Susceptibility of Wheat and *Aspergillus niger* Phytases to Inactivation by Gastrointestinal Enzymes

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The activity of wheat and *Aspergillus niger* phytases was determined following preincubation for 60 min at 37 °C alone or in the presence of pepsin or pancreatin to examine their ability to survive in the gastrointestinal tract. At pH 3.5 both phytases were stable, but at pH 2.5 wheat phytase rapidly lost activity. Following preincubation at pH 3.5 in the presence of 5 mg of pepsin/mL, *A. niger* phytase retained 95% of its original activity, whereas only 70% of the wheat phytase activity was recovered. The stability of *A. niger* phytase in the presence of pepsin was the same at pH 2.5 as at pH 3.5. Results similar to those with pepsin at pH 3.5 were obtained following preincubation of the phytases in the presence of pancreatin at pH 6.0.

Keywords: *Phytase; pepsin; pancreatin; wheat; Aspergillus*

INTRODUCTION

Dietary phytases are promoted for humans and animals for fundamentally different reasons. Degradation of phytate (*myo*-inositol hexakisphosphate; $InsP_6$) increases the bioavailability of minerals important to human nutrition. In animal feeds phytase replaces the inorganic phosphate supplement while reducing the amount of undigested phytate excreted by animals as a potential water pollutant. As a variety of plant and microbial phytases become available for use, the need for comparative data on their effectiveness and limitations will increase.

Although yeast and bacterial phytases are also being evaluated to determine their most appropriate applications, plant and fungal phytases are currently available for industrial use. The phytases from wheat bran and *Aspergillus niger* are both active in the human gut (Sandberg and Andersson, 1988; Sandberg et al., 1996), but their enzymatic characteristics are significantly different. Wheat phytase has a single pH optimum at pH 5 and initially removes the phosphate at position 4 (the D-configuration is assumed) on the inositol ring (Phillippy, 1989). *A. niger* phytase has optima at pH 2.0 and 5.5 and preferentially attacks position 3 (Irving and Cosgrove, 1972).

As the contents of the stomach acidify during the digestive process, the pH would appear to favor *A. niger* phytase considerably over wheat phytase. Nevertheless, these phytases recently gave similar nutritional responses when fed to pigs (Han et al., 1997). One possible explanation for this unexpected result was that the wheat enzyme was more resistant to proteolysis in the stomach, where most of the phytate hydrolysis is thought to occur. The purpose of the following work was to compare the stabilities of wheat and *A. niger* phytases in the presence of pepsin or pancreatin, which are secreted into the stomach or duodenum, respectively.

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EXPERIMENTAL PROCEDURES

Materials. Wheat phytase (0.05 unit/mg; 1 unit releases 1 μmol of P_i from 1.5×10^{-3} M phytate per minute at pH 5.15 and 55 °C), *A. ficuum (niger)* phytase (2.9 units/mg; 1 unit releases 1 μmol of P_i from 4.2×10^{-2} M monomagnesium monopotassium phytate per minute at pH 2.5 and 37 °C), pepsin A from porcine stomach mucosa (480 units/mg), pancreatin from porcine pancreas (activity equivalent to 4 × U.S. Pharmacopeia specifications), and dodecasodium phytate from corn were from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade.

Stability Assays. To determine pH stability, 50 μg of wheat phytase was preincubated for 0, 15, 30, 45, or 60 min at 37 °C in 20 μL of 75 mM glycine plus 0.005% Triton X-100, pH 2.5 or 3.0, or 20 μL of 75 mM formate plus 0.005% Triton X-100, pH 3.5. The preincubated solutions were analyzed for residual phytase activity by adding 180 μL of 100 mM sodium acetate, 1 mM sodium phytate, and 0.001% Triton X-100, pH 5.5, and incubating for an additional 30 min at 37 °C to permit hydrolysis of ~5% of the total phosphate content of the phytate. The reaction was stopped by the addition of 800 μL of acetone/5 N H_2SO_4/10 mM ammonium molybdate (2:1:1), and P_i was determined by measuring the absorbance at 355 nm (Heinonen and Lahti, 1981).

Phytase inactivation by pepsin was determined by preincubating 50 μg of wheat phytase or 2 μg of *A. niger* phytase in 20 μL of 75 mM formate plus 0.005% Triton X-100, pH 3.5, containing 1, 5, 20, or 100 μg of pepsin for 60 min at 37 °C. These amounts of phytase were chosen as determined experimentally to give approximately equal activities under the conditions of the phytase assay. The reactions were stopped by analyzing the preincubated solutions for residual phytase activity as described above at pH 5.5, where the activity of pepsin was expected to be insignificant (Kitabatake et al., 1988).

Phytase inactivation by pancreatin was determined by preincubating 50 μg of wheat phytase or 2 μg of *A. niger* phytase in 20 μL of 75 mM MES, 0.5 mM cholic acid, 50 mM NaCl, pH 6.0, and pancreatin for 60 min at 37 °C. Because the pancreatin would not completely dissolve, the stock solution of 5 mg of pancreatin/mL of 150 mM MES, 1 mM cholic acid, and 0.1 M NaCl, pH 6.0, was centrifuged for 10 s at 16000*g* to sediment particulates, and aliquots of the supernatant solution were used corresponding to 0.5, 2.5, 10, or 50 μg of pancreatin in the original stock solution. The preincu-

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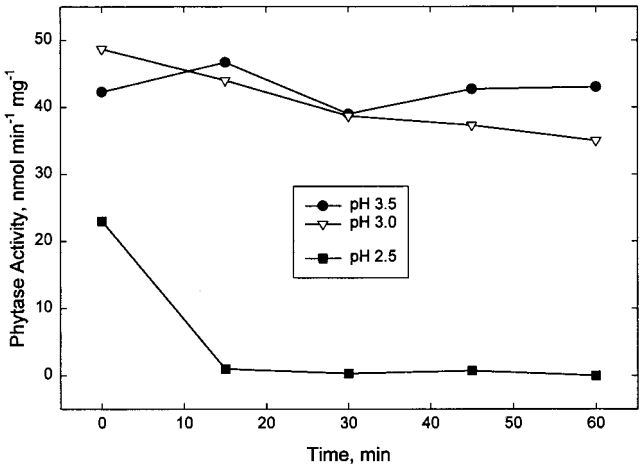

Figure 1. Effect of pH on the stability of wheat phytase. Data are the average from two experiments.

bated solutions were analyzed immediately for residual phytase activity as described above. The pancreatin reaction was not stopped after the 60 min preincubation and may have continued to some extent during the 30 min phytase assay.

RESULTS AND DISCUSSION

One hour preincubations were used in this study to roughly approximate a time when phytase may be present and active in a human or animal stomach or small intestine. Because the pH of the stomach content may fall to pH 3 or below, the stability of wheat phytase at low pH values in the absence of proteolytic enzymes was determined. As shown in Figure 1, at pH 3.5 the wheat phytase was completely stable for 1 h at 37 °C. At pH 3.0 there was a slight decrease in activity, whereas at pH 2.5 all activity was lost after 15 min. In a similar experiment, *A. niger* phytase was stable at pH 2.5 (results not shown). Previously we showed that *A. niger* phytase was stable for 2 h at pH 2.0 at 23 °C (Phillippy and Mullaney, 1997).

Phytase stability in the presence of pepsin was determined at pH 3.5 because both phytases were stable at that pH. Although pepsin is most active around pH 2, limited cleavage of ovalbumin was also observed at pH 4 (Kitabatake et al., 1988). The activity of wheat phytase decreased with increasing concentrations of pepsin, and 70% of the starting activity remained after 1 h of preincubation with 5 mg of pepsin/mL (Figure 2). In contrast, *A. niger* phytase retained 95% of its activity at the highest level of pepsin. The most likely explanation for the greater resistance of *A. niger* phytase to proteolysis was its higher level of glycosylation. Unglycosylated human lactoferrin was much more susceptible to degradation by trypsin than the glycosylated form (Van Berkel et al., 1995). Another possibility was that a component of the crude wheat phytase increased the activity of the pepsin. However, the data in Table 1 show that following preincubation with pepsin, a solution containing both phytases produced 45.8 nmol of P_i or 95% of the sum of the activities of solutions containing only one of the phytases, which formed a total of 48.4 nmol of P_i. Therefore, wheat phytase had little effect on the decrease in *A. niger* phytase activity caused by pepsin. *A. niger* phytase also retained >90% of its activity after 1 h of preincubation with 5 mg of pepsin/mL at pH 2.5 (results not shown).

Dietary *A. niger* phytase activity could not be recovered from the ileum of pigs (Jongbloed et al., 1992).

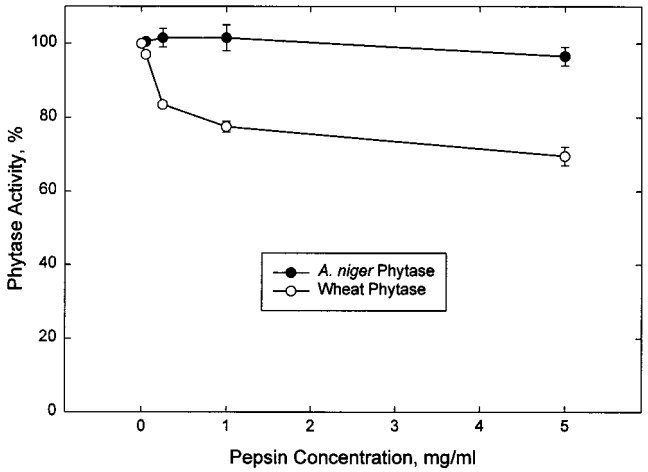

Figure 2. Phytase stability during preincubation with pepsin at pH 3.5. Data are the average from two experiments, and the error bars represent the ranges.

Table 1. Effect of Wheat Phytase on the Susceptibility of *A. niger* Phytase to Inactivation by Pepsin[a]

wheat phytase (μg)	*A. niger* phytase (μg)	pepsin (μg)	activity (nmol of P_i)	activity[b] (%)
25	0	0	29.0	100
25	0	100	18.1	62
0	1	0	31.2	100
0	1	100	30.3	97
25	1	0	54.4	100
25	1	100	45.8	84

[a] Data are the average from two experiments. [b] The activity of each solution without pepsin was assigned the value of 100% for comparison with the activity of the corresponding solution with pepsin.

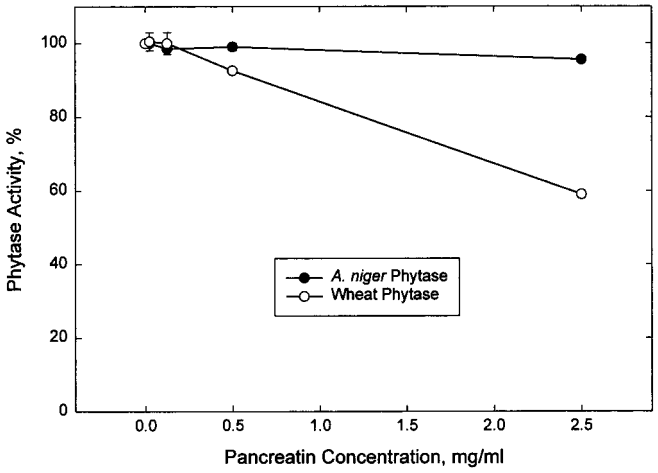

Figure 3. Phytase stability during preincubation with pancreatin at pH 6.0. Data are the average from two experiments, and the error bars represent the ranges.

Because it appears to resist the effects of low pH and pepsin, an alternate explanation for its disappearance may be cleavage by pancreatin hydrolases. Because the pH within the pig duodenum can remain above pH 3 5 h after feeding (Jongbloed et al., 1992), wheat phytase may also retain some activity as it enters the small intestine. Therefore, the susceptibility of wheat and *A. niger* phytases to inactivation by pancreatin was determined.

As shown in Figure 3, wheat phytase lost 40% of its activity following 1 h of preincubation with 2.5 mg/mL of pancreatin; *A. niger* phytase appeared to be stable.

Table 2. Phytase Activity following Preincubation with Pepsin or Pancreatin[a]

	wheat phytase activity		*A. niger* phytase activity	
secretion (μg)	nmol of P_i/50 μg	nmol of P_i min^{-1} mg^{-1}	nmol of P_i/2 μg	nmol of P_i min^{-1} mg^{-1}
pepsin				
0	56.0	37.4	55.6	927
1	54.5	36.3	55.8	930
5	46.9	31.3	56.3	939
20	43.4	28.9	56.2	937
100	38.9	25.9	53.5	892
pancreatin				
0	61.0	40.7	53.0	884
0.5	61.7	41.2	53.2	887
2.5	61.2	40.8	52.4	873
10	56.2	37.5	52.6	877
50	35.9	24.0	50.6	844

[a] Data are the average from two experiments.

Half as much pancreatin as pepsin was used due to the limited solubility of the pancreatin. Centrifugation of the pancreatin prior to use to remove particulates had no effect on the results of the subsequent phytase inactivation experiments.

In the preceding experiments the amount of wheat protein used was 25-fold the amount of *A. niger* protein to achieve similar amounts of phytase activity (Table 2). Because the phytase was only a minor component of the crude wheat enzyme, electrophoresis could not be used to prove that the activity loss was due to proteolysis as opposed to a noncatalytic interaction. However, the initial inactivation of wheat phytase by pepsin or pancreatin appeared to be linear over time (results not shown), which would be expected for an enzymatic reaction. Furthermore, the effects of pepsin and pancreatin were heat labile, with no phytase inactivation occurring during preincubation with pepsin or pancreatin that had been heated for 15 min at 74 or 66 °C, respectively (Figure 4).

The nutritional responses in pigs fed microbial or cereal phytases were similar (Han et al., 1997). *A. niger* phytase resulted in somewhat greater phosphorus absorption and retention than wheat phytase, but the opposite occurred for calcium and nitrogen. Because fungal phytase is more active than wheat phytase at low pH, the comparable efficiency of the wheat enzyme may have resulted from hydrolysis of most of the phytate before the pH became too acidic and also possibly as a consequence of the different isomeric inositol phosphates formed upon the hydrolysis of phytate. In addition, the wheat bran used as a source of phytase may have increased the level of intestinal fermentation, which was recently shown to counteract the effects of phytic acid on mineral utilization in rats (Lopez et al., 1998). Similarly, in pigs fed diets that were not supplemented with extra calcium, the endogenous cereal phytases degraded most of the phytate in the stomach and small intestine, and microbial phytases from the colon flora reduced the phytate levels to zero (Skoglund et al., 1997).

A. niger phytase was more stable in the presence of pepsin or pancreatin than wheat phytase. However, accumulation of Ins(1,2,3,6)P_4 may be higher in the InsP_4 fraction produced by wheat phytase compared to that produced by *A. niger* phytase, which prefers to hydrolyze InsP_6 starting at position 3 (Irving and Cosgrove, 1972). Ins(1,2,3,6)P_4 has a moderate ability to bind to the inositol 1,4,5-trisphosphate receptor, which results in the opening of calcium channels (DeLisle et al., 1994; Burford et al., 1997). Type 3 Ins(1,4,5)P_3 receptors have been reported in rat and human intestines (Blondel et al., 1993; Maranto, 1994) and may allow capacitative calcium entry from the external environment into cells through the plasma membrane (Putney, 1997). It is not known whether inositol phosphate receptors are bound by phytate degradation products in the intestine, but the ability of Ins(1,2,3,6)P_4 to enhance calcium absorption in rats (Shen et al., 1998) suggests such a possibility. Further evidence of this possibility was the increased calcium absorption by pigs fed wheat phytase compared to *A. ficuum* phytase

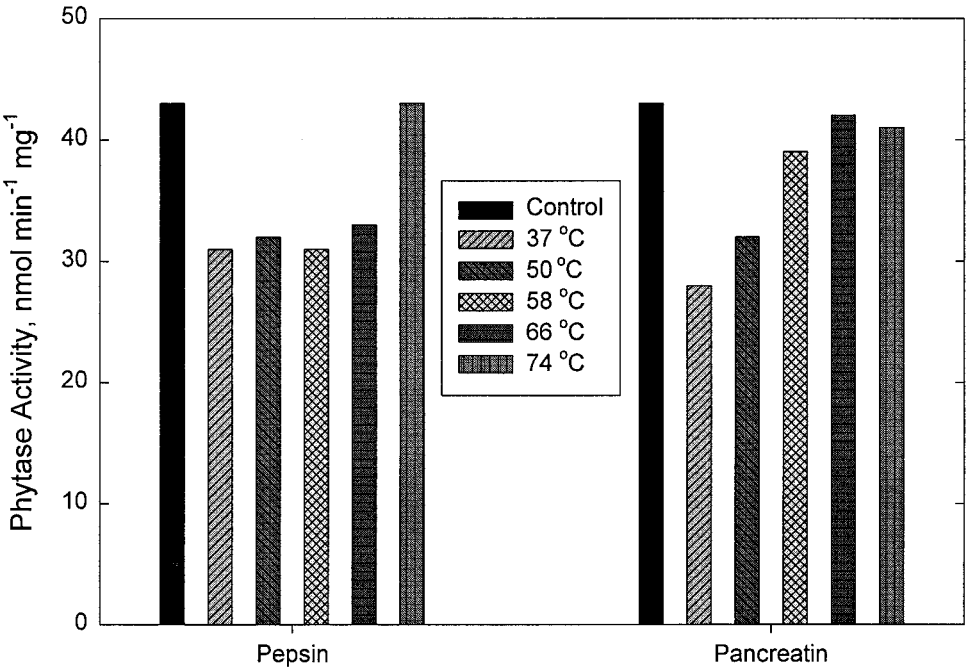

Figure 4. Effect of heat treatment of pepsin or pancreatin prior to preincubation with wheat phytase. Solutions of pepsin or pancreatin were heated for 15 min at the indicated temperatures in the preincubation buffer.

in the study by Han et al. (1997). If these data can be substantiated, the phytase in fresh vegetables (Phillippy, 1998) may also promote calcium absorption from diets containing phytic acid better than fungal phytase. More studies will be needed to provide additional evidence of the effectiveness of plant phytases and to determine the most efficient methods for incorporating sufficient amounts into diets to provide optimum phytate hydrolysis.

ABBREVIATIONS USED

Inositol polyphosphates are represented by Ins(X)P$_Y$, where X is the position number of the phosphates using the D configuration and Y is the total number of phosphates; P_i, inorganic phosphate; MES, 2-(N-morpholino)-ethanesulfonic acid.

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