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

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Abstract

The ability of representative members from three classes of phytases: histidine acid phosphatases, β-propeller phytases, and purple acid phosphatases, to hydrolyze metal-phytate salts and phytate adsorbed to aluminum precipitates, were compared. All three tested phytases were able to hydrolyze Ca^{2+}-, Mg^{2+}- and Mn^{2+}-phytates, but not Al^{3+}-, Fe^{2+}-, Fe^{3+}-, Cu^{2+}- and Zn^{2+}-phytates. In addition, when these ions were present, the hydrolysis of Ca^{2+}-phytate was prevented. Only higher concentration of citrate, but not malate nor oxalate, can partially solubilize some of these phytate salts for enzyme hydrolysis. Phytate adsorbed to aluminium precipitates were resistant to all three enzymes, except when organic acids were added (citrate > oxalate > malate). While high concentration of organic acids were inhibitory to enzyme activity (oxalate > citrate > malate), purple acid phosphatase was more resistant to citrate than histidine acid phosphatase. Since desorption of phytate from solid surface by organic acids is essential for phytase activity, genetic engineering of plants that enhance secretion of both citrate and phytases, preferably purple acid phosphatase, from the root, may be a feasible approach to improve soil phytate assimilation.

Keywords

beta-propeller phytase, citrate, histidine acid phosphatase, malate, oxalate, purple acid phosphatase, phytate.

Introduction

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

Inositol phosphates (IP), particularly inosital hexakisphosphate (IHP or phytate), is the predominant form of organic phosphorus in soil (Anderson et al., 1980). The preferential accumulation of inositol phosphates in soils is due to their adsorption on soil colloids, which hampers their biodegradation (Ognalaga, 1994). As the number of P increases, the interaction between inosital phosphates with soil becomes stronger due to their higher charge density. The typical proportion of inositol phosphates in soil is 83%:12%:4%:1% for IP6:IP5:IP4:IP3 (Anderson et al., 1980). They are either being adsorbed to clays or precipitated as insoluble salts, such as sesquioxides of Fe and Al in acid soils and insoluble calcium salts in alkaline soils (Turner et al., 2002).

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

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

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

Materials and methods

Chemicals and Enzymes

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

Verification of wheat bran phytase as a purple acid phosphatase

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

Phytase activity assays

The enzymes were first diluted with 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) buffer, pH 6.0 with or without 1 mM $CaCl_2$ and the reaction was initiation by addition of sodium phytate, in which the final concentration of sodium phytate in the reaction mix was 1 mM. To determine the pH optima of the enzymes, 100 mM 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, 8.5) with 1 mM $CaCl_2$ were used. All the enzyme assays were carried out at room temperature for thirty minutes before being stopped by adding equal volume of 4% (v/v) trichloroacetic acid (TCA). 150 μl TCA-mixture was then transferred to a microtiter plate and the liberated Pi was quantified by OD 650 nm measurement, taken ten minutes after addition of 24 μl molybdate reagent (Murphy and Riley, 1962). One unit of enzyme was defined as the amount of enzyme that liberates 1 μmol of Pi per minute under the assay conditions. For inhibition assays, the enzymes were incubated with different concentrations of the inhibitors citrate, malate, molybdate, and oxalate in 100 mM MES buffer, pH 6.0, with or without 1 mM $CaCl_2$. For phenylgloxal inhibition assay, the enzymes were dialysed into 50 mM sodium bicarbonate at pH 7.5. The enzymes were then incubated with various concentrations of phenylgloxal at 37^{o}C in the bicarbonate buffer (Ullah and Sethumadhavan, 1998).

After incubation for one hour, 50 μl aliquot was transferred to 950 μl 100 mM MES buffer, 1 mM $CaCl_2$, 1 mM phytate, pH 6.0. After incubation at room temperature for 30 minutes, the amount of released Pi was determined by method described above.

Preparation of insoluble phytate salts

To study the hydrolysis of metal phytates, 500 mM stock solutions of Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+} salts were prepared by dissolving $CaCl_2 \cdot 2H_2O$, $CuCl_2 \cdot 2H_2O$, $FeSO_4 \cdot 7H_2O$, $MgCl_2 \cdot 6H_2O$, $MnCl_2 \cdot 4H_2O$, $ZnSO_4 \cdot 7H_2O$, $FeCl_3 \cdot 6H_2O$ and $AlCl_3 \cdot 6H_2O$ in distilled water accordingly. The 500 mM stock solution was serially diluted to 250 mM, 100 mM, 50 mM and 25 mM, respectively. Equal volumes of 25, 50, 100, 250 and 500 mM salt solutions (Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{3+} and Al^{3+}) and 10 mM sodium phytate were mixed and incubated overnight at room temperature without shaking. The precipitated salts were centrifuged at 13,000 g at room temperature for five minutes. The supernatant was decanted off and the precipitated salts were washed thrice in two volumes of 50 mM MES buffer, pH 6.0, with 1 mM $CaCl_2$ and finally resuspended in one volume of the same buffer. Phytase assays were initiated by incubating 0.5 ml of phytate salts with 0.5 ml enzyme (5 mU/ml) at room temperature. Aliquots of the mixture were periodically taken at thirty minutes to twenty-four hours after enzyme addition. The salts were pelleted by

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

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

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

Determination of inositol hexakisphosphate

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

Preparation of Al and Fe(III) precipitates

The method for aluminum and iron (III) precipitates preparation was based on the method by Shang et al. in 1992. A 400 ml 0.17M $AlCl_3$ solution was titrated by 1.0M NaOH solution until the pH reached 6.5. The mixture was maintained at pH 6.5 for fifteen minutes and then centrifuged at 13,000 g for twenty minutes. The supernatant solution was decanted and the pellet left in the centrifuge bottle was resuspended in 300 ml distilled water to remove soluble ions. The salts were collected again by centrifugation and this washing step was done twice. Finally, the precipitates were then stored at -80ºC overnight and freeze-dried. To prepare iron (III) precipitates, a 0.17M $FeCl_3$ solution (400 ml) titrated by 1.0M NaOH solution until the pH reach 6.5. The iron (III) precipitates were then washed and prepared as described above.

IHP adsorption to Al and Fe(III) precipitates

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

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

The activity of three classes of phytases at pH6.0 was first determined and standardized. Phytases were diluted to 5 mU/ml in 50 mM MES assay buffer with 1 mM $CaCl_2$ at pH6.0.

1 ml of the Al-IHP solution prepared was transferred to a 1.5ml eppendorf tube, and then centrifuged at 13,000 g for five minutes. The supernatant (F1) was transferred to a 1.5ml eppendorf tube and the salt pellet was then resuspended in 1ml MES buffer at pH6.0 without any $CaCl_2$. After centrifugation at 13,000 g for five minutes, the supernatant was removed as F2. The amount of unbound IHP in F1 and F2 was determined by the molybdate method (Murphy and Riley, 1962) following acid-persulphate oxidation (US Environmental Protection Agency. 1993).

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

and S2. For the assay on Fe (III) precipitates, the experimental procedures were the same as above except that the supernatant (S2) was neutralized by 50 μl of 10M NaOH.

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

Results

Characterization of the three classes of phytases

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

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

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

Verification of wheat bran phytase as a purple acid phosphatase

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

pH optima of the three classes of phytase at 22^{o}C

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

Effect of organic acids on enzyme activities

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

Hydrolysis of various phytate salts

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

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

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

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

IHP adsorption to Al and Fe(III) precipitates

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

Secretion of organic acids from plant roots is a means to improve P acquisition. Under P stress, plant roots elevate the secretion of citrate and malate (Lipton et al., 1987; Hoffland et al., 1992; Johnson et al., 1996). Organic acids were shown to elevate 10-1000-fold higher soil solution Pi concentrations by desorbed Pi adsorbed to soil constituents (Earl et al., 1979; Traina et al., 1986). They either directly exchange Pi adsorbed to soil constituents, such as crystalline $Al(OH)_3$ or $Fe(OH)_3$, or chelate metal ions in cation-Pi complexes (Jones 1998). Our results showed that only citrate is effective in solubilizing Al^{3+}, Fe^{2+}, Fe^{3+} and Zn^{2+}-phytate for phytase hydrolysis, whereas the effects of malate and oxalate are not prominent. In contrast, all tested organic acids could enhance hydrolysis of phytate adsorbed to Al (III) precipitates by HAP and PAP, in the descending order citrate > oxalate > malate, presumably by releasing free phytate from the precipitates. While the Pi extraction efficiency of organic acids also follows the same order (Jones 1998), the result is slightly different from a study on the sorption of organic acids, where the amount of anions adsorbed to acid soils and ferric hydroxide was in the descending order oxalate > citrate > malate at pH 4-6 (Jones and Brassington 1998). Hence, for desorbing free phytate, ligand exchange could be the main mechanism for oxalate, while metal complexation could be a more important mechanism for citrate. Citrate carries three carboxyl groups in

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

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

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Figure legends

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

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

Figure 3. The pH profile of *Bacillus subtilis* phytase (■), fungal phytase (∀) and wheat bran phytase (▲). Their enzyme activities were assayed at room temperature in the following buffers with 1 mM $CaCl_2$: 50 mM glycine-HCl buffer between pH 2.0-3.5, 50 mM acetate buffer between pH 4.0-5.5, 50 mM Tris-Maleate buffer between pH 6.0-8.0 and 50 mM Glycine-NaOH between pH 9.0-9.5. Each point represents the mean of four experiments.

Figure 4. Effect of citrate (a), oxalate (b) and malate (c) on BPP (■), HAP (♦) and PAP (▲) with (……) or without 1 mM $CaC1_2$ (——). Each point represents the mean of four experiments.

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

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

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

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

Figure 1

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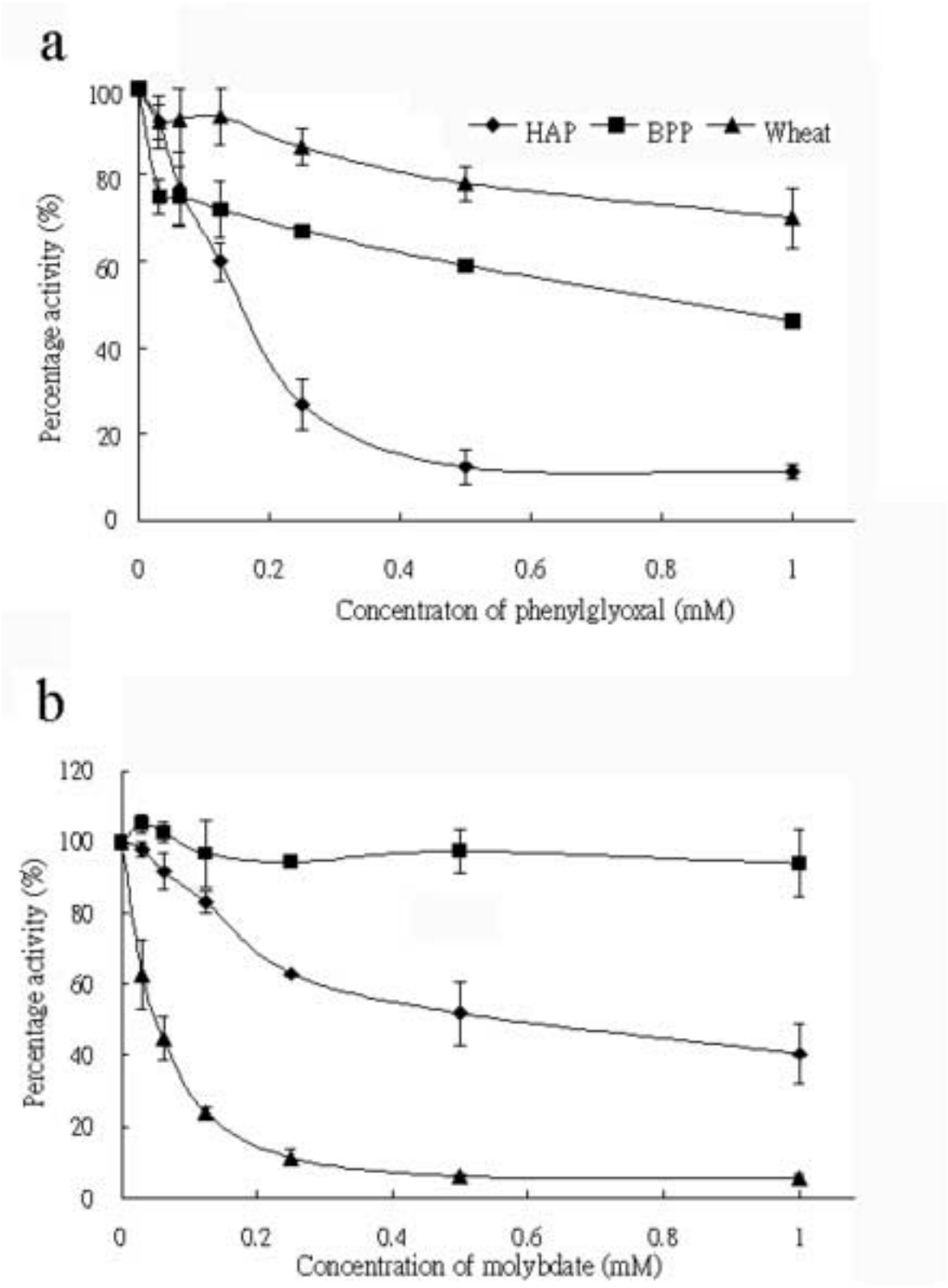

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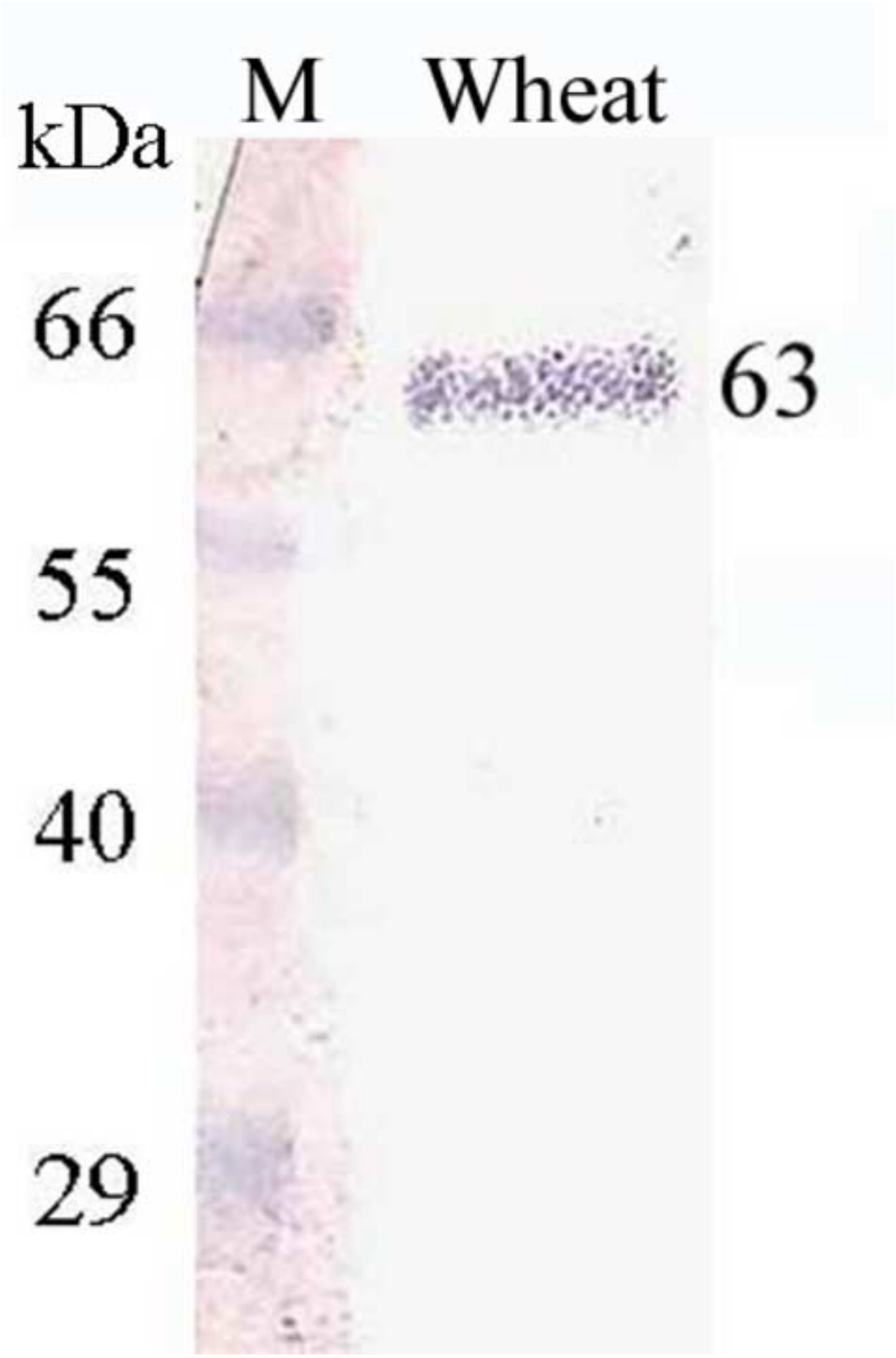

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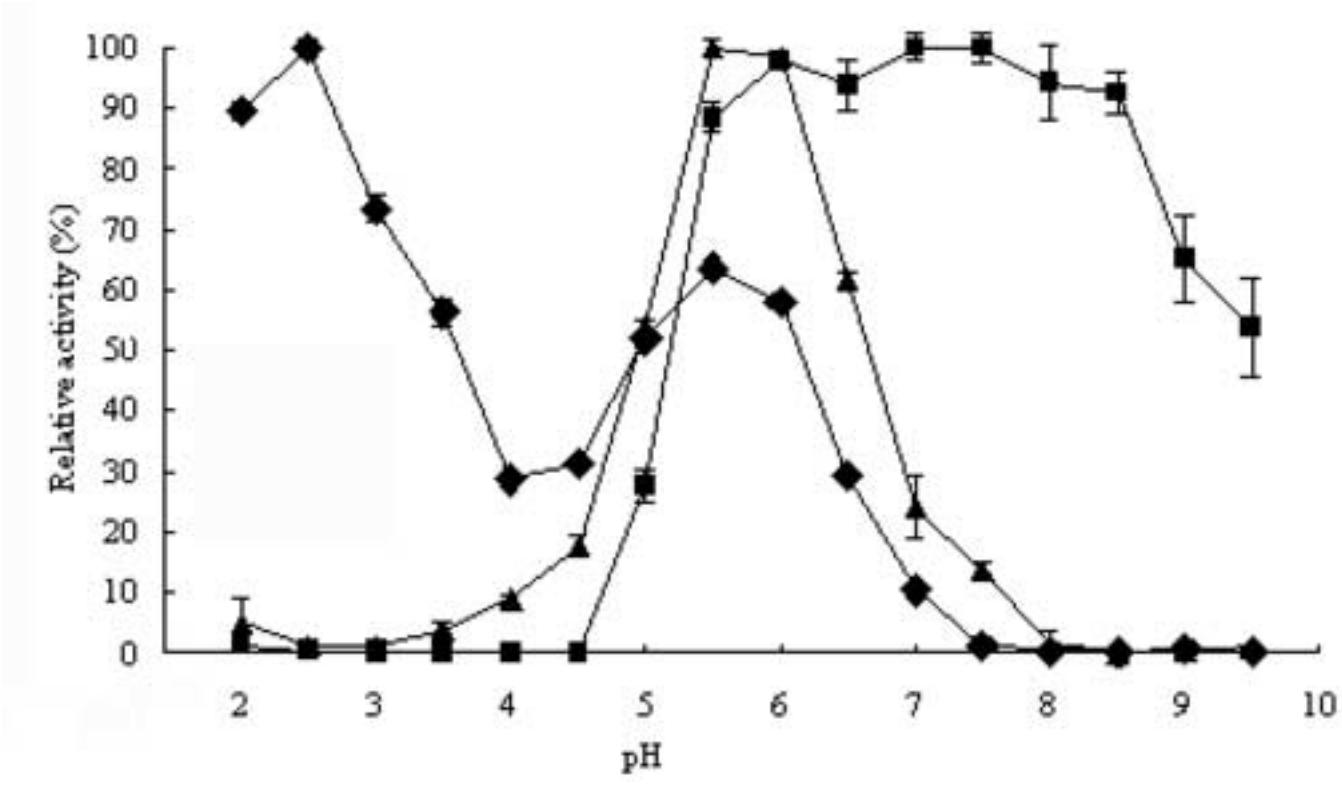

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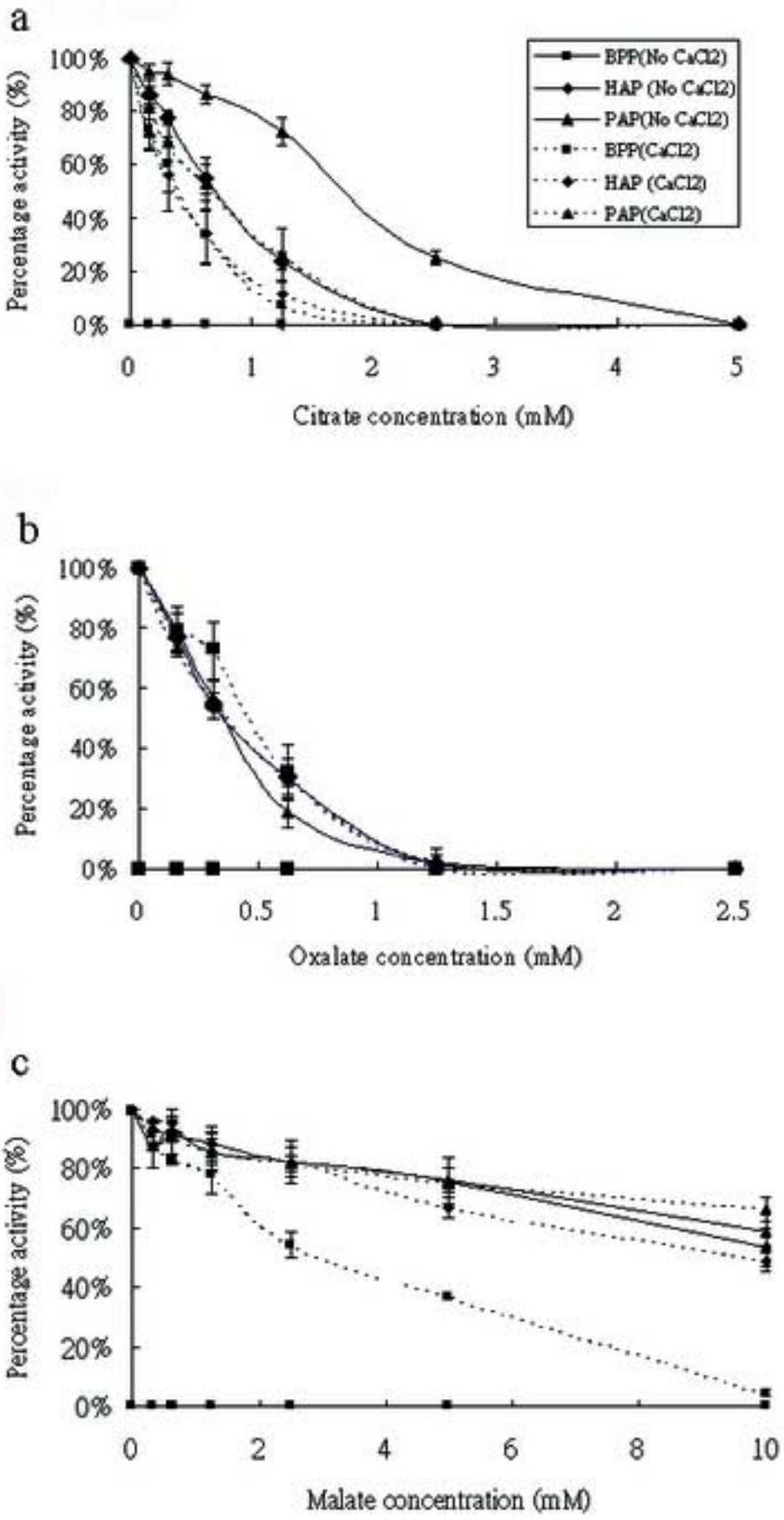

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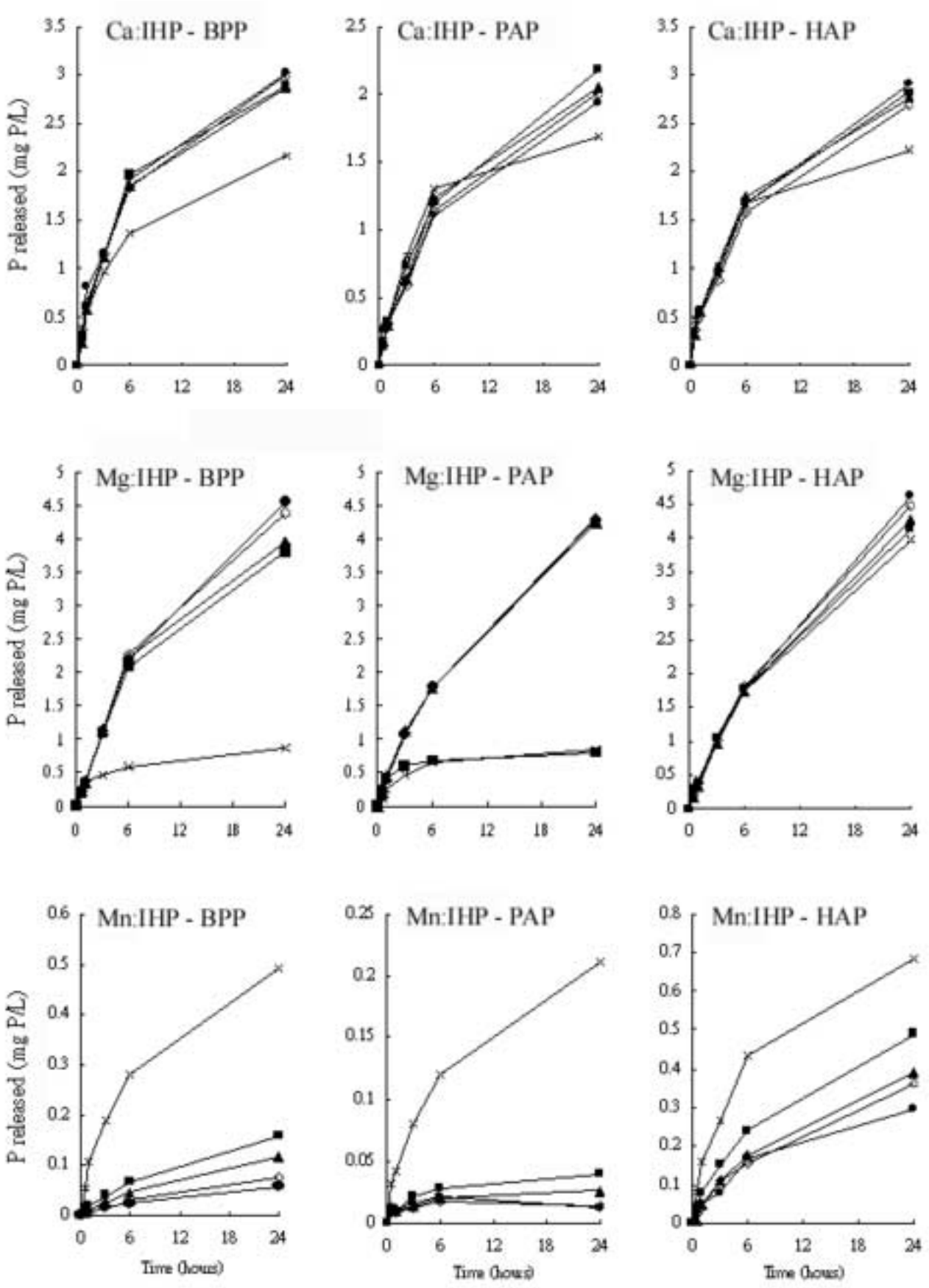

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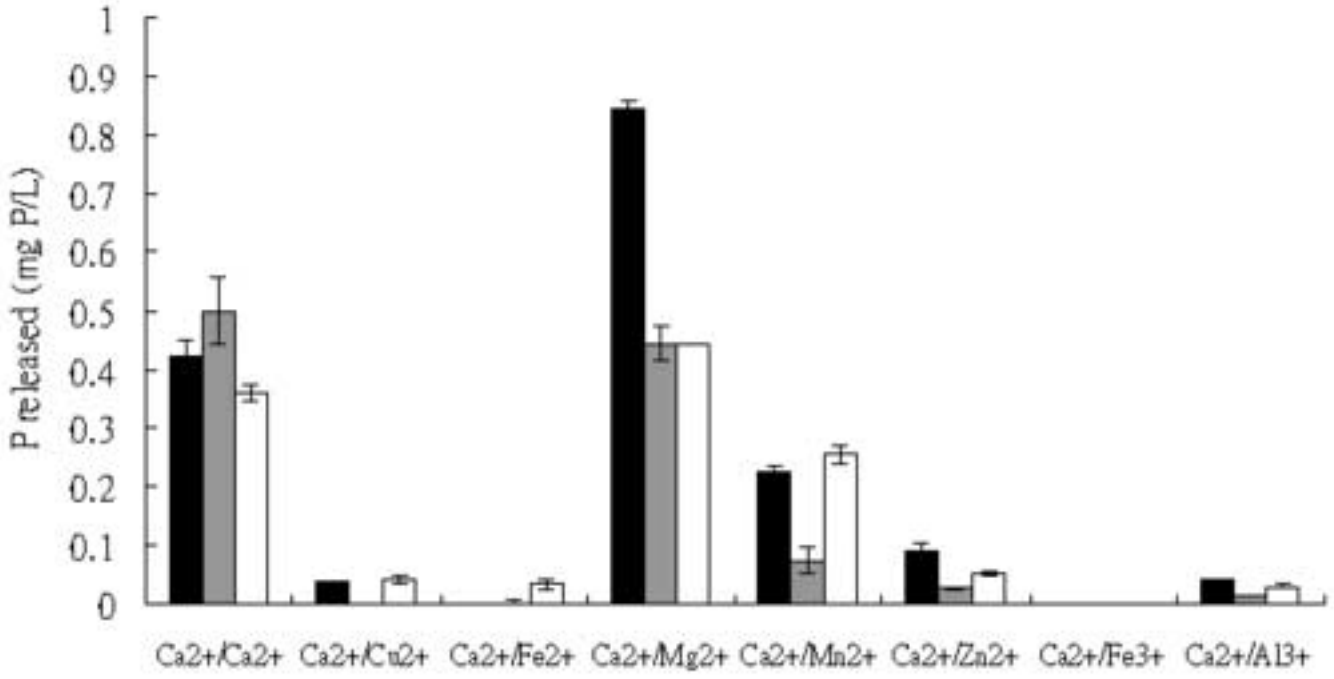

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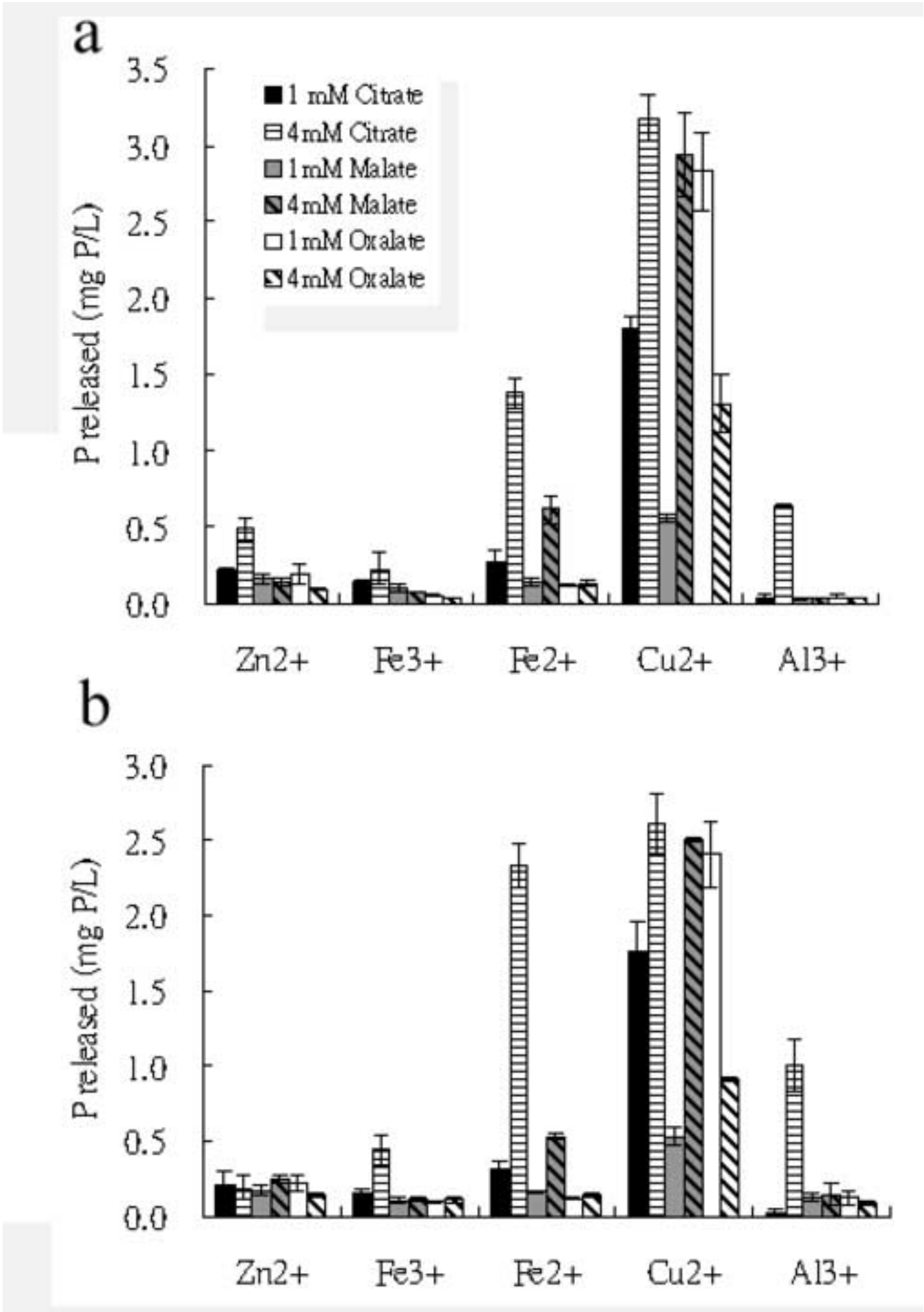

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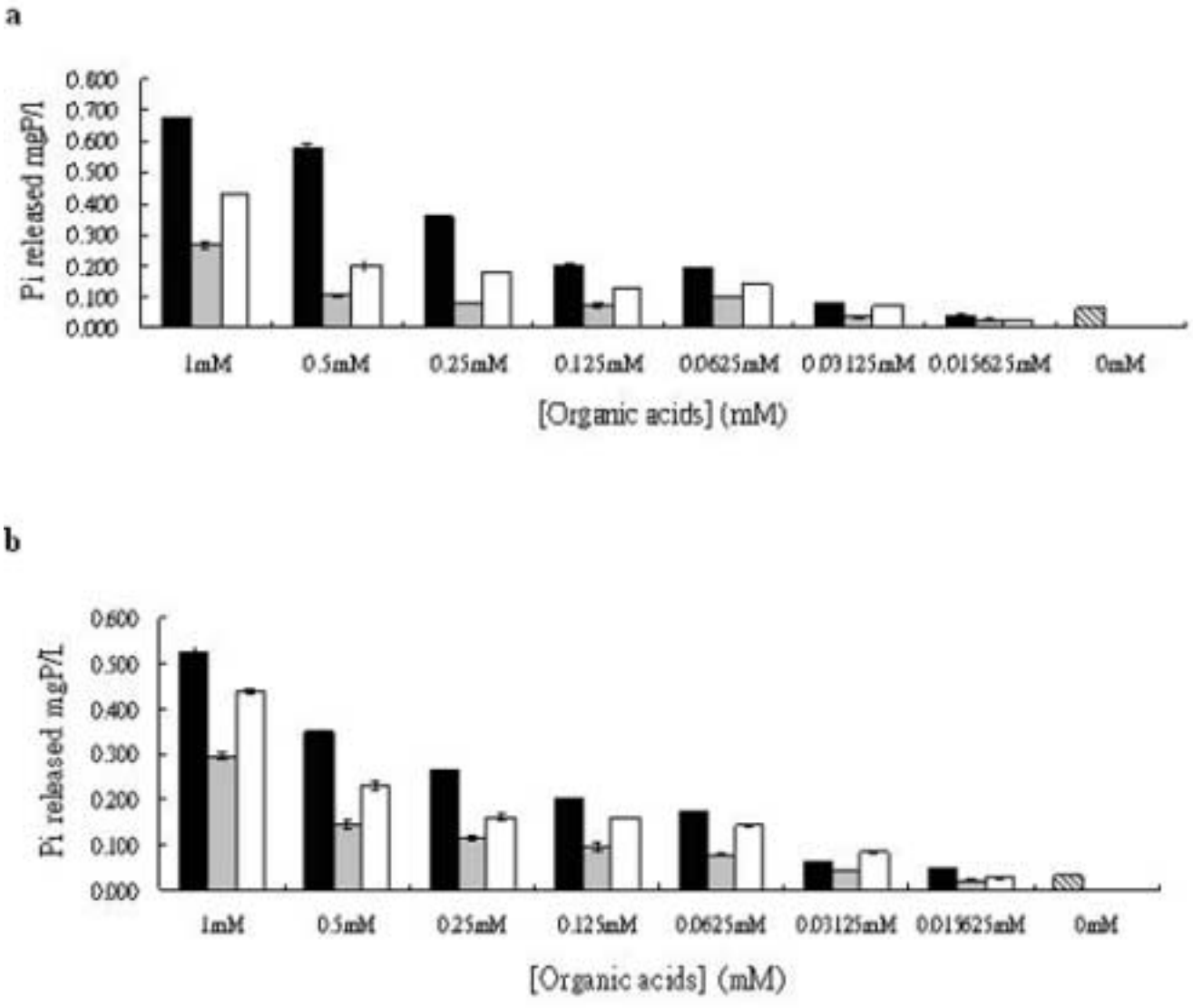

Table 1

Table 1. Adsorption of IHP by aluminum & 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

Table 2. Amount of Pi adsorbed by aluminum & 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.