Interactions of phytate and myo-inositol phosphate esters (IP_{1-5}) including IP_5 isomers with dietary protein and iron and inhibition of pepsin

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Interactions of phytate and myo-inositol phosphate esters (IP_{1-5}) including IP_5 isomers with dietary protein and iron and inhibition of pepsin[1]

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ABSTRACT: Phytic acid (IP_6) and *myo*-inositol phosphate esters (IP_{1-5}), including IP_5 isomers prepared chemically and enzymatically with bacterial and fungal phytases, were examined for their effects on protein aggregation of soy protein and β-casein, interaction with Fe^{3+}, and pepsin activity. The results indicated that the aggregating capabilities of IP esters (IP_{1-6}) on the 2 proteins decreased dramatically from IP_6 to IP_5 and became negligible with IP_{1-4}. Among the IP_5 isomers tested, $InsP_5$(1,2,3,4,5) produced by 6-phytase was slightly less powerful in aggregating protein than $InsP_5$(1,2,4,5,6) produced by 3-phytase (P = 0.001). For protein hydrolysis, IP esters of IP_{3-4} still showed inhibition of pepsin though to a lesser extent than IP_{5-6}. The in vitro data with IP_{1-5} generated with microbial 3- and 6-phytases indicate that, for complete alleviation of pepsin inhibition, IP_6 needs to be broken down to IP_{1-2}. In contrast to the aggregation with protein, the reactivity of IP_{1-6} toward Fe^{3+} decreased proportionally from IP_6 to IP_3. Based on the radical decrease in turbidity of IP_6 -protein complex observed, as a result of IP_6 dephosphorylation to IP_5, a novel qualitative and semi-quantitative phytase plate assay was established using IP_6-protein complex incorporated into an agarose petri-dish as substrate. Phytase activity was shown as the development of clear halos on the agarose plate with time. This simple phytase plate assay method can be used at animal farms, control laboratories, and even for the screening of engineered phytase variants. The current study, thus, stresses the importance of the efficient hydrolysis of IP_6 at lower pH range to alleviate the negative effect of phytic acid and its degradation products on protein and Fe^{3+} digestion.

Keywords: inositol phosphate, pepsin, phytase assay, phytic acid

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INTRODUCTION

It is well known that phytate (*myo*-inositol hexakisphosphate, **IP_6**) is the major form of inorganic phosphate storage in plant seed and is mobilized when plant seeds germinate. Phytic acid content in cereals is usually in the range of 0.8 to 4.0% of dry weight, and it can be a rich source of inorganic phosphate (**Pi**) for animals and humans (Reddy, 2002; Greiner and Konietzny, 2010). At acidic pH, IP_6 may interact with dietary protein, leading to the formation of IP_6-protein aggregates, which has been well documented in the literature (Reddy et al., 1989; Rajendran and Prakash, 1993). For example, Murthy and Rao (1984) reported the maximum interaction of IP_6 with mustard 12S protein occurred at pH 3.0 with the binding capacity of 170 mol Pi mol^{-1} protein. These aggregates may have decreased accessibilities to protease, possibly resulting in inefficient protein digestion in the gastric phase, thereby leaving inappropriate protein substrate for the pancreatic protease (Knuckles et al., 1989; Kies et al., 2006). Phytic acid-protein complex occurs as a result of masking of positive charges of the protein by IP_6 (Reddy et al., 1989).

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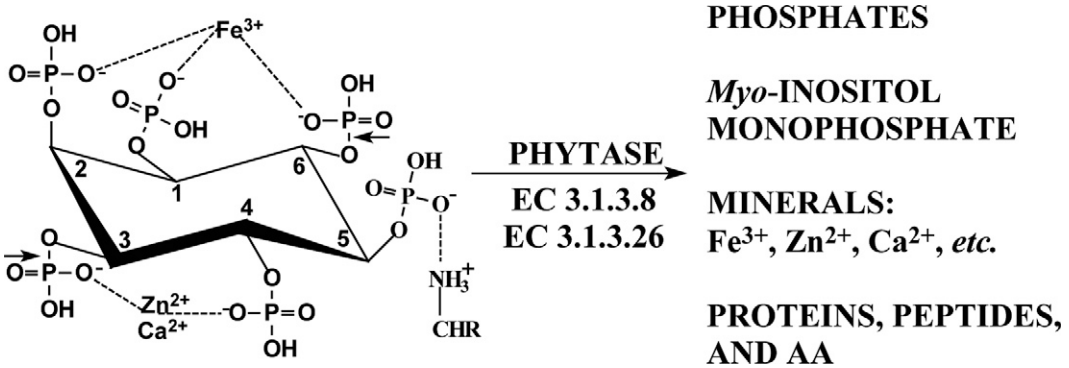

Figure 1. A sketch of the reactions catalyzed by 3-phytase (EC 3.1.3.8) and 6-phytase (EC 3. 1.3.26). The products released beside inorganic P and *myo*-inositol monophosphate can include metal divalent and trivalent ions, proteins, peptides, and AA (NH^{3+}-CHR). The arrows indicate that the ester bond is initially attacked by the 3- and 6-phytase, respectively. The *myo*-inositol molecule was drawn in the so-called "turtle" configuration (Agranoff, 2009).

Phytic acid also binds minerals, such as Fe^{3+}, Zn^{2+}, Ca^{2+}, and Mn^{2+}, which results in the formation of phytate (Weaver and Kannan, 2002; Tang et al., 2006) and other positively charged feed additives through electrostatic interactions (Figure 1). Such interactions can occur in cereals as protein bodies (globoids; Bohn et al., 2007), and also in the gastrointestinal tracts of animals and humans. Thus, the hydrolysis of IP_6 will not only release Pi, but also several positively charged nutrients (Figure 1). In this study, the effects of inositol phosphate ester ($IP_{1\text{-}6}$) and IP_5 positional isomers obtained chemically or prepared enzymatically using commercial microbial phytases were investigated for their effects on protein aggregation, pepsin activity, and interaction with Fe^{3+}.

MATERIAL AND METHODS

Chemicals and Materials

Bovine β-casein, porcine pepsin (P7000, 370 U≅ mg^{-1}; P7012, 2,500 to 3,000 U≅mg^{-1}), *myo*-inositol phosphate esters $IP_1(2)$, $IP_2(2,4)$, and IP_6 (sodium phytate) from rice, perchloric acid (60%, vol/vol), *myo*-inositol hexasulfate hexapotassium salt (**MIHS**) and sodium hexametaphosphate (Sigma-Aldrich, St Louis, MO), and *myo*-inositol tris-, tetrakis-, and pentakisphosphates [$IP_3(1,4,5)$; $IP_4(1,2,4,5)$, $IP_5(1,2,4,5,6)$, $IP_5(1,2,3,4,5)$, and $IP_5(1,3,4,5,6)$; Sichem, Bremen, Germany] were obtained from commercial companies. One pepsin unit is defined as the increase of ΔA280 by 0.001 absorbance unit per min at pH2.0 at 37°C, measured as TCA-soluble products using hemoglobin as substrate. The amounts of these IP_3, IP_4, and IP_5 esters in 0.1 and 1 mg portions were used without weighing because, according to the manufacturer, the weight deviation was less than 5%. Dyed casein in the form of tablets [Protazyme AK containing azurine cross-linked (**AZCL**) casein; Megazyme, Wicklow, Ireland] was used for pepsin assay. Casein with fluorescence labelling (BODIPY FL casein, E6638) was obtained from a commercial company (Invitrogen, Carlsbad, CA). The bacterial phytase [i.e., the *E. coli* K12 phytase (EC 3.1.3.26) *AppA* gene expressed in *Schizosaccharomyces pombe* (Phyzyme XP) and the subtilisin variant (EC 3.4.21.62; P3000, Protex 30L; Danisco A/S, Brabrand, Denmark)], the fungal phytase [i.e., the *Aspergillus ficcum* phytase expressed in *A. niger* (EC 3.1.3.8; Natuphos; BASF, Ludwigshafen, Germany)], and soy [*Glycine max* (L.) Merr.] protein isolate (BodyLab Nutrition, Hadsund, Denmark) were obtained from commercial companies. Microplates of different formats (Nunc A/S, Roskilde, Denmark; Corning Inc., Corning, NY) and the corn (*Zea mays* L.) and soy based feed (Danish Technological Institute, Kolding, Denmark; Supplemental Table 1) were obtained accordingly. The feed was dosed with different amounts of *E. coli* phytase. The phytase found in the feed was 184, 223, 442, 1,129, and 2,301 phytase unit (**FTU**) ≅kg^{-1}. Phytase activity was determined according standard phytase assay method (Engelen et al., 2001). That is, commercial phytase or phytase feed extract was reacted with phytic acid at 37°C and pH 5.5 for 60 min. The reaction was stopped by adding acid vanadate-molybdate reagent, which produces colored complex with Pi produced. The optical density (**OD**) of the yellow colored complex was monitored at 415 nm. Commercial feed samples with unknown composition were obtained (Danisco A/S).

Protein Aggregation with Inositol Phosphate Esters

Soluble soy protein fraction was prepared from soy protein isolate by the following procedure: soy protein isolate (0.5 g) was added to 25 mL 0.25 *M* Gly≅HCl with pH 2.5 (**Buffer G**), pH was adjusted to 2.5 or 3.0 with NaOH, centrifuged at 4,500 × *g* at 25°C for 20 min, and filtered through a glass fiber filter (Whatman GF/B; Maidstone, Kent, UK). Protein concentration in the filtrate was determined by a dye binding method using BSA as standard according to the protocol from the manufacturer (Bio-Rad Laboratories, Hercules, CA). For assaying aggregation of *myo*-inositol phosphate ester (IP_x, where x denotes the

number of phosphate ester bonds) with soy protein, the reaction mixture contained 5 μL of 1 of the IP_x (IP_{1-6}) at 3 m*M* in water, 100 μL soy protein, and Buffer G or 0.1 *M* sodium acetate to a final volume of 120 μL. The final concentration of IP_x was 125 μ*M* and the final soy protein concentration was 1.5 mg mL^{-1}. In certain experiments, this system also contained NaCl (0.3 *M*), and the pH of the system varied between 1.6 and 4.5, buffered by Gly≅HCl or sodium acetate. For testing IP_6 hydrolyzate formed by *E. coli* and *A. niger* phytase, 5 μL of IP_x were replaced with 10 μL hydrolyzate. After mixing the contents, aggregation in the form of turbidity was determined immediately at 25°C by following the increase in OD at 600 nm using a scanning microplate spectrophotometer (PowerWave$_x$; Bio-TEK Instruments, Inc., Winooski, VT). For testing aggregation of β-casein with chemically pure IP_{1-6}, the experimental conditions were the same as for soy protein, and the final concentration of β-casein was 1.5 mg mL^{-1}. For testing β-casein aggregation with IP_6 hydrolyzate, the reaction mixture contained 2 μL hydrolyzate, 80 μL β-casein in Buffer G, and water to a final volume of 96 μL. Final β-casein concentration was 1.5 mg mL^{-1} and final IP_6 hydrolyzate concentration at time 0 min was 107 μ*M* of IP_6. Optical density at 600 nm was read in a 384 well-microplate using a microplate reader (SpectraMax M5; Molecular Devices Corp., Sunnyvale, CA).

Assay of Phytase from Feed Samples

For assaying phytase from feed extract in a petri-dish plate assay, water was added to 2.5 to 12.5 g feed to a final volume of 50 mL and mixed until the feed pellet became disintegrated, which took usually 6 to 10 min. The supernatant obtained after standing still for 1 to 2 min was applied directly to the wells (20 μL) in an agarose plate containing IP_6-soy protein complex at pH 1.6 to 3.5, buffered by using 0.1 to 0.25 *M* Gly≅HCl, or at pH 3.5 to 4.5, buffered by using 0.1 *M* acetate. The agarose gel, which contained IP_6 (0.1 to 0.4 m*M*) or the phytase inhibitors of MIHS (0.1 m*M*) and NaF (10 m*M*), soy protein (1.5 mg mL^{-1}), and agarose [1.5% (wt/vol)] was prepared by melting agarose containing IP_6 or the inhibitor or both in a water bath at 100°C or in a microwave oven. The melted agarose solution was mixed with soy protein solution and poured directly into a 9-cm petri dish and gelled at room temperature (23°C). After gelling, wells with a volume of approximately 20 μL were made in the gel manually using pipette tips, and numbered. The development of clear halos on the agarose gel with phytase extract was followed by incubating the plate at temperatures between 15 and 35°C. In control, phytase extract was replaced with extract made from the same feed but containing no added microbial phytase. For standards, feed extracts with the same feed samples to be tested and containing known amount of phytase were used. For negative control, 20 μL phytase extract was added to the agarose gel containing 0.1 m*M* of MIHS or 10 m*M* of NaF. The area of clear halos was measured in square centimeters for semi-quantification of phytase activity.

Interaction of Inositol Phosphate Esters with Fe^{3+}

Ferric sulfosalicylic acid solution (also called Wade's solution) was made as described earlier (Rounds and Nielsen, 1993; Kwanyuen and Burton, 2005). It consisted of 0.015% (wt/vol) of $FeCl_3 \cdot 6H_2O$ and 0.15% (wt/vol) of sulfosalicylic acid. The *myo*-inositol ester (IP_{1-6}), 2.5 μL at 3 m*M*, was added to 75 μL of 10 m*M* methyl piperazine (pH 4.0) and 80 μL Fe^{3+}-sulfosalicylic acid solution and mixed. The mixture had a final pH of 2.4. The final volume was 157.5 μL, and the final IP_x concentration was 47.6 μ*M*. Decrease in OD at 500 nm as a result of Fe^{3+} interaction with IP_{1-6} was read spectrophotometrically 2 min after mixing at 23°C against a blank containing no IP_x. A standard curve was made by mixing IP_6 standards (1.5 to 21 nmol) with 75 μL Buffer G and 80 μL Fe^{3+}-sulfosalicylic acid solution to ensure that OD data obtained were in the linear range.

Ferric perchloric acid solution was prepared as described earlier (Phillippy and Bland, 1988; Skoglund et al., 1998). The *myo*-inositol ester (IP_{1-6}), 1.5 μL at 3 m*M*, was added to 75 μL Buffer G and 40 μL Fe^{3+}-perchloric acid solution containing 0.1% (wt/vol) $Fe(NO_3)_3$ $9H_2O$ and 2% (vol/vol) $HClO_4$. The final volume was 116.5 μL, and the final concentration of IP_x was 38.6 μ*M*. Increase in OD at 290 nm, as a result of the interaction between Fe^{3+} and IP_x, was read spectrophotometrically 2 min after mixing at 23°C. A standard curve was made by mixing IP_6 standards (1.5 to 28 nmol) with 75 μL Buffer G and 40 μL Fe^{3+}-perchloric acid solution to ensure that OD data obtained were in the linear range.

Preparation of Phytic Acid Hydrolyzates

The dry weight loss of sodium phytate [P3168, Lot 123k1413, purity of 95% (wt/wt); Sigma-Aldrich] was measured to be 14.74%, with an environmental humidity of 37%. It was determined by increasing the temperature from 23 to 160°C until a constant weight was reached using a HB43 halogen moisture analyzer (Mettler Toledo; Greifensee, Switzerland). The reaction mixture for measuring IP_6 hydrolysis consisted of 30 mL of 8.526 m*M* of sodium phytate, 4 FTU of the *E. coli* phytase, or 10 FTU of the *A. niger* phytase in Buffer G and Buffer G to a final volume of 50 mL. One phytase unit was defined as the activity of phytase that generates 1 μmol of Pi min^{-1} from 5.1 m*M* of sodium phytate at pH 5.5 in 0.25 *M* sodium acetate at 37°C. Complete hydrolysis of IP_6 by the bacterial and fungal phytase would generate stoichiometrically

5.116 m*M* *myo*-inositol monophosphate (IP_1). The hydrolysis reactions were started by adding phytase to the rest of the reactants that had been incubated at 37°C for 10 min. The reaction was continued for 23 h and samples of 5 mL were taken at 0, 5, 10, 15, 20, 30, 40, and 50 min and 1, 1.5, 2, 3, and 23 h, heated in a boiling water bath for 5 min, cooled, and saved at −20°C before analyzing for Pi (Engelen et al., 2001) and IP_x by high performance ion exchange chromatography (**HPIC;** Skoglund et al., 1997). The hydrolysis samples (i.e., IP_6 hydrolyzates) collected were also tested for their effects on protein aggregation and inhibition of pepsin-catalyzed casein hydrolysis.

Analysis of Inositol Phosphate Esters

The separation and detection of IP_{1-6} were achieved by HPIC as described earlier (Skoglund et al., 1997, 1998) using the column (4 × 250 mm with a 4 × 50 mm pre-column; CarboPac Guard PA-100; Dionex, Sunnyvale, CA). MilliQ water and 1 *N* HCl prepared in water were used as Solvents A and B, respectively. The gradient program was: 2.5 to 50% (vol/vol) Solvent B in 30 min followed by isocratic at 50% (vol/vol) Solvent B for 3 min and isocratic at 2.5% (vol/vol) Solvent B for 2 min. The flow rate was 0.8 mL min^{-1}. Phytic acid hydrolyzate sample of 0.5 mL in Buffer G was filtered and 20 μL or 40 μL with or without added internal IP_{2-6} standards at 1.5 m*M* were injected into the column. The *myo*-inositol phosphate esters IP_{1-6} standards (Sichem and Sigma-Aldrich) prepared in Buffer G were also analyzed under the same conditions with an injection volume of 40 μL. The eluant from the PA-100 column was reacted in-line in a T-tube with Fe^{3+} perchloric acid solution at a flow rate 0.4 mL min^{-1}. Phytic acid IP_6 and IP_{1-5} isomers were detected at 290 nm as positive peaks resulting from the formation of IP_6-Fe^{3+}-ClO_4^- complex. Peaks were identified by their retention time compared with standards and by their relative retention times compared with IP_6 for peaks that lack standards (Skoglund et al., 1998). Quantification of IP_{5-6} was performed by the comparison of the peak area with the respective IP_{5-6} standards (Sichem). The linear range for IP_6 was 10 to 250 μg in an injection volume of 40 μL.

Pepsin Assay

In a typical chromophore labeled casein assay, 1 AZCL-casein tablet, 1 mini-magnetic bar, 1 mL Buffer G, and 0.5 mL IP_6 hydrolyzate or 0.5 mL Buffer G (positive control) were added to a test tube. After pre-incubation at 40°C for 5 min for the hydration of the AZCL-casein, 0.5 mL pepsin solution (12.5 μg mL^{-1}; Sigma P7000; Sigma-Aldrich) in Buffer G, or 0.5 mL Buffer G (blank) were added to start the reaction. The reaction was allowed to proceed at 40°C for 60 min and was stopped by adding 10 mL 2% (wt/vol) of trisodium phosphate. After centrifugation at 3,000 × *g* for 10 min at 23°C, the supernatant was filtered and OD of the filtrate was measured at 590 nm against the blank.

In a typical fluorophore labeled casein assay, the reaction mixture contained casein labeled with the fluorescence dye BODIPY FL (Invitrogen) at 5 μg mL^{-1} in 10 m*M* HCl, 0.125 units of porcine pepsin (Sigma P7012; Sigma-Aldrich), and increasing amounts of IP_6 (0 to 1.5 mg mL^{-1}) in a final volume of 160 μL. The reaction was performed at 30°C. Fluorescence was monitored by using a microplate reader (SpectraMax M5; Molecular Devices Corp.) with excitation set at 485 nm and emission set at 524 nm. Reading sensitivity was set at 6, and 6 readings were recorded on average at 1-min intervals. The microplate was shaken before each reading. A half-area 96-well ultraviolet microplate was used for top reading.

A detailed experiment design flow chart is given in Figure 2 to illustrate the plans and goals set for the current study. A computer program (Excel 2007; Microsoft Danmark ApS, Hellerup, Denmark) was used for data processing and preparation of the figures. Mean ± SD are presented in all figures. Statistical comparisons for differences between IP_5(1,2,3,4,5) and IP_5(1,2,4,5,6) isomers

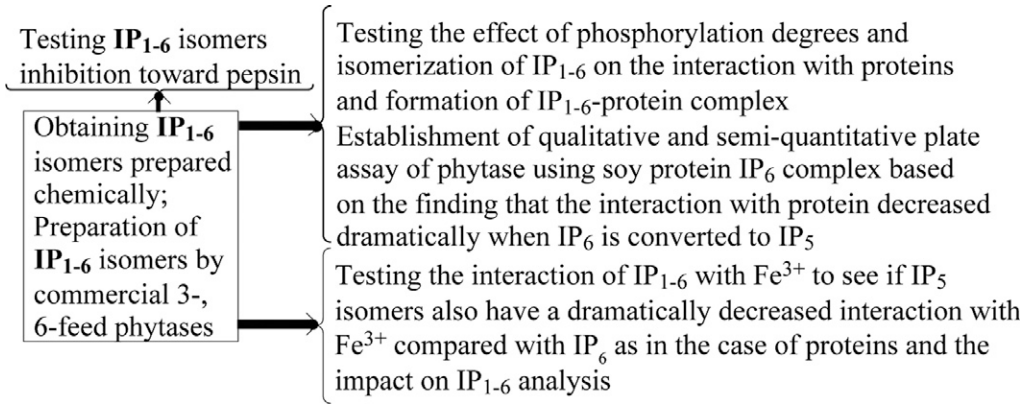

Figure 2. Experimental design flow chart.

in their aggregation of soy protein and β-casein were performed by Student *t*-test.

RESULTS AND DISCUSSION

Protein Aggregation by Inositol Phosphate Ester

Soy protein and bovine β-casein were studied for their aggregate formation with IP_{1-5} esters compared with IP_6. Figure 3 shows the aggregation of soy protein as a function of IP_6 concentration. Under the assay conditions, the aggregation reached its maximum, as observed from the turbidity (OD at 600 nm) at an IP_6 concentration of about 0.08 mg mL^{-1}, and further increasing the IP_6 concentration did not lead to increased turbidity. The amount of protein remaining in solution after centrifugation of the reaction mixture decreased rapidly at increased IP_6 concentration up to 0.1 mg mL^{-1}. It is noted, however, that even at 0.33 mg mL^{-1} of IP_6, there were still 29.4 ± 2.4% soy protein in solution. Interaction and formation of aggregate were also observed between IP_6 and β-casein. In contrast to soy protein, β-casein was more liable in forming aggregate at the same IP_6 concentration. For instance, to reach a turbidity of 1.0, the IP_6 concentration needed for β-casein was 1.80 times less than that for soy protein under the same conditions. The IP_6 concentration needed to aggregate these 2 proteins is relevant in vivo for food and feed, which can have 0.5 mg IP_6 mL^{-1} slurry, assuming that food and feed have a reduced concentration of IP_6 of 0.2% (wt/wt; 2 mg mL^{-1}), and, in the digestive tract, they are diluted by a factor of 4. Several factors, including pH and salts, affected the interaction between IP_6 and protein. For example, in the case of soy protein, aggregates were formed in a pH range of 2.5 to 3.8 in Gly≅HCl buffer, whereas at pH lower than 2.0, the solution was clear (OD at 600 nm ≤ 0.05). This is due to the fact that IP_6 has 6 dissociable protons with a pKa of approximately 2 (Reddy et al., 1989). Salts like $CaCl_2$ (Murthy and Rao, 1984) and NaCl affected IP_6-protein interaction negatively. For instance, the turbidity of IP_6-soy complex was abolished in the presence of 0.3 M NaCl (OD ≤ 0.05). Beside IP_6 that forms aggregates with positive charged proteins, other poly-anions such as MIHS, the analogue of IP_6, and hexametaphosphate also formed aggregates with soy protein at 0.1 m*M* (data not shown).

By using the IP_6-protein aggregate system as control, the aggregation capabilities of IP_{1-4} and IP_5 isomers with defined structures with the 2 proteins were examined. It was observed that the turbidity of the system decreased radically with a decrease in phosphorylation from IP_6 to IP_3 (Figure 4). The 2 IP_5 isomers, IP_5(1,3,4,5,6) and IP_5 (1,2,4,5,6), showed a 4.6 ± 0.1 fold decrease in aggregating soy protein compared with IP_6. *myo*-Inositol pentakis-phosphate IP_5(1,2,3,4,5), an intermediate product of *E. coli* 6-phytase, showed the least aggregation degree (6.6 ± 0.5-fold turbidity reduction) compared with IP_5(1,3,4,5,6) and IP_5 (1,2,4,5,6) generated by *A. niger* 3-phytase ($P = 0.001$). It was noted that, under the same conditions, the ability of IP_4(1,2,4,5), IP_3(1,4,5), IP_2 (2,4), and IP_1(2) to aggregate soy protein was negligible, as the OD of these mixtures was ≤0.007 ± 0.002. As with soy protein, a similar interaction pattern with IP_{1-4} and IP_5 isomers, compared with IP_6, was also observed with β-casein (data not shown). These observations were in

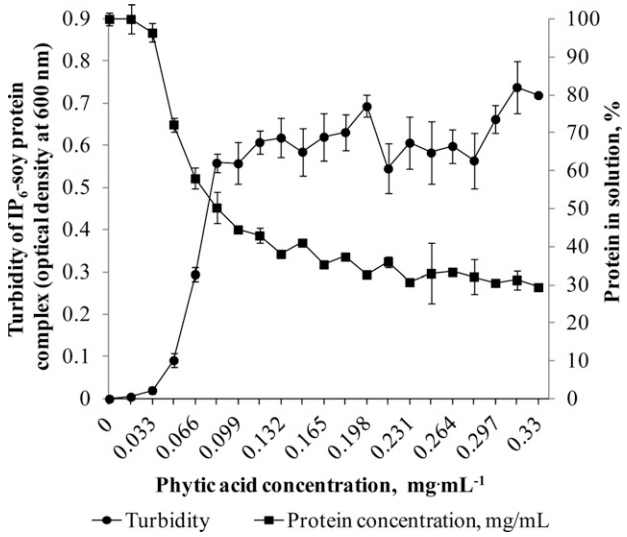

Figure 3. Aggregation of soy protein as a function of phytic acid (IP_6) concentrations. The assay mixture contained 0 to 20 μL IP_6 (1 mg mL^{-1}), 0 to 20 μL 0.25 M Gly≅HCl (pH 2.5, Buffer G), and 100 μL soy protein made in Buffer G with a final protein concentration of 0.6 mg mL^{-1}. Turbidity [optical density (OD) 600 nm] was measured at 23°C, and each data point is the average of 4 measurements with SD.

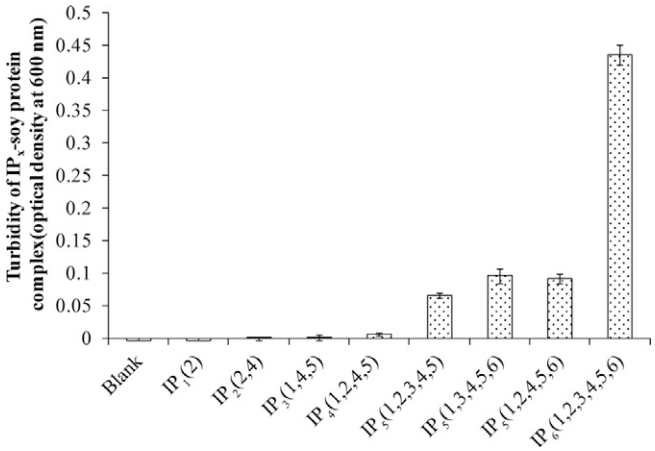

Figure 4. The aggregation of soy protein by *myo*-inositol phosphate esters (IP_{1-6}) and IP_5 positional isomers. The assay contained 5 μL IP_{1-6} (3 mM), Buffer G, and 100 μL soy protein (1.8 mg mL^{-1}). Turbidity [optical density (OD) 600 nm] was measured at 23°C after a 1 min mixing, and each data point is the average of 4 measurements with SD.

contrast to our assumption that phosphorylation degrees of *myo*-inositol (i.e., IP_{1-6}) were directly proportional to their protein aggregation capabilities.

Inositol Phosphate Esters on Casein Digestibility

Under in vivo conditions, IP_{1-6} occurs as a mixture of various esters and isomers because of the action of phytases of plant, microbial, or mucosal origin (Greiner and Konietzny, 2010). It was, therefore, essential to examine various IP_{1-6} mixtures generated by commercial microbial phytases for their aggregation of dietary proteins and inhibition of pepsin-catalyzed protein hydrolysis. The presence of IP_6 at 0.911 mg mL^{-1} resulted in 86.9 ± 0.5% loss in pepsin activity (Figure 5). The IP_6 concentration used (0.9 mg mL^{-1}) is relevant in vivo in feed slurries as discussed before. Phytic acid inhibited protein hydrolysis by pepsin was also observed with fluorophore-labeled casein as substrate (BODIPY FL casein), pepsin activity being lost by 24.7 ± 9.4% at IP_6 concentration of 0.027 mg mL^{-1}. The inhibition of pepsin catalyzed AZCL-casein hydrolysis by IP_6 was reversed by the addition of either *E. coli* phytase (Figure 6) or *A. niger* phytase (data not shown).

The hydrolysis of IP_6 by phytases results in the formation of inositol pentakis-, tetrakis-, tris-, bis-, and monophosphate in a stepwise manner (Wyss et al., 1999; Greiner et al., 2000). Commercial phytases are bacterial and fungal origin, which produce *myo*-inositol monophosphate IP_1 and Pi as end products after thorough hydrolysis (Greiner, 2007; Selle and Ravindran, 2007). Figure 6 shows the time course for the degradation of IP_6 in the presence of 0.08 FTU mL^{-1} of the *E. coli* phytase at pH 2.5. At 3 h after incubation, IP_1 and IP_2 could be detected, while at 23 h (data not shown), only IP_1 was detected.

From Figure 6, it can be observed that a mixture of Pi, IP_1, and IP_2 produced at 3 h gave no longer inhibition on pepsin activity (106.6 ± 1.1 vs. 100.0% in the absence of IP_6 hydrolyzate) and the sample collected at 23 h containing solely Pi and IP_1 (pepsin activity being 108.2 ± 2.9%). The reaction mixtures containing other IP esters including IP_3, IP_4, IP_5, and IP_6 all inhibited pepsin catalyzed protein hydrolysis to various degrees. For instance, at 2 h with a mixture containing IP_3 along with Pi, IP_1, and IP_2, the pepsin activity was reduced to 64.8 ± 9.6% (Figure 6). Decreased pepsin inhibition with the progress of IP_6 hydrolysis was also observed with *A. niger* phytase (data not shown). These results, showing that IP_{3-6} (and not IP_{1-2}) still inhibited pepsin activity (Figure 6), are coincidently in agreement with those observed earlier using IP_{1-5} preparations prepared chemically with unknown structures (Knuckles et al., 1989).

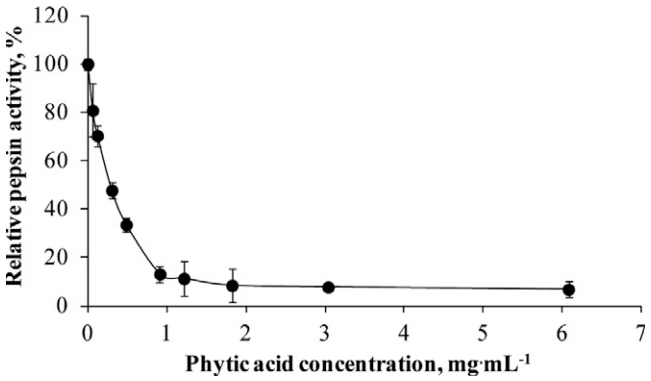

Figure 5. Phytic acid inhibition of porcine pepsin-catalyzed hydrolysis of azurine cross-linked casein. The assay was performed at 40°C. Each data point is the average of duplicate.

Interaction of Inositol Phosphate Esters with Fe^{3+}

Based on the observations that when IP_6 was converted to IP_5 and IP_{1-4}, the capability to interact with proteins decreased (Figure 4), we set out to examine if such a radical change would also be observed in their chelating with Fe^{3+} using the same IP_1–IP_6 and IP_5 isomer preparations. The Fe^{3+} is known to form a complex with IP_6, which is indigestible by phytase (Tang et al., 2006). From Figure 7, one can see that there was no dramatic changes in chelating power from IP_6 to IP_5, expressed either as the Fe^{3+} bound by IP_6 or the OD decrease caused by the formation of Fe^{3+}-IP_6 complex, which had a molar ratio of 2 to 1 under the assay conditions. The 3 IP_5 isomers, IP_5(1,3,4,5,6), IP_5(1,2,4,5,6), and IP_5(1,2,3,4,5), had 76.0 ± 8.2, 70.4 ± 9.6, and 73.0 ± 5.7% Fe^{3+} reactivity relative to IP_6, respectively. The IP_4(1,2,4,5) and IP_3(1,4,5) had 35.0 ± 6.7 and 29.5 ± 5.7% reactivity, respectively, while IP_2(1,4) and IP_1(2) had no detectable reactivity (Figure 7). A standard curve between the OD decrease at 500 nm and IP_6 concentration (1.5 to 21 nmol) gave good linearity (R^2 = 0.997; data not shown), indicating the direct correlation between the OD and the Fe^{3+} bound. The OD value obtained with 7.5 nmol of IP_6 used in Figure 7, with which all IP_{1-5} were compared, was in the linear range. These data indicate that, in sulfosalicylic acid solution at pH 2.4, the binding of Fe^{3+} with inositol phosphate esters decreased with a decrease in the degree of inositol phosphorylation, and IP_3 was still capable of binding Fe^{3+} at 29.5 ± 5.7% strength of IP_6. The observation shown in Figure 7 is in line with the results obtained with IP_{3-6} using human intestinal cell lines (Han et al., 1994) and in human studies (Sandberg et al., 1999), which showed that inositol phosphate esters with a phosphorylation degree as low as IP_3 still contributed to the poor digestion of iron.

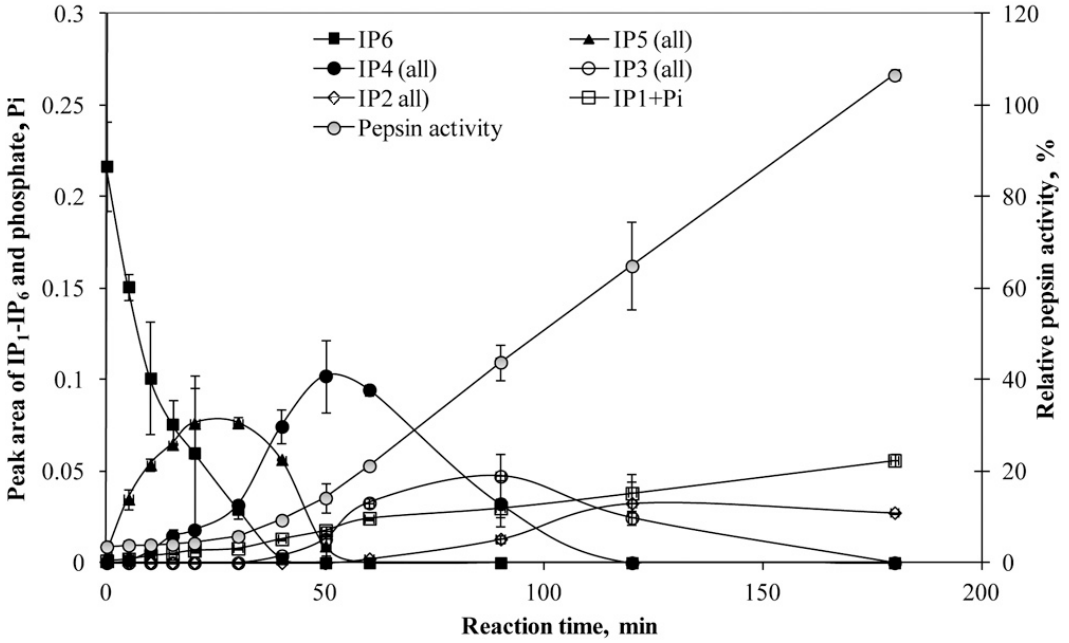

Figure 6. Time course of phytic acid hydrolysis by *E. coli* phytase (Phyzyme XP, Danisco A/S, Brabrand, Denmark; 0.08 phytase unit≅mL^{-1}) and inhibition of porcine pepsin catalyzed azurine cross-linked casein hydrolysis by the hydrolyzates. Phytic acid hydrolysis was performed at 37°C; pepsin activity assay was carried out at 40°C. Each data point is an average of 2 separate experiments.

The interaction of IP_{1-6} with Fe^{3+} complexed with perchloric acid was further examined at pH 2.0 to confirm the aforementioned results when Fe^{3+} was complexed with sulfosalicylic acid at pH 2.4. Just as the Fe^{3+}-sulfosalicylic acid reagent, the reagent of Fe^{3+} complexed with perchloric acid is commonly used in-line in the detection of IP_2-IP_6 after chromatographic separations (Skoglund et al., 1998). When reacted with Fe^{3+}-perchloric acid solution at pH 2.0, the 3 IP_5 isomers IP_5(1,3,4,5,6), IP_5(1,2,4,5,6), and IP_5(1,2,3,4,5) had 66.4 ± 5.2, 65.7 ± 6.7, and 58.4 ± 3.8%, reactivity relative to IP_6, respectively. The IP_4, IP_3, IP_2, and IP_1 had 24.1 ± 4.2, 16.2 ± 3.5, 9.2 ± 2.1, and 4.6 ± 2.4% reactivity of IP_6, respectively. A standard curve of OD and IP_6 concentration (1.5 to 28 nmol) was made, which showed a linear response (R^2 = 0.997; data not shown).

Ferric ion was chosen in the current study for its interaction with IP_1−IP_6 instead of Ca^{2+} and Zn^{2+} to address the dual issue of iron nutrition and IP_{2-6} analysis. The Fe^{3+} reagents have been traditionally used for the quantification of IP_6 by weighing the ferric phytate precipitate formed (Kikunaga et al., 1985). Two Fe^{3+} reagents are currently used for the quantification of IP_{2-6} in HPIC (Rounds and Nielsen, 1993; Skoglund et al., 1998, Oberleas and Harland, 2007). The results from our 2 studies described indicate that errors in quantification of IP_{1-5} could occur if one assumes that the relative reactivity of IP_1−IP_5 to IP_6 to be 83.3 (5/6), 66.7 (4/6), 50 (3/6), 33.3 (2/6), and 16.7% (1/6) of IP_6, respectively, which was assumed by investigators in quantifying IP_1−IP_5 (Skoglund et al., 1997).

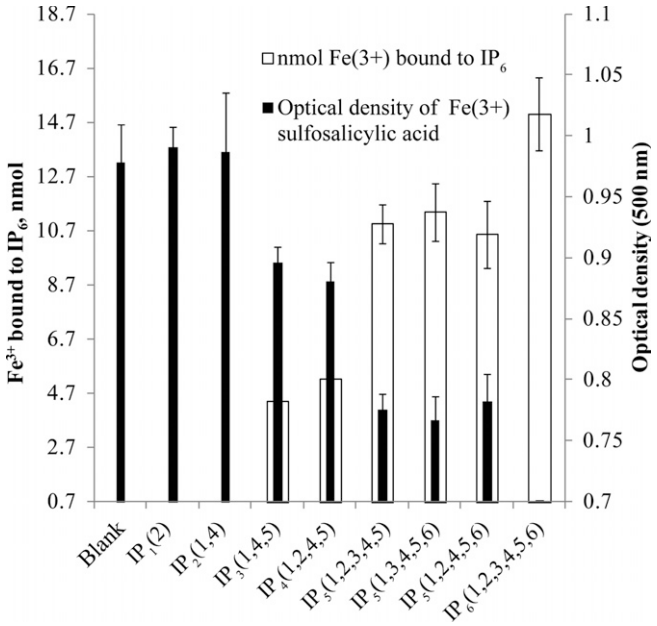

Figure 7. Interaction of *myo*-inositol phosphate esters (IP_1−IP_6) and IP_5 positional isomers with Fe^{3+}. The ferric reagent used was Fe^{3+} sulfosalicylic acid solution. The reaction mixture had a final pH of 2.4. Each data point measured at 23°C is the average of 4 measurements with SD.

Phytase Plate Assay Development

Based on the observation that the turbidity of IP_6-protein complex decreased radically when IP_6 was converted to IP_5 (Figure 4), and the correlation between the phytase dose and the time needed to clarify the IP_6-soy protein solution (R^2 = 0.984; data not shown), a phytase

plate assay was designed. The plate assay was based on the incorporation of 1.5% (wt/vol) agarose to the IP_6-protein system such that an agarose gel plate was formed with a whitish background because of the complex formation between IP_6 and soy protein. Addition of phytase to the wells in the gel created a clear halo around the wells after incubation (Figure 8; Supplemental Figure 1). The transparent halo was usually observed in 10 to 20 min at 23°C for a phytase dose of 1,000 to 2,000 FTU kg^{-1} feed. For a dose of 250 to 800 FTU kg^{-1}, clear halos were observed at 23°C in 3 to 7 h or overnight. The incubation temperature was chosen between 20 and 35°C. Feed sample with no added phytase was used as negative control. Agarose gels containing phytase inhibitors (10 m*M* NaF or 0.1 m*M* MIHS) were also found to be good controls as addition of phytase did not result in the development of halos in gels (data not shown).

It can be seen from Figure 8, the detection limit for phytase can be down to 193 FTU≅kg^{-1} feed for an incubation time of 7 and 20 h at 23°C, a dosage usually used for laying hen diets. The relationship between phytase dosage used (223 to 2,301 FTU≅kg^{-1} feed) and the halo area developed can also be expressed mathematically (R^2 = 1.0, Figure 8). Factors that may affect the phytase plate assay include buffers, buffer pH, IP_6 concentrations, ratio of feed to extraction solution, and extraction time. The optimal conditions for the phytase plate assay established in this study were: 0.25 *M* of Gly or acetate buffer at pH 3.0 to 3.5, IP_6 at 0.10 to 0.15 m*M*, water to feed ratio of 4:1, and an extraction time of 10 to 30 min at 23°C in the case of *E. coli* phytase (Phyzyme XP; Danisco A/S).

The assay method for feed phytase is designed to be both qualitative and semi-quantitative. The CV for intra-assay variation was up to 16% when tested for the phytase dose range of 233 to 2,301 FTU≅kg^{-1} (Figure 8). The CV for inter-assay variation was up to 13% when tested for the dose of 442 FTU≅kg^{-1} at different days.

From Figure 8, it can be seen that there was a good relationship between the phytase plate assay method and the standard method based on Pi release (Engelen et al., 2001). However, for certain feed samples, such relationship was not always clear. For example, among 9 feed samples collected from different feed mills, 2 of them, which contained 276 and 433 FTU≅kg^{-1} by the standard method showed no halo development. This could be due to the interference to the standard method caused by endogenous Pi (Kim and Lei, 2005). It should be noted the lack of halo development for these 2 samples was not due to possible phytase inhibitors in the feed, as all 9 feed samples gave good response to the externally added phytases at 500 and 1,000 FTU kg^{-1} (data not shown).

Conclusions

There are numerous reports describing the interactions of IP_6 with proteins and iron in the last several decades, but few of them have examined the interactions between proteins and defined IP_6 degradation products. The present

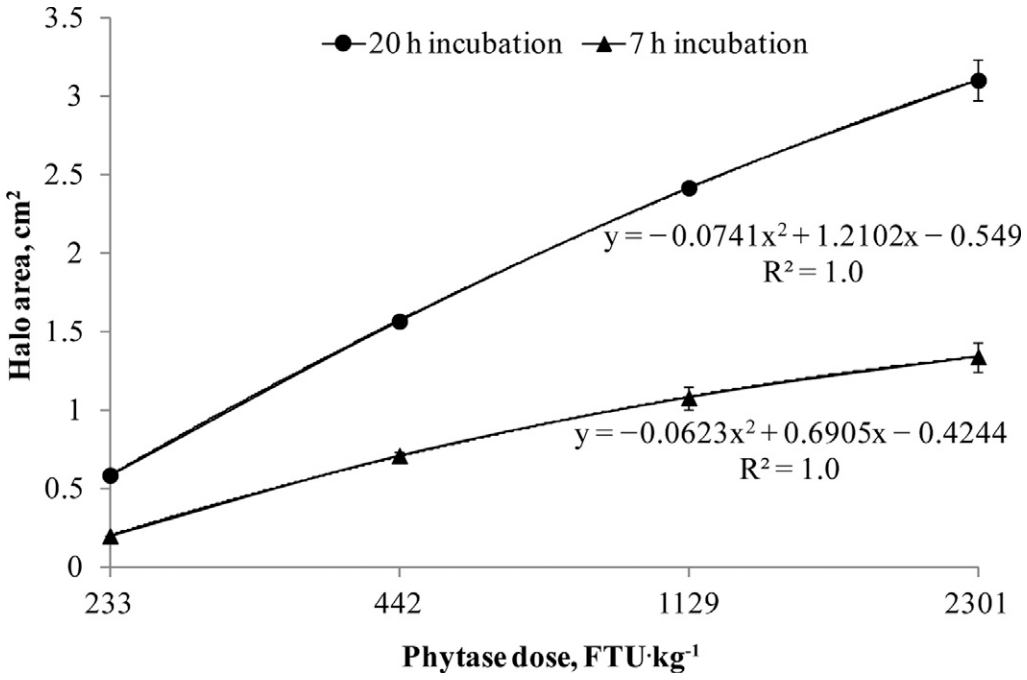

Figure 8. The plate assay of phytase and its correlation to the standard phytase assay method based on inorganic P (Pi) release. The *x*-axis, the amount of *E. coli* phytase assayed by the standard method based on Pi release; *y*-axis, halo area, which was measured after incubation for 7 (▲) and 20 h (●) at 23°C with the phytase feed extract. Each data point is the average of 4 assays with SD. Standard deviation bars are shown when they are larger than the symbols. FTU = phytase unit.

in vitro study, using pure IP_{1-4}, IP_5 isomers, and IP_6 hydrolyzate containing IP_{1-5} generated by commercial bacterial and fungal 6- and 3-phytases, indicates for the first time that the solubility of soy protein and β-casein increased radically when IP_6 was converted to IP_5 and further to IP_4. Based on these observations, a novel, simple, yet phytase-specific, qualitative and semi-quantitative assay using agarose-petri dishes suitable for microbial phytase screening in phytase protein engineering, as well as for use in feed laboratories and at animal farms for controlling phytase activity, was developed. This method is complementary to the tedious, lengthy, yet quantitative phytase standard method based on the assay of Pi released. The *myo*-inositol phosphate esters IP_{1-3}, which had no influence on protein solubility, still interacted with Fe^{3+}, and IP_3 inhibited pepsin catalyzed protein hydrolysis, though to a much lesser extent than IP_6. These studies indicate that an early and thorough hydrolysis of phytate by phytase in the upper digestive tract is essential for an improved phosphorus, minerals (e.g., Fe^{3+}), and protein digestion.

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