usage between *phyA* and that of dicotyledonous plant genes is insignificant. Third,

the *phyA* from *Aspergillus niger* might be more stable than the t168phyA in tobacco. It has been reported that the stability and activity of beta-propeller phytases (BPPs) are dependent on the calcium concentration in the environment [27, 28]. The depletion of calcium ions will result in a decrease in thermostability and the irreversible loss of its activity. The cytosolic calcium concentration is only at the micromolar level, and the calcium concentration in the extracellular fluid varies. Although transgenic 168phyA in tobacco reduced the seed IP6/IP5 level [29], and in our study the t168phyA had a specific activity and thermostability which was comparable to that of b168phyA *in vitro*, the effect of the low calcium concentration on the enzyme's activity and stability *in vivo* is unknown.

In this study, phytase activity was undetectable in the wild-type tobacco leaf extracts. This was in contrast to another report, which detected 280 nKat phytase activity per gram of leaf from wild-type tobacco [12]. This discrepancy can be explained by the differences in activity assay conditions. In this study, the activity assay was carried out at pH 7.0 and 37°C, whereas in the other study, the assay was carried out at pH 5.0 and 58°C, the optimum pH and temperature of *phyA* from *Aspergillus niger*. The phytase activity that was detected in the wild-type tobacco leaves in the other study [12] could have been due to endogenous phytase. It should be noted that most known phytases of plant origin belong to the families of histidine acid phosphatases and purple acid phosphatases, which usually have acidic pH optima. For example, the pH optimum for soybean phytase is 4.1-4.6 [18] and the optima for the wheat bran phytases are 4.5-5.0 and 5.5-6.0 [19]. These phytases generally show very low activity at neutral pH. Although phytases with alkaline pH optima have also been detected in plants, such as the phytase from Lily pollen that exhibited an optimum pH of 8.0 [33], their expression is usually localized in certain plant organs such as seeds and pollens, but rarely found in leaves. Therefore, we carried out our activity assays at pH 7.0, and no endogenous phytase activity was detected in the wild-type tobacco leaves.

Three potential glycosylation sites are present in the peptide sequence of *168phyA*. We showed that *168phyA* was glycosylated when expressed in tobacco (Fig. 2). This resulted in an increase in the molecular size of *t168phyA*. By the 3D-PSSM fold recognition server [34], the fold of a thermostable phytase from *Bacillus amyloliquefaciens* (TS-Phy) (protein data bank ID, 1POO) was identified as the closest to that of the *168phyA*, with an E-value of $4.12e^{-05}$. The structure of TS-Phy was used to model the 3-dimensional structure of the *168phyA* using the Deep-view software package. The final model showed a structure-based sequence identity of 73% to TS-Phy (Fig. 6). From this model, we can see that all three potential glycosylation sites are located at the surface of the protein and in close proximity to the active sites of the enzyme. The effect of the glycosylation on the structure and folding of the enzyme is still unknown. However it is clear that the biochemical properties of the enzyme are affected in several ways, which are summarized in Table 3. Although the pH and the temperature profiles of *t168phyA* are different from those of the native phytase, their thermostabilities are similar, which indicates that the stability of *168phyA* is not severely affected by the glycosylation. However the K_m of *t168phyA* towards phytate was higher than that of *b168phyA*, while the turnover number of *t168phyA* against phytate was found to be lower than that of *b168phyA*, indicating that *t168phyA* had lower catalytic efficiency than *b168phyA* against phytate as substrate.

Nevertheless, the plant-derived *168phyA* is an active enzyme in plant [29]. Its secretion from the roots of tobacco and *Arabidopsis* were shown to enable assimilation of phytate in agar [30]. This improvement in phosphorus mobilization in crops would be beneficial to agriculture by reducing the consumption of phosphate fertilizer and eutrophication.

Acknowledgement

This research was supported by the Research Grants Council of the Hong Kong Special Administrative Region Government, China (project HKU 7335/04M).

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Figure legends

Fig. 1. SDS-PAGE (A) and Western blot (B) of the purified *t168phyA* from Tobacco A3SP-4. Lane M shows the molecular weight standards; Lane A3 and Wt is the crude leaf extracts from line A3SP-4 and wild-type tobacco, respectively. Lane T is the purified *t168phyA*. Lane B is the purified *b168phyA*. As shown in the figure, *t168phyA* is ~4kDa larger than *b168phyA*.

Fig. 2. Immunoblot for glycoprotein detection. Lane M is a protein marker. Lane 1 is the purified *t168phyA*. Lane 2 is *b168phyA*. The negative controls were not oxidized with periodate. There was no band in Lane 2 whether or not it was oxidized. For Lane 1, only the membrane that was oxidized with periodate showed a band at the same size as *t168phyA*, indicating that *t168phyA* is a glycoprotein.

Fig. 3. A comparison of the activity of *t168phyA* and *b168phyA* at different pH. The reactions were conducted at 37°C, with 1 mM sodium phytate as substrate (n = 3)

Fig. 4. A comparison of the temperature profile of *t168phyA* and *b168phyA*. The enzyme assays were performed at pH 7.0 with 1 mM sodium phytate as substrate (n = 3)

Fig. 5. A comparison of the thermostability of *t168phyA* and *b168phyA*. The enzymes were incubated at various temperatures for 10 minutes and then allowed to cool down to room temperature for 1 hour before being assayed for activity. The activity of the enzymes incubated at room temperature (25°C) was taken as 100%. The enzyme assays were conducted at pH 7.0 with 1 mM sodium phytate as substrate (n = 3).

Fig. 6. Structural model of *168phyA* based on the structure of TS-phy from *Bacillus amyloliquefaciens*. (A) A stereo view of *168phyA*. The active site of the enzyme is located at the top of the molecule. (B) A ribbon diagram of *168phyA*. The yellow balls represent the Ca ions in the structure. The Green residues represent the three Asn residues of the three potential glycosylation sites in the enzyme. The phosphate groups of the substrate are shown in orange.

Table 1. Phytase activity in the leaves of Tobacco A3SP-4.

| | |
|------------------------------------|---------|
| Amounts of leaves ground | 10.3 g |
| Volume | 37 ml |
| Total protein in mg | 70.9 |
| Phytase activity per ml | 15.8 mU |
| Phytase activity per mg protein | 8.2 mU |
| Phytase activity per g of leaves | 56.7 mU |
| Total activity | 584 mU |
| Phytase in mg | 0.254 |
| % phytase in total soluble protein | 0.4 % |

The amount of phytase (mg) and the percentage of phytase in the total soluble protein of tobacco leaves were estimated based on the specific activity of 2.3 U/mg.

Table 2. A summary of the Purification of *t168phyA* from Tobacco A3SP-4.

| Procedure | Volume (ml) | Total activity (mU) | Total protein (μ g) | Specific activity (U/mg) | Yield (%) | Purification fold |
|-----------------------|-------------|---------------------|--------------------------|--------------------------|-----------|-------------------|
| Crude leaf extract | 37 | 584 | 70875 | 0.008 | 100 | 1 |
| Ethanol precipitation | 13 | 378 | 3688 | 0.102 | 64.7 | 12.8 |
| Hitrap Q | 5 | 77 | 424 | 0.182 | 13.2 | 22.7 |
| Gel filtration | 3 | 31 | 13 | 2.302 | 5.2 | 287.8 |

Table 3. Comparison of biochemical properties of t168phyA and b168phyA.

| | t168phyA | b168phyA |
|--------------------------------------|--|---|
| Molecular weight (based on SDS-PAGE) | 44kDa | 40kDa |
| Glycosylation | Yes | No |
| pH optimum | 6.0 | 5.5 |
| Temperature optimum | 45-50°C | 60°C |
| Km | 0.340 mM | 0.192 mM |
| Kcat | 107 min ⁻¹ | 310 min ⁻¹ |
| Kcat/Km | 314 mM ⁻¹ min ⁻¹ | 1614 mM ⁻¹ min ⁻¹ |
| Specific activity | 2.3 U/mg | 11.7 U/mg |

Figure 1
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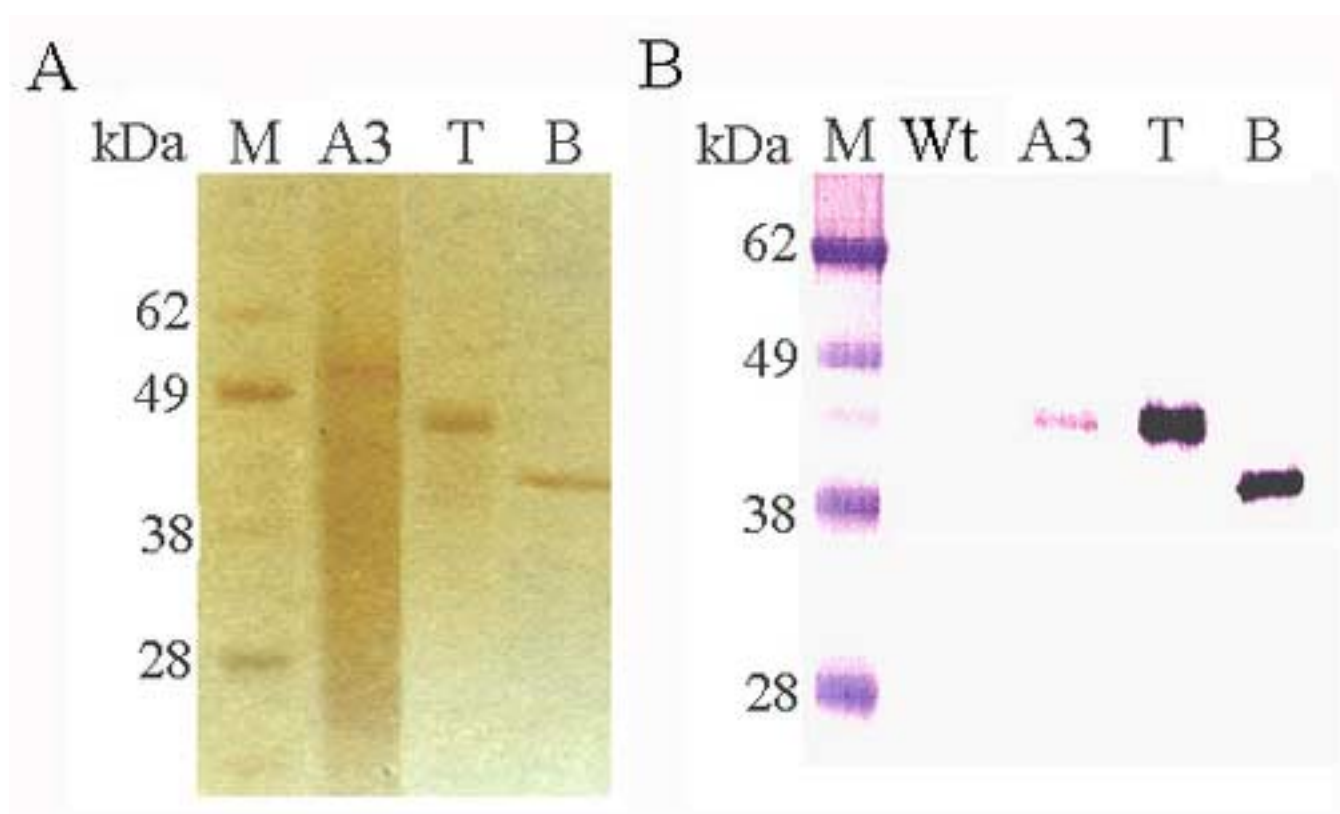


Figure 2

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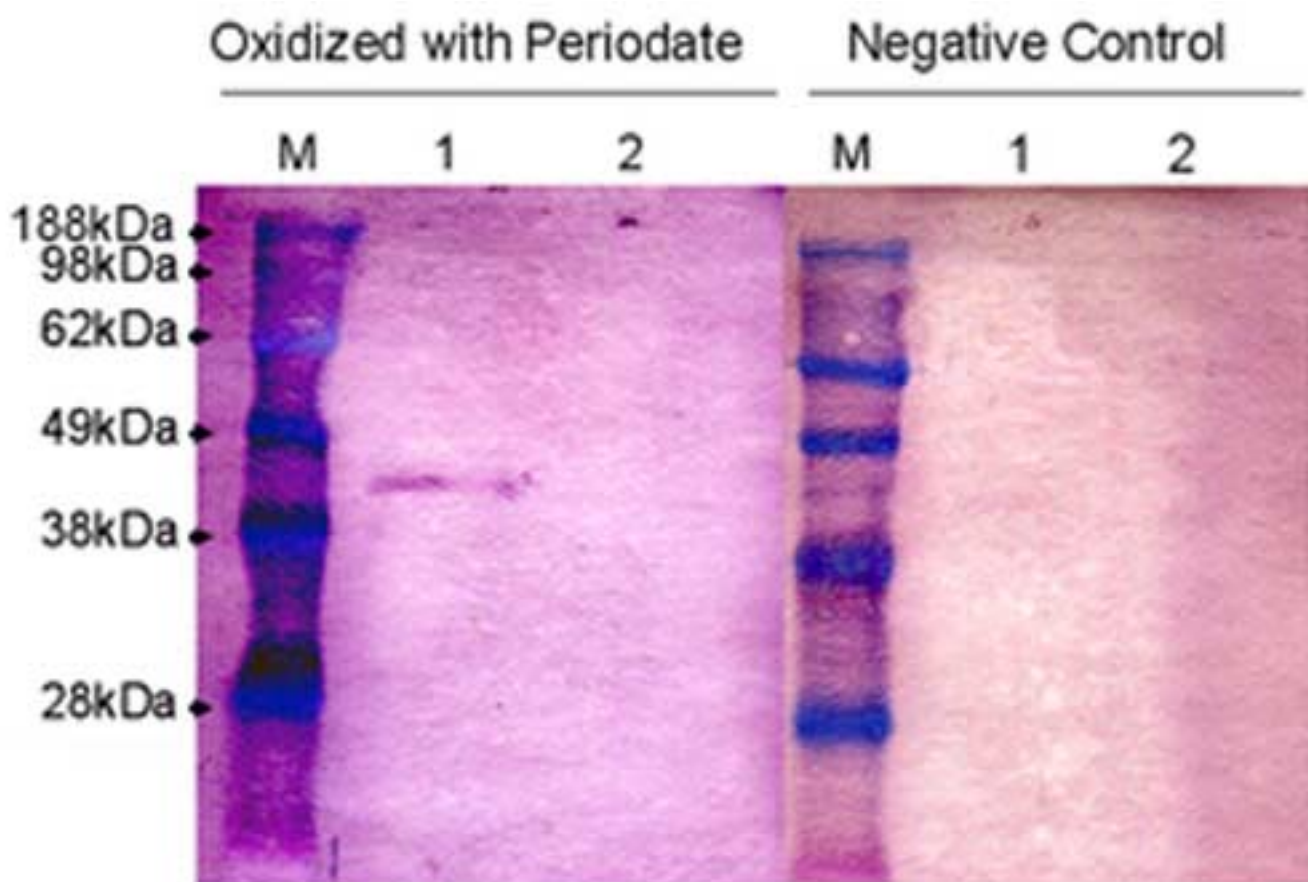


Figure 3
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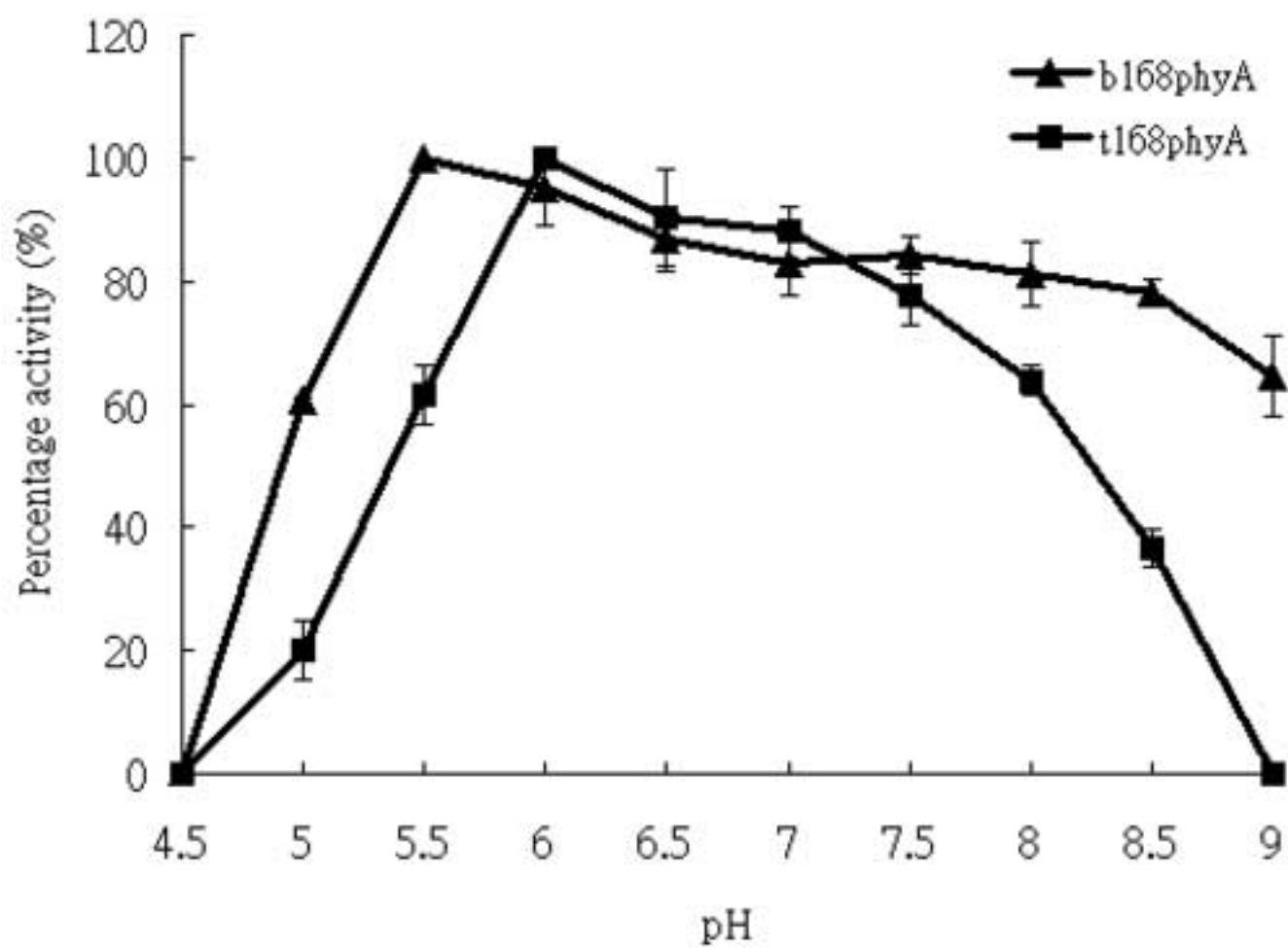


Figure 4
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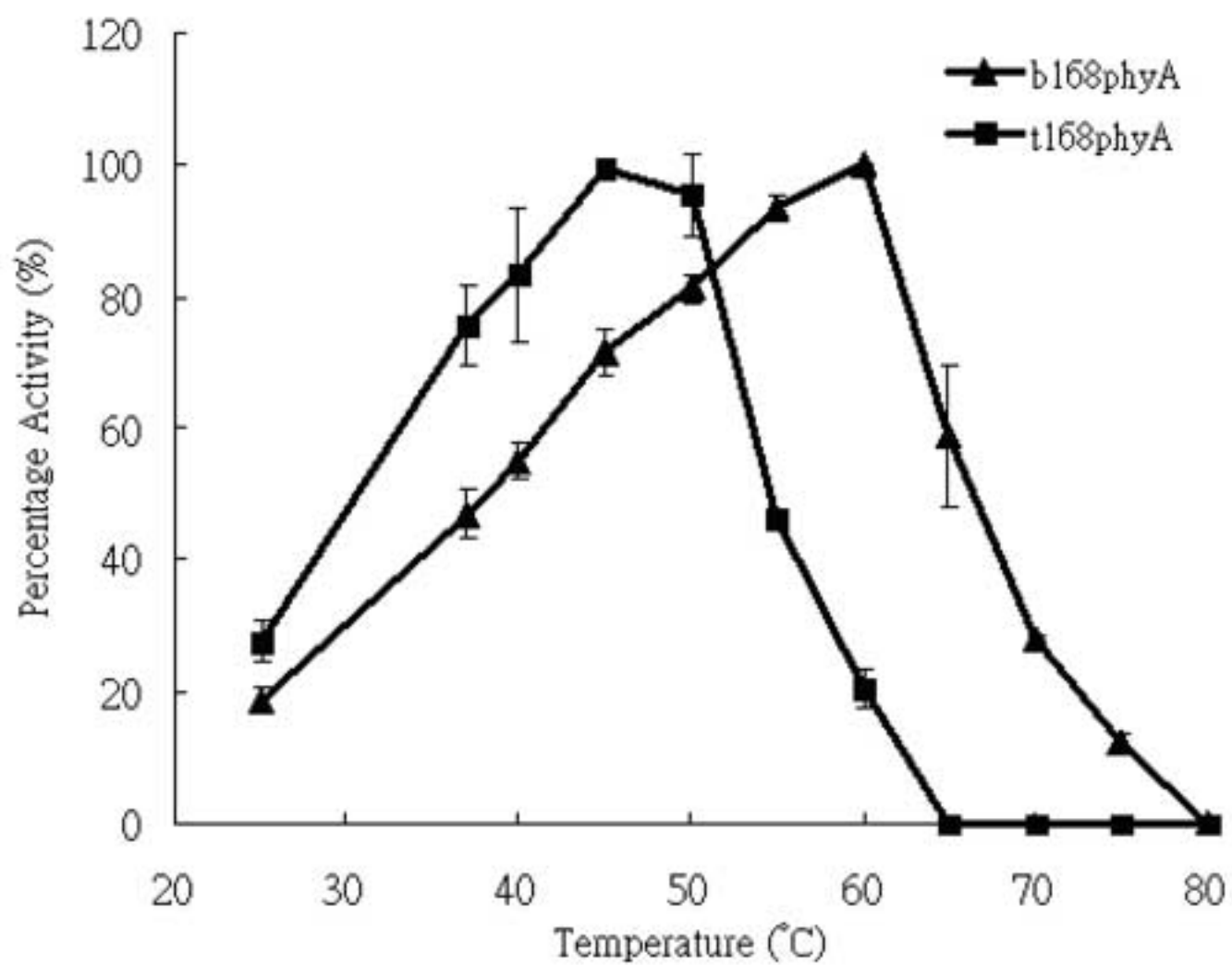


Figure 5
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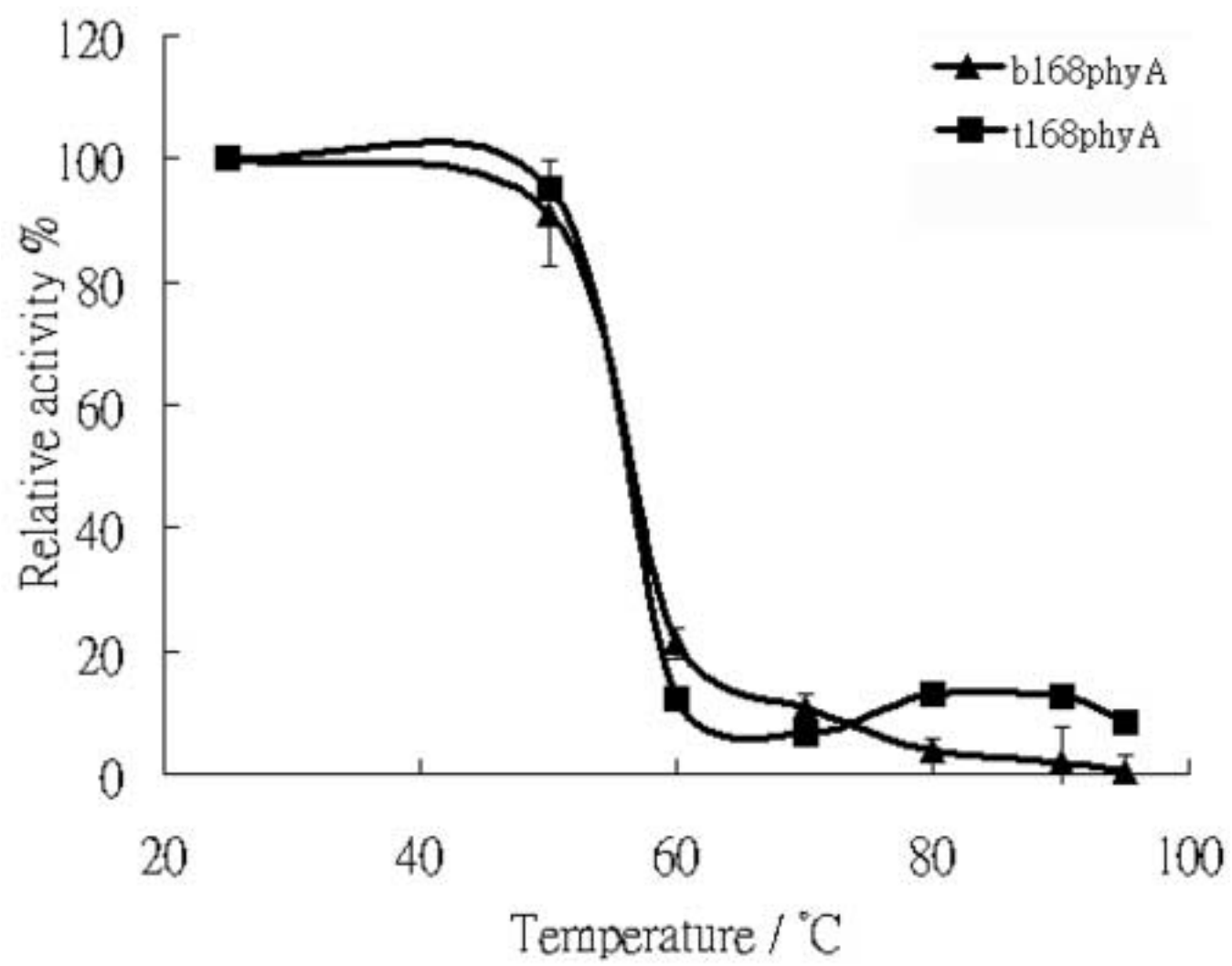


Figure 6
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