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Dual Effects of Sodium Phytate on the Structural Stability and Solubility of Proteins

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ABSTRACT: The interaction between sodium phytate and three proteins was studied using solubility experiments and differential scanning calorimetry (DSC) to assess structural stability. Lysozyme, which is positively charged at neutral pH, bound phytate by an electrostatic interaction. There was evidence that phytate cross-linked lysozyme molecules forcing them out of solution. Myoglobin and human serum albumin, which were neutral or negatively charged, respectively, displayed association rather than binding, and there was no complex formation. All of the proteins were structurally destabilized by the presence of phytate but were not denatured. From these findings, we predict that phytate would bind electrostatically to a wide variety of positively charged proteins in the stomach as well as to trypsin and chymotrypsin in the duodenum. Both binding reactions may compromise the digestion of the protein component in feed stuffs. Because the interaction between phytate and protein is electrostatic, the presence of anions, such as chloride, would nullify the antinutritional effect of phytate.

KEYWORDS: *Hofmeister, unfolding, precipitation, phytase, digestion*

INTRODUCTION

Phytate concentrations in the order of 10 g/kg are usually present in pig and poultry diets, where they are derived from plant-sourced feed ingredients, such as maize, wheat, and soybean meal. The polyanionic molecule phytate (*myo*-inositol hexaphosphate, IP6) is believed to possess an array of antinutritive properties because it has the capacity to chelate positively charged nutrients, including proteins, in binary protein−phytate complexes at pH levels less than the isoelectric points of the protein.[1] While this may present economically important challenges to pig and poultry producers, the underlying mechanisms of these antinutritive properties are only partially understood, although several theories have been advanced to explain the role of phytate in animal nutrition. In relation to protein use, phytate-bound protein may be refractory to pepsin digestion because of structural and solubility changes induced by complex formation.[2] It has been proposed that compensatory gastric hypersecretions of pepsin and hydrochloric acid trigger a chain of physiological events that may compromise both digestion of protein and absorption of amino acids.[3] Phytate is also reported to inhibit the activity of endogenous digestive enzymes. Phytate may inhibit the activities of amylase,[4,5] trypsin, and chymotrypsin.[6,7] Amylase, trypsin and chymotrypsin play pivotal roles in the digestion of starch and protein, respectively. It is generally believed that the protein−phytate complexes are formed because of salt-like linkages (electrostatic interactions) between negatively charged phytate and the positively charged basic amino acid residues of arginine, lysine, and histidine.[1,8]

A recent alternative theory is that phytate acts as a kosmotrope, like the closely related phosphate anion, and reduces the solubility of the protein indirectly.[9] The kosmotropes are at one end of the Hofmeister series of salts and are responsible for indirectly reducing protein solubility and increasing their structural stability.[10,11] At the other end of the Hofmeister series of salts are the chaotropes, which are responsible for increasing protein solubility and decreasing their structural stability. The mechanisms by which Hofmeister series salts alter protein solubility and stability is currently subject to reappraisal.[12] The hydrogen bond "structure maker−breaker" theory for the indirect actions of salt and osmolytes on protein stability has been contradicted using different experimental approaches, pressure perturbation calorimetry and femtosecond pump−probe spectrscopy.[13,14] The field has been further challenged by the discovery of an inverse Hofmeister effect of salts on protein solubility, suggesting that salt−protein interactions are more complex than previously thought.[15] If the kosmotrope theory is correct, it would challenge the current understanding of the antinutritional properties of the phytate, reviewed in depth in ref 3.

In this paper, the protein−phytate complex and kosmotrope theories were challenged experimentally using three soluble proteins and sodium phytate. The three proteins used were lysozyme, myoglobin, and human serum albumin (with octanoate in the fatty acid binding sites) (Table 1). Each of these proteins is well-characterized, their structures are known and available in accessible databases, which makes interpretation of the data less problematic. This experiment did not attempt to mimic the environment of the digestive tract, which is complex and changeable, but used well-defined controlled conditions to study the fundamental interactions that take place between the anion phytate and different proteins. Differential scanning calorimetry (DSC) was used to detect the effect of

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Table 1. Number of Basic Amino Acid Residues, Isoelectric Point, Stoichiometry, and ΔT_m of Phytate Binding for Lysozyme, Myoglobin, and Human Serum Albumin[23−25]

	number of arginine, histidine, and lysine residues	isoelectric point (pH)	ΔT_m
lysozyme	18	11.4	−6.5
myoglobin	22	7.3, 6.8	−7.0
human serum albumin	66	4.8	−3.1

phytate on protein stability. The increased sensitivity of modern DSCs has enabled the thermodynamics of heat-induced protein unfolding to be studied,[16] heat-induced aggregation,[17] and the role of small molecules on protein stability to be investigated.[18,19] DSC enabled the stability part of the kosmotrope theory to be challenged. Protein solubility was assessed using standard precipitation experiments.

MATERIALS AND METHODS

Proteins, sodium phytate, sodium octanoate, and the salts were sourced from Sigma-Aldrich, Gillingham, U.K.

Solubility Studies. A 12 mg/mL stock solution of protein in 100 mM NaCl at pH 7 and a 200 mM sodium phytate stock solution in 100 mM NaCl at pH 7 were prepared. The protein concentration in each sample run was 6 mg/mL; the sample was diluted 2 times by adding 100 mM NaCl solution and sodium phytate solution to give the desired sodium phytate concentration. Samples were equilibrated for 20 min at room temperature before being centrifuged at 10000*g* for 20 min at 4 °C using a Sigma Laboratory centrifuge 4K15 (SciQuip, Ltd., Merrington, U.K.). After centrifugation, the supernatant was collected, dilution of the sample was made with 100 mM NaCl buffer at pH 7, and the dilution was varied to achieve an absorbance between 0.1 and 0.8. The absorbance of each sample was then measured at 280 nm 3 times, and an average was taken using a Jenway 6305 spectrophotometer (Bibby Scientific, Stone, U.K.). Absorbance at 280 nm is due to tryptophan and tyrosine residues in the protein.

DSC. A 0.5 mg/mL protein in 100 mM NaCl at pH 7 and varied sodium phytate concentrations were used. DSC was undertaken with a Nano-DSC (TA Instruments, New Castle, DE) at a heating rate of 1.5 °C/min from 30 to 100 °C in forward scans. The instrument was held at a constant temperature for 15 min between each scan. Data evaluation used the software was provided by the manufacturer. Buffer−buffer baselines were subtracted from sample data. The T_m value (the temperature with the maximum heat capacity value on the DSC scan) was used as a measure of protein structural stability.

Salting-off Study. The experiment to study whether binding of phytate to protein was an electrostatic interaction used the following conditions: 0.5 mg/mL protein in Milli-Q water at pH 7 and sodium chloride concentrations from 0 to 1 M. The DSC operation was unchanged.

RESULTS AND DISCUSSION

Lysozyme (6 mg/mL) solubility was unaffected until the phytate concentration reached 0.4 mM, and then the absorbance dropped to 33% of its starting absorbance at 4 mM phytate and then rose until full solubility was regained at 30 mM phytate (panel i in Figure 1).

There are two possible explanations for the change in solubility of lysozyme with a rising phytate concentration: complex formation and behavior as a chaotrope (as opposed to a kosmotrope). The pI of lysozyme is 11.4; therefore, at pH 7.0, the protein is positively charged.[20] It is probable that the negatively charged phytate molecule interacts with the positively charged protein by electrostatic interaction. Because the phytate and protein both have multiple charged groups,

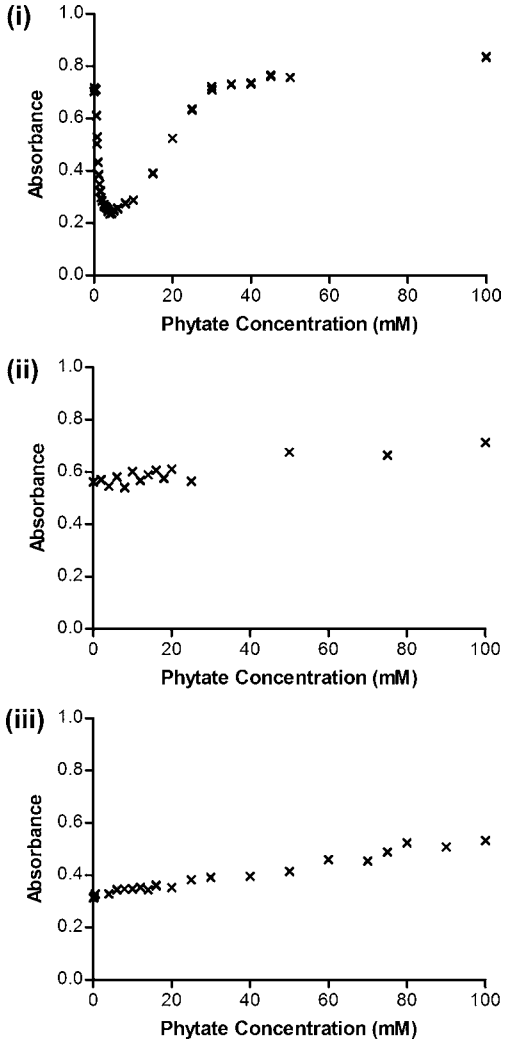

Figure 1. Absorbance at 280 nm measured after the addition of phytate followed by centrifugation as a measure of protein solubility with varying sodium phytate concentrations and (i) lysozyme, (ii) myoglobin, or (iii) human serum albumin in 10 mM NaCl at pH 7.0 (all proteins at 6 mg/mL).

there is the possibility of it forming a network. The drop in solubility between 0.4 and 30 mM phytate is consistent with a network forming composed of interlinked protein and phytate reducing the solubility of the protein, but over 4 mM phytate, the positively charged residues of the protein become increasingly saturated with phytate ions, reducing the opportunity for cross-linking to take place and resulting in a rise in solubility (Figure 2). The solubility curve of lysozyme with phytate (panel i in Figure 1) could also be interpreted as phytate acting like a chaotropic anion. Cloud point studies of lysozyme with different anions demonstrated that binding of a chaotropic anion at low concentrations (such as iodide or perchlorate)[10,11] reduced the solubility of the protein, and this was followed by an increase in solubility with the addition of further salt.[15] This could account for the two phases in the lysozyme solubility curve. The initial binding of the anion to positively charged amino acid residues neutralizes the electrostatic repulsion of the positively charged proteins and allows the proteins to aggregate. The indirect resolubilization of the protein is less well-understood but may be related to alteration of the hydrogen-bond population in the bulk water. If this

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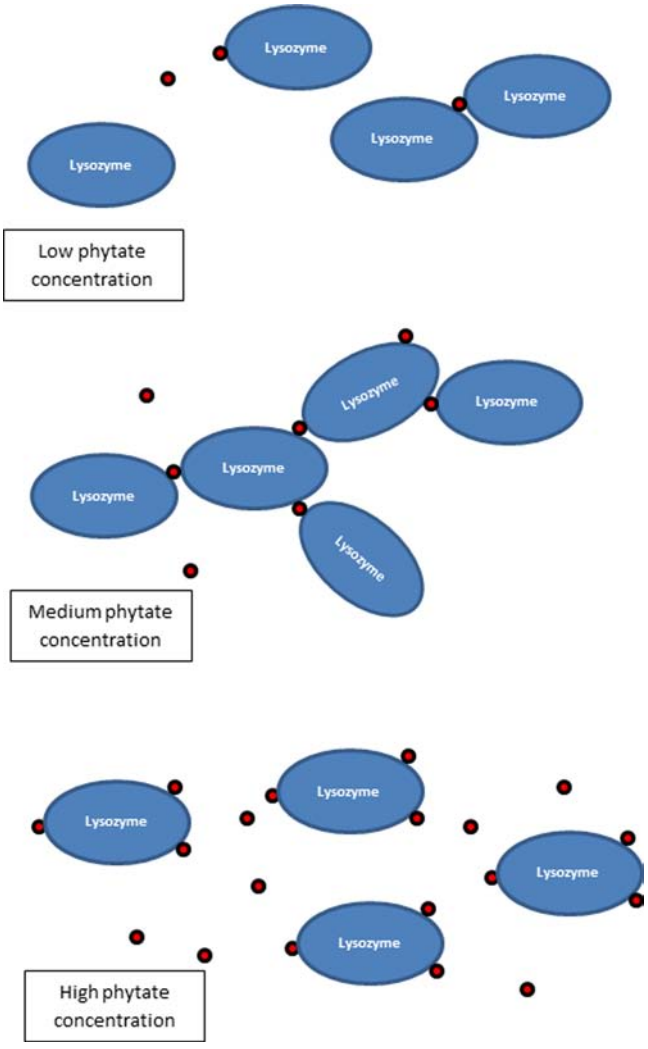

Figure 2. Interaction between proteins and phytate at pH < pI.

interpretation is correct, phytate is a chaotrope and not a kosmotrope. These experimental data are consistent with the protein−phytate complex theory and inconsistent with the kosmotrope theory. It is worth noting that both explanations for lysozyme solubility suggest binding of the phytate to the protein by electrostatic interaction.

Solubility of both myoglobin and human serum albumin were unaffected by the presence of phytate (panels ii and iii in Figure 1). The key difference between the three proteins is their respective pI values. Myoglobin and human serum albumin have pI values of 7.0 ± 0.3 and 4.7; therefore, they are neutral and negative, respectively.[21,22] These proteins would not be susceptible to cross-linking by negatively charged phytate. The negatively charged phytate ion would also be incapable of negating the repulsion of like (negatively charged) human serum albumin molecules.

DSC was conducted with the three proteins (0.5 mg/mL protein in 100 mM NaCl at pH 7.0). The structural stability of the proteins is presented as the temperature where the protein is unfolding at its maximum rate (the T_m value). The higher the T_m value, the more stable the protein. The effect of phytate on the structural stability of lysozyme is illustrated in Figure 3, where it causes a drop in the T_m value. The T_m value of lysozyme dropped from 73.7 to 67.2 °C with the addition of just 5 mM sodium phytate (panel i in Figure 4). Above 5 mM,

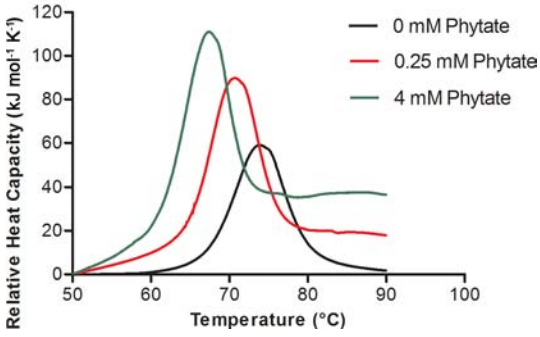

Figure 3. DSC scans of lysozyme with varying sodium phytate concentrations in 100 mM NaCl at pH 7.0.

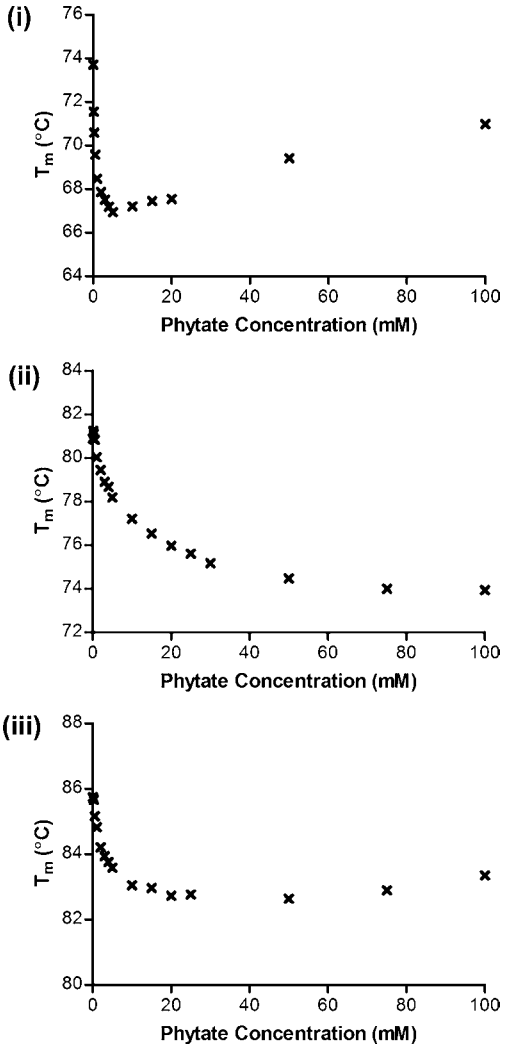

Figure 4. Temperature of maximum unfolding (T_m) values measured by DSC with varying concentrations of phytate of (i) lysozyme, (ii) myoglobin, or (iii) human serum albumin at 0.5 g/L and 100 mM NaCl at pH 7.0.

the T_m value slowly rose, achieving 71.0 °C by 100 mM phytate. The low phytate concentration required to achieve maximum destabilization of the protein structure suggests a low stoichiometry of around 10 phytate molecules per lysozyme molecule required to reduce the stability of lysozyme. This is consistent with direct binding of the phytate with the 18 basic residues of lysozyme. Phytate could bind to more than one

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basic residue at a time if they are close together, and some residues would not be on the protein surface, making them unavailable for binding. The presence of bound phytate could destabilize the structure of the protein by two possible mechanisms. The localized negative charge where phytate is bound would attract neighboring basic residues toward it and repel the acidic residues. This would apply force on the peptide backbone, destabilizing it. The presence of phytate bound to the surface of a protein would also alter the hydration layer around the protein and the Gibb's free energy associated with this structure.[19] The Gibb's free energy associated with the hydration layer is believed to play an important role in the structural stability of proteins and to be modulated by the presence of small molecules, such as sugars and amino acids.[19] The DSC data are consistent with binding of phytate to lysozyme (as proposed earlier), followed by an indirect stabilization of the protein, suggesting the phytate is acting like a kosmotrope.

The structural stability of myoglobin and human serum albumin were reduced by the addition of sodium phytate (panels ii and iii in Figure 4). The T_m value of myoglobin dropped from 80.9 to 73.9 °C with the addition of 100 mM sodium phytate. The T_m value of human serum albumin dropped from 85.7 to 82.7 °C with the addition of 20 mM sodium phytate. The drops in the T_m values were consistent with an adsorption isotherm rather than a uniform drop observed with chaotropes, like guanidinium chloride. It is worth noting that, while the reduction in stability of the proteins in the presence of phytate is marked, it was not enough to denature the three proteins studied at physiological temperatures. These data are not evidence of any loss of functionality of these proteins caused by sodium phytate.

To confirm whether the interaction between lysozyme and phytate was electrostatic, NaCl was added to lysozyme plus 4 mM phytate (the phytate concentration that gave a minimum T_m value for lysozyme; see panel i in Figure 4) and the structural stability was monitored using a DSC. The chloride anion would compete with the phytate for basic residues on the protein surface, and the DSC would detect the recovery of structural stability associated with the displacement of phytate from the lysozyme by the chloride anions. Consistent with the theory that it was an electrostatic interaction that caused the initial destabilization of lysozyme, NaCl was able to restore the T_m value of lysozyme until it matched the negative control (panel i in Figure 5). Sodium chloride was also able to nullify the destabilization of myoglobin and human serum albumin by phytate, suggesting that the association of phytate with these proteins was also electrostatic in nature, even though these proteins did not have a positive charge (panels ii and iii in Figure 5).

The experimental findings presented in this paper illustrate a picture of the interaction between phytate and proteins that is more complex than previously thought. When the pI of the protein is greater than the pH of the solution, the protein has a net positive charge. Under these conditions, phytate binds directly with the basic amino acid side chains by electrostatic interaction. Because phytate has six negatively charged phosphate groups, the molecule can cross-link basic amino acid side chains from different proteins to form an insoluble network or complex.

The kosmotrope theory was too simplistic. The DSC stability experiment with lysozyme (panel i in Figure 4) contradicted the solubility experiment (panel i in Figure 1). Phytate did

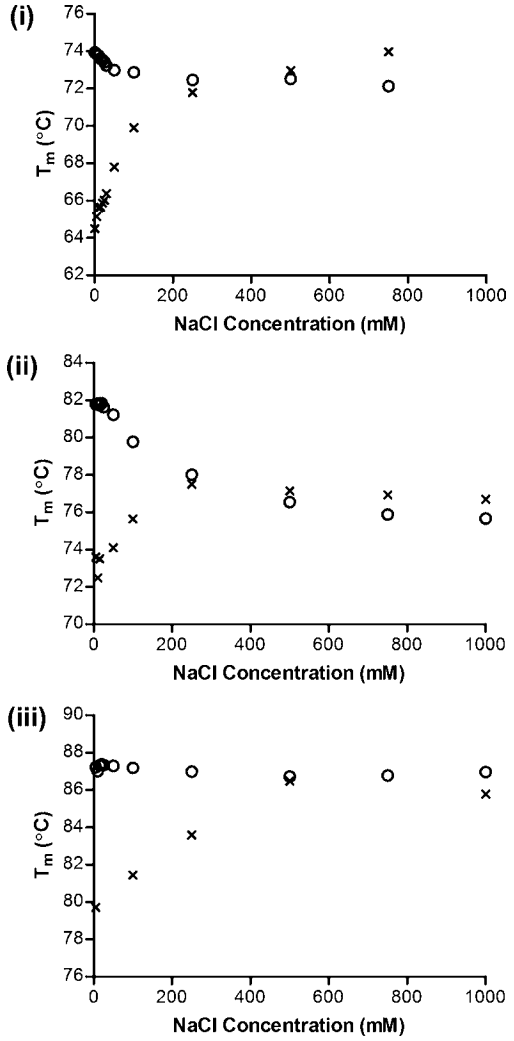

Figure 5. Salting-off of phytate bound to (i) lysozyme (4 mM phytate and 0.5 g/L protein), (ii) myoglobin (50 mM phytate and 0.5 g/L protein), or (iii) human serum albumin (20 mM phytate and 0.5 g/L protein), by the addition of NaCl (×) and control without phytate present (○). Measured by DSC and presented as the temperature of maximum unfolding (T_m) values versus NaCl concentration.

stabilize lysozyme indirectly (at concentrations over 5 mM), like a typical kosmotrope. Below 5 mM, phytate did destabilize lysozyme. It is possible that the destabilization of lysozyme was due to phytate binding to the protein and multiple negative charges of the phytate in close proximity to the protein destabilizing its structure. The disparity between the stability and solubility data suggests that phytate has a dual character; it may be a kosmotrope, but the solubility experiment suggests that it is also capable of acting as a cross-linking agent, which dominates any behavior at lower concentrations.

The interaction between phytate and negatively charged (or neutral) proteins is less clear. This occurs whenever the pI of the protein is less than the pH of the solution. The fact that chloride anions nullify the destabilizing properties of phytate for myoglobin and human serum albumin indicates that this is an electrostatic interaction but differs from direct binding detected with the positively charged protein in its strength. The experimental evidence suggests that phytate weakly interacts with positively charged side chains and possibly the partially charged parts of the protein (the exposed backbone or polar

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side chains) in an ion−dipole interaction. This type of interaction would be reduced by the presence of chloride anions. In this paper, we refer to this weak interaction as association. The presence of phytate near the protein was sufficient to reduce the T_m value measured by DSC but was too transient or weak to allow for network formation, hence, no observed precipitation. It is also worth noting that there was no evidence of ternary protein complexes under the defined (divalent cation-free) conditions used in this experiment.

It is worth noting that trypsin, chymotrypsin,[23] and rapeseed napin[24] have similar solubility curves to lysozyme when exposed to different concentrations of phytate, suggesting that lysozyme is not unique in its behavior. Both trypsin and chymotrypsin were positively charged (like lysozyme); therefore, the similarity in their solubility behavior is likely to be due to the same mechanism.

It is worth considering what implications this experimentation has on the role of phytate in digestion. While digesta is a complex changeable mixture, the fundamental interactions between phytate and proteins are likely to be unchanged, which does allow for some qualified discussion of what happens in the digestive tract of an animal. First, the destabilization by phytate in this study was not enough to denature any of the proteins used. It is possible that phytate could help denature substrate proteins during digestion, which could aid digestion. Destabilization of digestive enzymes through association or binding would only be a problem if the T_m for a digestive enzyme is sufficiently close to 37 °C for the presence of phytate to denature the enzyme. Second, the weak association of phytate with negative or neutrally charged proteins is unlikely to have any detrimental effects on digestion. Third, the stronger binding of phytate to positively charged proteins could interfere with digestion by either blocking digestive enzyme active sites or the cleavage sites on the food protein (trypsin cleaves next to an arginine or lysine, both potential sites for phytate binding).[25] There have been several papers that suggest that the antinutritional properties of phytate may be due to interference with the function of critical digestive enzymes. These enzymes operate in digesta at differing pH values (Table 2). Each digestive enzyme has an individual pI value (Table 3). This means that the interaction of phytate with some digestive enzymes could switch from direct binding (and complex formation) to weak association as they pass down the digestive tract. Proteins originating from a diverse range of grains (have pI values between 4 and 7)[3] are likely to be subject to binding by phytate at the acidic pH values in the stomach, although this is less likely to occur further down the digestive tract.

Table 2. pH in the Digestive Tract of Weaned Pigs[26]

organ	pH
stomach	3.2
duodenum	5.7
jejunum	6.0
ileum	6.9

In the past, there has been much made of the relationship between cations and phytate. Phytate isolated from grain is rich in the divalent cations magnesium and calcium. The presence of divalent cations will affect the interaction of phytate with proteins because the cations will compete with the basic side chains of the protein for the phytate anion. The presence of divalent cations will also influence phytate solubility and can interact directly with proteins. The research presented here indicates that the presence of anions also has to be considered. Anions compete with phytate for the binding sites on proteins (including the digestive enzyme trypsin and the substrate proteins). It is also possible that higher salt (NaCl) diets for pigs and poultry may counter phytate binding to digestive enzymes, protein substrates, or any other positively charged material in the digesta, minimizing any antinutritional effect.

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Notes

The authors declare no competing financial interest.

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Table 3. Isoelectric Point of Digestive Enzymes with Predicted Interaction between the Digestive Enzyme and Phytate and Published *in Vitro* Enzyme Activity Results

enzyme	isoelectric point (pH)	stomach (pH 3.2)	ileum (pH 6.9)	*in vitro* enzyme activity in the presence of phytate
α-amylase (procine pancreatic)	7.5 and 6.4[27]	binding	probable association	inhibition at pH 4 low inhibition at pH 7[28]
lipase (porcine pancreatic)	5.3[29]	binding	association	low inhibition at pH 6.5−8[30]
trypsin	10.5[31]	binding	binding	slightly increased activity and precipitation at pH 3 less effect at pH 7.8[5]
chymotrypsin	7.7[32]	binding	probable association	increased activity and precipitation at pH 3 less effect at pH 7.8[5]
pepsin	1.0[33]	association	association	low inhibition of small substrate digestion at pH 3[2]

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