The effect of minerals and mineral chelators on the formation of phytase-resistant and phytase-susceptible forms of phytic acid in solution and in a slurry of canola meal

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Abstract

Minerals can readily bind to phytic acid and thus have the potential to form mineral–phytate complexes that may be resistant to hydrolysis by phytase activity of animal, plant and microbial origin. In simple solution, at pH 7.0, mineral concentrations from 0.053 mM for Zn2+ up to 4.87 mM for Mg2+ caused a 50% inhibition of phytate-P hydrolysis by microbial phytase. The rank order of mineral potency as inhibitors of phytate hydrolysis was Zn2+ > Fe2+ > Mn2+ > Fe3+ > Ca2+ > Mg2+ at neutral pH. Acidification of the media to pH 4.0 decreased the inhibitory potency of all of the divalent cations tested. The inhibitory potency of Fe3+ showed a moderate increase with declining pH. Inclusion of 25 mM ethylenediamine-tetraacetic acid (EDTA) completely blocked Ca2+ inhibition of phytate hydrolysis at pH 7.

Inorganic P comprised 0.20–0.25 of the total P in a slurry of canola meal. Incubation with microbial phytase increased inorganic P up to 0.50 of total P levels. Supplementation with chelators such as EDTA, citrate and phthalate increased the efficacy of microbial phytase in hydrolyzing phytic acid. Incubation of canola meal with 100 mM phthalic acid plus microbial phytase resulted in complete hydrolysis of phytate-P. Competitive chelation by compounds such as EDTA, citric acid or phthalic acid has the potential to decrease enzyme-resistant forms of phytic acid and thereby improve the efficacy of microbial phytase in hydrolyzing phytic acid. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Plant-based diets fed to production animals generally contain substantial quantities of phytic acid. Digestion and utilization of dietary phytate-P by monogastric animals requires hydrolysis of phytate by phytase of plant, animal or microbial origin. Some plants such as wheat contain a phytase activity that does contribute to the utilization of the phytate-P in the diet. Barrier-Guillot et al. (1996) found a linear correlation between wheat phytase activity and P retention in broiler chicks. In pigs fed cereal-based diets, P availability ranged from 0.20 to 0.60 with a clear dependence on the plant phytase activity in the diet (Pointillart, 1994). Kemme et al. (1998) measured a 3% gastric degradation of phytic acid in pigs fed corn-based diet with a low intrinsic phytase activity and a 47% gastric degradation when pigs were fed a high intrinsic phytase diet based on wheat and barley.

In recent years, dietary supplementation with sources of microbial phytase has become a common practice to improve phytate-P digestibility. Ravindran et al. (1995) reviewed a large body of literature and stated that microbial phytase supplementation generally results in a 20–45% improvement in phytate-P utilization in diets fed to poultry. In weanling pigs, phytase supplementation resulted in a 50% increase in P retention and a 42% decrease in fecal P (Lei et al., 1993). Similar improvements were reported when measuring the effect of phytase supplementation of tapioca-soybean meal-based diets feed to sows (Kemme et al. (1997a) and growing-finishing pigs (Kemme et al., 1997b). Bruce and Sundstol (1995) found that phytase supplementation of oat-based diets fed to growing pigs increased ileal P digestibility coefficient from 0.194 to 0.268.

Intrinsic phytase of plant or animal origin or supplemental microbial phytase does not result in complete hydrolysis of dietary phytic acid and a substantial portion of total phytate-P remains undigested even with high levels of phytase in the diet. The mineral content of the diet is known to influence the phytate-P digestibility. Increasing dietary calcium levels has been shown to decrease phytate-P digestibility in rats (Nelson and Kirby, 1979), poultry (Scheideler and Sell, 1987) and pigs (Sandberg et al., 1993). Decreasing calcium levels from 10 to 5 g/kg in low P diets resulted in a 15% increase in phytate-P digestibility in chicks (Mohammed et al., 1991). A progressive increase in dietary calcium decreased the coefficient of phytate hydrolysis from 0.42 to 0.03 in chicks (Ballam et al., 1984). Increasing Ca : P ratios decreased apparent P utilization from dietary calcium phytate in chicks (Waldroup et al., 1964). With turkeys, an increase in Ca : P ratios from 1.1 : 1 to 2.0 : 1 decreased the efficacy of microbial phytase in promoting phytate-P digestibility (Qian et al., 1996). In pigs fed a corn–soybean meal based diet supplemented with microbial phytase, further inclusion of Ca to normal dietary levels had a negative effect on parameters of performance (Lei et al., 1994) and on plasma P concentration and apparent P absorption (Lantzsch et al., 1995).

Phytic acid can readily form chelates with divalent minerals, such as calcium, iron, zinc and magnesium (Morris, 1986). Formation of mineral–phytate complexes is associated with a reduction in mineral bioavailability. High levels of dietary phytic acid and calcium decrease iron (Ellis et al., 1982) and zinc (Forbes et al., 1984) bioavailability in rats. High gastric pH, such as occurs in infants, facilitates the formation of phytate–calcium–zinc chelates (Champagne and Phillippy, 1989).
Insoluble phytate–mineral precipitates and soluble mineral–phytate complexes may be resistant to hydrolysis by intrinsic and supplemental phytase in the diet. This hypothesis is consistent with the incomplete utilization of dietary phytate-P and the negative effect of minerals on phytate-P digestibility. However, very little is known of the susceptibility of soluble or insoluble phytate–mineral chelates to hydrolysis by microbial phytase. This study provides a systematic evaluation of the effects of minerals, pH and mineral chelators on the susceptibility of phytate to hydrolysis by microbial phytase in simple solution and in a slurry of canola meal under defined in vitro conditions.

2. Materials and methods

2.1. Partial purification of microbial phytase

Phytase activity was partially purified from crude microbial phytase (Natuphos, Gist-Brocades, Delft, The Netherlands) as described by Newkirk and Classen (1998). The enzyme was purified to remove inorganic P and side activities that could complicate interpretation of the results. A solution of 20% (weight/volume) crude microbial phytase in 0.1 M NaCl, 20 mM Tris-HCl pH 8.0 was centrifuged at 30 100 x g for 20 min. The supernatant was filtered and applied to a Q Sepharose column. The column was washed with 1 l of 0.1 M NaCl, 20 mM Tris-HCl pH 8.0 and the phytase containing protein material eluted with 1 l of 0.2 M NaCl, 20 mM Tris-HCl pH 8.0. This material was dialyzed against water, freeze-dried and resuspended in a minimal volume of water. Protein in this material could be separated into three major peaks after passage through Sephadex G-200. The second major peak contained a 11.2-fold enrichment in the specific activity of phytase relative to the crude microbial phytase starting material.

Protein was assayed by complexing with coomassie blue and reading the change in absorbance at 595 nm (Sigma microprotein diagnostic kit No. 610) with human serum albumin as the standard.

2.2. Phytate hydrolysis in the presence of excess microbial phytase

Partially purified microbial phytase (0.042 mg protein/ml final concentration) was added as the last ingredient to a solution of 0.142 mM phytic acid, buffered to the required pH with 50 mM N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid-Tris(hydroxymethyl)amionmethane (hepes-Tris) (pH 7 and 7.5) or 50 mM 2[N-morphlino]ethane-sulphonic acid-Tris(hydroxymethyl)amionmethane (mes-Tris) (pH 4, 5, 6, 6.5, 7) and any other compounds as required. The assays were performed in 96 well microtitre plates with a volume of 120 µl per well. The plates were incubated at 60°C for 30 min and the reaction stopped with 80 µl of 50% ice cold trichloroacetic acid. Precipitant material was pelleted by centrifugation at 540 x g for 10 min and 160 µl of the supernatant was assayed for inorganic P. The concentrations of minerals used in the reported experiments were based on preliminary work indicating an effective range for rendering a portion of the total phytate-P as resistant to hydrolysis.
Inorganic P was measured by formation of reduced phosphomolybdate using the Sigma diagnostic kit No. 670 (Sigma, St. Louis, MO).

2.3. Inorganic P in canola meal

A slurry of 0.5 g of canola meal in 1.5 ml of solution containing 0.05 mM hepes-Tris pH 6 plus any other additions as required for the particular experiment was incubated for the indicated period of time at 50°C. The reaction was stopped with 4 ml of ice cold 0.625 M HCl. The slurry was ultrasonicated for 1.5 min and then centrifuged at 44 000 × g for 15 min at 4°C. The supernatant was filtered through 0.45 μm filters and assayed for inorganic P.

2.4. Total P in digests of canola meal

A digest consisting of 0.5 g of canola meal in a 5.0 ml solution of 2.1 M perchloric acid and 10 M nitric acid was prepared. The mixture was placed at room temperature for 2 h, and then digested at 100°C for 2.5–3 h, and 180°C for 4–4.5 h. The mixture was cooled, diluted with 50 ml of H2O and assayed for inorganic P.

2.5. Statistical analysis

When appropriate, one-way ANOVA was performed to test for significant differences between mean values of soluble inorganic P concentrations. Duncan’s multiple comparisons test was used to compare treatments effects on mean values.

The Hill equation \(y = X_{\text{min}} + \left(\frac{X_{\text{max}} - X_{\text{min}}}{1 + (x/X_{50})^p}\right)\) was used to describe the relationship between mineral concentration and concentration of inorganic P formed at particular pH condition. The variables in the equations are defined as follows: \(X_{\text{min}}\) = minimum x value, \(X_{\text{max}}\) = maximum x value, \(X_{50}\) = concentration of mineral resulting in an x value that is 50% of \(X_{\text{max}} - X_{\text{min}}\), and \(p\) = a value that describes the direction and degree of curvature of line. The model was fit to the data by non-linear regression analysis the values of \(X_{50}\) and \(p\) are reported.

3. Results

3.1. Development of an assay system for determining phytase-susceptible and phytase-resistant forms of phytic acid in solution

The objective of the assay system was to demonstrate the principle of phytase-susceptible and phytase-resistant forms of phytic acid in solution and not to mimic conditions of gastro-intestinal passage. As such conditions of enzyme concentration, temperature and incubation period were selected to maximize the rate of phytate hydrolysis and the data is reported as the results of an end-point assay terminated well after all of enzyme-susceptible substrate had been hydrolyzed. In the absence of added mineral, the molar concentration of inorganic P in the supernatant was six times the initial
concentration of phytic acid indicating total hydrolysis by microbial phytase (Fig. 1). Any effects of minerals are restricted to rendering a portion of the total phytate as unavailable to the hydrolytic process.

3.2. Effect of Ca$^{2+}$ at varying pH on phytate hydrolysis by excess microbial phytase

Fig. 1 shows the effects of two concentrations of CaCl$_2$ at six different pH values on the proportion of phytate-P hydrolyzed to inorganic P in the presence of excess microbial phytase. At the highest concentrations of 1000 and 500 mM CaCl$_2$, precipitants were visible in the solution. At pH 4 in the presence of 1000 mM CaCl$_2$, only 50% of the phytate-P added to the wells was hydrolysed by the phytase. Lower concentrations of CaCl$_2$ were required to inhibit phytate hydolysis as the pH of the solution was increased from 6 to 7.5. A 30% reduction in phytate-P hydrolysis occurred with 25 mM CaCl$_2$ at pH 6 while concentrations of 50, 10 and 5 mM CaCl$_2$ caused complete inhibition of hydrolysis at pH 6.5, 7, and 7.5, respectively.

3.3. Effect of Ca$^{2+}$ concentration on phytate hydrolysis by excess microbial phytase

Fig. 2 shows the effects of 0 to 5 mM CaCl$_2$ at pH 7.5 on the portion of phytate-P hydrolyzed to inorganic P. The Hill equation converged to the data with a Hill coefficient of $-6.84$ and a half maximal $X_{50}$ of 1.13 mM CaCl$_2$. 

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**Fig. 1.** Effect of pH and selected concentrations of CaCl$_2$ on inorganic P formation from phytic acid in the presence of excess microbial phytase. Microbial phytase was added as the last ingredient to a solution containing the indicated concentrations CaCl$_2$ plus 142 $\mu$M phytic acid at the indicated pH as described in Section 2. The bars indicate the mean of four determinations of inorganic P. Shared letters indicate no significant difference in means at the indicated pH of the reaction ($p = 0.05$). The hatched line shows concentration of phytate-P present in the mixture prior to the start of the reaction.
3.4. Effect of Ca\(^{2+}\), Mg\(^{2+}\), Mn\(^{2+}\), Fe\(^{2+}\), Fe\(^{3+}\), and Zn\(^{2+}\) at varying pH on phytase hydrolysis by excess microbial phytase

The procedure of fitting the Hill equation to data obtained for phytate hydrolysis was used to determine the concentration of minerals at which 0.50 of the phytate-P was resistant to hydrolysis by microbial phytase (Table 1). At neutral pH all of the minerals were effective inhibitors of phytate hydrolysis. The molar ratios of mineral : phytate at half maximal inhibitory concentration of mineral at pH 7 are shown in Fig. 3. Zn\(^{2+}\) was the most potent divalent cation tested as an inhibitor of phytate hydrolysis. A molar ratio for Zn\(^{2+}\) : phytate of 0.35 : 1 resulted in 50% inhibition of phytate-P hydrolysis. Molar ratios at neutral pH for 0.50 inhibition ranged from 2 : 1 up to 34 : 1 for the other cations.

Table 1

<table>
<thead>
<tr>
<th>pH</th>
<th>Ca(^{2+})</th>
<th>Mg(^{2+})</th>
<th>Mn(^{2+})</th>
<th>Fe(^{2+})</th>
<th>Fe(^{3+})</th>
<th>Zn(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>–</td>
<td>–</td>
<td>274 ± 8</td>
<td>0.52 ± 0.034</td>
<td>0.36 ± 0.091</td>
<td>1.4 ± 0.14</td>
</tr>
<tr>
<td>5.0</td>
<td>125 ± 31</td>
<td>–</td>
<td>99.8 ± 24.5</td>
<td>0.43 ± 0.063</td>
<td>0.36 ± 0.083</td>
<td>0.77 ± 0.025</td>
</tr>
<tr>
<td>6.0</td>
<td>27 ± 5.4</td>
<td>200 ± 5</td>
<td>1.53 ± 0.12</td>
<td>0.36 ± 0.024</td>
<td>0.51 ± 0.031</td>
<td>0.51 ± 0.092</td>
</tr>
<tr>
<td>6.5</td>
<td>5.1 ± 0.14</td>
<td>48 ± 2.3</td>
<td>0.77 ± 0.022</td>
<td>0.32 ± 0.018</td>
<td>0.67 ± 0.046</td>
<td>0.47 ± 0.064</td>
</tr>
<tr>
<td>7.0</td>
<td>1.6 ± 0.04</td>
<td>4.9 ± 0.10</td>
<td>0.54 ± 0.030</td>
<td>0.28 ± 0.041</td>
<td>0.64 ± 0.056</td>
<td>0.05 ± 0.007</td>
</tr>
<tr>
<td>7.5</td>
<td>1.1 ± 0.03</td>
<td>2.7 ± 0.05</td>
<td>–</td>
<td>0.13 ± 0.018</td>
<td>–</td>
<td>0.05 ± 0.031</td>
</tr>
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\(^a\) X\(_{50}\) values obtained from fitting the Hill equation to the data on inorganic P formation from phytic acid with varying concentration of mineral and excess microbial phytase in the media as described in Fig. 2.
At acidic pH, the minerals could be classified as low or high affinity inhibitors in terms of the concentration of mineral required to impair phytate hydrolysis (Table 1). Fig. 4 shows the effect of pH on the molar ratios of Mn$^{2+}$, Ca$^{2+}$, and Mg$^{2+}$ to phytic acid at half maximal mineral concentration. High concentrations of these low affinity minerals were required to impair phytate hydrolysis at acidic pH. The molar ratios for Zn$^{2+}$ and Fe$^{2+}$ at half maximal mineral concentration showed a modest increase as the pH of the media decreased from 7.5 to 4.0 (Fig. 5). A drop in pH was associated with a tendency toward a lower concentration of Fe$^{3+}$ required to inhibit phytate hydrolysis. (Fig. 5).
3.5. Effect of EDTA on calcium inhibition of phytate hydrolysis at pH 7 by excess microbial phytase

Fig. 6 shows the effect of EDTA on the capacity of CaCl₂ to impair phytate hydrolysis. In the absence of calcium, EDTA had no effect on the hydrolysis of phytate-P. With no chelator present, inclusion of 5 mM CaCl₂ caused complete inhibition of phytate hydrolysis. 5 mM EDTA had no effect on calcium inhibition of phytate hydrolysis.

Fig. 6. Effect of Ca²⁺ and EDTA at pH 7.0 on inorganic P formation form phytic acid in the presence of excess microbial phytase. The bars represent the mean of four replicate determinations of inorganic P. Common letters indicated no significant difference between means (p = 0.05).
However, inclusion of 25 mM EDTA in the media completely reversed the effects of calcium such that all of the phytate-P was hydrolyzed by microbial phytase.

3.6. Effect of EDTA on the efficacy of microbial phytase in a slurry of canola meal

Fig. 7 shows the effects of microbial phytase, EDTA and microbial phytase plus EDTA on the concentration of inorganic P in a slurry of canola meal incubated for 60 min at 50°C. Incubation with the phytase doubled the concentration of inorganic P in the slurry. 5 mM EDTA had no effect on the inorganic P level in the meal. However, 5 mM EDTA increased the inorganic P level in phytase-supplemented canola meal from 74 to 96 nmol P/g of meal.

3.7. Effect of phthalic acid and citric acid on the efficacy of microbial phytase in a slurry of canola meal

Fig. 8 shows the effect of graded levels of phthalic acid and citric acid on the concentration of inorganic P in slurries of canola meal supplemented with microbial phytase and incubated for 15 min at 50°C. Inclusion of 5 mM phthalic acid or citric acid had no effect on inorganic P levels relative to the enzyme-supplemented control canola meal slurry. Increasing the concentration of phthalic acid or citric acid to 25 mM increased the inorganic P to a level comparable to 5 mM EDTA plus enzyme-supplemented canola meal. Further increases in the level of phthalic acid and citric acid in the slurry increased the formation of inorganic P in enzyme-supplemented canola meal. Incorporation of 100 mM phthalic acid resulted in a fourfold increase in inorganic P relative to the unsupplemented canola meal slurry and over a twofold increase in inorganic P relative to the enzyme-supplemented slurry.
Fig. 8. Effect of sodium EDTA, sodium citrate and potassium phthalate on the inorganic P levels in slurries of canola meal supplemented with microbial phytase (Enzyme). The slurries were incubated for 15 min with the indicated concentrations of chelators. The bars represent the mean of eight replicate determinations with the indicated supplementations to the slurries. Common letters indicated no significant difference between means ($p = 0.05$).

Fig. 9. Time course for inorganic P formation in slurries of 0.5 g of canola meal with no supplementation (CM), with 0.66 mg of microbial phytase (CM + ENZ), with microbial phytase plus 5 mM sodium EDTA (CM + ENZ + EDTA) and with microbial phytase plus 100 mM potassium phthalate (CM + ENZ + PHTHALATE). The reaction stopped at the indicated times, and inorganic P determined as described in Section 2. Data points represent the mean of eight replicate determinations. The dashed line represents the mean of five assays of total P in 0.5 g of canola meal. Common letters indicated no significant difference between means ($p = 0.05$) at the indicated time points.
3.8. Effect of microbial phytase, phytase plus EDTA and phytase plus phthalic acid on the time course for the formation of inorganic P in a slurry of canola meal

Incubation of a slurry of unsupplemented canola meal for 150 min at 50°C did not affect the level of inorganic P in the meal (Fig. 9). Inorganic P comprised 0.20–0.25 of the total P in the unsupplemented canola meal. Supplementation of the meal with microbial phytase resulted in a time-dependent increase in the concentration of inorganic P. In the presence of the enzyme, formation of inorganic P reached a plateau whereby inorganic P comprised \( \approx 50\% \) of the total P in the meal after 60 min of incubation. Further supplementation of the meal with EDTA or phthalic acid had no obvious effect on the time course of inorganic P formation. However, both compounds increased the final plateau concentration of inorganic P in the slurry. In the presence of 5 mM EDTA inorganic P comprised 70% of the total P in the meal. Inclusion of 100 mM phthalic acid in enzyme-supplemented canola meal resulted in inorganic P comprising nearly 100% of the total P in the meal at the conclusion of the incubation period.

4. Discussion

The capacity of phytate to form chelates with minerals is well documented. Cheryan (1980) in summarizing available literature on formation of mineral–phytate chelates concluded that Zn\(^{2+}\) formed the most stable complexes followed by Cu\(^{2+}\), Ni\(^{2+}\), Co\(^{2+}\), Mn\(^{2+}\), Ca\(^{2+}\) and Fe\(^{2+}\) in decreasing order of stability. Interestingly, the physical status of the complexes as either soluble or insoluble chelates is dependent upon the duration of incubation, pH of the media, molar ratio of mineral to phytate and presence of multiple cations in the solution. Copper at a concentration of 1 mM forms soluble chelates with 10 mM phytate while 1 mM zinc forms phytate chelates that slowly precipitate out of solution over a 48 h period (Champagne and Fisher, 1990). Insoluble phytate–mineral chelates tend to exist in a fine colloidal state that requires relatively high centrifugal forces to pellet out of solution (Champagne et al., 1990). As such incomplete pelleting may tend to overestimate the soluble and underestimate the insoluble fractions of phytate–mineral chelates.

In this study, we made no attempt to distinguish between fully soluble chelates and insoluble chelates that would require extensive centrifugation to pellet out of solution. Further, we did not measure the free phytate or mineral bound phytate in our assay system. Our interests were confined to characterizing the effects of minerals and pH on the susceptibility of phytate to hydrolysis by microbial phytase.

At neutral pH, Zn\(^{2+}\) was the most potent divalent cation tested in forming phytase-resistant complexes with phytate. Champagne and Fisher (1990) found that relatively low ratios of Zn\(^{2+}\) : phytate were required to form complexes and these workers proposed that a single Zn\(^{2+}\) atom forms a bridge between two molecules of phytate. Chemical shifts in the \(^{31}\)P NMR spectrum support a model whereby Zn\(^{2+}\) preferentially binds to the P5 phosphate group of the 5 axial/1 equatorial phytate conformer at neutral pH (Champagne et al., 1990). This type of binding may then facilitate Zn\(^{2+}\) bridging between two phytate molecules. Bridging in the presence of relatively low concentrations of Zn\(^{2+}\) may thus
convert phytate to a conformation that is inaccessible to hydrolysis by phytase. Cu$^{2+}$ binds to the P5 phosphate group of phytate at neutral pH, however, chemical shift data indicates additional involvement of the P4 and P6 phosphate groups of the molecule (Champagne et al., 1990). One could speculate that divalent cations other than Zn$^{2+}$ may have a similar binding mechanism as observed for Cu$^{2+}$ and thus have more of a tendency to associate with a single phytate and less tendency to form molecular bridges between two phytate molecules. Subtle differences in the mechanism of metal binding to phytate may account for the substantial difference in the potency of Zn$^{2+}$ in comparison to the other metals tested in rendering phytate inaccessible to microbial phytase at neutral pH.

Diets are composed of a complex mixture of minerals which likely results in interactive effects in forming complexes with phytic acid. In simple solution at high Ca : Zn ratios Ca$^{2+}$ enhances Zn incorporation into phytate–mineral complexes (Byrd and Matrone, 1965). Presumably, the mixed mineral Ca–Zn–phytate complex is more stable than simple Zn–phytate complexes at low Zn concentration. In rats, Zn utilization decreased with increasing phytate and with increasing Ca levels and a constant level of dietary phytate (Forbes et al., 1984).

Dropping the pH of the media increases the concentration of Zn$^{2+}$, Fe$^{2+}$, Ca$^{2+}$, Mn$^{2+}$ and Mg$^{2+}$ required to achieve a 0.50 inhibition of phytate hydrolysis by microbial phytase. Protonation of the phosphate groups of the phytate molecule will greatly reduce the affinity for divalent cation binding. The cations tested in this study can be divided into low and high affinity groups when comparing the concentrations required to inhibit phytate hydrolysis at low pH. The dramatic differences in the concentrations of Mn$^{2+}$, Ca$^{2+}$ and Mg$^{2+}$ in comparison to the concentrations of Zn$^{2+}$ and Fe$^{2+}$ required to cause a 50% inhibition of phytate-P hydrolysis is likely a function of the mechanism of binding to the various phosphate groups at acidic pH. At this time no detailed studies are available as to the mineral–phytate binding mechanisms at acidic pH.

The molar ratio for metal inhibition of phytate hydrolysis does not fully correlate with information on the molar ratios required to form insoluble phytate complexes at acidic pH. Less than 5% of phytic acid remained in a soluble state at pH 5 at a Ca : phytate ratio of 12.67 : 1 (Grynspan and Cheryan, 1983). In our system, under similar conditions, Ca$^{2+}$ had no inhibitory effect on phytate hydrolysis and a molar ratio of 900 : 1 was required to cause a 50% inhibition of phytate-P hydrolysis at pH 5. At pH 6, a molar ratio of Mg : phytate of 6 : 1 reduced the solubility of phytate-P by 80% (Cheryan et al., 1983). These same conditions had no effect on phytate-P hydrolysis by microbial phytase in our study. A Mg : phytate ratio of 1400 : 1 was required to inhibit phytate-P hydrolysis by 50% at pH 6 in our system. The discrepancy between the Ca$^{2+}$ and Mg$^{2+}$ concentrations required to form insoluble chelates and the concentrations required to impair phytate hydrolysis by microbial phytase are consistent with a model whereby the chelates formed at acidic pH remain accessible to the enzyme. One could speculate that at extremely high concentrations, the minerals will form additional low affinity associations with the chelates that impair access of the enzyme to the functional groups on the phytate molecule.

Fe$^{3+}$ is a potent inhibitor of phytate hydrolysis by microbial phytase over the full range of pH tested in this study. Increasing the pH of the media reduced the potency of Fe$^{3+}$ as an inhibitor of phytase. Trivalent cations may well form a tighter association with the
A phytate molecule that is resistant to displacement by a drop in the pH of the media. This may account for the difference in the effects of varying pH on the inhibitory potency of divalent and trivalent cations as seen in this study.

Addition of 5 mM EDTA completely overcame the inhibitory effect of Ca$^{2+}$ as an inhibitor of phytic acid hydrolysis by microbial phytase. Presumably, EDTA functioned as a competitive chelator removing calcium from the media and shifting equilibrium’s to minimize formation of enzyme-resistant mineral–phytate complexes.

Poorly absorbed phytic acid mineral complexes are likely to occur as natural components of feedstuffs. Supplementation of a diet containing soy protein isolate with EDTA decreased the mineral requirement of the diet (Kratzer et al., 1958). In chicks (Nielsen et al., 1966) and turkey poults (Vohra and Kratzer, 1964) supplementation of zinc-deficient diets with various chelators such as EDTA promoted growth and overcame the symptoms of zinc deficiency. Low molecular weight pectins characterized by a high degree of esterification are known to complex with minerals and have been shown to improve Fe solubility and absorption in rats (Kim and Atallah, 1993). Ascorbic acid and citric acid enhance Fe bioavailability in humans consuming vegetarian diets (Anand and Seshadri, 1995) and ascorbic acid increases Fe utilization in soy-based infant formulas (Davidsson et al., 1994). A model of competitive chelation may account for the effects of chelators on improving mineral bioavailability. In this model, the chelators complex with minerals and thereby decrease the pool of minerals that complex with phytate. Presumably, mineral–chelator complexes exists in a soluble form that can be absorbed intact or can release minerals to binding sites on the brush border membrane of the intestinal epithelium.

Prolonged incubation of a canola meal slurry with microbial phytase results in a partial increase in inorganic P levels up to 50% of the total P content of the meal. These results imply that the organic P component of the meal exists in enzyme-susceptible and enzyme-resistant forms. Binding of divalent cations to phytic acid may render a portion of dietary phytic acid resistant to hydrolysis by microbial phytase. Further, minerals bound to phytic acid may be unavailable to the animal and thus increase the dietary requirements. Addition of a chelator may serve to remove cations from binding to phytic acid and as such may increase the relative level of enzyme-susceptible phytic acid in the meal. In this study, EDTA, citrate and phthalic acid all improved the efficacy of microbial phytase in forming inorganic P. High concentrations of phthalic acid plus microbial phytase resulted in a 100% hydrolysis of phytate-P to inorganic P in the slurry of canola meal. Zyla et al. (1995) achieved complete dephosphorylation of a maize–soybean meal based diet using an enzyme cocktail that contained phytase, acid phosphatase, fungal acid protease, pectinase and citrate. The authors speculated that citrate in the cocktail may function to enhance the efficacy of phytase by promoting the breakdown of mineral–phytate complexes.

In theory dietary supplementation with chelators has the potential to improve the efficacy of microbial phytase and thus improve P and mineral retention in production animals. An ideal ‘competitive chelator’ must have a higher affinity than phytic acid for mineral binding. Further, the chelator must be non-toxic and the minerals bound to the chelator should be available to the animal either by absorption of the mineral–chelate complex or by dissociation and absorption of the minerals. The phosphate group on the
The phytate molecule is a comparatively weak chelator of minerals. Amino acids and carboxylic acids such as citric acid have a higher affinity than phytic acid for mineral binding (Cheryan, 1980). Chelators such as EDTA with stability constants for zinc between 13 and 17 were effective in overcoming Zn\(^{2+}\) deficiency in turkeys (Vohra and Kratzer, 1964). In chickens, similar types of chelators with Zn\(^{2+}\) stability constants between 11.1 and 18.2 were effective as growth promoters in Zn\(^{2+}\) deficient diets (Nielsen et al., 1966). In broiler chicks, supplementation of corn–soybean based diets with 3% citrate significantly increased protein, calcium and P retention (Gentesse et al., 1994). Zyla et al. (1996) found that incorporation of an enzyme cocktail composed of phytase, acid phosphatase, acid protease, pectinase and citric acid in a corn–soybean meal based diet with no supplemental P resulted in improved performance, bone mineralization, and calcium and P retention in turkeys when compared to results obtained with a phytase only supplemented diet. Recently, Li et al. (1998) found that incorporation of 1.5% citrate in a corn–soybean meal based diet supplemented with microbial phytase tended to further increase dry matter, P and calcium digestibility in pigs relative to the treatment group fed the same diet with no citrate addition.

The effectiveness of competitive chelators as a method of improving the efficacy of microbial phytase as a dietary supplement likely will vary with the ingredient composition of the diet. Yi et al. (1996a) found that increasing the Ca : P ratio while maintaining a constant level of supplementation in corn–soybean meal-based diets correlated with a decrease in Ca retention. As such one could hypothesize that any beneficial effect of a competitive chelator such as citrate on the efficacy of microbial phytase will be greatest in diets characterized by high levels of phytate, low levels of intrinsic phytase plus relatively high levels of multivalent minerals. The actual effects of competitive chelation with in-feed microbial phytase supplementation are largely hypothetical and further research involving chelators added to various diets fed to monogastric animals is needed to understand the interactions between minerals, phytic acid, phytase and competitive chelators during the digestive absorptive process. Further characterization of competitive chelation may serve to identify an ideal ‘competitive chelator’ that when added to the diet could enhance the activity of exogenous microbial phytase and thereby increase phytate-P retention and mineral bioavailability.

The potential for batch-scale dephytinization of feed ingredients using enzyme cocktails under controlled conditions in vitro is unknown at present. Zhu et al. (1990) used a two stage procedure that involved pretreatment of a corn–soybean meal mixture with citric acid at pH 3.1 followed by treatment with wheat bran to achieve 0.81 dephytinization. Zyla et al. (1995) achieved complete dephytinization of a corn–soybean meal based feed in a liquid slurry containing microbial phytase and various co-factor enzymes plus citrate. In both of these procedures citrate likely functioned by increasing the susceptibility of the substrate to hydrolysis by the enzyme.

**References**


