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Science and Technology

Meri-Rastilantie 3 B, FI-00980
Helsinki, Finland
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Journal of Food, Agriculture & Environment Vol.6 (3&4) : 500-505. 2008

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Hydrolysis of organic phosphates by commercially available phytases: Biocatalytic potentials and effects of ions on their enzymatic activities

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Received 7 June 2008, accepted 18 August 2008.

Abstract

Commercially available phytases from wheat and fungus *Aspergillus ficuum* have been used in environmental and agricultural phosphorus study. In order to better understand the biochemical properties of these two phytases, *in vitro* experiments were conducted to study their catalytic potentials to hydrolyze a number of representative organic phosphates [phytate; *p*-nitrophenyl phosphate (PNP); *p*-nitrophenyl phosphate di-2-amino-2-ethyl-1-3-propanediol (PNP2A2E); *p*-nitrophenyl phosphate bis-cyclohexylammonium (PNPBC); bis-p-nitrophenyl phosphate (Bis-PNP); D-glucose 6-phosphate sodium salt (DG6PNa); and D-glucose 6-phosphate disodium salt (DG6P2Na)]. The results showed that the activity of wheat phytase in hydrolyzing these substrates was in the order: phytate > PNPBC > PNP2A2E > PNP > DG6P2Na > DG6PNa > Bis-PNP. Substrate preference for the fungal phytase followed the pattern: phytate > PNP > PNP2A2E > PNPBC. The kinetic constants of the two enzymes on these substrates demonstrated that the binding affinity for the fungal phytase with phytate was the highest. We further observed that As, Ba, Br and I ions enhanced the fungal phytase activity, whereas wheat phytase activity was suppressed by most ions we tested. Information obtained in this research is helpful in assessing the application of the two phytases in phosphate pollution research under various environmentally relevant conditions.

Key words: Phytase, organic phosphorus, wheat phytase, *Aspergillus ficuum*.

Introduction

Phytases (*myo*-inositol hexakisphophate phosphohydrolases, EC 3.1.3.8 and 3.1.3.26) are produced by plants and microorganisms [1-3]. These enzymes hydrolyze phytate (*myo*-inositol hexakisphophate) in a stepwise formation of myo-inositol pentakis-, tetrakis-, tris-, bis- and monophosphate to release inorganic phosphate [2, 4]. Commercially available phytases have been widely used in agricultural and environmental research [5, 6]. For example, these enzymes can be added to animal feedstuff to improve phosphate bioavailability of monogastric animals [6, 7]. Commercially available phytase also shows potential to improve bread processing in food industries [8]. Phytase could be used as an environmental-friendly biological agent in pulp and paper industries to hydrolyze plant phytate [9]. Commercially available phytases have also been used as an analytical tool to characterize organic P forms in such environmental samples as soil [10-12], animal manure [13-15], plant exudates [16] and lake waters [17]. Bishop *et al.* [18] incubated a Chilean volcanic soil with several commercially available phosphatases that included fungal phytase to investigate enzymatic organic phosphorus mineralization. Their results suggested that treatment of soils with phytase could increase P availability to crops. However, with only 50% of the total organic P extracted from these soils, they cautioned that a significant amount may be resistant to phosphatase hydrolysis. Apparently, more understanding of the effects of environmental factors (such as pH and metal ion) on their enzymatic activities would be helpful to the better utilization of these commercially available enzymes in agricultural and environmental fields.

Generally, phytase from fungi is 3-phytase as it initially hydrolyzes the P-O bond at the C-3 of inositol ring (EC 3.1.3.8); while those from plants are 6-phytase which initially hydrolyzes the P-O bond at the C-6 of inositol ring (EC 3.1.3.26) [19]. However, the hydrolytic activity of phytase is not only limited to phytate, as other phosphomonoester compounds can be hydrolyzed by phytase enzymes [2, 4, 20]. To demonstrate maximum efficacy of utilizing commercially available phytases, Boyce and Walsh [6] characterized the physicochemical properties of four commercial phytase products. To further enhance our understanding of the biochemical characteristics of commercial phytases relevant in identifying and quantifying hydrolyzable organic phosphorus in the environment, we (i) determined the kinetic parameters of phytases from wheat and fungus *Aspergillus ficuum* and (ii) evaluated the effects of anions and cations on their activities.

Materials and Methods

Organic phosphate compounds: Inositol hexakisphosphate (phytic acid sodium salt), *p*-nitrophenyl phosphate disodium hexahydrate (> 97%) (PNP); *p*-nitrophenyl phosphate di (2-amino-2-ethyl-1,3 propanediol) (PNP2A2E); *p*-nitrophenyl phosphate bis (cyclohexylammonium) (PNPBC); bis-*p*-nitrophenyl phosphate sodium (Bis-PNP); D-glucose 6-phosphate sodium salt

(98%) (DG6PNa) and D-glucose 6-phosphate disodium hydrate (98-100%) (DG6P2Na) were purchased from Sigma-Aldrich, St. Louis, Mo.

Reagents: Acetate buffer (144 mM, pH 5.15) was prepared by dissolving 19.6 g sodium acetate trihydrate (Sigma product # S-8625) and 0.493 g $MgSO_4{\cdot}7H_2O$ (Sigma product # M-1880) in 1-L volumetric flask. The mixture was titrated to pH 5.15 with 1 M glacial acetic acid, and final volume adjusted to 1 L with deionized water. Glycine-HCl buffer (200 mM, pH 2.5) was prepared by dissolving 22.306 g glycine hydrochloride (Sigma product # G-2879) into 800 mL deionized water in a volumetric flask and pH adjusted to 2.5 with 2 N NaOH and the final volume (1 L) with deionized water.

Enzyme preparations: Crude phytase enzymes from wheat (EC 3.1.3.26, 0.03 U mg^{-1} activity) and fungus *Aspergillus ficuum* (EC 3.1.3.8, 1.1 U mg^{-1} activity) was purchased from Sigma-Aldrich, St. Louis, Mo. The wheat phytase was purified to remove orthophosphates by dissolving 0.91 g of the enzyme in 5 mL of 10 mM NaAc buffer (pH 5.0) and dialyzed three times using a Spectra/Por Float-A-Lyzer (MWCO: 10,000, Spectrum Laboratories, Inc) for 12 h with 1 L buffer. The dialyzed enzyme solution was then centrifuged at 10,000 rpm for 10 min at 4°C. One mL of the enzyme was then pipetted into 1.5 mL micro-centrifuge tubes and stored (-20°C) until use. The fungal phytase was used without further purifications.

Assay conditions: The optimal pH for the commercially available crude phytase reported by the supplier is 2.5 (*Aspergillus ficuum* phytase) and 5.15 (wheat phytase), while the optimal temperature reported is 37°C (*Aspergillus ficuum* phytase) and 55°C (wheat phytase). One unit (U) of the enzyme is reported to liberate 1.0 μmol orthophosphate with appropriate substrate at the appropriate pH and temperature. We used 0.133 U mL^{-1} of the enzyme to hydrolyze phytate and 0.033 U mL^{-1} for the other substrate compounds. The effect of pH, temperature and time, on each enzyme was determined by exposing each enzyme to a wide range of temperature ranging from 10 to 80°C, pH ranging from 2-9, and time ranging from 1-10 hr.

Kinetic determination: Using a 3 mL centrifuge tube, phosphate substrate was dissolved in 144 mM acetate buffer to which was added wheat phytase. In another 3 mL centrifuge tube, phosphate substrate was dissolved in 200 mM glycine-HCl buffer and *Aspergillus ficuum* phytase added. The resulting final enzyme concentration was 0.133 U mL^{-1} for reactions with phytic acid and 0.033 U mL^{-1} for reactions with the other substrates. All reaction mixtures were carried out in a final volume of 3 mL and incubated for 1 hr. At the end of the incubation period, the reaction was stopped using 10% sodium dodecyl sulfate. Controls were set up by incubating the substrate without enzyme to correct for the P_i released due to chemical hydrolysis. Amount of P_i released was measured colorimetrically [21] and results plotted against substrate concentrations as described by the three linear Michaelis-Menten transformations plots (Lineweaver-Burk plot (1/V vs 1/[S]); Hanes-Wolf plot ([S]/V vs [S]); Eadie-Hofstee plot (V vs V/[S]). The K_m, V_{max}, K_{cat} and K_{cat}/K_m for each enzyme were calculated using these linear transformations plots.

Determination of effects of ions: The effects of anions and cations on phytase activities were determined at established conditions (2 mM phytate, 144 mM sodium acetate; pH 5.0; temperature 55°C for wheat phytase) and (2 mM phytate, 200 mM glycine-HCl; pH 2.5; temperature 37°C for *Aspergillus ficuum* phytase). One hundred mM stock solutions of Pb^{2+}, Cd^{2+}, Cr^{2+}, Co^{2+}, Mo, Ni^{+}, As^{3+}, Ba^{2+}, Li^{+}, F^{-}, Br^{-} and I^{-} ions was prepared by dissolving $PbCl_2$, $CdCl_2$, K_2CrO_4, $CoCl_2{\cdot}6H_2O$, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, $Ni(NO_3){\cdot}6H_2O$, $NaAsO_2$, $BaCl_2$, LiCl, NaF, KBr and KI in deionized water respectively.

Various concentrations of each ion ranging from 1 to 10 mM were mixed with 2 mM phytate and equilibrated at the appropriate temperature. Two-tenths enzyme unit mL^{-1} of the appropriate enzyme was added to give a total volume of 3 mL. After incubating the mixture for an hour, the reaction was terminated using 0.5 mL of 10% trichloroacetic acid (w/v). The reaction mixture was centrifuged at 10,000 rpm for 5 minutes and the clear supernatant aliquot was analyzed colorimetrically for phosphate [21].

All data points represents the average of two replications, the numbers in parenthesis represent the standard deviations of these points and the error bars represents the standard errors of these points.

Results and Discussion

Kinetic parameters: All the phytase enzymes assayed obeyed the Michaelis-Menten kinetics (plots not shown). Table 1 lists the kinetic (K_m, V_{max}, K_{cat}, K_{cat}/K_m) values obtained for the enzyme activities with the various substrates. The values for *Aspergillus ficuum* using Bis-PNP, DG6PNa and DG6P2Na were not determined because of very high variability. The K_m for *Aspergillus ficuum* was higher than that of wheat phytase, while the catalytic efficiency (K_{cat}/K_m) of wheat phytase was higher than that of

Table 1. Calculated V_{max}, K_m, K_{cat}, K_{cat}/K_m values of phytase activities.

	Wheat				*Aspergillus ficuum*			
	V_{max} (mg L^{-1} hr^{-1})	K_m (mM)	K_{cat} (hr^{-1})	K_{cat}/K_m (hr^{-1} mM^{-1})	V_{max} (mg L^{-1} hr^{-1})	K_m (mM)	K_{cat} (hr^{-1})	K_{cat}/K_m (hr^{-1} mM^{-1})
Phytate	278 (±15)	0.96(±0.2)	2090(±39)	2177(±96)	318(±5)	2.1(±0.1)	2391(±13)	1139(±10)
PNP	191 (±11)	4.05(±0.43)	5788(±119)	1429(±19)	602(±20)	17.1(±0.7)	18224(±50)	1068(±1.1)
PNP2A2E	193 (±13)	3.65(±0.48)	5849(±139)	1603(±30)	545(±44)	15.65(±2)	16515(±110)	1055(±2)
PNPBC	171(±6.3)	3.10(±0.24)	5194(±63)	1676(±22)	971(±60)	32.46(±23)	29424(±151)	907(±7)
Bis-PNP	16.2(±0.1)	0.42(±0.02)	490(±1.1)	1167(±18)	nd	nd	nd	nd
DG6PNa	91.8(± 2)	2.51(±0.14)	2779(±23)	1107(±11)	nd	nd	nd	nd
DG6P2Na	66.5(±0.3)	1.52(±0.02)	2000(±3)	1316(±3)	nd	nd	nd	nd

PNP (*p*-nitrophenyl phosphate disodium hexahydrate); PNP2A2E (*p*-nitrophenyl phosphate di [2-amino-2-ethyl-1, 3 propanediol]); PNPBC (*P*-nitrophenyl phosphate bis cyclohexylammonium); Bis-PNP (Bis p-nitrophenyl phosphate sodium); DG6PNa (D-glucose 6-phosphate sodium salt); DG6P2Na (D-glucose 6-phosphate disodium hydrate); nd (not determined); number in parenthesis are standard deviations.

Aspergillus ficuum. Wheat phytase had a lower K_m and higher K_{cat}/K_m with phytate than with PNP, PNP2A2E, PNPBC, Bis-PNP, DG6PNa, DG6P2Na.

The lowest K_m value (0.42 mM) observed was with wheat phytase using Bis-PNP but, the K_{cat} value was 10 times lower using PNP, PNP2A2E and PNPBC and 5 times lower using phytate and DG6PNa as substrates. The higher K_{cat} signifies greater turnover of the enzyme in catalyzing the substrate. Berg *et al.* [22] summarized the turnover numbers of most enzymes with their physiological substrates between 1 and 10^4 s^{-1}. The ratio (K_{cat}/K_m) is used as a measure of catalytic efficiency and to compare an enzyme's preference for various substrates. Higher values indicate more preference for a particular substrate.

Phosphate hydrolysis by wheat phytase proceeded at a linear rate for two hours with phytate, PNP, PNP2A2E and PNPBC while with Bis-PNP, DG6PNa and DG6P2Na it was linear up to an hour. A linear rate up to an hour has been reported by Kerovuo *et al.* [23] when phytase from *Bacillus subtilis* was used to hydrolyze phytate. This is in contrast to the two hours we observed with phytase from wheat and *Aspergillus ficuum*. Our findings showed that the enzymes have clear differences in activity toward phytate or the other substrates.

The activity of wheat phytase using various substrates showed phytate > PNPBC > PNP2A2E > PNP > DG6P2Na > DG6PNa > Bis-PNP, although the binding affinity (K_m) for fungal phytase with phytate was higher than that for wheat phytase. The substrate preference for this enzyme showed phytate > PNP > PNP2A2E > PNPBC. The high K_{cat} and K_{cat}/K_m values observed for PNP, PNP2A2E and PNPBC suggest the presence of acid phosphatase and other phosphate hydrolyzing enzymes as impurities in the crude extracts. While commercially available crude extracts hydrolyzed phytate, homogenous wheat germ acid phosphatase has been reported to show a much lesser substrate specificity [24].

Effects of temperature, time, and pH on activity: Wheat phytase was stable between 50 and 60°C for all the substrates used while fungal phytase appeared to be stable to 60°C with phytate, PNP, PNP2A2E, PNPBC and Bis-PNP, and to 70°C with DG6PNa and DG6P2Na (Fig. 1). Boyce and Walsh [6] reported four commercial phytases with maximum activities at 39°C.

Wheat phytase activity was linear with time for up to two hours using phytate, PNP, PNP2A2E and PNPBC (Fig. 2). The activity of *Aspergillus ficuum* phytase was linear for up to 3 hours using phytate and DG6PNa (Fig. 2). The observed linear relationships indicate that the activity was not complicated by accumulation of released inorganic phosphorus (P_i) in the medium. However, with a longer incubation time, the reaction velocity became nonlinear. The incubation time used allows ample time for accumulation of inorganic phosphates.

The activity of each enzyme was also compared at different pH from 2 to 9 and incubation times from 1 to 10 hours using a substrate concentration 5 times greater than the K_m [25]. Our results showed wheat phytase with optimal activity at pH 5.0 (Fig. 3) was similar to that from wheat bran [26, 27]. *Aspergillus ficuum* phytase showed the optimal activity at pH 2.0, with all the substrates used (Fig. 3). Ullah [28] reported an optimal activity at pH 2.5 and Dao [29] reported a pH of 1.9 for phytase from *Aspergillus ficuum* while Wyss *et al.* [2] reported an optimum pH range of 2.5 to 7 by 10 different phytases or phosphomonoesterases.

Effects of ions on activity: Cations and anions can either increase, decrease or nearly have no effect on an enzyme activity. Ions that tend to decrease the activity of an enzyme does so by binding to the enzyme active sites, binding to the enzyme substrate complex or substituting for such metal cofactors as Ca or Mg [30]. Several research groups [23, 29, 31-33] have demonstrated that di- and trivalent cations do modulate phytase activity.

In this study, we observed some significant effects, when phytase activity was determined in the presence of several cations and anions ranging in concentration from 1 to 10 mM. Wheat phytase activity was depressed by both cations and anions at increasing concentrations (Fig. 4). The degree at which this activity was suppressed differed with ion species. Pb^{2+}, Cr^{6+}, Mo^{6+} and Cd^{2+} decreased the activity greatly with increasing concentration compared to Co^{2+}, Li^{+}, Ba^{2+}, Ni^{2+} and As^{3+}, while F^- effects were greater than that of Br^- and I^- (Fig. 4).

Aspergillus ficuum phytase activity was affected by both cations and anions with increasing concentrations. This fungal enzyme

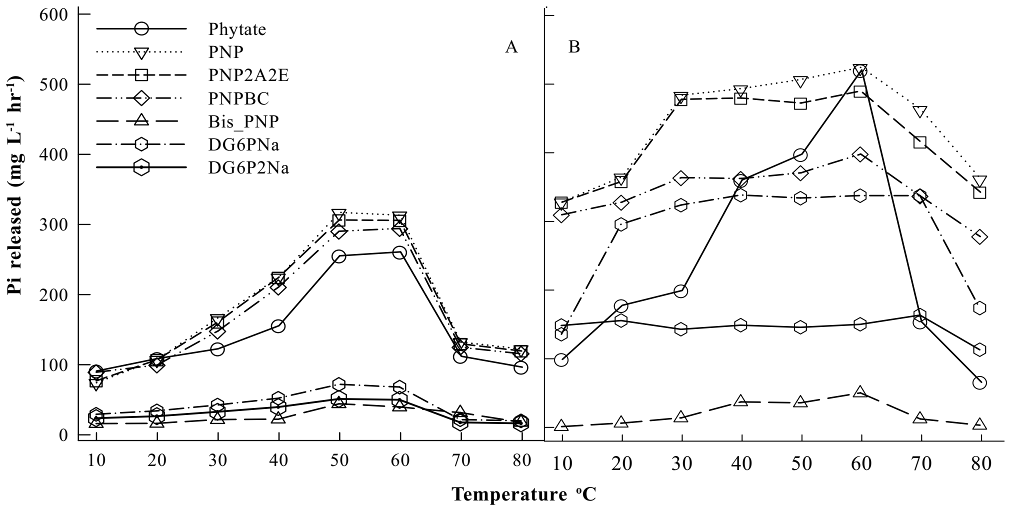

Figure 1. Effect of temperature on phytase activity: (A) wheat phytase; (B) *Aspergillus ficuum* phytase.

was more sensitive to Mo^{6+}, Pb^{2+} and Cr^{2+} than the other cations (Fig. 5). Halide ions also reduced the fungal enzyme activity (Fig. 5). The enzyme was sensitive at lower concentrations of F^- than that of Br^- and I^-. The activity of the enzyme increased with increasing As concentration for up to 8 mM, while that of Ba^{2+} increased up to 10 mM. The inhibitory effect of Pb^{2+}, Cd^{2+} and Co^{2+} on phytase may not have been due to binding of the metal ions to the enzyme, but rather the metal ions may have formed poorly soluble or insoluble complex with phytate [34, 35], thereby decreasing the active concentration of phytate from solution.

Conclusions

The reaction kinetics, substrate specificity, catalytic rate, optimum temperature and optimal pH on several organic P compounds were determined for commercially available phytases from wheat and fungus *Aspergillus ficuum*. This study observed a broad range of substrates for these two commercial enzyme products. *Aspergillus ficuum* phytase was stable to 70°C for reactions involving DG6PNa and DG6P2Na, and 60°C for reactions involving the other compounds. The optimum pH for *Aspergillus ficuum* phytase activity was pH 2 for most of the compounds tested, except with DG6PNa. Wheat phytase was stable between 50 and 60°C, and showed an optimum pH of 5.0. Wheat phytase activity was suppressed by both cations and ions, while *Aspergillus ficuum* phytase activity was enhanced by As^{3+}, Ba^{2+}, Br^- and I^- and suppressed by the other cations and an anion (F^-). The studies enhanced our understanding of the biochemical properties of two commercial enzyme products and their applications in phosphate chemistry research under various environmentally relevant conditions.

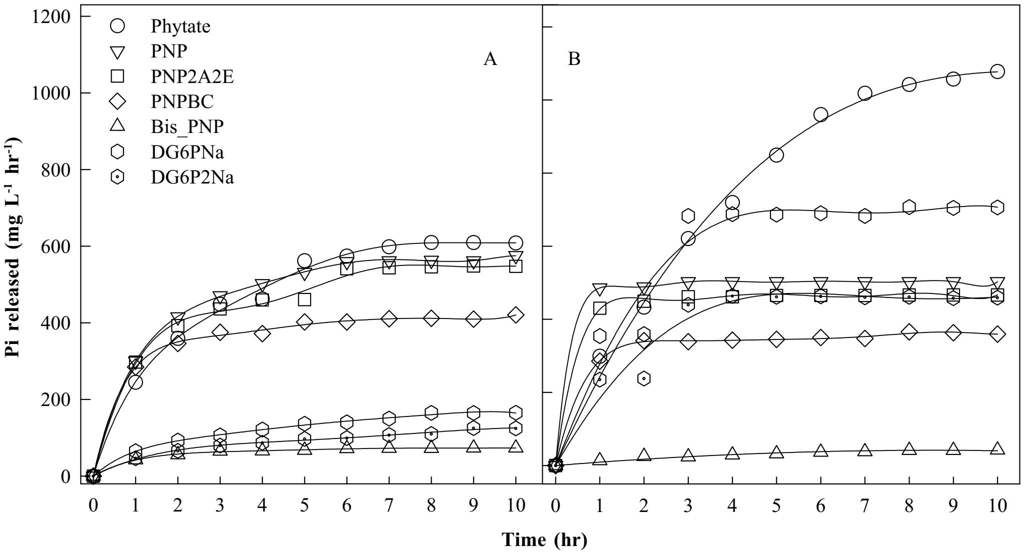

Figure 2. Effect of incubation time on phytase activity: (A) wheat phytase; (B) *Aspergillus ficuum* phytase.

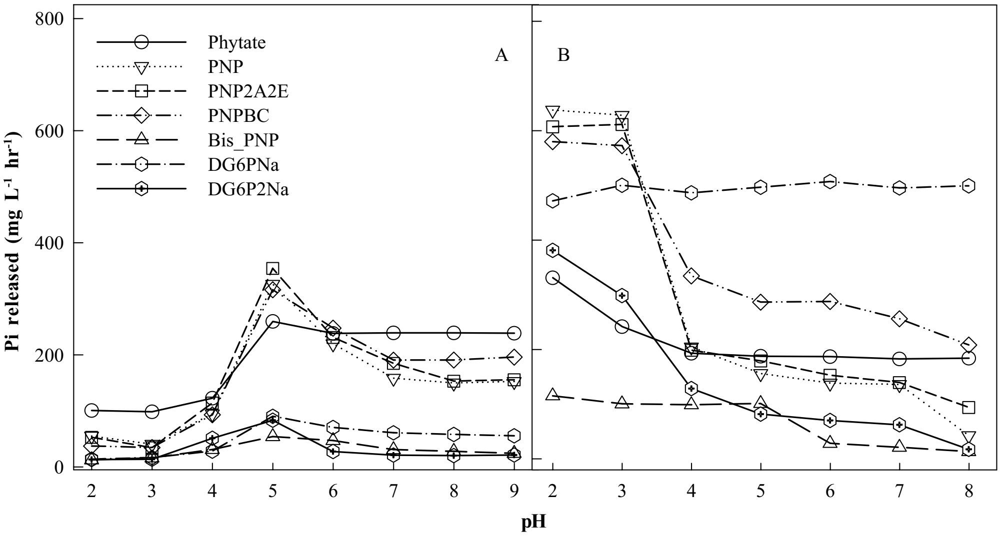

Figure 3. Effect of pH on phytase activity: (A) wheat phytase; (B) *Aspergillus ficuum* phytase.

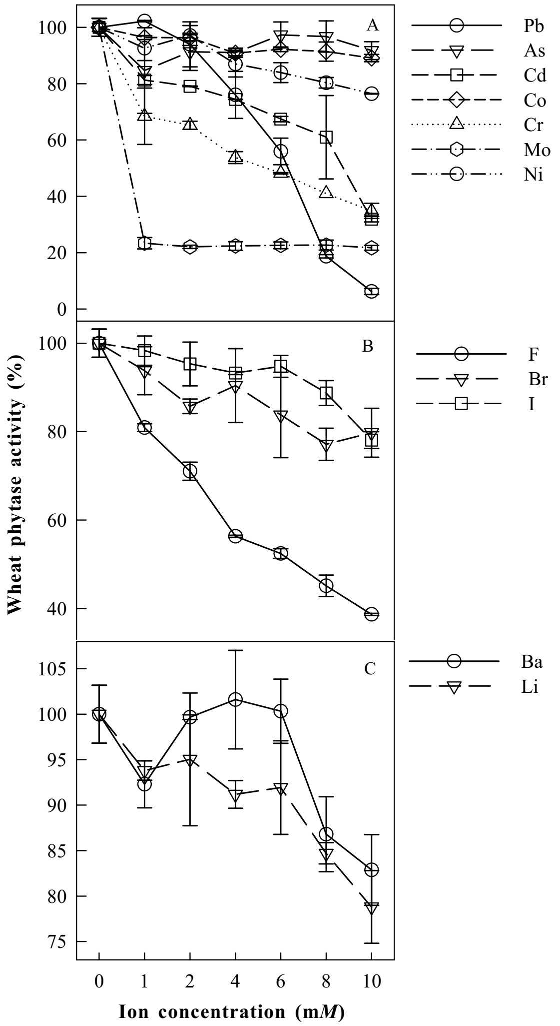

Figure 4. Effect of ions on wheat phytase activity.

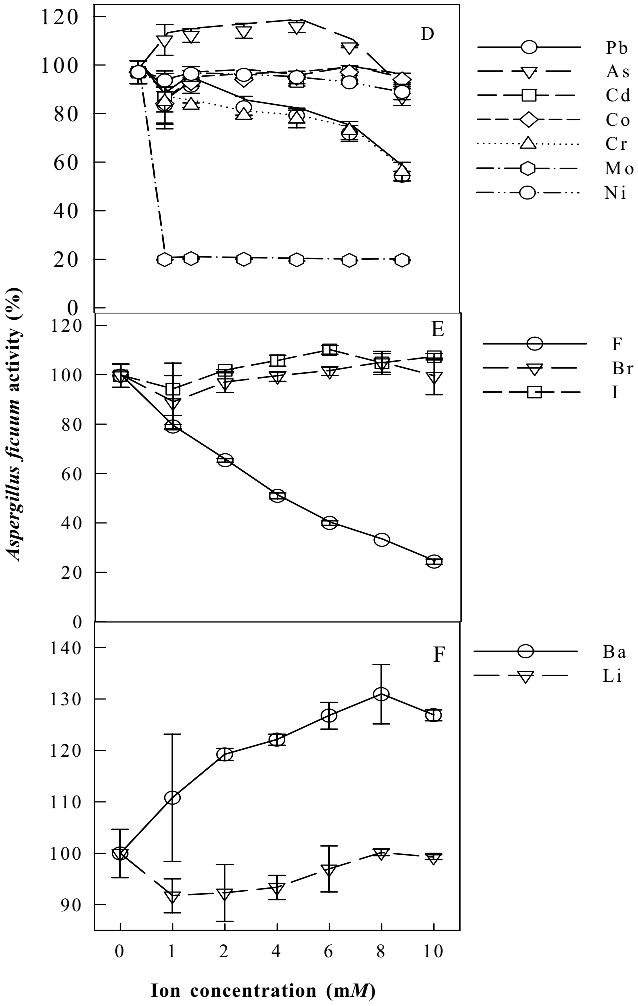

Figure 5. Effect of ions on *Aspergillus ficuum* phytase activity.

Acknowledgements

This work is contributed by the Winfred Thomas Agricultural Research Station, Alabama A&M University, Normal, AL. Journal # 619. Trade or manufacturers' names mentioned in the paper are for information only and do not constitute endorsement, recommendation, or exclusion by Alabama A&M University and/ or USDA-ARS. The research was supported in part by USDA-CSREES, Evans-Allen Grant # ALAX 011.

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