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7 Title: Hydrolysis of precipitated phytate by three distinct families of phytases.

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1    **Abstract**

2    The ability of representative members from three classes of phytases: histidine acid  
3    phosphatases,  $\beta$ -propeller phytases, and purple acid phosphatases, to hydrolyze  
4    metal-phytate salts and phytate adsorbed to aluminum precipitates, were compared.

5    All three tested phytases were able to hydrolyze  $\text{Ca}^{2+}$ -,  $\text{Mg}^{2+}$ - and  $\text{Mn}^{2+}$ -phytates, but  
6    not  $\text{Al}^{3+}$ -,  $\text{Fe}^{2+}$ -,  $\text{Fe}^{3+}$ -,  $\text{Cu}^{2+}$ - and  $\text{Zn}^{2+}$ -phytates. In addition, when these ions were

7    present, the hydrolysis of  $\text{Ca}^{2+}$ -phytate was prevented. Only higher concentration of  
8    citrate, but not malate nor oxalate, can partially solubilize some of these phytate salts

9    for enzyme hydrolysis. Phytate adsorbed to aluminium precipitates were resistant to  
10   all three enzymes, except when organic acids were added (citrate > oxalate > malate).

11   While high concentration of organic acids were inhibitory to enzyme activity (oxalate  
12   > citrate > malate), purple acid phosphatase was more resistant to citrate than histidine

13   acid phosphatase. Since desorption of phytate from solid surface by organic acids is  
14   essential for phytase activity, genetic engineering of plants that enhance secretion of

15   both citrate and phytases, preferably purple acid phosphatase, from the root, may be a  
16   feasible approach to improve soil phytate assimilation.

17   **Keywords**

18   beta-propeller phytase, citrate, histidine acid phosphatase, malate, oxalate,

19   purple acid phosphatase, phytate.

## 1 **Introduction**

2           Organic forms of phosphorus (P) constitute a large proportion of soil P, often  
3 50%-80% of total soil P (Turner, 2002). Although organic P is present in soil solution  
4 in higher concentrations than inorganic phosphate (Seedling and Jungk, 1996), direct  
5 uptake of organic phosphate compounds by plants is unlikely. Rather, P is acquired by  
6 plant roots as inorganic phosphate (Raghothama, 1999). Therefore, soil organic  
7 phosphorus compounds must first be dephosphorylated by phosphatases or phytases  
8 before they can be assimilated.

9           Inositol phosphates (IP), particularly inositol hexakisphosphate (IHP or  
10 phytate), is the predominant form of organic phosphorus in soil (Anderson et al.,  
11 1980). The preferential accumulation of inositol phosphates in soils is due to their  
12 adsorption on soil colloids, which hampers their biodegradation (Ognalaga, 1994). As  
13 the number of P increases, the interaction between inositol phosphates with soil  
14 becomes stronger due to their higher charge density. The typical proportion of inositol  
15 phosphates in soil is 83%:12%:4%:1% for IP<sub>6</sub>:IP<sub>5</sub>:IP<sub>4</sub>:IP<sub>3</sub> (Anderson et al., 1980).  
16 They are either being adsorbed to clays or precipitated as insoluble salts, such as  
17 sesquioxides of Fe and Al in acid soils and insoluble calcium salts in alkaline soils  
18 (Turner et al., 2002).

19           Inorganic phosphates (Pi) can be released from phytates by the action of

1 phytases. Based on their cDNA sequences, three-dimension structures and reaction  
2 mechanisms, four classes of phytases have been characterized. They are histidine acid  
3 phosphatases (HAPs),  $\beta$ -propeller phytases (BPPs), purple acid phosphatase (PAPs)  
4 and a novel phytase from *Selenomonas ruminantium*. Several groups have engineered  
5 genetically modified (GM) plants that secrete histidine acid phosphatases (Richardson  
6 et al., 2001; Zimmermann et al., 2003). They reported that the secretion of HAPs from  
7 GM plant roots enabled the plants to grow in agar supplemented with IHP.  
8 Zimmerman et al. (2003) also showed that the GM plants growing in sand exhibited a  
9 better growth performance than the control plants when the plants were irrigated with  
10 sodium phytate solution. In addition, we have also created transgenic plants that  
11 secreted  $\beta$ -propeller phytase, which were able to utilize IHP in agar (Lung et  
12 al. ,2005). While these studies proved that the secreted phytases could act on soluble  
13 phytates, whether they could release Pi from phytate fixed to various soil components  
14 is in question.

15       Since IHP is precipitated as insoluble phytate salts in soil and its sorption was  
16 dependent on the contents of amorphous Al and Fe in acid soils (Anderson et al., 1980;  
17 Shang et al., 1992), it is the objective of this study to examine whether the three  
18 classes of phytases could release inorganic phosphates from phytate salts, as well as  
19 from IHP adsorbed to aluminum and iron (III) precipitates, under various conditions.

## 1 **Materials and methods**

### 2 *Chemicals and Enzymes*

3 Citric acid (C-7129), maleic acid (M-0375), phenylglyoxal (142433), phytic acid  
4 (P-3168), sodium oxalate (O-0626), sodium molybdate (M1651), phytase from  
5 *Aspergillus ficuum* (P-9792) and phytase from wheat bran (P-1259) were purchased  
6 from Sigma-Aldrich (USA). The wheat phytase were further purified by ion-exchange  
7 chromatography according to the procedures described by Nakano et al., 1999.  
8  $\beta$ -propeller phytase (BPP) of *Bacillus subtilis* strain168 were overexpressed by a  
9 prophage expression system and purified as described previously (Tye et al., 2002).

10

### 11 *Verification of wheat bran phytase as a purple acid phosphatase*

12 The cDNA of PAP15 gene from *Arabidopsis thaliana* (Li, et al. ,2002) was  
13 obtained by RT-PCR and subcloned into the pRsetA vector (Invitrogen) for  
14 overexpression in *E. coli*. The recombinant protein, which was expressed in the  
15 inclusion bodies, was purified by a nickel column under denaturing conditions. The  
16 denaturant was removed by dialysis and the precipitated proteins were further  
17 separated by SDS-PAGE. A 57 kDa band, corresponding to PAP15, was excised for  
18 rabbit immunization. Antiserum obtained after the second booster was collected for  
19 western blotting analysis.

1

2 *Phytase activity assays*

3       The enzymes were first diluted with 100 mM 2-(N-morpholino)ethanesulfonic  
4 acid (MES) buffer, pH 6.0 with or without 1 mM CaCl<sub>2</sub> and the reaction was initiation  
5 by addition of sodium phytate, in which the final concentration of sodium phytate in  
6 the reaction mix was 1 mM. To determine the pH optima of the enzymes, 100 mM  
7 glycine (at pH 4.0) and 100 mM Tris-maleate (pHs 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0,  
8 8.5) with 1 mM CaCl<sub>2</sub> were used. All the enzyme assays were carried out at room  
9 temperature for thirty minutes before being stopped by adding equal volume of 4%  
10 (v/v) trichloroacetic acid (TCA). 150 µl TCA-mixture was then transferred to a  
11 microtiter plate and the liberated Pi was quantified by OD 650 nm measurement,  
12 taken ten minutes after addition of 24 µl molybdate reagent (Murphy and Riley, 1962).  
13 One unit of enzyme was defined as the amount of enzyme that liberates 1 µmol of Pi  
14 per minute under the assay conditions. For inhibition assays, the enzymes were  
15 incubated with different concentrations of the inhibitors citrate, malate, molybdate,  
16 and oxalate in 100 mM MES buffer, pH 6.0, with or without 1 mM CaCl<sub>2</sub>. For  
17 phenylgloxal inhibition assay, the enzymes were dialysed into 50 mM sodium  
18 bicarbonate at pH 7.5. The enzymes were then incubated with various concentrations  
19 of phenylgloxal at 37°C in the bicarbonate buffer (Ullah and Sethumadhavan, 1998).

1 After incubation for one hour, 50  $\mu$ l aliquot was transferred to 950  $\mu$ l 100 mM MES  
2 buffer, 1 mM  $\text{CaCl}_2$ , 1 mM phytate, pH 6.0. After incubation at room temperature for  
3 30 minutes, the amount of released Pi was determined by method described above.

4

#### 5 *Preparation of insoluble phytate salts*

6 To study the hydrolysis of metal phytates, 500 mM stock solutions of  $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  
7  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$  salts were prepared by dissolving  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ,  
8  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ ,  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ ,  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  and  
9  $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$  in distilled water accordingly. The 500 mM stock solution was serially  
10 diluted to 250 mM, 100 mM, 50 mM and 25 mM, respectively. Equal volumes of 25,  
11 50, 100, 250 and 500 mM salt solutions ( $\text{Ca}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{3+}$  and  
12  $\text{Al}^{3+}$ ) and 10 mM sodium phytate were mixed and incubated overnight at room  
13 temperature without shaking. The precipitated salts were centrifuged at 13,000 g at  
14 room temperature for five minutes. The supernatant was decanted off and the  
15 precipitated salts were washed thrice in two volumes of 50 mM MES buffer, pH 6.0,  
16 with 1 mM  $\text{CaCl}_2$  and finally resuspended in one volume of the same buffer. Phytase  
17 assays were initiated by incubating 0.5 ml of phytate salts with 0.5 ml enzyme (5  
18 mU/ml) at room temperature. Aliquots of the mixture were periodically taken at thirty  
19 minutes to twenty-four hours after enzyme addition. The salts were pelleted by



1 centrifugation and the amount of Pi in supernatant was determined by the molybdate  
2 method described above.

3 To study whether or not the other cations could interfere with the hydrolysis of  
4 calcium phytate, 25 mM salt solution ( $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Al}^{3+}$ )  
5 was premixed with 25 mM  $\text{Ca}^{2+}$  solution. For control, 25 mM  $\text{Ca}^{2+}$  solution was  
6 premixed with 25 mM  $\text{Ca}^{2+}$  solution. Equal volume of the premixed salt solution was  
7 then added to 10 mM sodium phytate. The preparation of salt precipitates and the  
8 enzyme assays were carried out as described above.

9 To study the effects of organic anions on the hydrolysis of  $\text{Al}^{3+}$ -,  $\text{Cu}^{2+}$ -,  $\text{Fe}^{2+}$ -,  
10  $\text{Fe}^{3+}$ - and  $\text{Zn}^{2+}$ -phytate salts, 0.5 ml of 100 mM salt solutions and 0.5 ml of 10 mM  
11 sodium phytate were mixed in eppendorf tubes and incubated overnight at room  
12 temperature. The precipitated phytate salts were pelleted at 13,000 g and washed  
13 thrice in assay buffer (50 mM MES, pH 6.0, without calcium ions) before  
14 resuspended in 0.5 ml phytase (5 mU/ml in assay buffer). 0.5 ml organic acid (0, 0.5,  
15 2 and 8 mM in 50 mM MES, pH 6.0) was then added to each tube. The mixtures were  
16 incubated overnight at room temperature with gentle shaking. The amount of Pi  
17 liberated into the supernatant was determined as described above.

18

19 *Determination of inositol hexakisphosphate*

1           A standard curve of inositol hexakisphosphate (IHP) was first made by  
2   dissolving 0.2486g phytic acid (Dodecasodium salt) into 500ml distilled water to  
3   make a 0.1gP/L stock solution and then diluted to 1mgP/L. Standards curve within the  
4   applicable range 0-1 mgP/L was constructed by serial 2-fold dilution. The method  
5   used was based on the protocol of the determination of phosphorus by  
6   semi-automated colorimetry (U.S. Environmental Protection Agency, 1993).

7

#### 8   *Preparation of Al and Fe(III) precipitates*

9           The method for aluminum and iron (III) precipitates preparation was based on  
10   the method by Shang et al. in 1992. A 400 ml 0.17M  $\text{AlCl}_3$  solution was titrated by  
11   1.0M NaOH solution until the pH reached 6.5. The mixture was maintained at pH 6.5  
12   for fifteen minutes and then centrifuged at 13,000 g for twenty minutes. The  
13   supernatant solution was decanted and the pellet left in the centrifuge bottle was  
14   resuspended in 300 ml distilled water to remove soluble ions. The salts were collected  
15   again by centrifugation and this washing step was done twice. Finally, the precipitates  
16   were then stored at  $-80^\circ\text{C}$  overnight and freeze-dried. To prepare iron (III) precipitates,  
17   a 0.17M  $\text{FeCl}_3$  solution (400 ml) titrated by 1.0M NaOH solution until the pH reach  
18   6.5. The iron (III) precipitates were then washed and prepared as described above.

19

1 *IHP adsorption to Al and Fe(III) precipitates*

2        Adsorption of IHP to the aluminum and iron (III) precipitates were based on the  
3 method by Shang et al. (1992). First, 0.05g Al precipitate was stirred in 80 ml 10 mM  
4 MES buffer, pH 6.0, in a 250ml flask for sixteen hours for pH adjustment. Then, 20  
5 ml IHP solution (3.25 mM IHP, 0.05M NaCl in 10 mM MES buffer, pH 6.0) was  
6 added to the flask. The initial reaction concentration of IHP in suspension was  
7 therefore 0.65 mM, the concentration of NaCl was 0.01M, and the solid content was  
8 0.5mg ml<sup>-1</sup>. After overnight stirring, 1 ml aliquots of salt suspension were transferred  
9 to 1.5 ml eppendorf tubes for centrifugation at 13,000g for five minutes. The amount  
10 of unbound IHP in the supernatant and the subsequent washes were then determined  
11 by the molybdate method (Murphy and Riley, 1962) following acid-persulphate  
12 oxidation (US Environmental Protection Agency, 1993). The amount of IHP adsorbed  
13 by aluminum and iron (III) precipitates was calculated by taking the difference  
14 between the initial amount of IHP in solution and the amount in the supernatant after  
15 overnight adsorption and subsequent washing. For the study on iron (III) precipitate,  
16 the experimental procedures were the same as above except that 0.1 g of Fe  
17 precipitate was used and the concentration of the precipitate was therefore 1 mg ml<sup>-1</sup>.

18

19 *Phytase activity towards IHP adsorbed to Al and Fe(III) precipitates*

1           The activity of three classes of phytases at pH6.0 was first determined and  
2 standardized. Phytases were diluted to 5 mU/ml in 50 mM MES assay buffer with 1  
3 mM CaCl<sub>2</sub> at pH6.0.

4           1 ml of the Al-IHP solution prepared was transferred to a 1.5ml eppendorf tube,  
5 and then centrifuged at 13,000 g for five minutes. The supernatant (F1) was  
6 transferred to a 1.5ml eppendorf tube and the salt pellet was then resuspended in 1ml  
7 MES buffer at pH6.0 without any CaCl<sub>2</sub>. After centrifugation at 13,000 g for five  
8 minutes, the supernatant was removed as F2. The amount of unbound IHP in F1 and  
9 F2 was determined by the molybdate method (Murphy and Riley, 1962) following  
10 acid-persulphate oxidation (US Environmental Protection Agency. 1993).

11          1ml of diluted phytase was then added to resuspend the pellet, and incubated at  
12 room temperature for various time intervals. The incubation periods were set at 0, 2, 4  
13 and 6 hours. At each time interval, the salt was pelleted by centrifugation at 13,000 g  
14 for five minutes, and the amount of Pi released by phytases in the supernatant (S1)  
15 was determined by the molybdate method. Since a portion of released Pi might be  
16 adsorbed to the salt precipitates, the salt was extracted by 1ml of 0.5M sulfuric acid  
17 for sixty minutes. After centrifugation, the supernatant (S2) was collected and  
18 neutralized by 100µl of 10M NaOH. The extracted Pi was then determined by the  
19 molybdate method. The total amount of Pi released by phytases was determined in S1

1 and S2. For the assay on Fe (III) precipitates, the experimental procedures were the  
2 same as above except that the supernatant (S2) was neutralized by 50  $\mu$ l of 10M  
3 NaOH.

4 To study the effect of organic anions on phytase activities towards IHP adsorbed  
5 to Al precipitates, enzymes were standardized to 5mU/ml in 50 mM MES assay buffer,  
6 pH 6.0, with various concentrations of organic acids. The amount of Pi released by  
7 phytases in the supernatant (S1) was determined by the molybdate method (Murphy  
8 and Riley, 1962).

## 1 **Results**

### 2 *Characterization of the three classes of phytases*

3 *Aspergillus ficuum* is known to produce two phytases, phytase A (phyA) and  
4 phytase B (phyB) (Ullah and Sethumadhavan, 1998). Both phytases contain the  
5 RHGXRRP active site and are therefore regarded as members of the histidine acid  
6 phosphatase family. Since phyB only has activity at pH < 5.0, the enzyme activity  
7 exhibited by the fungal phytase in this study, in which pH was always > 5.0, was  
8 contributed by phyA. Nonetheless, the identity of fungal phytase as a histidine acid  
9 phosphatase was confirmed by its sensitivity towards phenylgloxal (Fig 1a).

10 Nakano et al. (1999) purified and characterized two phytases (PHY1 and PHY2)  
11 from wheat bran. The N-terminal amino acid sequences of both phytases are  
12 EPAXTLTGPSRPV, which are almost identical to the amino acid sequence residues  
13 22-34 (EPASTLEGPSRPV) of a wheat phytase cDNA (Genbank accession no.  
14 AX298209). This cDNA encodes a polypeptide of 540 a.a. residues and the calculated  
15 molecular weight, excluding the first 21 a.a. putative signal peptide, is 57.7 kDa,  
16 which is very close to the apparent molecular weight of PHY1 (68kDa) and PHY2  
17 (66kDa). The difference could be attributed to glycosylation since the putative  
18 polypeptide contains five potential N-glycosylation sites. The encoded polypeptide  
19 does not carry the RHGXRRP motif of HAP, but has a high homology to the other

1 plant purple acid phosphatases, including *Oryza sativa* (Genbank accession  
2 NP910086). Therefore, it should be classified into the PAP family. To confirm this,  
3 the sensitivity of the wheat phytase towards several inhibitors was tested. It was found  
4 to be resistant to phenylgloxal, but was sensitive to molybdate (Fig. 1b). The activity  
5 of PAP is generally suppressed by molybdate (Vogel et al., 2002).

6

#### 7 *Verification of wheat bran phytase as a purple acid phosphatase*

8 An antiserum directed against recombinant *Arabidopsis* PAP15 was employed in  
9 Western blotting studies. As shown in figure 2, a cross-reactive band of 63 kDa was  
10 recognized by the antiserum in the wheat bran phytase preparation.

11

#### 12 *pH optima of the three classes of phytase at 22°C*

13 The pH profiles of all three classes of phytases were tested at ambient  
14 temperature (22°C) because it is close to the normal temperature in soil. As shown in  
15 figure 3, the fungal and the wheat phytases both exhibited activity at acidic to neutral  
16 pH (pH 4.0-6.5), whereas BPP exhibited activity at slightly acidic to alkaline pH (pH  
17 5.5-8.5).

18

#### 19 *Effect of organic acids on enzyme activities*

1 Plant roots can secrete several organic anions, including citrate, malate, oxalate  
2 and succinate, for the solubilization of minerals (Lipton et al., 1987). Therefore,  
3 whether the secretion of anions might inhibit the activities of phytases was examined.  
4 As shown in fig. 4a and b, increasing concentrations of citrate and oxalate inhibited  
5 the activity of all three classes of phytases, whereas malate had relatively low  
6 inhibiting effect even at 5 mM (Fig. 4c). It was found that the removal of calcium  
7 from the buffer completely abolished the activity of BPP, while it has negligible effect  
8 on the activities of HAP and PAP (Fig. 4). When calcium was absent, 50% inhibition  
9 of HAP and PAP activities were observed at citrate concentrations at 0.664 and 1.745  
10 mM respectively (Fig. 4a). Regarding oxalate, 50% inhibition of HAP and PAP was  
11 observed at 0.34-0.36 mM acid (Fig. 4b).

12

### 13 *Hydrolysis of various phytate salts*

14 As shown in figure 5, all BPP (*Bacillus* phytase), PAP (wheat phytase) and HAP  
15 (*A. ficuum* phytase) were able to release inorganic orthophosphate from calcium,  
16 magnesium and manganese phytates at all cation:IHP ratios. However, none of them  
17 can hydrolyze  $Al^{3+}$ ,  $Cu^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$  and  $Zn^{2+}$  salts. High calcium or magnesium to  
18 IHP ratio up to 50:1 did not affect the dephosphorylation of phytate by all three  
19 classes of phytases. BPP and PAP progressively released more phosphate ions at



1 increasing  $Mg^{2+}$  to IHP ratio. In contrast, for manganese phytate, higher  $Mn^{2+}$  to IHP  
2 ratio significantly suppressed the release of  $P_i$  by all three classes of enzymes.  
3 *Bacillus* phytase could not utilize magnesium phytate as the true substrate as it  
4 showed no activity to magnesium phytate in the absence of  $Ca^{2+}$  ions.

5 Figure 6 shows the hydrolysis of calcium/cation phytate complexes when  $Ca^{2+}$   
6 was co-precipitated with other cations during the formation of phytate salts. With  
7 reference to the hydrolysis of calcium phytate, co-precipitation of  $Fe^{2+}$ ,  $Fe^{3+}$ ,  $Cu^{2+}$ ,  
8  $Al^{3+}$  with  $Ca^{2+}$  in the phytate complex completely inhibited the hydrolysis of phytate  
9 by all three classes of phytases. Reduced activity was observed when  $Mn^{2+}$  or  $Zn^{2+}$   
10 was coprecipitated with  $Ca^{2+}$ . Only  $Mg^{2+}$  did not show any interference.

11 Since organic anions can chelate calcium ions, calcium ions were omitted in the  
12 assay buffer and as a result, BPP lost its activity. Therefore, the effect of organic  
13 anions on the hydrolysis of  $Al^{3+}$ -,  $Cu^{2+}$ -,  $Fe^{2+}$ -,  $Fe^{3+}$ - and  $Zn^{2+}$ -phytate salts were only  
14 studied on HAP and PAP (Fig. 7). In general, citrate was more effective than malate  
15 and oxalate in solubilizing cation-phytate salts, which led to the release of phytate for  
16 enzyme hydrolysis (Fig. 7). In the case of Cu-phytate, higher oxalate concentration  
17 resulted in lower P release, presumably due to its stronger inhibitory effect to both  
18 HAP and PAP (Fig. 4b).

19

1 *IHP adsorption to Al and Fe(III) precipitates*

2 After freeze drying, the amount of Al and Fe precipitates prepared from 400 ml  
3 of 0.17 M salt solution were 5.3 and 7.3 g, respectively. The amounts of IHP adsorbed  
4 to Al and Fe(III) precipitates after overnight incubation at pH 6.0 are shown in table 1.  
5 The amount of IHP adsorbed to Al precipitates was ten times more than that adsorbed  
6 to Fe(III) precipitates, which was consistent with the data from Shang et al. (1992).

7

8 *Phytase activity towards IHP adsorbed to Al and Fe(III) precipitates*

9 When the enzyme assays were carried out without the addition of organic acids,  
10 the total amount of Pi released by three distinct enzymes slightly increased as the  
11 reaction proceeded. However, the figures were very insignificant and unsteady despite  
12 of long reaction time (data not shown). In fact, the barely detectable Pi released  
13 during enzyme incubation might be attributed from the hydrolysis of soluble IHP that  
14 was desorbed over the long period of shaking. We can concluded that none of the  
15 three enzymes was able to hydrolyze IHP adsorbed to Al and Fe (III) precipitates.

16 Since plants are able to secrete organic acids from their roots, the effect of  
17 organic acids was examined. As shown in figure 8, both PAP and HAP released more  
18 Pi from IHP adsorbed to aluminum precipitates at increasing organic acid  
19 concentrations (from 0.015625 mM to 1 mM). Among all three organic acids, citrate

1 contributed the best enhancing effect on releasing Pi, and oxalate exhibited greater  
2 effect than malate. However, the difference between the three organic acids  
3 diminished as the concentration of organic acids decreased.

4 In the case of IHP adsorbed to Fe (III) precipitates, none of the three organic  
5 acids can significantly augmented Pi liberation by PAP or HAP, when compared with  
6 the control without organic acids. The insignificant reading could be due to (1) Low  
7 amount of IHP adsorbed to Fe (III) precipitates (Table 1); (2) The Pi released from the  
8 enzymatic reaction, if any, was adsorbed by the Fe (III) precipitates. To test the extend  
9 of Pi adsorption by the precipitates, 1 mg P/L Pi solution was added to the precipitates.  
10 After 3 hours of incubation, the amounts of free Pi that remained in the solution were  
11 measured. As shown in table 2, more Pi was adsorbed to the FeOH-IHP, FeOH and  
12 AlOH compounds (77.7-84.1%) than to AlOH-IHP (39.4%). The Pi adsorbed to these  
13 compounds could not be recovered by acid extraction. Only 19% and 38% of Pi  
14 adsorbed to FeOH-IHP and AlOH-IHP were desorbed by 0.5M sulfuric acid (data not  
15 shown).

16

17

18

## 1 **Discussion**

2        Although phytate constitutes the major proportion of phosphorus in soil (Turner  
3 et al., 2002), it is not directly available to plants. One approach in plant biotechnology  
4 for enabling plants to assimilate external phytate is the excretion of phytases from  
5 their roots. Transgenic plants that secrete BPP (Lung et al., 2005), HAP (Richardson et  
6 al., 2001) and PAP (Xiao et al., 2005) from the roots have been generated. All of them  
7 were reported to be able to assimilate soluble phytate in agar. However, whether the  
8 excreted phytases can utilize soil phytate is questionable. Due to its dense negative  
9 charges, IHP is tightly absorbed to clays, or precipitated as insoluble salts with Ca, Fe  
10 and Al in soil (Turner et al., 2002). This is the major constraint that restricts the  
11 applicability of this technology in agriculture.

12        The three-dimension structures of BPP (Shin et al., 2001), HAPs (Lim et al.,  
13 2000; Liu et al., 2004) and plant PAPs (Strater et al., 1995; Schenk et al., 2005) have  
14 been revealed by X-ray crystallography. The substrate binding site in HAP is located  
15 in a deep indentation inside the molecule, which limits its access to phytate salts in  
16 soil. The binding of IHP to *E. coli* HAP involved the interaction of all six  
17 deprotonated phosphate groups on the inositol ring with the side chains of thirteen,  
18 mostly basic, amino acid residues, implying a need for the solubility of the IHP salts  
19 before substrate binding (Lim et al., 2000). While the substrate binding sites in

1  $\beta$ -propeller phytase (Shin et al., 2001) and PAP (Strater et al., 1995; Schenk et al.,  
2 2005) are located at the enzymes' surfaces, it is unlikely for them to hydrolyze phytate  
3 adsorbed to soil surface, as shown by our results on Al (III) and Fe (III) precipitates.

4 Phytate in soil is either complexed with cations or adsorbed to soil components.  
5 Our results indicated that all three enzymes could hydrolyse phytate complexed with  
6  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  ions, but not phytate complexed with  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  nor  $\text{Zn}^{2+}$ .  
7 The major source of soil phytate is from phytins of plant seed remains, which are  
8 complexes of phytate with  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  ions. Our results showed that the availability  
9 of  $\text{Ca}^{2+}$ -or  $\text{Mg}^{2+}$ -phytates to these enzymes were greatly hampered by the presence of  
10  $\text{Al}^{3+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  nor  $\text{Zn}^{2+}$  ions. This phenomenon can be explained by the  
11 stabilities of these cations with phytate, which is in the order of  $\text{Fe}^{2+} \sim \text{Zn}^{2+} \sim \text{Fe}^{3+} >$   
12  $\text{Mn}^{2+} > \text{Ca}^{2+} > \text{Mg}^{2+}$  (Maenz et al., 1999). The implication is that once plant phytin  
13 interacts with other cations in soil, their availability to phytase secreted from plant  
14 roots is interfered.

15 In addition to phytate salts, the adsorption of inositol phosphates to clays or to  
16 insoluble salts such as Fe and Al oxides and hydrous oxides (Shang et al., 1992) also  
17 diminished the phytate availability. Greaves and Webley (1969) reported that the  
18 hydrolysis of sodium phytate was generally reduced in the presence of the clay  
19 minerals kaolinite and montmorillonite. Our results showed that IHP was not

1 susceptible to phytase hydrolysis once it was adsorbed to Al and Fe precipitates.

2        Secretion of organic acids from plant roots is a means to improve P acquisition.

3 Under P stress, plant roots elevate the secretion of citrate and malate (Lipton et al.,

4 1987; Hoffland et al., 1992; Johnson et al., 1996). Organic acids were shown to

5 elevate 10-1000-fold higher soil solution Pi concentrations by desorbed Pi adsorbed to

6 soil constituents (Earl et al., 1979; Traina et al., 1986). They either directly exchange

7 Pi adsorbed to soil constituents, such as crystalline  $\text{Al}(\text{OH})_3$  or  $\text{Fe}(\text{OH})_3$ , or chelate

8 metal ions in cation-Pi complexes (Jones 1998). Our results showed that only citrate is

9 effective in solubilizing  $\text{Al}^{3+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Zn}^{2+}$ -phytate for phytase hydrolysis,

10 whereas the effects of malate and oxalate are not prominent. In contrast, all tested

11 organic acids could enhance hydrolysis of phytate adsorbed to Al (III) precipitates by

12 HAP and PAP, in the descending order citrate > oxalate > malate, presumably by

13 releasing free phytate from the precipitates. While the Pi extraction efficiency of

14 organic acids also follows the same order (Jones 1998), the result is slightly different

15 from a study on the sorption of organic acids, where the amount of anions adsorbed to

16 acid soils and ferric hydroxide was in the descending order oxalate > citrate > malate

17 at pH 4-6 (Jones and Brassington 1998). Hence, for desorbing free phytate, ligand

18 exchange could be the main mechanism for oxalate, while metal complexation could

19 be a more important mechanism for citrate. Citrate carries three carboxyl groups in

1 comparison to malate and oxalate, which carries two carboxyl groups, so it has greater  
2 ability to complex cations. For example, at pH 6, oxalate and malate mainly carry two  
3 negative charges, while the ratio of citrate<sup>2-</sup> and citrate<sup>3-</sup> is approximately 1:1 (Jones  
4 and Brassington, 1998).

5 To conclude, in order to fulfill the goal of enabling plants to assimilate  
6 precipitated phytate by genetic engineering, both approaches involving enhancement  
7 of phytase secretion and organic acids excretion should be adopted. Enhanced  
8 secretion of organic acids has been accomplished by overexpression of malate  
9 dehydrogenase (Tesfaye et al., 2001), citrate synthase (Koyama et al., 2000) and  
10 malate transporter (Delhaize et al., 2004). While fungal phyA (HAP) and wheat bran  
11 phytases (PAP) have comparable specific activities (190-225 vs. 260-290 U/mg) and  
12 kcat values (300-348 vs 144-270 s<sup>-1</sup>), fungal phyA has higher km for phytate (27-50  
13 vs 0.48-0.77 μM) (Nakano et al., 1999; Ullah et al., 2002). Hence, PAP might be a  
14 better choice than HAP, as it can tolerate higher citrate concentration, which could  
15 accumulate locally at the root-soil interface. Other factors, like the expression level  
16 and the stability of recombinant phytase in plant, should also be taken into  
17 consideration. BPP, in contrast, is not a suitable candidate for this purpose, due to its  
18 calcium dependence and sensitivity to organic acids.

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3 the Hong Kong Special Administrative Region Government, China.

4

5



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- 12

1 **Figure legends**

2 **Figure 1. Sensitivity of *Bacillus subtilis* phytase (■), fungal phytase (∇) and wheat**  
3 **bran phytase (▲) towards phenylglyoxal (a) and molybdate (b).** Each point  
4 represents the mean of four experiments.

5 **Figure 2. Western blotting of purified wheat bran phytase by anti-PAP15**  
6 **antiserum.**

7

8 **Figure 3. The pH profile of *Bacillus subtilis* phytase (■), fungal phytase (∇) and**  
9 **wheat bran phytase (▲).** Their enzyme activities were assayed at room temperature  
10 in the following buffers with 1 mM CaCl<sub>2</sub>: 50 mM glycine-HCl buffer between pH  
11 2.0-3.5, 50 mM acetate buffer between pH 4.0-5.5, 50 mM Tris-Maleate buffer  
12 between pH 6.0-8.0 and 50 mM Glycine-NaOH between pH 9.0-9.5. Each point  
13 represents the mean of four experiments.

14

15 **Figure 4. Effect of citrate (a), oxalate (b) and malate (c) on BPP (■), HAP (◆) and**  
16 **PAP (▲) with (.....) or without 1 mM CaCl<sub>2</sub> (—).** Each point represents the  
17 mean of four experiments.

18

19 **Figure 5. Hydrolysis of phytate salts at different cation:IHP ratios.** The  
20 cation:IHP ratios were 2.5:1 (x), 5:1 (■), 10:1 (▲), 25:1 (◊) and 50: 1 (#). (N = 3).

21

22 **Figure 6. Hydrolysis of co-precipitated calcium/cation phytate.** The amounts of P  
23 released by BPP (black), PAP (grey) and HAP (white) are shown. The enzyme  
24 reaction was incubated at room temperature for 30 mins. (N = 3).

25

1 **Figure 7. Effects of organic anions on the hydrolysis of phytate salts.** The amounts  
2 of Pi release by PAP (a) and HAP (b) in the presence of organic anions are shown. (N  
3 = 3).

4

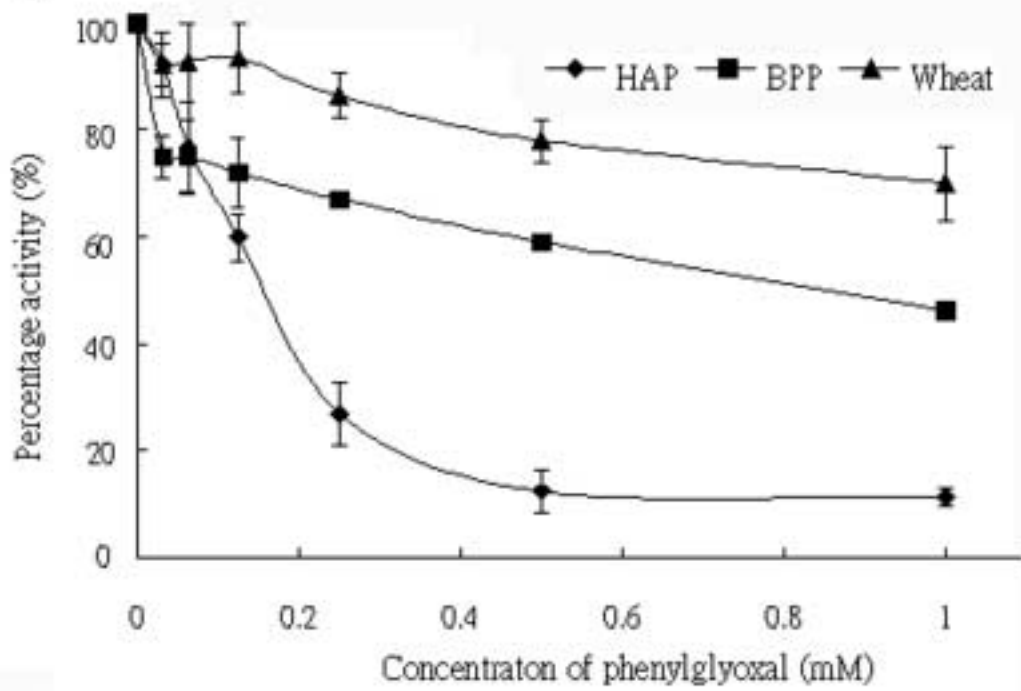
5 **Figure 8. Pi released from IHP adsorbed to Al ppt.** The amounts of Pi released by  
6 PAP (a) and HAP (b) in the presence of citrate (black), malate (grey), oxalate (white)  
7 or without organic acid (striped) are shown. Each data represents the mean of three  
8 observations with standard deviation.



Figure 1

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**a**



**b**

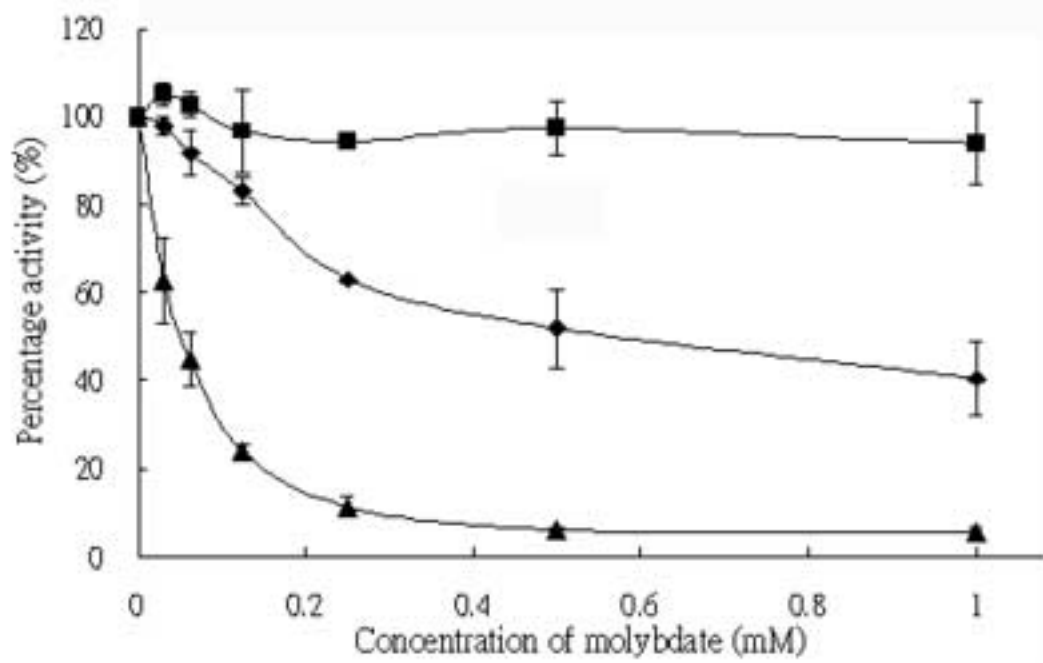


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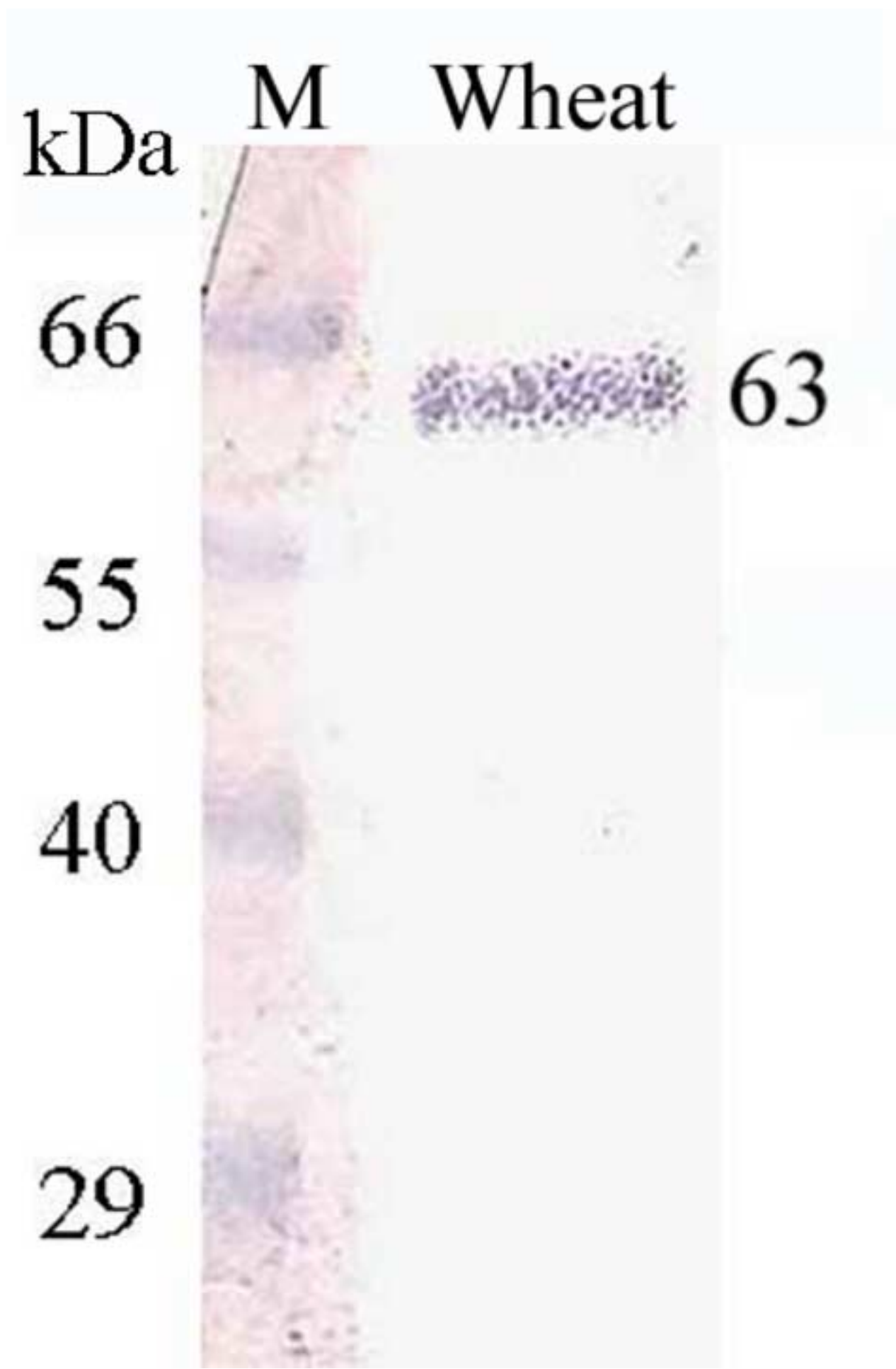


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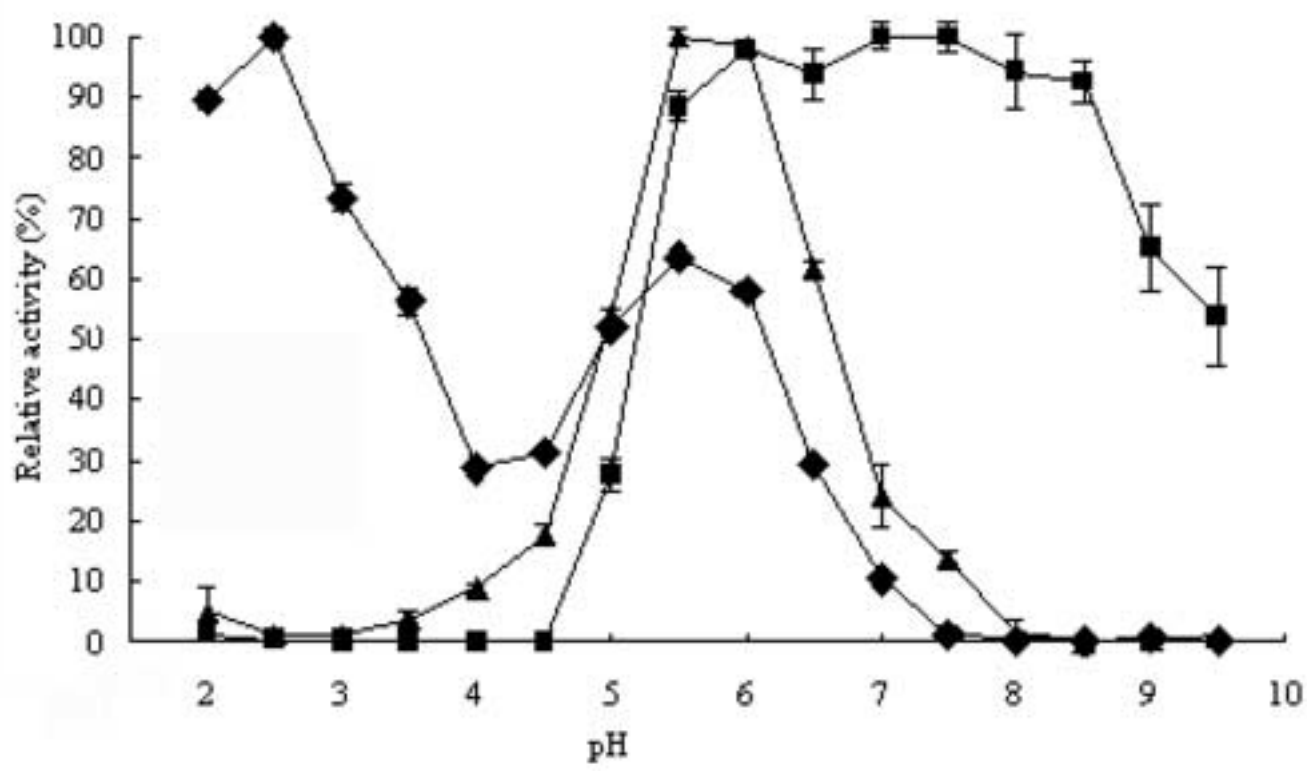


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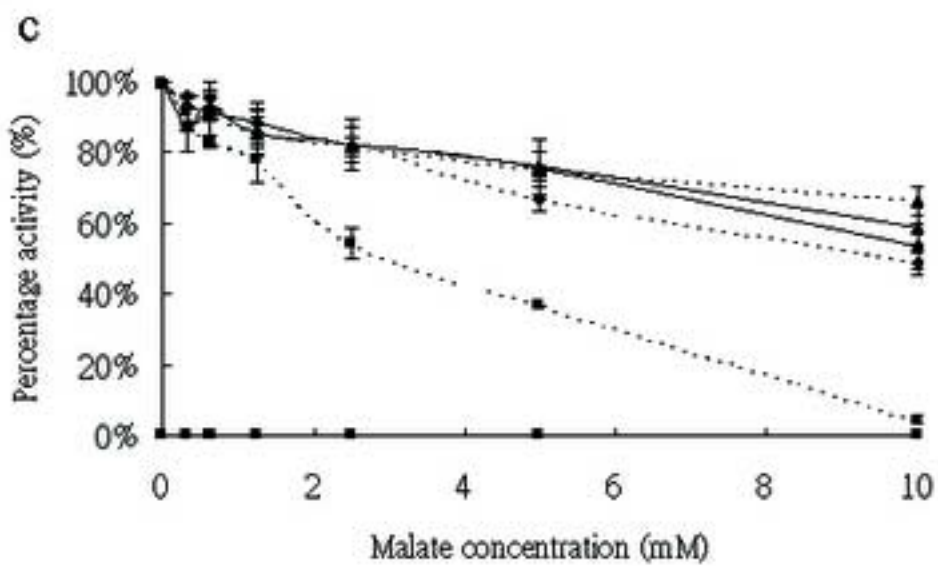
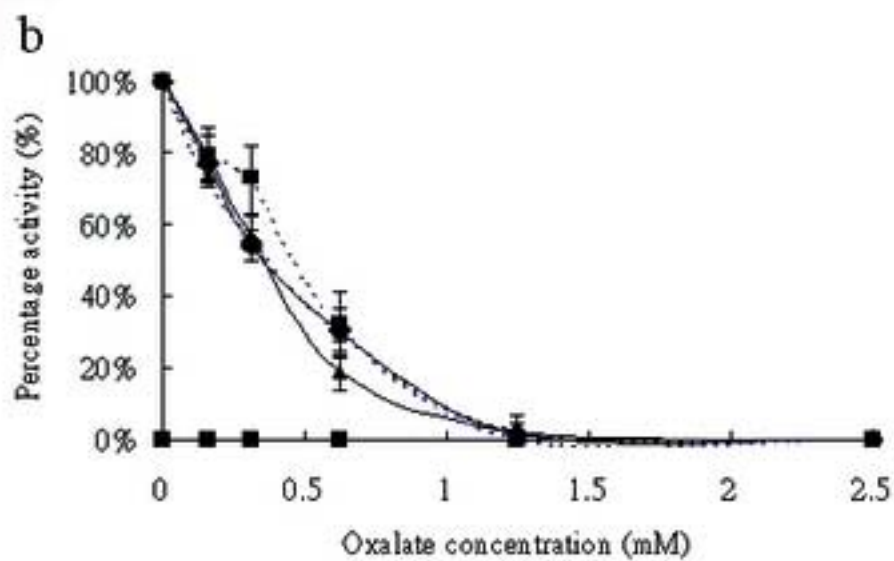
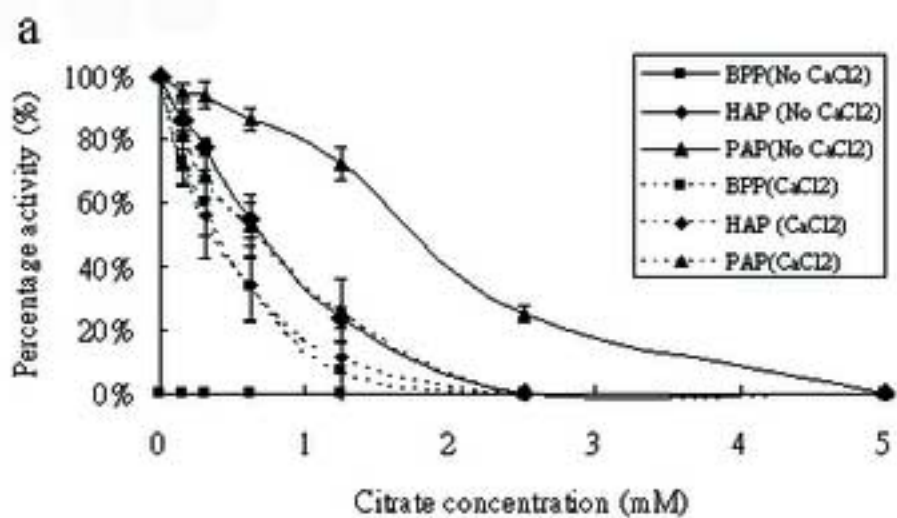


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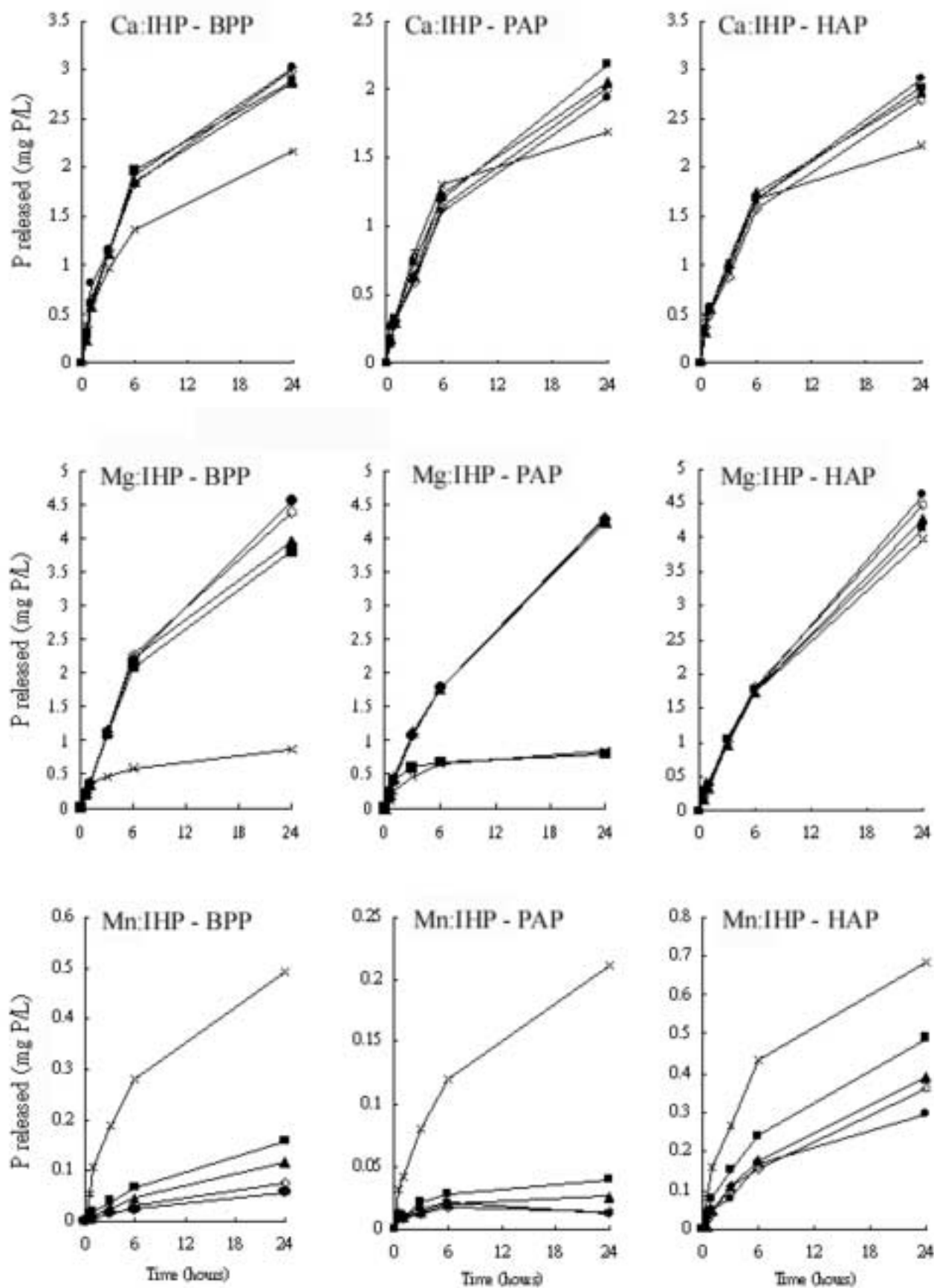


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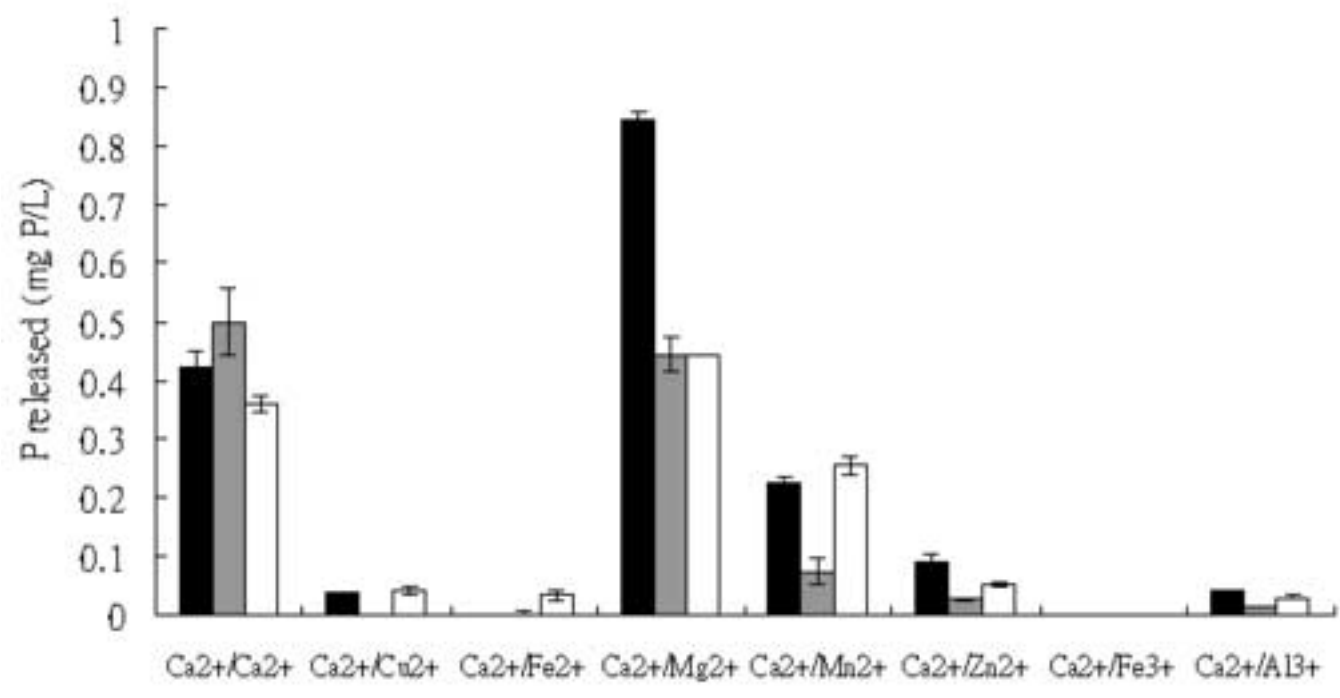


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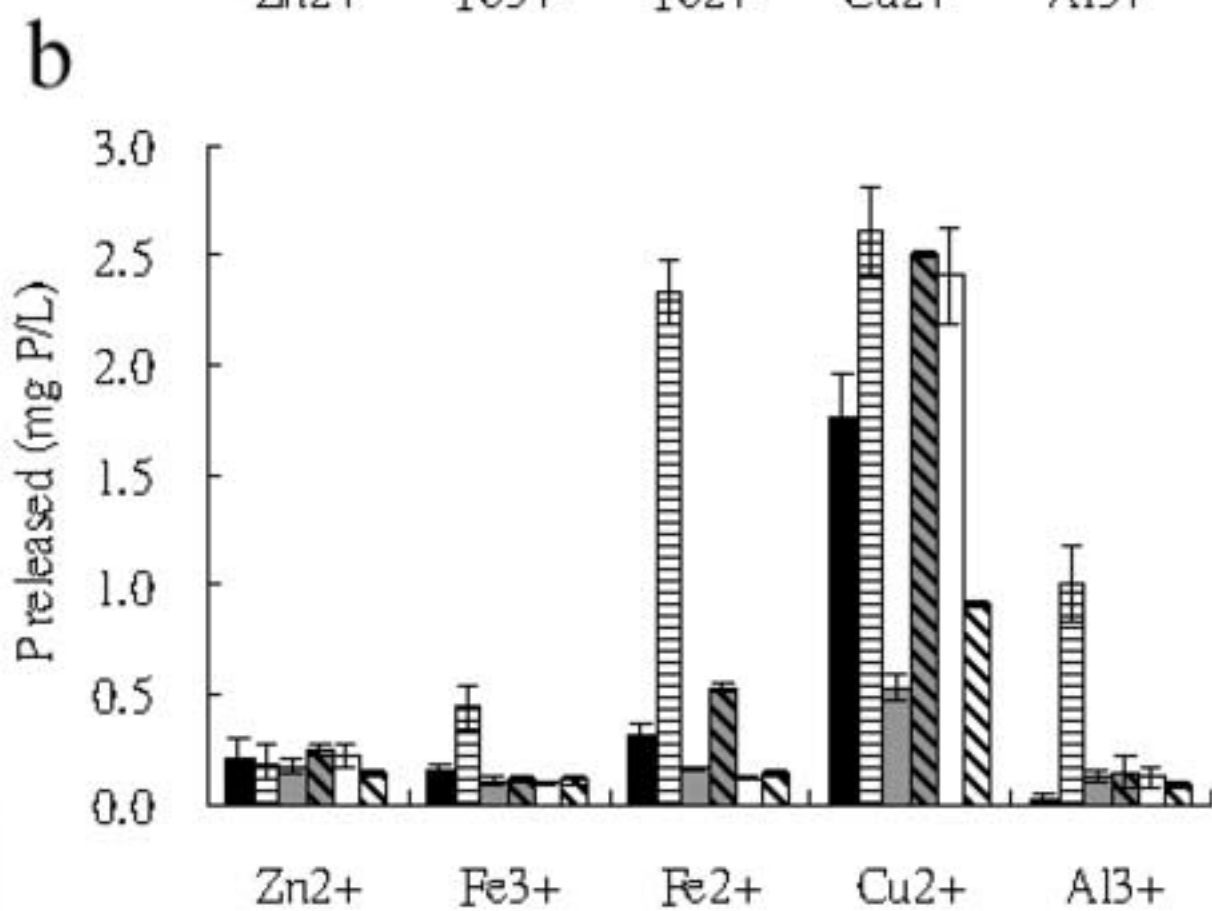
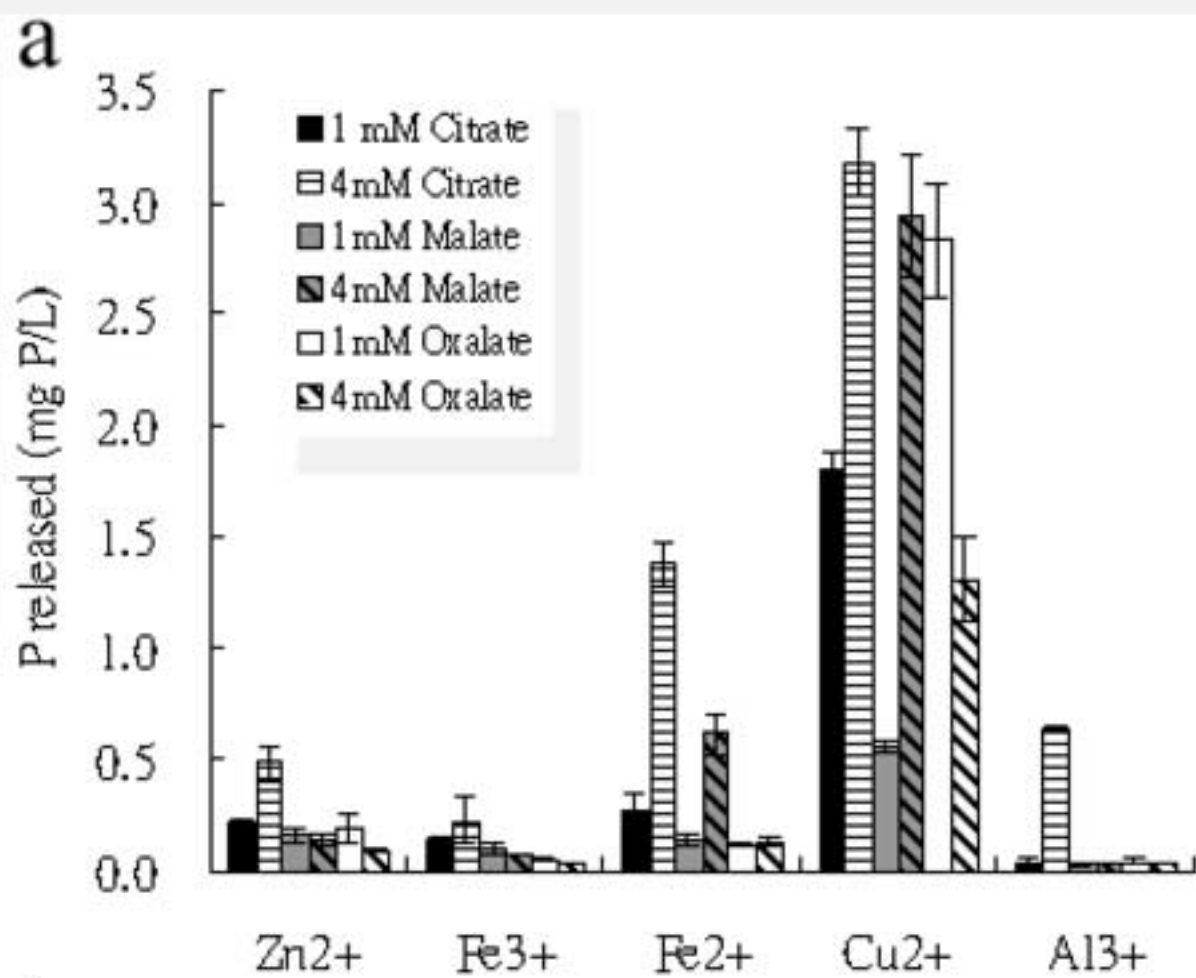


Figure 8

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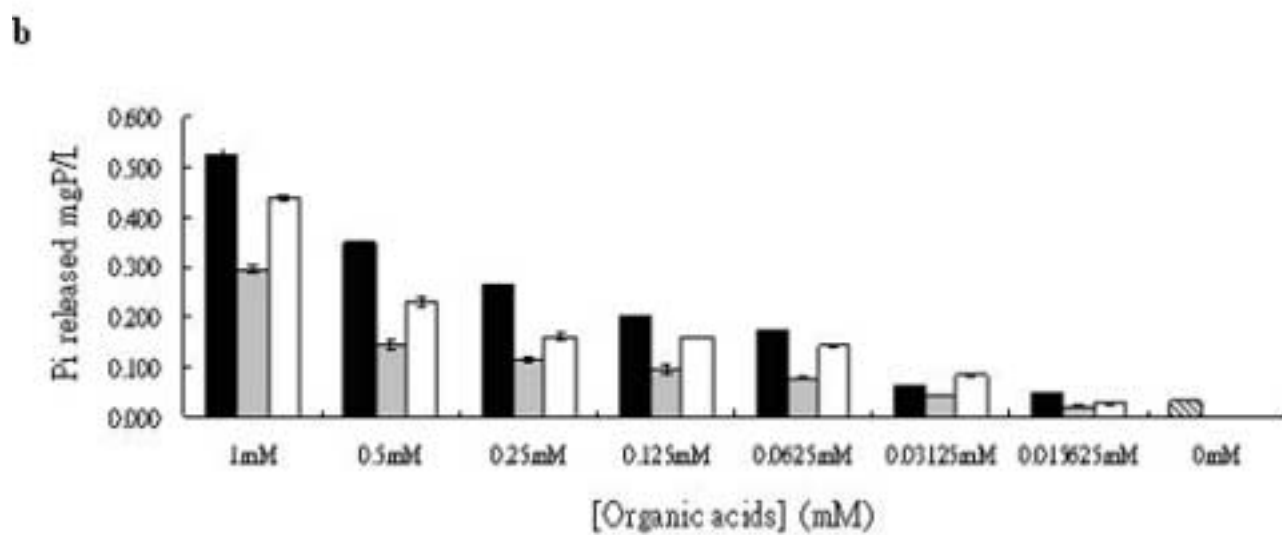
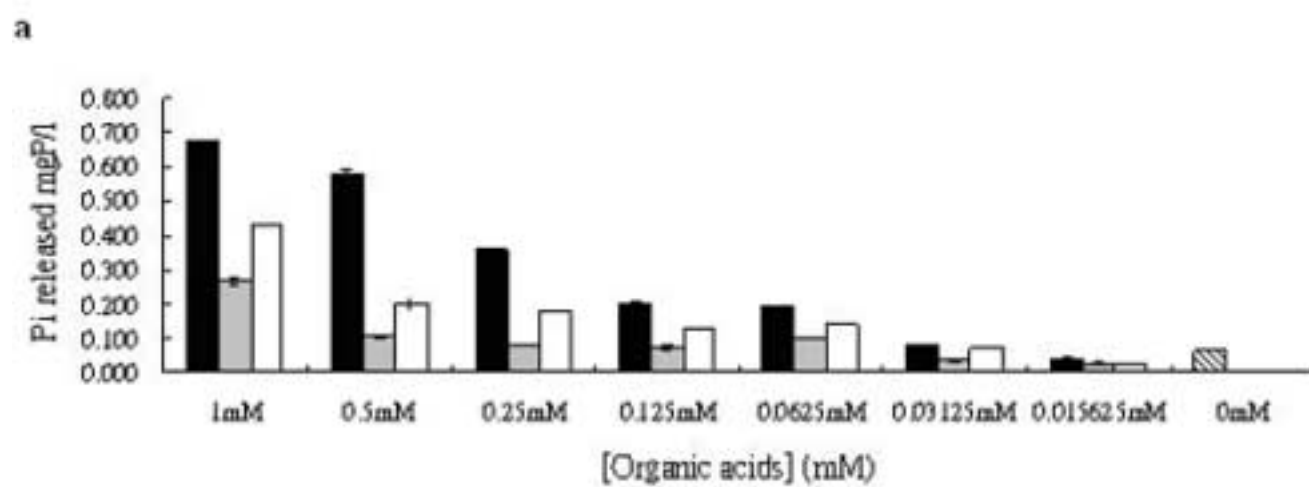




Table 1. Adsorption of IHP by aluminum &amp; iron (III) precipitates.

IHP added (mgP/L)	20	40	60	80	100	120
IHP bound (cmol P/ kg Al ppt.)	21.92±0.36	29.22±0.62	29.39±1.09	28.35±0.65	35.26±0.05	29.48±3.09
IHP bound (cmol P/ kg Fe ppt.)	1.26±0.18	1.35±0.31	1.33±0.22	0.63±0.32	1.94±0.03	2.11±0.05

Each point presents the mean of three experiments with standard deviation.

Table 2. Amount of Pi adsorbed by aluminum &amp; iron (III) compounds.

	FeOH-IHP	Fe-OH	AlOH-IHP	Al-OH
Pi added (mgP/L)	1.0	1.0	1.0	1.0
Pi adsorbed after 3hr (mgP/L)	0.777±0.016	0.783±0.018	0.394±0.021	0.841±0.016
% adsorption	77.7±1.6%	78.3±1.8%	39.4±2.1%	84.1±1.7%

Each data represents the mean of three experiments with standard deviation.