Complete Enzymic Dephosphorylation of Corn–Soybean Meal Feed under Simulated Intestinal Conditions of the Turkey†

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A crude laboratory phytase preparation, a commercial phytase, and a commercial experimental phytase were used *in vitro* to study the extent of corn–soybean feed (P_{tot}: 4.83 ± 0.06 g/kg) dephosphorylation under simulated intestinal conditions of the turkey. The phytases had different specific activities; varied in side activities of acid protease, acid phosphatase, and pectinase; and revealed different abilities for feed dephosphorylation. Acid phosphatase was shown to be a key activity in feed dephosphorylation, while fungal acid protease enhanced dephosphorylation by stimulating gastric digestion. An "enzymic cocktail" that completely dephosphorylated phytates present in corn–soybean feed also contained pectinase and citric acid. Complete dephosphorylation was accompanied by 12–29% increase in dialyzable protein, and 45–81% increase in the concentration of reducing sugars liberated from feed. The potential use of the "cocktail" as a feed additive for monogastric animals may result in improved digestibility of phytate phosphorus, protein, and carbohydrates.

Keywords: *Phytate; phytase; dephosphorylation; acid phosphatase; acid protease; "enzymic cocktail"*

INTRODUCTION

Phytates are known to be a principal storage form of phosphorus in plant seeds and, because of their strong chelating properties, decrease bioavailability of minerals (Ca^{2+}, Mg^{2+}, Fe^{3+}, and Zn^{2+}) from the gastrointestinal tract of monogastric animals and humans (Kratzer and Vohra, 1986; Reddy, 1989). The action of plant (EC 3.1.3.26) or microbial phytases (EC 3.1.3.8) on phytates may deprive food and feed components of antinutritional properties, increase available phosphorus, and phosphorus retention by simple-stomached animals, as well as increase retention of calcium and micronutrients. Phytate dephosphorylation by phytase has been studied both outside (enzymic pretreatment of feed, Rojas and Scott, 1969) and inside the intestinal tract of animals (Nelson et al., 1971; Simons et al., 1990; Ketaren et al., 1993; Lei et al., 1993; Schöner et al., 1993). Soaking, germination, and natural lactic acid fermentation of grains, foods, and feedstuffs were proposed as an alternative way of phytate hydrolysis which takes advantage of the intristic phytases present in plant seeds or biosynthesized by contaminating bacteria (Chompreeda and Fields, 1984; Larsen, 1993). Transgenic tobacco seeds containing engineered *Aspergillus niger* phytase were claimed by Pen et al. (1993) as a novel feed additive for improved phosphorus utilization.

In the presence of *Aspergillus* sp. phytase the extent of phytate hydrolysis in the intestine of poultry (expressed as phosphorus digestibility, availability, or utilizability) has been reported to be no more than 55% (Simons et al., 1990; Schöner et al., 1993). This amount of phytate degradation allows for the substitution of phytase at no more than 1–1.2 g of inorganic phosphorus per kilogram of practical diets for pigs and poultry (Vogt, 1992; Yi et al., 1994). Phytase may have limited access to phytate because of phytate occlusion by starch, lipids, and protein. Metal ions may stabilize complexes of phytate with other tissue components, whereas conditions of the intestinal tract (pH changes and proteolysis) might be detrimental to the stability of phytase. Consequently, phytate dephosphorylation *in vivo* is incomplete.

Han (1988) studied the impact of proteinase (bromelain), cellulase, and hemicellulase on soybean meal phytate dephosphorylation by *Aspergillus ficuum* phytase. Using a single 3-h incubation at 37 °C and one unit of each enzyme per 1 g of the substrate (3.3% w/v), Han (1988) found that cellulase and hemicellulase improve the rate of phytate hydrolysis, whereas proteinase was not effective. Acid phosphatase in *Aspergillus niger* phytase accelerated the hydrolysis of a sodium phytate solution (Żyła, 1993) and rapeseed phytates (Żyła and Koreleski, 1993). This activity had a vital role in a corn–soybean meal (CSM) feed dephosphorylation by a commercial phytase under simulated intestinal conditions of the turkey (Żyła et al., 1994). The positive role of acid phosphatase (pH optimum 2.5, biosynthesized by *A. ficuum*) in the hydrolysis of phytate and lower phosphate esters of *myo*-inositol has also been reported (Ullah and Phillippy, 1994).

The objective of this investigation was to design a "cocktail" of several enzymes to completely dephosphorylate phytate under simulated intestinal conditions of the turkey.

MATERIALS AND METHODS

Reagents. Dodecasodium phytate, *p*-nitrophenyl phosphate, and soybean trypsin inhibitor (type I-S) were purchased from Sigma Chemical Co. (St. Louis, MO). Dinitrosalicylic acid was purchased from Aldrich (Milwaukee, WI). All other chemicals used were of analytical grade. Feed ingredients were obtained from commercial suppliers.

Enzymes. Microbial phytases (EC 3.1.3.8) used were the following: a commercial preparation (phytase, C), a laboratory

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Table 1. Composition and Nutrient Content of CSM Diet

ingredient	g/kg
soybean meal (44% CP)	580.41
corn (ground, shelled)	354.25
corn oil	46.13
calcium carbonate (38% Ca)	10.69
salt (NaCl)	4.00
methionine hydroxy analog	2.19
trace mineral premix	1.10
selenium premix	0.52
vitamin premix	0.52
choline HCl	0.19

nutrient content	g/kg
Ca[a]	6.00
$P_{available}$[a]	1.90
P_{total}	4.83
protein[a]	288.70
metabolizable energy[a] (MJ/kg)	12085.00
crude fat[a]	62.7
fiber[a]	4.88

[a] By calculation ("Feed Formulation", The Brill Corp., Norcross, GA).

preparation (phytase, L), and a commercial experimental phytase (CE). Phytase L was prepared from a waste *A. niger* mycelium and was in a liquid form. *A. niger* mycelium was obtained from the Department of Food Biotechnology, University of Agriculture in Krakow, Poland. The mycelium was washed free from medium, dried, and milled in a laboratory grinder. A weighed portion of mycelium powder was wetted with cold 0.3 M acetic acid solution (pH 3.0) and homogenized. The 50 g/L suspension was centrifuged (3000*g*, 2 °C, 0.5 h) and the supernatant was mixed with a protease inhibitor solution (20 g/L) prepared from potato juice (Żyła et al., 1989). The resulting solution was concentrated 10-fold at 45 °C in a laboratory vacuum evaporator and mixed with glycerol (20 g/L) before storage (4 °C).

Fungal acid protease (EC 3.4.23.6) type XIII, from *Aspergillus saitoi*, acid phosphatase (EC 3.1.3.2) from potato, *A. niger* pectinase (EC 3.2.1.15), pepsin, and pancreatin (activity: 8 × USP) were purchased from Sigma Chemical Co., St. Louis, MO. The supplier of the experimental phytase also provided experimental preparations of *A. niger* acid phosphatase, fungal acid protease, and pectinase, which were tested in analogous fashion as "Sigma" enzymes, and denoted by EXP subscripts throughout this work.

Experimental Diet Composition. The detailed composition of the corn–soybean meal (CSM) diet used in the experiments is given in Table 1.

Assays. Total phosphorus in feed was determined colorimetrically by the molybdovanadate method (AOAC, 1970) in duplicate samples which were digested by a wet-ash procedure. The procedure was validated by including standard reference material 1572 (citrus leaves) from the National Institute of Standards and Technology. The protein content of different phytases was determined by the method of Lowry et al. (1951).

***In Vitro* Digestions and Measurements.** The *in vitro* procedure of Żyła et al. (1994), designed for simulating the intestinal tract of the turkey was used. A 1 g (± 0.001 g) sample of CSM, ground through a 1 mm screen, was weighed into a 5 mL plastic syringe. The syringes were prepared by cutting off their Luer-lock tips. The feed sample was hydrated with double distilled water and HCl solution so that a concentration of 0.03 M HCl in a final volume of 1.5 mL was obtained. When the enzyme solution was applied, water was partly (or completely) substituted for by the enzyme solution. The contents of each tube were vortexed, the tubes were sealed with parafilm and incubated in a water bath at 40 °C for 30 min. Then, 0.5 mL of 1.5 M HCl and 3000 units of pepsin were added to each tube, mixed well, vortexed, sealed with parafilm, and reincubated for 45 min at the same temperature. At the end of this period, 0.65 mL of 1 M $NaHCO_3$ containing 3.7 mg/mL pancreatin was added dropwise with constant stirring into each tube. The slurry was transferred quantitatively to segments of dialysis tubing (molecular weight cutoff 12000–14000, diameter 16.0 mm, Sigma Chemical Co.) by means of the syringe piston. Segments were placed in 250 mL Erlenmeyer flasks containing 100 mL of 0.1 M NaCl in a 0.05 M succinate buffer (pH 6.0) and incubated in a shaking water bath at 41.1 °C (temperature of dialysate was 40 °C). A ratio of about 25:1 (v/v) between the dialyzing medium and segment contents was maintained. Samples of the dialysate were withdrawn after 240 min for determining inorganic phosphate (Lowry and Lopez, 1946), reducing sugars (Miller, 1959), and protein (Lowry et al., 1951). Samples were analyzed by the *in vitro* procedure in triplicate. In this model, pepsin and pancreatin digestion periods are preceded by a preincubation at pH 5.25 to simulate digestion in the crop of poultry. Pancreatin digestion was carried out in dialysis tubings in order to simulate gradient absorption from the duodenum. The feed/water ratio was kept within physiological limits and a constant feed weight to digestive enzymes was maintained. The response of the model to increasing phosphate concentration in feeds was linear, with the the recovery of phosphorus ranging from 93 to 113% (mean = 104; SE = 1.39). The model was validated also by *in vivo* study (Żyła et al., 1994) with growing turkeys fed diets containing 0, 500, and 1000 phytase units/kg of feed in a factorial arrangement with 0, 1, 2, and 3 g/kg of supplemental phosphate (from KH_2PO_4). After a simple transformation of data the amounts of phosphorus hydrolyzed from feed samples by *in vitro* digestions correlated with the 3 week body weight gains ($r = 0.986$ $p < 0.0001$), feed intake ($r = 0.994$ $p < 0.0001$), feed efficiency ($r = 0.992$ $p < 0.0001$), and toe ash ($r = 0.952$ $p < 0.0001$).

Enzyme Activity Measurements. Phytase activity was determined at 40 °C using 2 mmol of sodium phytate in 100 mmol acetate buffer (pH 4.5) as a substrate. The incubation mixture contained 3 mL of substrate and 0.5 mL of enzyme solution; incubation time was 60 min. The reaction was terminated by adding a 0.5 mL aliquot of the reaction mixture to 4 mL of acetate–acid–molybdate reagent; liberated phosphorus was determined spectrophotometrically (Heinonen and Lahti, 1981). One unit of phytase activity (FU) was defined as the amount of enzyme required to liberate 1 μmol of inorganic phosphorus in one minute under the above conditions.

The activity of acid phosphatase (EC 3.1.3.2) was assayed at 40 °C using 5.5 mmol of disodium *p*-nitrophenylphosphate in 100 mmol of acetate buffer (pH 4.5). The final volume of the solution containing the enzyme and the substrate was 1.05 mL. After 10 min of incubation, the reaction was terminated by the addition of 5 mL of 40 mM NaOH, and the amount of *p*-nitrophenol released was determined spectrophotometrically at 405 nm. One unit of acid phosphatase activity (APU) was equal to 1 μmol min^{-1} of *p*-nitrophenol liberated under the described conditions.

Acid protease (EC 3.4.23.6) activity was assayed in 0.1 M borate–acetate–phosphate buffer pH 2.8 at 40 °C. One milliliter of enzyme solution was added to 5 mL of 2.5% (w/v) hemoglobin (pH adjusted to 2.8), and the incubation proceeded for 30 min. The reaction was stopped by the addition of 10 mL of 110 mmol TCA solution; the contents were filtered, and the liberated amino acids and peptides were determined with Folin–Ciocalteu phenol reagent. One unit of acid protease (PRU) was defined as the amount of enzyme which hydrolyzed hemoglobin to produce color equivalent to 1.0 μmol of tyrosine/min at pH 2.8 and 40 °C.

The activity of pectinase (EC 3.2.1.15) was determined in 0.05 M acetate buffer pH 4.5 at 40 °C. 1.4 mL of 0.678% (w/v) polygalacturonic acid solution was mixed with 0.1 mL of enzyme solution and incubated for 10 min. The reaction was terminated by the addition of 2 mL of dinitrosalycylic acid reagent, and the reducing sugars released were determined by the method mentioned above. For the colorimetric measurement of carbohydrates with the dinitrosalicilic acid reagent, known amounts of glucose (0.25 mg) were added to samples, blanks and standards as described by Slominski et al. (1993). One unit of pectinase (polygalacturonase) activity (PCU) was the amount of enzyme which released 1.0 μmol of

Table 2. Specific Activity of Phytase and Secondary (Side) Activities in Preparations C, L, and CE

phytase	protein content (mg/g)[b]	phytase activity (FU/g[b])	acid phosphatase activity (APU/g[b])	pectinase activity (PCU/g[b])	acid protease activity (PRU/g[b])	phytase specific activity (FU/mg[c])	APU/FU	PCU/FU	PRU/FU
C	128	4440	2401	15	49	34.69	0.54	0.003	11
L[a]	23	15	134	7	6	0.65	8.93	0.45	393
CE	221	375	395	5550	138	1.70	1.05	14.80	368

[a] Phytase L was in a liquid form. The protein concentration and enzymes activities were expressed per milliliter. [b] Of sample. [c] Of protein.

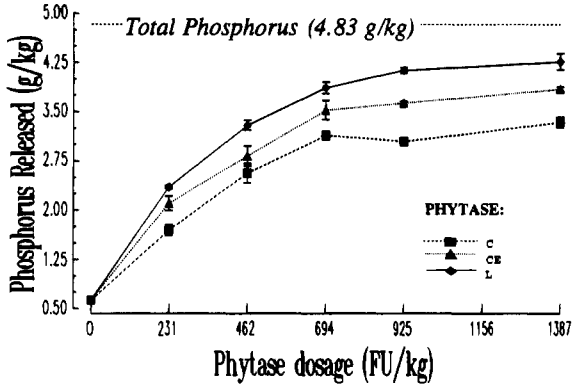

Figure 1. Influence of the phytase dosage and the preparation type on the inorganic phosphorus released from 1 g of CSM. Error bars indicate standard errors.

reducing sugars measured as D-galacturonic acid from polygalacturonic acid per min at pH 4.5 and 40 °C.

RESULTS AND DISCUSSION

Side Activities Present in Phytase Preparations. Enzyme preparations for the food industry are characterized in terms of main as well as secondary (side) activities (Godfrey and Reichelt, 1983). Secondary activities are of essential importance when complete conversion of complex substrates is required, or when substrates are occluded by other tissue components. Enzymatic saccharification of wheat starch by *A. niger* glucoamylase is a good example of such a process (Konieczny-Janda and Richter, 1992).

In the present study, three different preparations of phytase (main activity) were used to achieve complete dephosphorylation of a feed based on CSM. Preliminary characteristics of these preparations revealed different purities of main activity as well as different levels at which phytase was accompanied by secondary (acid phosphatase, acid protease, and pectinase) activities (Table 2). Commercial phytase (C) was of a high purity and had low side activities. Laboratory-made phytase (L) had low specific phytase activity, but was high in acid phosphatase. Phytase CE had a level of acid phosphatase similar to phytase C and protease activity comparable to that found in phytase L. Phytase CE had the highest pectinase activity among the phytases.

Efficacy of Phytases in Feed Dephosphorylation. In the first experiment, equivalent activities of phytase ranging from 0 to 1387 FU/kg of feed, from preparations C, L, and CE were applied to 1 g samples of feed and digested *in vitro*. Amounts of dialyzable phosphorus released from feeds are depicted in Figure 1. Although there were significant differences in dephosphorylation rate among phytases, there were no linear responses to increasing phytase concentrations, and dephosphorylation was not complete. This indicates that phytase activity alone is not able to completely dephosphorylate phytates in corn–soybean feed. The same conclusion was reported in feeding trials with turkey poults (Ledoux et al., 1994). In this study, when the phytase level in feed exceeds 700–750 units/kg of feed, plateaus occur in the response curves. In a few *in vivo* studies that have been done with poultry, the metabolic responses (weight gain, bone mineralization, phosphorus utilization) to increasing phytase concentration in diets high in phytate phosphorus are saturated when certain concentration of enzyme in feed is reached (Simons et al., 1990; Vogt, 1992; Yi et al., 1994). Dephosphorylation rates with phytases C, L, and CE differed significantly at most levels of enzyme dosage (Figure 1). A negative relationship between the specific activity of the phytases (Table 2) and their ability to release phosphorus from CSM proved to be significant (r ranged from −0.90 at 1387 FU/kg of feed to −0.94 at 231 FU/kg of feed). These findings confirm an important role of secondary activities other than phytase, in the dephosphorylation process. The negative influence of phytase purification upon the dephosphorylation of rapeseed phytates has been reported previously (Żyła and Koreleski, 1993).

Effect of Acid Phosphatase$_{EXP}$ (4 APU/g) and Pectinase$_{EXP}$ (0.05% w/w) on CSM Dephosphorylation. The positive effect of acid phosphatase activity on the dephosphorylation of corn–soybean meal phytates under simulated conditions of the turkey intestine has been observed earlier (Żyła et al., 1994). In the present study, fungal acid phosphatase$_{EXP}$ alone, or in combination with an *A. niger* pectinase$_{EXP}$ (0.05% w/w) was tested in terms of its influence on phytase C efficacy. Acid phosphatase$_{EXP}$ addition to the feed (4 APU/g) increased the amount of inorganic phosphorus released from feed over controls by 33% , while acid phosphatase$_{EXP}$ plus phytase C (462 FU/kg of feed), resulted in a 10% increase in dephosphorylation as compared to phytase C alone (Table 3). There were no significant differences in dephosphorylation when the experimental preparation of acid phosphatase (derived from mold) was substituted for the same amount of APU from potato acid phosphatase (purchased from Sigma; data not shown). Acid phosphatase alone, and in combination with pectinase$_{EXP}$ enhanced hydrolysis of polysaccharides as compared to the control (Table 3). Both phytase C and L significantly increased the concentration of reducing sugars liberated from feed. Saccharification was further improved by simultanous application of phytase C and pectinase$_{EXP}$. Proteolysis was adversely affected by phytase L in spite of much higher PRU/FU than that of phytase C. It seems possible that protease inhibitors, which were present in potato juice used to prepare phytase L, inhibited pancreatic, but not fungal proteases. Furthermore, a negative correlation (r = −0.91) was found between mean values of IVDP and dialyzable protein in treatments containing phytase. This indicates that proteolysis either in the gizzard or in the intestine is detrimental to phytate hydrolysis by phytase.

Impact of Fungal Acid Protease on Feed Dephosphorylation. Phytate was reported to form com-

Table 3. Influence of Acid Phosphatase$_{EXP}$ (4 APU/g) and Pectinase$_{EXP}$ (0.05% w/w) on IVDP, Dialyzable Protein, and Reducing Sugars Concentration in the Dialysates from CSM Feed

treatment	IVDP (g/kg[a])	dialyzable protein (mg/g[a])	reducing sugars (mg/100 g[a])
control	$0.630^{a} \pm 0.021$	$43^{b} \pm 0.6$	$199^{a} \pm 5$
acid phosphatase	$0.840^{c} \pm 0.012$	$42^{b} \pm 0.6$	$222^{b} \pm 4$
pectinase	$0.714^{b} \pm 0.009$	$44^{bc} \pm 0.8$	$216^{ab} \pm 7$
acid phosphatase + pectinase	$0.938^{d} \pm 0.010$	$47^{c} \pm 0.7$	$248^{c} \pm 11$
phytase C	$2.562^{e} \pm 0.043$	$46^{c} \pm 1.5$	$221^{b} \pm 7$
phytase C + acid phosphatase	$2.843^{f} \pm 0.009$	$45^{bc} \pm 1.0$	$231^{b} \pm 8$
phytase C + pectinase	$2.723^{f} \pm 0.019$	$47^{c} \pm 0.6$	$239^{c} \pm 4$
phytase C + acid phosphatase + pectinase	$2.824^{f} \pm 0.045$	$46^{c} \pm 1.5$	$233^{b} \pm 9$
phytase L	$3.295^{g} \pm 0.060$	$38^{a} \pm 1.4$	$231^{b} \pm 9$

[a] Of the diet. Means with different superscripts are significantly different ($p < 0.05$, t-test).

Table 4. Dephosphorylation of CSM by Phytases C, CE, and L in the Presence of Acid Protease (Sigma, 14 PRU/g)

	C		CE		L		
phytase dosage (FU/g[a])	IVDP (g/kg[a])	p[b]	IVDP (g/kg[a])	p[b]	IVDP (g/kg[a])	p[b]	SEM
231	2.453	0.002	2.513	0.02	2.5119	0.07	0.05
462	3.247	0.02	3.557	0.001	3.7521	0.001	0.05
694	3.577	0.02	3.789	0.005	4.0091	0.008	0.06
925	3.517	0.003	3.930	0.05	4.4392	0.08	0.06
1387	3.583	0.002	4.183	0.07	4.3040	0.716	0.08

[a] Of the diet. [b] Probability that the IVDP mean for samples with acid protease (Table), and without fungal acid protease (see Figure 1) are not different (paired t-test).

plexes with proteins which are resistant to pepsin digestion (Kratzer and Vohra, 1986). Strong pepsin inhibition in the presence of phytate at pH 2–3 has been also reported by Vaintraub and Bulmaga (1991). These low pH values favor dephosphorylation since *Aspergillus* sp. phytase and acid phosphatase are very active at pH 2.5–3.5 (Ullah and Gibson, 1987; Żyła, 1990). Since pepsin in the stomach likely does not digest phytate-degrading enzymes, enhanced gastric proteolysis could contribute to releasing more phytate from its complexes with protein and possibly accelerate dephosphorylation. Acid protease activity was found in the aleurone grains of plant seeds (Yatsu and Jacks, 1968), along with phytates, and thus can be suspected to influence phytate dephosphorylation during seed germination.

In order to intensify *in vitro* gastric proteolysis, fungal acid protease ("Sigma" preparation) was applied to feed samples along with phytase (231–1387 FU/kg of feed). A preliminary optimization of acid protease dosage showed that 14 PRU/g of feed along with 463 FU of phytase C/kg of feed was optimal, as further increase did not result in significant changes in the rate of dephosphorylation (data not shown). Dephosphorylation of CSM was improved by acid protease at each level of phytase C (Table 4). With high concentrations of phytases L and CE (925 or 1387 FU/kg of feed) no significant effects of acid protease on IVDP were observed. Also, coapplication of the protease and 231 FU of phytase L/kg of feed did not increase IVDP. The changes in the concentration of protein and reducing carbohydrates in dialyzates were associated with the extent of dephosphorylation. The concentration of dialyzable protein increased when the amount of phytase C was increased up to 694 FU/kg, but declined when the activity of phytase L was increased (Figure 2). Ketaren et al. (1993) credited phytase for "proteolytic activity", as it increased live-weight gain, protein retention, and daily protein deposition in pigs. The coaplication of acid protease increased the extent of proteolysis, which at 925 FU/kg was similar for both phytases. In contrast to our findings, Han (1988) did not observe any positive influence of bromelain (plant protease) on soybean meal dephosphorylation. Soybean meal was evaluated by Han (1988) with a single incubation at pH

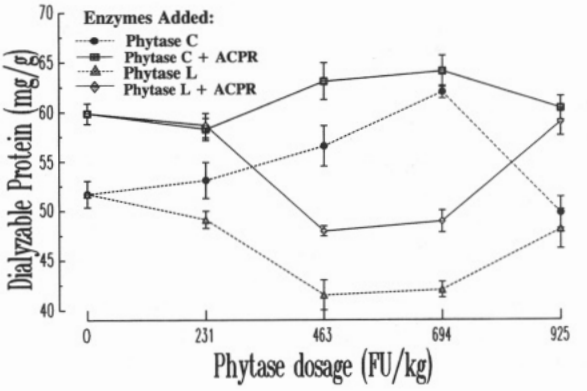

Figure 2. Dialyzable protein released from 1 g of CSM as a result of increasing dosages of phytases C and L, with and without fungal acid protease (ACPR) 14 PRU/g.

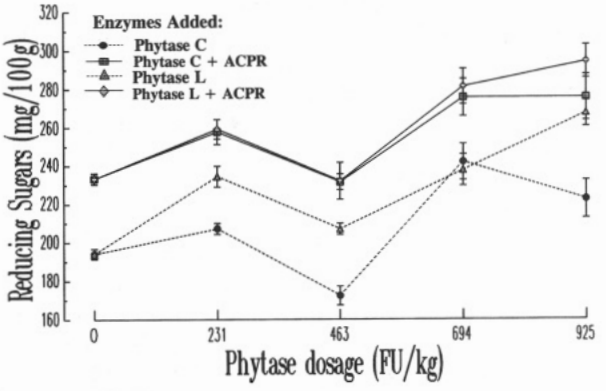

Figure 3. Reducing sugars released from 1 g of CSM as a result of increasing dosages of phytases C and L, with and without fungal acid protease (ACPR) 14 PRU/g.

5.4, producing certainly a different substrate than the feed formulation which underwent multiple digestions in the present studies.

Phytase L, which negatively influenced CSM proteolysis, promoted its saccharification. The response to acid protease addition was similar for phytase L and C (Figure 3). The efficacy of *Aspergillus saitoi* acid protease ("Sigma" preparation) in accelerating dephosphorylation of corn–soybean feed by phytase, was compared

Table 5. Effects of Two Acid Proteases on the Dephosphorylation of CSM Supplemented with 694 FU, Phytase CE

acid protease activity[a]	IVDP (g/kg)	
	source	mean ± SE
14	SIGMA	3.789^{c} ± 0.037
3.5	experimental prep	3.575^{a} ± 0.042
7	experimental prep	3.681^{b} ± 0.043
14	experimental prep	3.649^{ab} ± 0.040
28	experimental prep	3.774^{c} ± 0.026
42	experimental prep	3.846^{c} ± 0.043
70	experimental prep	3.976^{d} ± 0.091

[a] (PRU/g of feed). Means with different superscripts are significantly different ($p < 0.05$, t-test).

with an experimental preparation of *Aspergillus* acid protease. Although activity (14 PRU/g, plus 694 FU/kg) was the same in both preparations, twice as much enzyme from the experimental preparation was needed to reach the same level of dephosphorylation as for the Sigma source (Table 5). Proteases are known to differ in their ability to hydrolyze proteins of different origin. The determination of protease activity using hemoglobin as a substrate does not appear to give a good estimate of its ability to hydrolyze plant proteins.

Trypsin Inhibitor and Citric Acid Assist the Process of Dephosphorylation. Trypsin inhibitor was applied to feed samples (500 μg/g) to protect phytate-degrading enzymes from pancreatic digestion. It increased the rate of phytate hydrolysis by 694 FU of phytase C from 3.1353 to 3.4403 g/kg ($p < 0.001$), but had no effect on phytase L or a mixture of phytase C and fungal acid protease ($p > 0.05$).

Hazell and Johnson (1987) found citrate (24 μmol/g) to be effective in promoting iron diffusibility from plant foods under simulated intestinal conditions. It seemed possible that such an action might promote phytate hydrolysis either by assisting the breakdown of metal–phytate complexes, e.g. by binding calcium, or indirectly by protecting pancreatic lipase from inhibition caused by iron ions (Iwai and Tsujisaka, 1984). Indeed, the application of citric acid (24 μmol/g) was effective in enhancing CSM dephosphorylation with 694 FU of phytase L (3.8934 vs 4.3171 g of P/kg of feed), phytase C (3.1353 vs 3.5116 g of P/kg of feed) and phytase CE (3.5244 vs 3.8416 g of P/kg of feed). This has practical importance. Gentesse et al. (1994) found that CSM diet supplemented with 3% citric acid had significant increases in the metabolizability of protein, calcium, and phosphorus when fed to broilers.

Simultaneous Coapplication of Enzymes to Feed–Enzyme Mixtures ("Cocktails"). The coapplication of 14 PRU/g of acid protease ("Sigma" preparation), 24 μmol/g of citric acid monohydrate, 694 FU/kg of phytase from preparation C, L, and CE, along with 147 μL/g of *Aspergillus* pectinase (optimal dosage), caused a release of 4.037 ± 0.045, 4.704 ± 0.043, and 4.424 ± 0.08 mg of phosphorus from 1 g feed samples, respectively. Subsequent coapplication of acid phosphatase (42 APU/g) with phytase L or CE increased hydrolysis to 100 ± 1% of total phosphorus content in CSM. This "enzymic cocktail", however, required a very high level (14.7% v/w) of fungal pectinase addition to the feed. Dephosphorylation dropped with each decrease in the level of pectinase supplementation with both phytase L and CE. Taking into account that side pectinase activity differs substantially between these phytases (Table 2), it seemed obvious that an activity other than pectinase was crucial for dephosphorylation.

Table 6. Effects of a "Dephosphorylating Cocktail" [Fungal Acid Protease$_{EXP}$ (42 PRU/g); *A. niger* Pectinase, Sigma (147 μL/g); Acid Phosphatase$_{EXP}$ (42 APU/g; Citric Acid, 24 μmol/g)] on CSM Dephosphorylation, in the Presence of Varied Phytase (CE) Activity

phytase activity (FU/kg[a])	conversion rate[b] (%)
0	57 ± 1
231	84 ± 1
347	91 ± 1
462	93 ± 2
694	100 ± 1
925	102 ± 2

[a] Of the diet. [b] Percentage of total phosphorus content in CSM.

Table 7. Dephosphorylation of CSM (P_{tot}: 4.83 ± 0.06 g/kg) Phytase [925 FU/kg (from Different Preparations)], Fungal Acid Protease$_{EXP}$ (42 PRU/g; Citric Acid, 24 μmol/g) with Varied Activities of Acid Phosphatase$_{EXP}$ and Pectinase (Sigma)

phytase	treatment		N	IVDP (g/kg[a]) (mean ± SE)
	APU/g[a]	pectinase (μL/g)		
CE	100	0	10	4.639^{a} ± 0.043
CE	100	7.4	10	4.855^{c} ± 0.040
CE	100	14.7	13	4.832^{c} ± 0.033
C	75	7.4	10	4.719^{ab} ± 0.047
C	100	7.4	8	4.800^{bc} ± 0.051
C	75	14.7	8	4.809^{bc} ± 0.093
C	100	14.7	8	4.864^{c} ± 0.099
L	75	0	8	4.866^{c} ± 0.060
L	75	7.4	14	4.859^{c} ± 0.031

[a] Of the diet. Means with different superscripts are significantly different ($p < 0.05$, t-test).

This hypothesis was further supported when different activities of phytase (0–925 FU/kg) were evaluated with the "cocktail" components described above. As much as 57 ± 1% of the total phosphorus content was hydrolyzed when no phytase activity was added (Table 6), suggesting the presence of phytate-degrading enzymes in the crude pectinase preparation used. Subsequently, the pectinase ("Sigma" preparation) was found to have high activity of both phytase and acid phosphatase. On the basis of these results, a new "enzymic cocktail" was designed with phytase activity of 925 FU/kg and acid phosphatase 75 or 100 APU/g. The activity of acid protease as well as the concentration of citric acid in the new "cocktail" was the same as before. In spite of the high pectinase activity in phytase CE it was not possible to achieve complete dephosphorylation of phytates in CSM without addition of *A. niger* pectinase to the "cocktail". However, with acid phosphatase activity 100 APU/g, 7.4 μL of pectinase/g of feed was sufficient for complete dephosphorylation (Table 7). With phytase C similar rate of dephosphorylation was reached with the pectinase level of 14.7 μL/g and with acid phosphatase 100 APU/g. Phytase L did not require any pectinase addition when 75 APU/g of acid phosphatase was added to bring the dephosphorylation rate to 100%.

The enzymic "cocktail" composed of phytase (925 FU/kg), acid phosphatase (75–100 APU/g), fungal acid protease (14 PRU/g), citric acid (24 μmol/g), and pectinase (0–14.7 μL/g) completely dephosphorylated CSM feeds. It seemed obvious, however, that there was a nonidentified factor present in phytase L as well as in the pectinase which stimulated the process. Among the identified activities, phytate-degrading enzymes were of utmost importance. Attempts to lower acid phos-

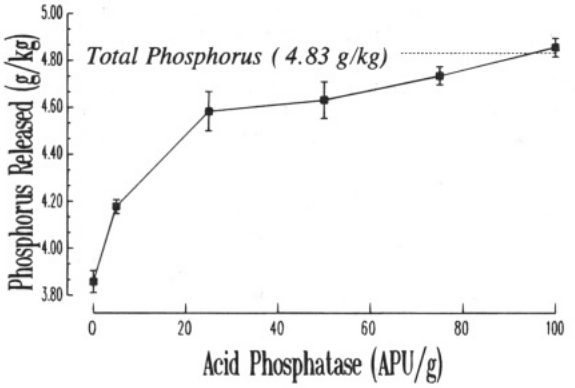

Figure 4. Influence of acid phosphatase activity on the dephosphorylation of CSM in the presence of 925 FU/kg (from preparation CE); fungal acid protease, 42 PRU/g; citric acid, 24 μmol/g; and pectinase 7.4 μL/g.

phatase activity, when phytase CE and all the other "cocktail" components were present, resulted in significant decreases in the dephosphorylation rate (Figure 4). Among tissue-degrading activities, fungal acid protease and pectinase proved to be significant in assuring the access of phytate degrading enzymes to their substrate. The extent of tissue degradation resulting from the application of the enzymic "cocktail" was measured as an increase in concentration of reducing sugars and dialyzable protein. Increase in the concentration of reducing sugars ranged from 45% with phytase L up to 81% with phytase C in the presence of all the "cocktail" components. The "cocktail" with phytase CE increased the concentration of dialyzable protein by 12%, and with phytase L by 29% (Figure 5). This is in agreement with the observation of Ritter et al. (1987) suggesting that the removal of phytate from soy proteins improves its digestibility.

Although the results presented need to be confirmed in *in vivo* nutritional studies with turkey poults, there is a potential for improved digestibility of phytate phosphorus, protein, and carbohydrates when the "cock-

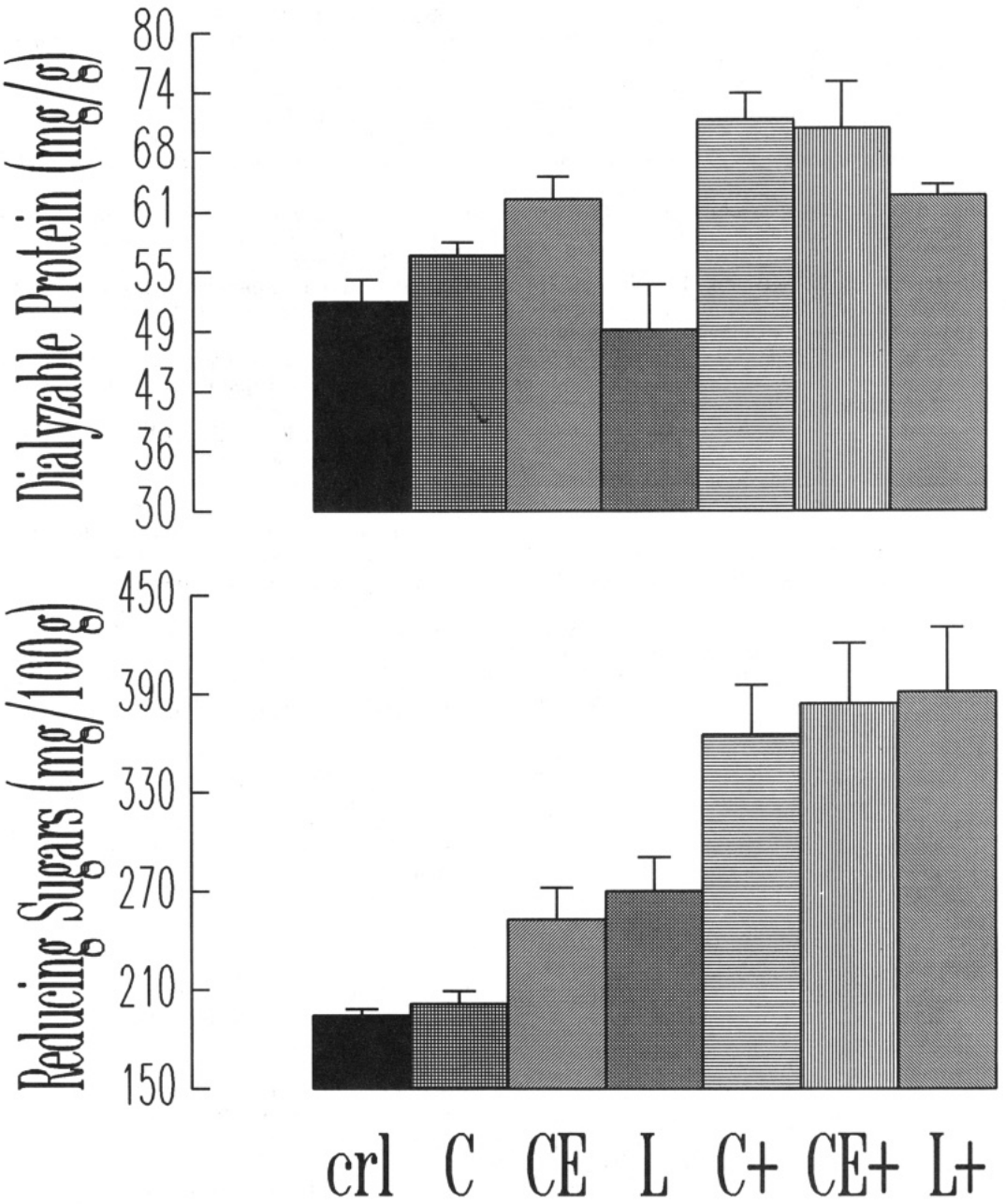

Figure 5. Dialyzable protein and reducing sugars released from CSM by phytases C, CE, and L, accompanied by an "enzymic cocktail": C+, CE+, L+. Ctrl denotes the values when no enzymes were added to the feed.

tail" is used as feed additive for monogastric animals. The issue of an economic benefit from using such an additive remains an open question as of now.

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