Characterization of phytase from three ferns with differing arsenic tolerance

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Phytase is involved in many physiological activities in plants including phosphorus metabolism and stress response. The effects of arsenic on phytase activities in arsenic-hyperaccumulator Pteris vittata were determined. Two arsenic-sensitive ferns (Pteris ensiformis and Nephrolepis exaltata) were included for comparison purpose. Fern phytase was extracted with Tris–HCl buffer (pH 7.6) followed by ammonium sulfate partial purification to characterize its properties and arsenic stress responses. The phytase showed an optimum pH of 5.0 and temperature of 40 °C except for P. vittata with 40–70 °C. Phytase from P. vittata was the first plant-phytase showing high heat resistance with no loss of activity by heating it at 70 °C, which may have application in feed industry. Phytase activity was inhibited by arsenate but not by arsenite. The fact that P. vittata phytase was the most heat-tolerant (40–70 °C) and had the highest resistance to arsenate among the three ferns suggest that phytase may play a role in arsenic detoxification and arsenic hyperaccumulation in P. vittata.

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1. Introduction

Phytic acid (myo-inositol hexakis dihydrogen phosphate) is a phosphorus-rich organic acid, which is widely present in plant seeds, roots, fruits and pollens. Phytase (myo-inositol hexaphosphate phosphohydrolase) hydrolyzes phytic acid into inorganic phosphate and a series of myo-inositol phosphates. There are two general classes of phytase, 3-phytase and 6-phytase, which vary greatly in their activity [29]. For example, among the 36 plants tested by Davidsson et al. [5], rye (Secale cereale; 6.9 U g⁻¹), triticale (Triticosecale; 4.8 U g⁻¹) and wheat (Triticum aestivum; 3.1 U g⁻¹) exhibited the highest phytase activity while sorghum (Sorghum sudanensis; 0.11 U g⁻¹) showed the lowest. Most plant phytase has similar biochemical properties to fungus phytase, with optimum temperature of 45–60 °C and optimum pH of 4.5–6.0 [13,18,30].

Phytase plays a major role in phosphorus metabolism. Hydrolysis of phytic acid by phytase releases inorganic phosphate to maintain phosphate balance during plant growth [11]. In addition, cell phosphate starvation promotes phytase activity, thereby increasing hydrolysis of phosphate ester to satisfy P demand by cells [15,27,33]. Plant phytase also plays a role in its P acquisition from soils [35]. For example, plant roots exudate phytase into rhizosphere to hydrolyze organo-phosphorus ester, thereby releasing more bioavailable inorganic phosphate [6,19,24]. In addition to being influenced by P status in plants, phytase also responds to arsenic stress in plants. This is partially because phosphate and arsenate are chemical analogues. A study shows that phytase activity in pea plants (Pisum sativum) is reduced after exposure to arsenate [26]. A separate report shows that a tyrosine phosphatase regulates the activity of arsenate reduce in velvetgrass (Holcus lanatus) [3]. However, the relationship between phytase and arsenic tolerance is still unclear, especially its role in arsenic hyperaccumulation by plants.

Phosphatase played an important role in arsenic detoxification and arsenic hyperaccumulation in Chinese brake fern (Pteris vittata) [37] and other plants [9]. There was significant amount of phytic acid in fern plants, such as P. vittata, Wolfiella floridana and Lemna minor, which may help its P uptake during arsenic stress, or antioxidative response [32,38,39]. In fact, we showed that arsenic induced phytase uptake by P. vittata [21]. Under arsenic exposure, P. vittata maintained higher concentrations of P in the roots compared to the non-arsenic hyperaccumulating plants (the control), which helps to mitigate arsenic toxicity in P. vittata.
We hypothesize that phytase might participate in arsenic detoxification by regulating plant P metabolism. To address our hypothesis, we conducted a series of experiments 1) to characterize phytase from three ferns with different arsenic tolerance levels, and 2) to examine phytase inhibition by arsenate and arsenite.

2. Materials and methods

2.1. Plant materials and plant growth condition

Three ferns of different arsenic tolerance were used, including *P. vittata*, *Pteris ensiformis*, and *Nephrolepis exaltata*. While *P. vittata* is an arsenic hyperaccumulator and highly tolerant to arsenic [22], *P. ensiformis* and *N. exaltata* are sensitive to arsenic [34]. All fern plants were procured from a nearby nursery (Milestone Agriculture, Inc., FL, USA).

Six-month-old fern plants were transferred to 3-L opaque plastic containers with 0.2-strength Hoagland solution [12] as the growth medium. The solution was aerated vigorously and replenished every three days. The ferns were cultivated hydroponically in a growth room at 23–28 °C and 70% humidity. A 14-h photoperiod with a daily photosynthetic photon flux of 350 μmol m⁻² s⁻¹ at plant canopy was supplied by an assembly of both cool-white and warm-white fluorescent lamps.

2.2. Enzyme preparation

The fronds were harvested after 2 weeks of growth, washed with tap water and rinsed thoroughly with deionized water. The leaflets from the fronds (~50 g) were homogenized in a Waring blender for 1 min in an ice-cold 50 mmol L⁻¹ Tris–HCl buffer (pH 7.6) containing 1 mmol L⁻¹ Titriplex III and 0.5 mmol L⁻¹ mercaptoethanol. The homogenate was kept on a magnetic stirrer for 30 min at 4 °C, and centrifuged at 10,000 g for 30 min at 4 °C.

The clear supernatant was subjected to precipitation using solid (NH₄)₂SO₄ [7]. All steps were carried out at 4 °C. Briefly, the supernatant was adjusted to 24% (wt/v) (NH₄)₂SO₄ saturation. After centrifugation at 10,000 g for 30 min, the supernatant was adjusted to 55% (wt/v) (NH₄)₂SO₄ saturation. The 25–55% saturated (NH₄)₂SO₄ precipitate was solubilized in approximately 10 ml of extraction buffer and extensively dialyzed in a dialyzing tube (MW12,000) against 5-L of 0.2-strength extraction buffer (pH 7.6) containing 1 mmol L⁻¹ NaOH (pH 9 and pH 10). Incubation was carried out at 37 °C for 2 h with 2 mmol L⁻¹ of substrate phytate in the same buffer.

Unless specified otherwise, phytase activity was determined in the assay mixture at 37 °C and pH 5 after 2 h incubation with 2 mmol L⁻¹ of substrate phytate. The effect of substrate concentration was determined using 0–4 mmol L⁻¹ phytate. The effects of incubation time on phytase activity were determined after incubating the phytase assay mixture for 60, 120, and 240 min, whereas the effects of temperature were determined after incubating the mixture at 30, 40, 50, 60, 70, 80, 90, and 100 °C. Arsenate and arsenite at concentrations from 0 to 2000 μmol L⁻¹ were used to determine arsenic inhibition of phytase activity.

2.5. Statistical analysis

Data were analyzed using the statistical functions of SAS 8.0 software. Plots were graphed using SigmaPlot 7.0 and MS EXCEL 7.

3. Results

3.1. Effect of pH

Different buffers with pH 2.0 to 10.0 were used to determine optimum pH for phytase (Fig. 1), which was around pH 5.0 for all three ferns. However, it differed slightly with ferns, with optimum pH 5.11, 5.23, and 5.31 for *P. vittata*, *P. ensiformis* and *N. exaltata*, respectively. They were calculated using maximum values of quadratic equation of enzyme activity vs pH in Fig. 1 (data not shown). On either side of the optimal pH value, phytase activity decreased sharply. For example, compared to optimal pH of 5, enzyme activity determined at pH 4.5 decreased by 33%, 32% and 52% for *P. vittata*, *P. ensiformis* and *N. exaltata*, respectively. In addition, fern phytase showed no activity at pH > 7, indicating that it was an acid phosphatase.

3.2. Effect of substrate concentrations, incubation time, and temperature

As expected, the activity of phytase from three ferns increased with phytate concentrations, with a linear relationship being observed at <2 mmol L⁻¹ phytate (Fig. 2). In addition, incubation time also influenced the specific activity of phytase assayed under the condition of pH 5, 37 °C and phytate concentration of

![Fig. 1. Effect of pH on activity of phytase extracted from fronds of *P. vittata*, *P. ensiformis* and *N. exaltata* grown in hydroponic conditions. The activity was assayed at 37 °C for 2 h at phytate concentration of 2 mmol L⁻¹. The data were the means of four replicates and the SE indicated by bars.](image-url)
2 mmol L⁻¹ (Fig. 3). However, phytase activity was relatively stable during 2 h incubation.

Temperature significantly affected phytase activity from the three ferns, with an optimum temperature of 40 °C (Fig. 4). It was interesting that phytase activity of P. vittata remained relatively unchanged in incubation temperature from 40 to 70 °C, whereas those from the other two ferns, especially N. exaltata decreased sharply at incubation temperature above 40 °C. For instance, compared to the phytase activity at 40 °C, the phytase activity decreased by 55.6% and 60.0% for P. ensiformis and N. exaltata, respectively. All phytase activity reduced sharply when incubation temperature was >80 °C.

3.3. Inhibiting effect of arsenic on phytase

In vitro assay of phytase activity showed that phytase from three ferns responded differently to exposure to arsenate (oxidized form-As(V)) and arsenite (reduced form-As(III)) (Fig. 5).

Inhibition of phytase activity by arsenate was expected since arsenate is an analog of phosphate. However, P. vittata phytase was much more tolerant to arsenate than those from P. ensiformis and N. exaltata. For instance, compared to the control with no arsenate, P. vittata phytase activity decreased by 31% compared to 70% and 100% for P. ensiformis and N. exaltata at 500 μmol L⁻¹ of arsenate (Fig. 5a). The phytase of all three ferns lost the activity when arsenate was 2000 μmol L⁻¹. It was interesting to note that arsenite did not inhibit the phytase activity of the three ferns (Fig. 5b).

4. Discussion

To better understand phytase-mediated phytate hydrolysis, it is important to know the optimum pH, temperature, incubation time, and substrate concentration to determine phytase activity from the three ferns. The optimum pH for commercially available phytase varies from 2.5 to 5.5; at pH greater than 6, its activity is essentially lost [14]. Plant phytase works better at 45–60 °C whereas microbial phytase works at wider temperature ranges (35–63 °C) [11].

The optimal conditions for phytase from three ferns were at pH 5 and 40 °C, which is in good agreement with previous results (Table 1). It was noteworthy that the optimum temperature for P. vittata phytase ranged from 40 to 70 °C. It was different from other plant phytase (Fig. 4, Table 1) and had a greater range than microbial phytase [1]. This may result from different substrate accessibility and thermal stability among phytases [2]. The fact that P. vittata phytase was stable to a treatment at 70 °C indicated that it was much more heat-tolerant than phytases from other plants.

To our knowledge, P. vittata phytase was the first plant phytase that exhibited such high stability to heat. This might be partially due to its sun-loving growth preference, which is different from other ferns mostly preferring shady environment [22]. This may have implication for application of P. vittata phytase in animal feeds. Since the optimum temperature for most plant phytase is 45–60 °C, the higher heat-stability of P. vittata phytase could make it more efficient when used as feed supplement.

Phosphorus is a key component of many biomolecules, such as DNA, RNA, proteins, enzymes and ATP etc. Arsenic toxicity results from its interference with P metabolism since arsenate and phosphate are analogues [37]. In plant, phytase hydrolyzes phytic acid into inorganic phosphate and a series of myo-inositol phosphates, which is showed as following:
As phytate-P accounts for relatively large proportion of plant P, this scheme indicates that phytate-P derived from phytase is essential to plant P metabolisms. Since arsenate and phosphate are chemical analogues, more P in a plant helps its As detoxification.

In this *in vitro* experiment, as expected that phytase from all three ferns was inhibited by arsenate, an analog of phosphate, and the result was in agreement with those of Päivöke and Simola [26] who used *P. sativum* phytase. However, compared to *P. ensiformis* and *N. exaltata*, *P. vittata* phytase was much more tolerant to arsenate (Fig. 5). Among the three ferns, *P. vittata* is the most tolerant to arsenic and is the most efficient in arsenic hyper-accumulation [22]. This result indicated that as a key enzyme in P metabolism, phytase is essential to maintain healthy plant growth and development. The high resistance to arsenic may help the plant to take up both arsenite and arsenate [40] but arsenite is the most tolerant to arsenic stress, which might be one of the mechanisms for arsenic detoxification and hyperaccumulation in *P. vittata*.

Unlike arsenate, phytase activity from ferns was not inhibited by arsenite (Fig. 5b). Instead, the phytase activity seemed to be enhanced by arsenite under *in vitro* conditions. Since arsenite is not an analog of phosphate, it is not surprising that arsenite didn’t interfere with phytase activity. The fact that *P. vittata* phytase was most tolerant to arsenate and was not inhibited by arsenite was of significance in arsenic detoxification by *P. vittata*. This is because *P. vittata* takes up both arsenite and arsenate [40] but arsenite is the dominant species in the fronds. This way, *P. vittata* could minimize arsenic toxicity by not impacting its P metabolism, which may help its arsenic hyperaccumulation ability.

However, in vivo inhibition of phytase activity of *P. sativum* under arsenate stress is different as reported by Päivöke and Simola [26]. The effect of soil arsenate addition on phytase activity of *P. sativum* depends on the soil arsenate levels and sampling time. In our previous study, >90% of arsenate taken by *P. vittata* are reduced into arsenite in a short time [38]. However, in other plants such as *P. sativum*, arsenite may be the dominant form [21]. As demonstrated in this study, arsenate inhibited phytase activity whereas arsenite did not (Fig. 5). The difference in arsenic species in the two plants may be the main reason why the effect of arsenate on phytase activity in two plants is different.

**Fig. 5.** In *vitro* stress response of phytase of *P. vittata*, *P. ensiformis* and *N. exaltata* to As(III) and As(V). The phytase activity was assayed at pH 5, 37 °C and phytate concentration of 2 mmol L⁻¹ for 2 h. The data were the means of four replicates and the SE indicated by bars.

### Table 1

<table>
<thead>
<tr>
<th>Plants</th>
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<th>T (°C)</th>
<th>C (mmol L⁻¹)</th>
<th>References</th>
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<tr>
<td>California white beans</td>
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<td></td>
<td></td>
<td>[4]</td>
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<tr>
<td>(Phaseolus vulgaris) Navy beans (P. vulgaris)</td>
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<td>1.0</td>
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<td>60</td>
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