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Interaction between Protein, Phytate, and Microbial Phytase. In Vitro Studies

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The interaction between protein and phytate was investigated in vitro using proteins extracted from five common feedstuffs and from casein. The appearance of naturally present soluble protein−phytate complexes in the feedstuffs, the formation of complexes at different pHs, and the degradation of these complexes by pepsin and/or phytase were studied. Complexes of soluble proteins and phytate in the extracts appeared in small amounts only, with the possible exception of rice pollards. Most proteins dissolved almost completely at pH 2, but not after addition of phytate. Phytase prevented precipitation of protein with phytate. Pepsin could release protein from a precipitate, but the rate of release was increased by phytase. Protein was released faster from a protein−phytate complex when phytase was added, but phytase did not hydrolyze protein. Protein was released from the complex and degraded when both pepsin and phytase were added. It appears that protein−phytate complexes are mainly formed at low pH, as occurs in the stomach of animals. Phytase prevented the formation of the complexes and aided in dissolving them at a faster rate. This might positively affect protein digestibility in animals.

KEYWORDS: Phytate; protein; phytate−protein complexing; phytase

INTRODUCTION

Phytates, salts of *myo*-inositol 1,2,3,4,5,6-hexakis dihydrogen phosphate or phytic acid, are found commonly in vegetable feedstuffs (*1*, *2*). On average about 67% of P in such feedstuffs is present in phytate. Monogastric animals, like pigs and poultry, degrade phytate poorly. Phytase from vegetable feedstuffs or from the intestinal microflora may degrade phytate partly (*3*, *4*), but in practical diets phytate degradation in the upper gastrointestinal tract is low. Consequently P digestibility is low. Products containing microbial phytase were developed as a feed additive to degrade phytate and to increase P digestibility. Today, these products are widely used in animal feeds.

At pH values of 1−6, which is the normal acidity in the stomach of pigs and in the crop, proventriculus, and gizzard of poultry, phytate appears as an ion with three to six negative charges (*5*). As a result, complexes are formed with cations such as K, Ca, Mg, and Zn. Proteins can also bind to the phytate anion, either as a binary protein−phytate complex, where protein is bound directly to phytate, or as a ternary protein−phytate complex, where protein is bound to a mineral ion that itself is bound to phytate. The first form occurs mainly at a pH of 5 and lower and the second form at pH values above 7 (*6*).

Phytate−protein complexes may be present in plants, by nature (*2*). They can also be formed within the gastrointestinal tract of animals. The complexed proteins may, therefore, be of dietary or of endogenous origin, e.g. digestive enzymes (*7*−*9*). Also free lysine may bind to the phytate ion (*10*). If these protein−phytate complexes are insoluble in the aqueous environment of the gastrointestinal tract, it is more difficult for proteolytic enzymes to hydrolyze these proteins. Consequently, protein digestion may be reduced.

When phytase cleaves the phosphate groups from phytate, complexed cations and proteins are also liberated, resulting in an increase of their availability. With regard to protein, the addition of phytase to the diet improved digestibility in several experiments with pigs and poultry (*11*−*13*). Although the effect is often quite small (*14*), it may be of practical importance, because a small improvement of protein and amino acid digestibility can reduce feed costs considerably, due to a reduced need for addition of the first limiting amino acids.

The objective of current in vitro experiments was to investigate mechanisms for the effect of phytase on protein digestion. Studied was whether different feedstuffs contain natural soluble protein−phytate complexes, whether such complexes are formed

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under conditions similar to those in the stomach of monogastric animals, and what the effect of phytase thereon is. In addition, the effect of phytase on the hydrolysis of protein from a phytate−protein complex by pepsin was investigated.

MATERIALS AND METHODS

Materials. The experiment involved studies with six feedstuffs: corn, canola meal, rice pollards, soybean meal, sunflower seed meal, and casein. Apart from casein, these are commonly used in animal feeds. Samples were obtained from feed compounders. They were air-dried at 70 °C and ground to pass a 1 mm sieve. From these feedstuffs protein extracts were prepared as follows:

(1) A 2.5-g portion of feedstuff was extracted with 25 mL water for 30 min at room temperature. After centrifugation (3000*g*, 30 min), the supernatant was removed ("extract 1"). The residue was extracted with 25 mL of 0.1 M NaOH during 30 min at room temperature. After centrifugation (3000*g*, 30 min), the supernatant was removed ("extract 2") and the remaining residue extracted with 25 mL of 70% ethanol during 30 min at room temperature. After centrifugation (3000*g*, 30 min), the supernatant was removed ("extract 3").

(2) In study 3 only extracts from casein and soybean meal were used. The extract was prepared by mixing 10 g of air-dried material with 100 mL of 0.1 M NaOH over a 2-h period. The supernatant was removed by centrifugation (3000g, 30 min), and the pH of the solution adjusted to 4.7 (1 N HCl) to precipitate the proteins. The precipitate was freeze-dried ("extract 4"). The concentrations of protein and phytate were measured in this material.

(3) In study 6, only casein was used. Ten milliliter solutions were constituted (0.1 M citrate buffer (pH 2.4) containing 25 mg of casein). Where required, 1.25 mg of phytic acid (PA) was added. The suspension was prepared 1 h before the incubation with enzymes started and was kept at 37 °C.

Sodium phytate and porcine pepsin were obtained from Sigma Chemical Co., St. Louis, MO (numbers P-8810 and P-6887, respectively). The amount of phytate in solutions was calculated as PA. pH of solutions was obtained using 0.1 M citrate or 0.1 M borate (for pH 8 or higher) buffer. The pH was adjusted with HCl or NaOH. All reagents used were of analytical grade. Microbial phytase (3-phytase, EC 3.1.3.8, from *Aspergillus niger*; Natuphos) was obtained from DSM Food Specialties, Delft, The Netherlands.

Methods. Protein contents of feedstuffs and extracts were measured using the Kjeldahl method (*15*). Relative protein content in solutions was measured with the Bio-Rad protein assay. Phytic acid analyses were performed by HPLC, using an OmniPac PAX-100 column and suppressed conductivity detection (Dionex) (*16*). Size exclusion chromatography (Bio-Gel P-100, Bio-Rad) was used to separate free and protein-bound phytate, using water as the eluent (*17*). Electrophoresis was applied to separate and quantify soluble proteins, according to their molecular weight. A homogeneous gel, type 12.5 (Pharmacia, Uppsala, Sweden), was used. Running conditions were 600 V, 50 mA, 30 W. After 1 h, Coomassie Blue R 250 was used to fixate the gel and for staining (*18*). Phytase was analyzed according to Engelen et al. (*19*). Phytase activity is expressed in FTU; one FTU is defined as the phytase activity that liberates 1 μmol orthophosphate from 5.1 mM sodium phytate per minute at 37 °C and at pH 5.5. All measurements were performed in duplicate.

Experimental Procedures. *Study 1*. The extent of proteins bound with phytate as appears naturally in the feedstuffs was studied by measuring protein and phytate contents in the extracts 1, 2, and 3. In extract 1, binding of protein and phytate was measured using size exclusion chromatography.

Study 2. Binding of protein with phytate in aqueous solution was studied at different pH's. Extract 2 was used, because (with the exception of rice pollards) this contained a high level of protein and a low level of phytate. The quantities of protein and PA in the solutions (10 mL) are reported. A quantity of phytate was added that took the natural level of PA into account, so that maximal precipitation occurred.

Study 3. In this study the ratio of protein (extract 4) to PA in the protein−phytate complex was measured dependent upon pH (2 and 3) and the protein:PA ratio (5, 10, and 20:1, w/w). After precipitation, the amounts of protein and PA in solution were measured, from which the quantities in the precipitate were calculated.

Study 4. The effect of phytase on the formation of protein−phytate complexes was investigated by adding 2.91 FTU phytase to a phytate solution (0.5 mg of PA), before adding that to 0.1 mL of protein extract (extract 2).

Study 5. Phytate (0.5 mg) was added to extract 2 (the quantity used was chosen to obtain a final protein:PA ratio of 10:1) at pH 2, to form a protein−phytate precipitate. To study the release of protein from this complex, pepsin (8 FIP-U), pepsin and phytase (4 FTU), or no enzyme was added to the solution. Solubility of protein and phytate was measured after incubating the mixture for 30, 60, 120, 180, or 240 min at 37 °C. One FIP unit is defined as the quantity of pepsin that changes the absorption at 280 nm with 0.01 unit/min at pH 2.0 and at 37 °C from the TCA-soluble fraction, using hemoglobin as the substrate.

Study 6. To the casein−phytate suspension pepsin (8 FIP-U), phytase (0.08 FTU), or both were added. After several time intervals (0, 1, 2, 3, 4, 5, 6, and 24 h) over the incubation period (37 °C), a sample of the total suspension was taken. These samples were divided into two equal parts. In one part the soluble phase was separated using ultracentrifugation (30000*g*) ("solution"). The other part was used as such ("suspension"). Proteins were determined by electrophoresis as described previously. Also solutions of the enzymes only were tested.

Table 1. Protein and Phytate Contents in Different Extracts of Feedstuffs,[a] Total Amounts of Crude Protein (CP; g/kg) and Phytate (as Phytic Acid, PA; g/kg), and the Percentage of Soluble Protein and Phytate in These Extracts Relative to the Content of the Feedstuffs (Study 1)

		extract				%
feedstuff	parameter	1	2	3	total	soluble[b]
casein	CP	18	552	55	880	71
	PA	–[c]	–	–	–	–
corn	CP	8	46	18	99	73
	PA	6.9	2.2	–	8.8	103
canola meal	CP	46	106	27	336	53
	PA	3.7	2.5	–	32.6	19
rice pollards	CP	49	56	10	140	82
	PA	19.1	11.0	–	98.0	31
sunflower seed meal	CP	27	128	21	300	59
	PA	4.9	–	–	35.4	14
soybean meal	CP	41	257	39	470	72
	PA	12.1	1.3	–	15.8	85

[a] See text for details. [b] In fractions 1–3, relative to total. [c] Not detectable.

RESULTS

Soluble protein extracts of the feedstuffs (study 1) contained between 53% (canola meal) and 82% (rice pollards) of the total protein present in the raw materials (**Table 1**). Recovery of phytate was low in sunflower seed and canola meals and high in corn. Overall, extract 2 contained most of the soluble protein and extract 1 most of the soluble phytate. In extract 1, proteins and phytate were not bound. No clear correlation was observed between protein and phytate contents in the different extracts.

Acidity of the solutions and phytate addition had a large impact on the solubility of protein (study 2; **Table 2**). At pH 2 and at pH 8 or higher, protein dissolved almost completely, whereas it mostly precipitated at pH 3−5. Addition of phytate decreased solubility of protein under acid conditions, especially at pH 2. The disappearance of dissolved phytate from the solution confirms that protein−phytate complexes were formed. The exception was rice pollards protein, which precipitated at pH 2, independent of phytate addition.

The ratio of protein to phytate in solution (study 3) affected protein precipitation. At pH 2, when this ratio was 10:1, protein precipitated, but with casein some precipitate was formed at a

Table 2. Relative Amount of Protein (%) in Solution with or without Addition of Phytate at Different pH's (Study 2)

feedstuff	addition	pH 2	pH 3	pH 4	pH 5	pH 8	pH 10
casein (13.75)[a]	–[b]	100	3	1	85	92	90
	+	1	0	1	91	86	83
corn (4.4)	–	100	42	36	34	97	84
	+	28	33	32	33	98	86
canola meal (2.65)	–	100	81	71	76	93	99
	+	63	78	73	74	97	100
rice pollards (1.4)	–	22	39	38	38	96	100
	+	16	33	36	35	91	97
sunflower seed meal (3.9)	–	100	20	20	22	88	98
	+	26	17	16	21	98	93
soybean meal (2.36)	–	91	60	17	71	87	100
	+	2	23	16	61	87	100

[a] mg of crude protein in solution per 10 mL of sample (extract 2). [b] –, no phytate added; +, phytate added (amounts per sample: casein, 1.0 mg; corn, 0.5 mg; canola meal, 0.2 mg; rice pollards, 0 mg; sunflower seed meal, 0.2 mg; soybean meal, 0.5 mg).

Table 3. Relative Amount of Protein and Phytic Acid (%) in the Precipitates Obtained at pH 2 or 3, with Different Amounts of Added Phytate (Expressed as Phytic Acid, PA) and Protein (CP)[a]

		amount added		in precipitate		
feedstuff	pH	CP (mg)	PA (mg)	CP (%)	PA (%)	ratio (w/w)
casein	2	25	5	99	48	10
			2.5	98	90	11
			1.25	36	56	13
	3	25	5	99	42	12
			2.5	98	66	15
			1.25	95	100	19
soybean meal	2	23.6	5	92	50	9
			2.5	87	88	9
			1.25	0	0	–
	3	23.6	5	95	39	11
			2.5	93	57	15
			1.25	90	67	25

[a] Experiment with extract 4 from casein and soybean meal (study 3).

ratio of 20:1 (**Table 3**). At pH 3 most protein precipitated when the ratio was 20:1. The protein:phytate ratio in the precipitate was about 10:1 with both feedstuffs at pH 2, but this ratio was higher at pH 3. This indicates that less phytic acid was required to form a precipitate at pH 3 than at pH 2. At pH 3, but not at pH 2, the protein:phytic acid ratio in the precipitate increased with increasing quantity of phytate added to the solution. For the other four feedstuffs, results were similar (not shown).

Addition of phytase to the phytate solution before addition to the protein extract (study 4) reduced precipitation of protein largely (**Table 4**). Phytase hydrolyzed phytate, preventing the formation of protein−phytate complexes.

The amount of protein in a soybean protein−phytate precipitate reduced slowly after incubation with pepsin at pH 2 (study 5; **Figure 1A**), but when phytase was also added, protein dissolved faster and to a larger extent. Without the addition of phytase, the amount of protein precipitated did not change (remained at 100%). At pH 3, protein already dissolved in part without phytate addition (**Table 2**). For that reason, the quantity of precipitated protein without addition of phytate decreased at pH 3 (**Figure 1B**). Protein precipitation increased after addition of phytate, but when phytase was also added, the level was lower than for the control treatment.

Degradation of protein from the protein−phytate precipitate by pepsin, with or without addition of phytase, was investigated

Table 4. Relative Amount of Protein (%) in Solution with or without Addition of Phytate to Feedstuffs Extract (Extract 2), in Combination with Phytase at pH 2 and 3 (Study 4)

	pH 2			pH 3		
feedstuff	P[a]	PP[b]	PPP[c]	P	PP	PPP
casein (13.75)[d]	100	1	93	3	0	4
corn (4.4)	100	28	100	42	33	42
canola meal (2.65)	100	63	95	89	81	82
rice pollards (1.4)	22[e]	16	57	39	33	47
sunflower seed meal (3.9)	100	26	90	34	23	28
soybean meal (2.36)	100	2	99	60	32	60

[a] P: protein extract only. [b] PP: protein extract with phytate (amounts per sample: casein, 1.0 mg; corn, 0.5 mg; canola meal, 0.2 mg; rice pollards, 0 mg; sunflower seed meal, 0.2 mg; soybean meal, 0.5 mg). [c] PPP: protein extract with phytate and phytase (2.91 FTU). [d] mg of crude protein in solution per sample. [e] Probably due to the high content of phytate in the protein extract.

in study 6. The electrophoresis gels after 1 and 2, 3 and 4, 5 and 6, and 24 and 1 h of incubation are shown in **Figure 2**, panels **A**, **B**, **C**, and **D**, respectively. The bands indicate the size of proteins in suspension and in solution. Pepsin and phytase themselves do not form bands (**Figure 2A**, lanes 2 and 3) and thus are not interfering with the results. After 1 h, protein bound to phytate resembles pure casein (**Figure 2A**, suspension, lanes 4, 5, 7 and 8 vs lane 1). When phytate was not added, pepsin hydrolyzed protein (lane 6, suspension) and proteins appeared in solution. Phytate formed a stable complex with casein: even after 24 h protein was in suspension, whereas no protein appeared in solution (**Figure 2D**, lane 4). Addition of phytase liberated part of the protein from the protein−phytate complex but did not degrade the protein into smaller units (lane 5). In contrast, addition of pepsin alone degraded the protein of the complex into smaller units but did not liberate these units from the complex until small units (ca. 12 kDa) were formed (lane 7 vs lane 4). When both phytase and pepsin were added, protein was liberated from the protein−phytate complex and degraded into smaller units (lane 8).

DISCUSSION

Protein−phytate complexes may already exist in plants or they might be formed within the gastrointestinal tract of monogastric animals. In present study, no clear correlation between the amount of protein and phytate in extracts 1−3 (water, dilute NaOH, and ethanol extracts) was observed. In extract 1, phytate levels were relatively high with soybean meal, corn, and rice pollards, but size exclusion chromatography indicated that they contained no protein−phytate complexes. Only in rice pollards was recovery of phytate in extract 2 high, indicating that this feedstuff may contain soluble protein−phytate complexes by nature. Low recovery of both protein and phytate in sunflower seed and canola meal prevents drawing a conclusion on the existence of natural protein−phytate complexes in these feedstuffs. These results suggest that soluble protein−phytate complexes are of minor importance in the investigated feedstuffs, with the possible exception of rice pollards.

In many plants phytate is stored in globoids. Globoids are particles that are usually incorporated in the protein bodies of plant cells (*20*). Consequently, phytate is usually stored in tissues that are rich in protein, for example, the germ or aleurone layer, which may lead to the assumption that protein is bound to phytate. This is also suggested by the similar solubility behavior of both proteins and phytate (*6*). Globoid crystals contain,

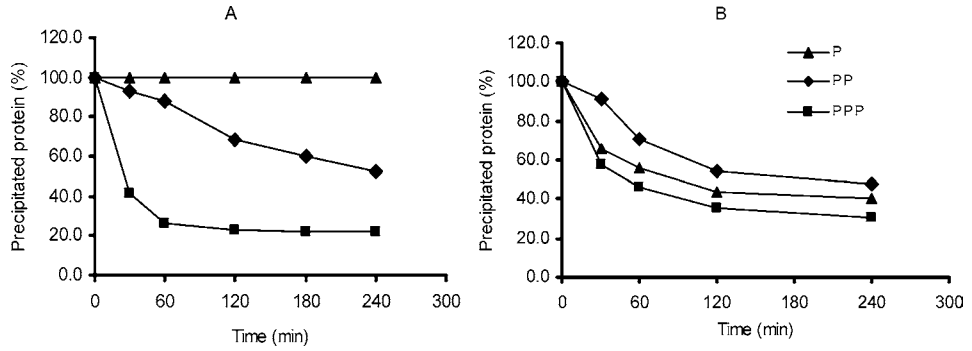

Figure 1. Relative amount of protein in a precipitate formed at pH 2 (from soybean meal protein extract), after addition of pepsin (8 FIP-U). P, control, i.e., no phytate or phytase added; PP, phytate added; PPP, phytate and phytase (4 FTU) added. Measurements at pH 2 (**A**) or 3 (**B**) (study 5).

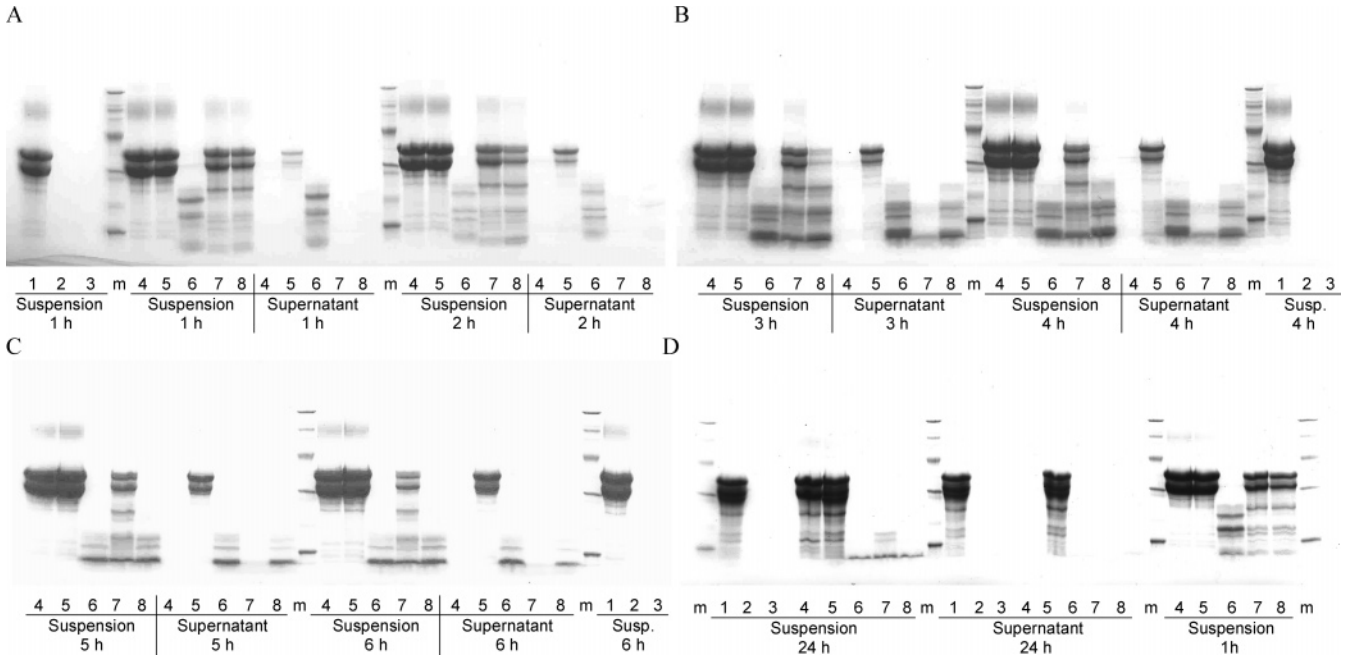

Figure 2. Study 6: electrophoresis gel of casein. Size of proteins in suspension and in solution is shown. After 1 or 2 h of incubation (**A**), 3 or 4 h (**B**), 5 or 6 h (**C**), or 24 or 1 h (**D**) (the latter is for comparison). Treatments: lane 1, buffer and casein; lane 2, buffer and phytase; lane 3, buffer and pepsin; lane m, marker; lanes 4–8, buffer and casein with the addition of phytate (lane 4), phytate and phytase (lane 5), pepsin (lane 6), phytate and pepsin (lane 7), and phytate, phytase, and pepsin (lane 8).

however, often only small amounts of protein (*21*), making direct binding of protein and phytate less obvious. Our results agree with this, although we studied soluble proteins only.

If protein is digested slower from a protein−phytate complex in the gastrointestinal tract of animals than when it is available in a dissolved form, complex formation may reduce protein digestibility. Because a high protein digestibility is key for efficient animal production, there is a high practical relevance of the investigated mechanisms. These practical aspects were studied in a number of digestibility experiments. Ileal amino acid digestibility increased in pigs (*11*, *22*) and poultry (*23*, *24*), when diets were supplemented with phytase. The improvement was not always significant in individual studies (*14*), but it was in a meta-analysis (*12*).

Because naturally soluble protein−phytate complexes are probably of minor importance (study 1), insoluble protein−phytate complexes must have been formed within the gastrointestinal tract of monogastric animals, most likely in the stomach because most proteins precipitated after addition of phytate at pH 2. No protein−phytate complexes were formed at pH values higher than 4 (**Table 2**), which is normal in the gut distally from the proximal duodenum. Phytase addition permitted dissolution of these complexes.

More than 85% of the protein precipitated when the protein:phytate ratio was 10:1 (at pH 2) or 20:1 (at pH 3; **Table 3**). The protein:phytate ratio in the precipitate was lower at pH 2 than at pH 3, probably because phytate has more negative sites available for binding to protein at pH 3 (*5*). A higher number of negative sites could result in a higher rate of formation of phytate−protein agglomerates. The "expansion" of this agglomerate continues when additional phytate is available, because the level of phytate in the complex increases with increasing phytate addition at pH 3. At pH 2, the possible binding sites may be saturated with protein bonds, when the precipitate is formed. Therefore, the relative amount of phytate in the protein−phytate complex varies little with increasing phytate addition. Looking to the practical implication, assume that a practical corn−soybean meal diets for monogastric animals contains about 20% crude protein and about 1% phytate (expressed as phytic acid), a ratio of 20:1. Accepting that the in vitro results are indicative for in vivo conditions, this means

that protein−phytate complexes may indeed be formed in the stomach of animals.

The protein:phytate ratio in the precipitates (**Table 3**) was higher than those measured by Lásztity and Lásztity (*25*). In soy glycenin, sunflower seed globulin, and wheat gluten, they measured protein:phytate ratios of 1.2−5 (w/w) at pH 2−5.5. Different characteristics of the proteins used and of the test conditions may explain this large deviation.

The protein:phytic acid ratio at pH 3 was higher in the precipitate of soybean meal protein than of casein, which is probably due to the amino acid composition. Basic amino acids may link best to the phytate ion. Soybean meal contains (on a protein basis) a higher level of arginine and about equal proportions of lysine and histidine, as compared to casein (*26*).

Phytase supplementation prevented protein−phytate complexing to a large extent (study 4). When such complexes had been formed, pepsin dissolved protein from them at a higher rate and to a larger degree when phytase was also added (**Figure 1**). Protein present in precipitates was hydrolyzed into smaller parts by pepsin (**Figure 2**). The pieces were only dissolved from phytate when they were smaller than about 12 kDa. When phytase was added to the protein−phytate precipitate together with pepsin, proteins were liberated from the complex, and hydrolyzed into smaller fragments at a faster rate than without phytase. Phytase itself, however, did not hydrolyze protein (**Figure 2**) and thus does not possess proteolytic activity to which the effect could be attributed.

For the digestion of protein by animals, the importance of the increased rate of protein hydrolysis into smaller fragments and the solubility of these fragments is presently unknown. Pepsin hydrolyzes protein at a lower rate from a protein−phytate precipitate than from soluble protein. This does not mean that there would be no digestion of these proteins. When digesta enter the small intestine and the pH rises, probably a large part of the protein fragments is dissolved. Proteolytic enzymes in the small intestine may further degrade these fragments. The digestion of amino acids depends on a dynamic system, which includes pH, residence time in the different compartments of the gastrointestinal tract, concentration and degree of solubility of proteins, and concentration of proteolytic enzymes.

Dietary phytate increases the formation of insoluble protein−phytate complexes in the stomach, with a risk that digestibility is reduced. Phytase prevents formation of these complexes or aids degrading them faster and further and may, consequently, improve protein digestibility. This mechanism could explain the small increase in protein digestibility (about 1−2%-units) observed in many experiments (*12*). Also the binding of proteolytic enzymes to phytate may explain part of this effect.

It is concluded that the feedstuffs studied contained only small amounts of soluble protein−phytate complexes. Insoluble protein−phytate complexes are formed at low pH, as found in the stomach of monogastric animals. Pepsin degrades protein from such complexes slower than from soluble protein, which may reduce protein digestibility slightly. Dietary phytase supplementation prevents the formation of protein−phytate complexes or aids in dissolving them faster. Therefore, phytase may improve protein digestibility.

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