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EFFECT OF METAL IONS ON THE ACTIVITY OF EXTRACELLULAR PHYTASE OF *Bacterium* SP.

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The effect of Ca^{2+}, Mg^{2+}, Cu^{2+}, and Fe^{3+} on the activity of Bacterium *sp. phytase is studied. The ions Ca^{2+} and Mg^{2+} at low concentrations act as activators whereas Cu^{2+} and Fe^{3+} are phytase inhibitors. Inhibition of phytase activity by Cu^{2+} is proposed to be competitive whereas Fe^{3+} noncompetitively inhibits sodium phytate.*

Most enzymes that transfer phosphate require divalent metal ions to manifest activity or to be activated. There is usually an optimal concentration, above which inhibition is observed. The magnitude of this optimal concentration depends on the nature of the metal. Phosphatases also usually require cations, although exceptions are known, for example, nonspecific phosphatase.

We previously isolated and purified phytase (myoinositolhexaphosphatephosphohydrolase, KF 3.1.3.8) from the culture medium of *Bacterium* sp. [1]. Studies of certain physicochemical properties showed that it is similar to analogous phytases isolated from other samples [2-11]. We studied the effect of certain ions on the activity of this enzyme because metal ions play an important role in the manifestation of phytase activity.

Figure 1 shows that Ca^{2+} and Mg^{2+} at concentrations of 0.5-1.0 mM act as phytase activators. Phytase activation reaches ~60% for Ca^{2+} and 35% for Mg^{2+}. Increasing the concentrations further causes phytase inhibition. This effect is explained in the literature by the precipitation of phytates as an insoluble metal salt [2, 4].

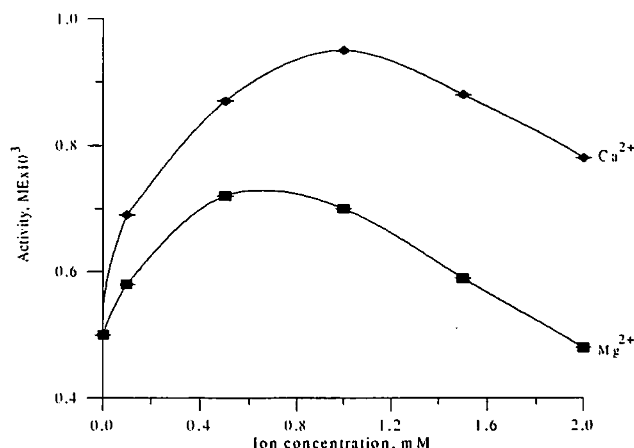

Fig. 1. Effect of Ca^{2+} and Mg^{2+} ions on activity of *Bacterium* sp. phytase. Conditions: salts were dissolved at the appropriate concentrations (0.1, 0.5, 1.0, 1.5, 2.0) in acetate buffer at pH 5.4; incubation temperature 50°C; incubation time 30 min; substrate Na phytate 10 g/l.

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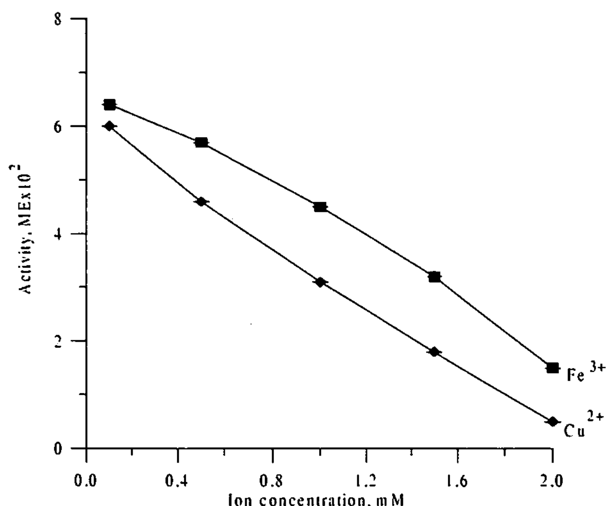

Fig. 2

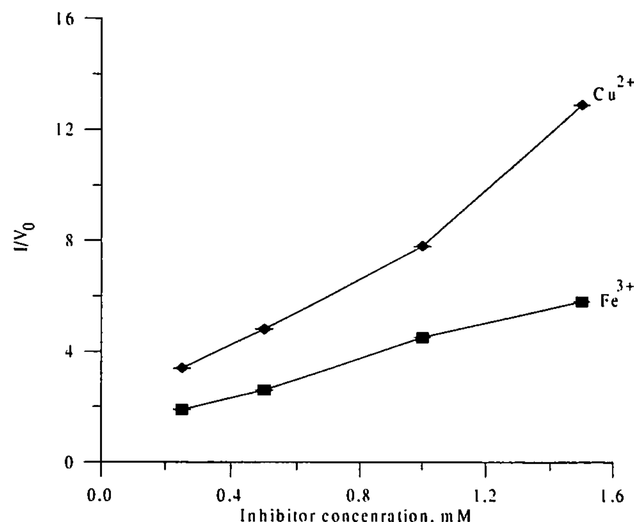

Fig. 3

Fig. 2. Effect of certain inhibiting ions on phytase activity (for conditions, see Fig. 1).
Fig. 3. Dixon diagram of Cu^{2+} and Fe^{3+} inhibition of *Bacterium* sp. phytase activity.

An excess of substrate and hydrolysis products inhibit certain phytases, including that studied by us. In particular, phytate concentrations above 2 mM inhibit phytase preparations from bean [3] and the fungus *Asp. ficuum* [4]. Only substrate levels above 0.3 M inhibit phytase from *Aerobacter aerogenes* [5]. Orthophosphate inhibits phytase from wheat and soy [6, 7]. The inhibiting concentrations, K_i, are 0.3 and 0.018 mM, respectively. Our experiments on the effect of orthophosphate ions formed through hydrolysis on bacterial phytase activity found that increasing the phosphate concentration to 2 mM inhibits the enzyme. The phytase activity falls to 83% of the initial value (Fig. 2).

According to the literature, Ca^{2+} activates many phytases at certain concentrations [2-7]. Activation of this phytase, similar to *Bacillus subtillus* phytase [7], is seen at a $CaCl_2$ concentration of 0.5 mM. Magnesium ions activate mung-bean phytase [2], but inhibit phytase from *Bacillus subtillus*, *Pseudomonas* sp., and *Lilium longiflorium* [8-10]. At low concentrations (10-50 μM) Ca^{2+} and Mg^{2+} weakly activate phytase from three-day cotton seedlings [11]. With increasing concentration of these cations in the incubation medium, slight inhibition is observed. Effective inhibitors of phytase from cotton seedlings are Fe^{3+}, Zn^{2+}, and Cu^{2+}.

According to our studies (Fig. 2), Cu^{2+} effectively inhibits extracellular phytase from *Bacterium* sp. Thus, phytase activity is 8% of the initial value in the presence of 2 mM Cu^{2+}; 29%, with Fe^{3+} at the same concentration.

Figure 3 shows 1/V as a function of the Cu^{2+} and Fe^{3+} concentration in the incubation medium. The inverse rate of phytin hydrolysis by phytase depends linearly on the concentration of inhibitor ions in the incubation medium. On the base of these diagrams we calculated the constants of phytase inhibition by these ions from the slopes of tangent angle: K_i = 0.040 (Fe) and 0.093 (Cu).

We also studied the effect of substrate concentration on inhibition by these ions. Figure 4a indicates that the substrate concentration (sodium phytate) depends linearly on the Cu^{2+} concentration in the incubation medium. Increasing the substrate concentration at constant Cu^{2+} concentration decreases phytase inhibition.

It should be noted that we obtained identical results from studies of inhibition by Fe^{3+} of phytase with varying substrate concentrations in the incubation medium (Fig. 4b).

Figure 4 shows that Cu^{2+} competes with substrate to inhibit phytase whereas Fe^{3+} acts noncompetitively. Therefore, it can be proposed that the observed competition appears owing to substitution by inhibiting ions of Ca^{2+}, which enhances the interaction between the substrate and enzyme. On the other hand, it is highly likely that an excess of phytin binds Cu^{2+} and Fe^{3+}, reducing their effective concentrations for inhibiting phytase.

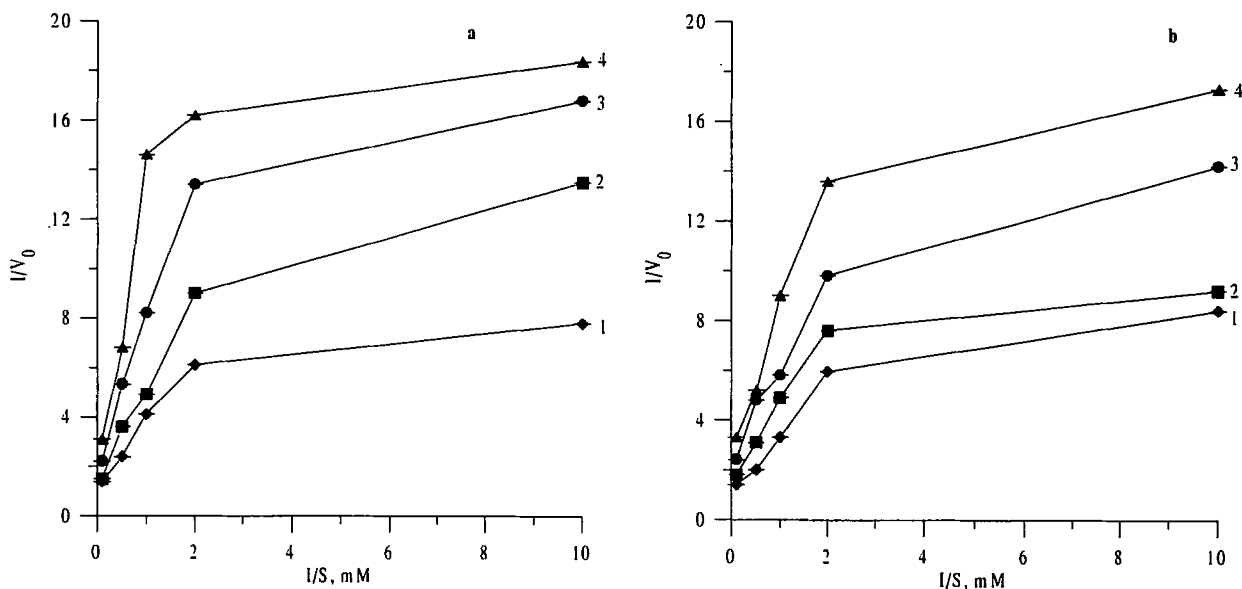

Fig. 4. Lineweaver—Burke diagram of Cu^{2+} (a) and Fe^{3+} (b) inhibition at various concentrations of *Bacterium* sp. phytase activity: without ions (1), 0.5 mM (2), 1.0 mM (3), 2.0 mM (4). Incubation medium: 0.2 M acetate buffer at pH 5.4 with added Cu^{2+} at the indicated concentrations; Na phytate 1 mM pH 5.4 at concentrations 0.1, 0.5, 1, 2, and 10; enzyme preparation was incubated for 30 min at 37°C.

EXPERIMENTAL

Extracellular phytase was obtained from culture medium of *Bacterium* sp. as previously described [12]. The three-day culture of microbes was centrifuged at 5000 g for 30 min at 4°C. The supernatant was lyophilized, dissolved in the minimal volume (10 mg/ml) of 0.2 M acetate buffer at pH 5.4, and precipitated stepwise by ammonium sulfate.

The precipitate was centrifuged (16,000 g, 30 min, 4°C), dialyzed against distilled water, concentrated, and placed on a column (2 x 62 cm) packed with Sephadex G-100 and previously equilibrated with 0.05 M NaOAc buffer at pH 5.0. The column was eluted by the same buffer at 8 ml/h.

The combined fractions that exhibited phytase activity were concentrated, dialyzed against distilled water, and placed on a column (2.5 x 31 cm) packed with DEAE-cellulose and previously equilibrated with 25 mM Tris-HCl buffer at pH 7.2. The column was eluted by this same buffer at 1 ml/min. Fractions were eluted by a linearly increasing gradient of NaCl (0-0.6 M). The enzyme purified in this manner was used to determine the phytase activity and the effect of the ions.

Phytase activity was determined by the amount of orthophosphate generated in an incubation medium of the following composition: 0.2 ml of 0.2 M NaOAc buffer at pH 5.4; 0.1 ml of 1 mM sodium phytate obtained by the literature method [12] and adjusted to pH 5.4 by addition of 1 M HCl, 0.2 ml of enzyme preparation. The mixture was incubated at 37°C and reaction was stopped, adding 0.5 ml of cooled 20% trichloroacetic acid. The same amount of enzyme preparation was added to control samples after addition of the trichloroacetic acid. Metal chlorides were used as sources of the ions. Orthophosphate was determined by the Taussky—Shorr method [13]. Protein was determined by the Lowry method [14].

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