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Phytate Degradation in a Mixture of Ground Wheat and Ground Defatted Soybeans during Feed Processing: Effects of Temperature, Moisture Level, and Retention Time in Small- and Medium-Scale Incubation Systems

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The optimal conditions for degradation of phytate (IP6, *myo*-inositol hexaphosphate) in a mixture of ground wheat and ground defatted soybeans (1:2, w/w) with added exogenous *E. coli* phytase were investigated at different temperatures (45, 60, 75, and 95 °C), moisture levels (25%, 35%, and 45%), and retention times (2−45 min). All treatment combinations were investigated in a small-scale mixer conditioner (experiment 1). The combined 45 °C and 45% moisture treatment was most efficient and reduced the content of IP6 by 86% during 45 min of incubation. This treatment combination was applied in a medium-scale mixer conditioner (experiment 2), and 76% reduction of IP6 at 45 min was obtained. During incubation, the content of lower groups of inositol phosphates, such as IP4 (*myo*-inositol tetraphosphate) and IP3 (*myo*-inositol triphosphate), increased significantly as the content of IP6 decreased. The major isomer formed was Ins(1,2,5,6)P_4.

KEYWORDS: Phytate; phytase; soybean; wheat; temperature; moisture; retention time; incubation

INTRODUCTION

Phytates or salts of phytic acid (*myo*-inositol hexaphosphate, IP6), consisting of an inositol ring with six covalently attached phosphate groups, are present in most vegetable feed ingredients in the range of 5−30 g kg^{-1} (*1*). Except for ruminants (*2*), most animals, including humans, have a limited capacity to release the phosphate groups from the inositol ring during digestion (*3*). This reduces the availability of phosphorus present in IP6. Furthermore, the phosphate groups are highly reactive and form strong bonds with divalent macro minerals and trace elements (*4*). Consequently, diets with high phytate content have been shown to reduce the availability of minerals in humans (*5*), pigs (*6*), rats (*7*, *8*), broiler chickens (*9*), Atlantic salmon (*10*), and Chinook salmon (*11*). Thus, dietary IP6 given at 2 g kg^{-1} reduced the digestibility of zinc in Atlantic salmon as compared to a diet without IP6 (*10*), whereas the derivatives of IP6, including IP5, IP4, and IP3, contributed to a reduced utilization of iron in humans (*5*).

Most vegetables containing IP6 also exhibit varying levels of endogenous phytase, the enzyme capable of liberating the covalently bound phosphate groups from the inositol ring. Processing methods such as soaking, hydrothermal processing, fermentation, and germination can reduce the content of IP6 by the activation of endogenous phytase (*12*). However, the most common way to remove IP6 is through the addition of exogenous and commercially available phytase, mainly produced from bacteria or yeast cultures. Usually, exogenous phytase is added to the dry feed mixture prior to pelleting/extrusion or sprayed onto finished pellets as a top coating. This has proved to be fairly efficient for warm-blooded monogastric animals (*9*, *13*−*15*), because the temperature optimum for phytase (40−50 °C) is close to the body temperature in the animals (pigs, 37−38 °C; poultry, 39−42 °C).

Because of the limited and unpredictable supply of fish meal, protein sources of vegetable origin are increasingly used in diets for farmed fish. Not only are coldwater fish such as salmon and cod carnivorous and poorly able to digest carbohydrate-rich vegetables, but also, because they are poikilothermic and their surrounding water temperatures normally range from 0 to 15 °C, added phytase would not have conditions for optimal activity in the digestive tract. Therefore, the content of IP6 should be reduced or removed from the diets before distribution to the fish. Diets for cod and salmonids are usually extruded, but despite the high temperatures and high pressure obtained, the short retention time in the extruder does not allow sufficient thermal degradation of IP6 (*16*). Cain and Garling (*17*) pretreated soybean meal with phytase and increased the inorganic P from 0.06% to 0.5% (IP6 not analyzed). However, in the latter study, the soybean meal had to undergo an extra drying step (24 h) before inclusion in the feed mixture. An extra drying step in the feed production line costs time and energy

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Table 1. Raw Material, Water, Formic Acid, and Phytase Levels in Experiments 1 and 2

	experiment 1				experiment 2			
moisture (%)	raw material[a] (kg)	water (mL)	formic acid[b] (mL)	phytase[c] (mL)	raw material[a] (kg)	water (L)	formic acid[b] (L)	phytase[c] (L)
25	2.5	191	270	1.1				
35	2.5	647	270	1.1				
45	2.5	1269	270	1.1	144	72.5	15.4	0.0628

[a] Mixture of ground wheat and soy (1:2, w/w). [b] 10% solution. [c] 2500 FTU kg^{-1} mixture.

and may negatively affect the nutritional value of the feed if the drying temperature is not adequately lenient (*18*). To our knowledge, there are no published studies on methods aiming at hydrolysis of IP6 under conditions that can be directly applied in an extrusion line for production of compound fish feed. In a conventional preconditioner, the temperature is 80−90 °C and the feed mash, holding 25−30% moisture, is retained for 1−3 min before entering the extruder barrel (*19*). The temperature in the extruder is normally in the range of 110−120 °C, and the retention time is commonly 15−20 s (*19*).

The aim of the present study was to investigate the effects of exogenous phytase on the degradation of IP6 in a mixture of ground wheat and ground defatted soybeans at a range of temperatures, moisture levels, and retention times that can be obtained/incorporated during industrial conditioning and subsequent extrusion of compound feed. The study comprised small-scale incubation as well as a follow-up study using medium-scale incubation in a mixer conditioner.

MATERIALS AND METHODS

Ingredients. Whole wheat (Felleskjøpet, Larvik, Norway) and hexane-extracted (defatted), toasted soybean meal with hulls (Denofa, Fredrikstad, Norway) were ground separately in a Münch hammer mill (HM 21.115, Wuppertal, Germany) through a 1 mm sieve at the Center for Feed Technology (Norwegian University of Life Sciences, Ås, Norway). Ground wheat and soybean meal were mixed in a 1:2 ratio (w/w).

Equipment and Materials. For the small-scale experiment (experiment 1), a twin shaft mixer of 6 L capacity (Forberg, Larvik, Norway) was equipped with a heating cap and a built-in sensor for monitoring the temperature of the feed mixture. In the medium-scale experiment (experiment 2), a modified twin shaft mixer (400 L) (Tatham, Rochdale, UK) was used. The modification involved insertion of a heating cap (Dinnissen, Sevenum, Holland), to maintain a desired and stable temperature in the feed during mixing. A built-in sensor monitored the temperature of the feed mixture. Formic acid (98%) was obtained from Merck (Darmstadt, Germany), and an *E. coli* phytase (Quantum 5000L) was provided by Zymetrics Inc. (Marlborough, UK).

Conditions for Phytate Degradation. *Experiment 1.* The mixture of wheat and soybean meal (2.5 kg, 88.6% dry matter) was incubated at different moisture levels (25%, 35%, and 45% total moisture), temperatures (45, 60, 75, and 95 °C), and retention times (2−45 min). The added water and the wheat−soy mixture were preheated to obtain the respective temperatures from the start of the incubation (**Table 1**). The water was acidified with the addition of formic acid to obtain a pH value close to the pH optimum of the enzyme. Liquid phytase was added to the acidified, preheated water and sprayed onto the mixture through a UniJet 4003 nozzle (Spraying Systems Co., Wheaton, IL) at a rate of 1.4 L min^{-1}. Application of the phytase-containing water lasted for 19, 39, and 65 s for the 25%, 35%, and 45% moisture treatments, respectively. All phytase treatments were conducted in duplicate ($n = 2$). In addition, a sham treatment ($n = 1$) was included. All treatments were randomly assigned.

Experiment 2. The treatment combination that proved to reduce IP6 most efficiently in experiment 1 (45 °C × 45% moisture) was repeated in the large mixer conditioner. Here, 144 kg of the mixture was conditioned, and formic acid was used to adjust the pH in the final mixture (**Table 1**). Two nozzles (UniJet5006 and Unijet6520, Spraying Systems Co., Wheaton, IL) were used, providing 4.2 L min^{-1}. Application of the phytase-containing water lasted for 19 min. The phytase treatments were conducted in triplicate ($n = 3$).

Both Experiments. The zero time point for the incubation time was set to the point when the entire amount of acidified phytase-containing water had been sprayed onto the mixture. From 0 min, the mixture was mixed continuously until 10 min (except at sampling times). Thereafter, the mixtures were mixed for about 10 s every third minute. The amount of phytase added was 2500 FTU kg^{-1} of the mixture of wheat and soybean meal (**Table 1**).

Sample Collection. *Experiment 1.* At 2, 6, 10, 15, 30, and 45 min, aliquots of about 20 g were taken from a defined compartment in the small mixer, transferred to a small plastic container, and immediately frozen in liquid nitrogen (−196 °C). The samples were stored at −20 °C until freeze-drying. Samples taken at 10 and 45 min were first analyzed for content of inositol phosphates.

Experiment 2. At 10, 25, 45, and 60 min, three individual samples were taken from three different compartments in the large mixer. Aliquots of about 20 g were taken into plastic containers, transferred to liquid nitrogen, and stored at −20 °C until freeze-drying.

pH Measurements. To obtain good estimates of the pH from the three different moisture levels, each moisture treatment was repeated five times ($n = 5$) in the laboratory. Raw material (100 g) was added to water and formic acid equivalent to the ratios given in **Table 1**. The moist mixtures (25%, 35%, and 45% moisture) were analyzed directly by a Freezetrode pH electrode (Hamilton, Bonaduz, Switzerland), suitable for direct measurements in doughs and moist flour mixtures. In addition, pH was measured according to AOAC (*20*) (method 943.02). Briefly, a 10 g sample was dissolved in 100 mL of deionized water (25 °C) and shaken frequently for 30 min. Thereafter the mixture was left standing for 10 min followed by measurement of the pH in the supernatant.

Analysis of Inositol Phosphates. Freeze-dried samples of 0.5 g were extracted in 10 mL of 0.5 M HCl and analyzed for content of inositol phosphates according to a method described by Carlsson et al. (*21*). The samples contained 90.3% DM ± 0.01, SEM, $n = 15$. Instead of using a mechanically driven pump for the flow of ($Fe(NO_3)_3 \times 9H_2O$) solved in 2% $HClO_4$, we used pneumatic pressure regulated by a Dionex PC-10 Pneumatic Postcolumn Delivery Module (Dionex, Sunnyvale, CA) to obtain a combined flow rate at 1.2 mL min^{-1}. To detect lower groups of inositol phosphates, *myo*-inositol bi-, tri-, tetra-, and pentaphosphates (IP2, IP3, IP4, and IP5, respectively), a hydrolyzed sample of sodium phytate was made according to Carlsson et al. (*21*) and used as a reference to the peaks detected at different retention times in our chromatograms. The amounts of inositol phosphates were quantified according to Skoglund et al. (*22*).

Statistics. A three-way ANOVA was applied to the dataset to determine the significance of the different treatments and interactions (*23*). The treatments were considered significant when $P < 0.05$. Significant differences between treatment means were ranked by applying Duncan's multiple range test.

RESULTS

pH. Both methods used to analyze pH showed that the addition of formic acid resulted in a pH that was relatively constant for all moisture treatments. When pH was measured directly in the dough, the pH was in the range of 4.7−4.8 (±≤0.03), whereas the AOAC method gave a pH of ap-

Table 2. Content of IP6 (g kg^{-1}) in the Sham[a] Treatment of Wheat and Soy[b] (1:2, w/w) Processed for 45 min at Different Temperatures (°C) and Moisture Levels (%) in Experiment 1

temperature, °C	moisture, %	IP6
45	25	9.97
45	35	8.61
45	45	7.62
60	25	9.39
60	35	7.33
60	45	7.04
75	25	9.08
75	35	9.56
75	45	9.62
95	25	7.91
95	35	9.57
95	45	11.48

[a] No exogenous phytase added. [b] The mixture of wheat and soy contained 11.3 ± 0.1 g IP6 kg^{-1}. $n = 1$.

proximately 5.0 ($\pm \leq 0.02$) for all moisture treatments. The pH values obtained by the AOAC method were significantly higher than those derived by direct measurement.

Experiment 1. The content of IP6 in the sham treatments varied from 7.0 to 11.5 g kg^{-1} (**Table 2**), whereas the content of IP6 in the raw material was 11.3 g kg^{-1}. The average value from all treatment combinations was 8.93 ± 1.28 g IP6 kg^{-1}.

Incubation of the raw material and added phytase for 45 min at 75 and 95 °C did not result in a major reduction in the content of IP6 for any of the three moisture levels (**Figure 1A**). At a moisture level of 25%, there was no significant difference between the four incubation temperatures investigated, but the differences increased as the moisture content increased. At a moisture level of 45%, there was a large and significant difference between the four incubation temperatures, where the content of IP6 was systematically reduced as the incubation temperatures decreased from 95 to 75, 60, and 45 °C. By omitting the two highest temperatures and the lowest moisture level, where no major hydrolysis was seen, the results of the treatment combinations presented in **Figure 1B** clearly indicate that major hydrolysis of IP6 had taken place already after 2 min. In the 45 °C × 45% moisture treatment, the content of IP6 was reduced by 68% already after incubation for 2 min, and reduced by 86% at 45 min. Results from analyses performed on samples with the combinations of 25%, 35%, and 45% moisture, 45 and 60 °C, and 10 and 45 min retention are shown in **Table 3**. The analysis of variance showed that temperature and moisture level contributed significantly to the liberation of phosphate from IP6. The effect of retention time was also significant, but it contributed less to the degradation of IP6 than temperature and moisture. This is supported by the tendency presented in **Figure 1B**. Apart from IP2, the interaction between moisture level and temperature was significant for all groups of inositol phosphates. The formation of IP3 and IP4 was affected by temperature, moisture, and the interaction between these effects, whereas retention time had no effect. The analyses showed that the raw material also contained some IP5 and IP2 in addition to IP6. The groups of IP5 were derived from soy, and we found that the isomers of IP5 were Ins(1,3,4,5,6)P_5, Ins(1,2,4,5,6)P_5, and Ins(1,2,3,4,5)P_5. The content of IP6 in soybeans was 12.9 g kg^{-1} and 8.2 g kg^{-1} in wheat. The chromatograms presented in **Figure 2** illustrate the disappearance of IP6, as well as the accumulation of IP4 and IP3. The main isomer formed was Ins(1,2,5,6)P_4.

Experiment 2. The results showed that the degradation of IP6 in experiment 1 was well reproduced in experiment 2 (**Figure 3**). Incubation for 10 and 25 min reduced the content of IP6 significantly as compared to the control, whereas incubation for 45 and 60 min was significantly more efficient than incubation for 10 and 25 min. Incubation of the mixture for 45 min resulted in a 76% reduction in the content of IP6, and prolonging the incubation time to 60 min increased the degradation of IP6 to 81%, although the difference observed at these time points was not significant. As observed in experiment 1, hydrolysis of IP6 resulted in a significant increase in the contents of IP3 and IP4 as the retention time increased (**Figure 3**). There were no significant changes in the amounts of IP5 and IP2.

DISCUSSION

The different pH values obtained with the two methods used was most likely caused by the dilution with 100 mL of water (pH = 7) in the AOAC method, but because of the logarithmic nature of the pH scale the difference was minor at the range of pH that was investigated. A pH ≈ 4.5 is optimal for most *E. coli* phytases (*24*), which is relatively close to the values obtained in our measurements. However, earlier reports (*25*, *26*) describe how the relative activity of *E. coli* phytases was reduced to 90% when the pH changed from 4.5 to 5.0. Thus, because both methods used for measuring pH in the present study resulted in a pH above 4.5, it is possible that the phytase efficiency could have been even better if the pH in the dough had been closer to 4.5.

The contents of IP6 in wheat and soy agreed well with previously reported values (*1*). Also, the isomers of IP5 in soy were similar to those reported by Phillippy and Bland (*27*).

The content of IP6 in the sham treatments indicates that the processing method used, a combination of moderate soaking and heating to different temperatures (hydrothermal processing), may have activated some of the endogenous phytase present in ground wheat and soybeans, and thus hydrolyzed some of the IP6 present. As compared to most vegetable and cereal feed ingredients, wheat contains a relatively high activity of endogenous phytase, whereas minor amounts of phytase are present in soybean meal (*28*). Carlson and Poulsen (*29*) found that endogenous wheat phytase reduced the content of IP6 considerably when wheat was soaked and incubated at 38 °C. In the present study, the sham treatments seemed to be less efficient at temperatures above 60 °C, and this agrees with results from Jongbloed and Kemme (*30*), who found that steam pelleting at 80 °C lowered the activity of wheat phytase, leading to a decreased P and calcium absorbability in pigs.

When the exogenous *E. coli* phytase was incubated with the raw material in the present study, temperatures above 60 °C did not reduce the content of IP6 to a large extent. According to Wang et al. (*31*), temperatures above 60 °C are too high, considering the optimal activity temperature of an enzyme such as phytase, and, in addition, the enzyme may denaturate, leading to a limited capacity of hydrolyzing the covalent bonds between the inositol ring and the phosphate groups. In a study by Greiner et al. (*25*), *E. coli* phytase lost most of its activity at temperatures above 60 °C. When Igbasan et al. (*26*) incubated an *E. coli* phytase for 60 min at 50, 60, and 70 °C, it retained 80%, 60%, and 5% of its initial activity, respectively. In the latter case, the enzyme activities were assayed with sodium phytate in a buffered aqueous solution. Hence, earlier studies support that the 45 °C treatment in our study was more efficient and lenient over time than the 60 and 75 °C treatments. Interestingly, disappearance of IP6 at 95 °C in the present study was higher at 25% moisture as compared to the other moisture levels

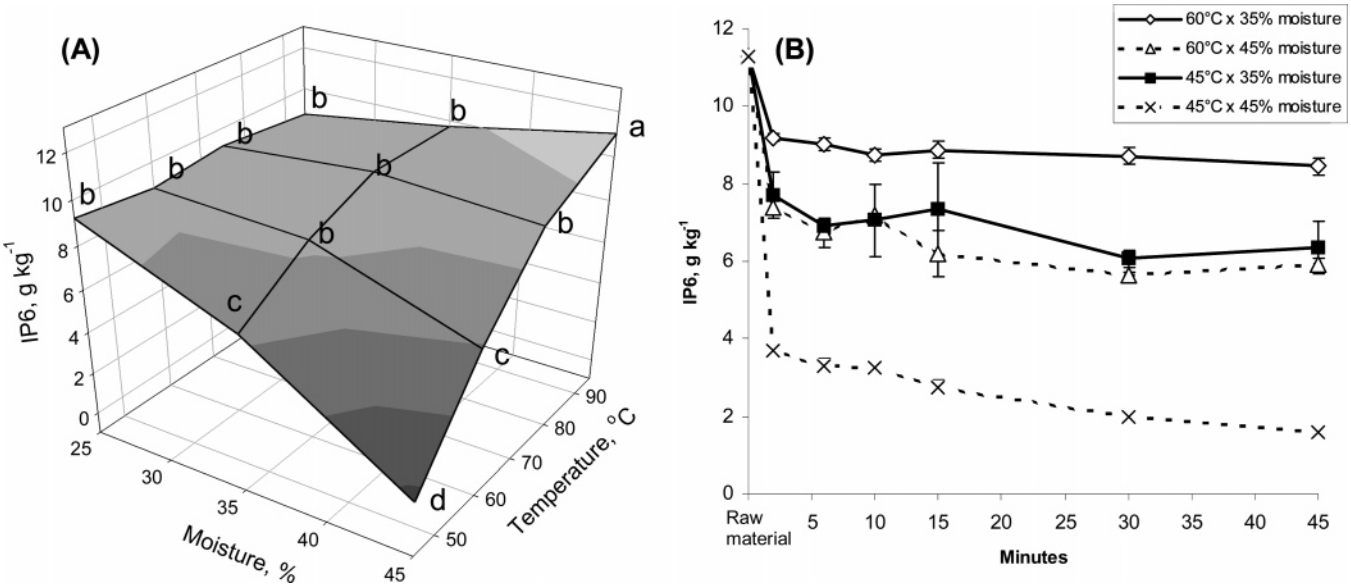

Figure 1. Content of IP6 (g kg^{-1}) in samples treated for 45 min at different temperatures (45, 60, 75, 95 °C) and moisture levels (25%, 35%, 45%) in experiment 1 (**A**). Content of IP6 (g kg^{-1}) in samples treated at various combinations of moisture levels and temperatures during the entire incubation period represented by increasing retention times (2, 6, 10, 15, 30, and 45 min) in experiment 1 (**B**). The data points in both figures are mean values of two observations ($n = 2$), where the error bars in (**B**) indicate SEM Different letters indicate significant differences ($P < 0.05$) between each of the treatment combinations in (**A**). The content of IP6 in the mixture of ground soybean and ground wheat was 11.3 g IP6 kg^{-1}.

Table 3. Content of Inositol Phosphates (g IP6–IP2 kg^{-1}) in Raw Material and Phytase-Treated Samples Processed at Different Temperatures (°C), Moisture Levels (%), and Retention Times (min) in Experiment 1

			inositol phosphate groups				
temperature, °C	moisture, %	min	IP6	IP5	IP4	IP3	IP2
raw material[a]			11.3 ± 0.1	1.2 ± 0.0	nd	nd	2.8 ± 0.2
45	25	10	8.8 ± 0.1	1.4 ± 0.1	nd	nd	3.2 ± 0.2
45	25	45	9.1 ± 0.2	1.2 ± 0.2	0.3 ± 0.0	nd	2.9 ± 0.0
45	35	10	7.1 ± 0.9	1.3 ± 0.2	1.0 ± 0.3	0.1 ± 0.1	3.2 ± 0.3
45	35	45	6.3 ± 0.7	1.0 ± 0.2	1.2 ± 0.3	nd	2.9 ± 0.2
45	45	10	3.3 ± 0.1	1.1 ± 0.3	3.2 ± 0.2	0.7 ± 0.0	3.6 ± 0.2
45	45	45	1.6 ± 0.1	0.2 ± 0.0	2.8 ± 0.6	1.1 ± 0.1	4.1 ± 0.9
60	25	10	9.6 ± 0.0	1.2 ± 0.1	nd	nd	3.1 ± 0.2
60	25	45	9.3 ± 0.4	1.1 ± 0.1	nd	nd	3.5 ± 0.2
60	35	10	8.7 ± 0.2	1.4 ± 0.1	0.4 ± 0.0	nd	3.2 ± 0.1
60	35	45	8.3 ± 0.2	1.4 ± 0.1	0.5 ± 0.1	nd	2.8 ± 0.1
60	45	10	7.2 ± 0.0	2.4 ± 0.1	1.2 ± 0.1	0.3 ± 0.1	2.9 ± 0.1
60	45	45	6.0 ± 0.0	2.1 ± 0.0	1.6 ± 0.1	0.7 ± 0.0	3.3 ± 0.2
three-way ANOVA							
temperature (*T*)			<0.0001	<0.0001	<0.0001	0.0003	NS
moisture (*M*)			<0.0001	NS	<0.0001	<0.0001	NS
retention time (R_t)			0.0222	0.0071	NS	0.0014	NS
$T \times M$			0.0002	<0.0001	0.0016	0.0005	NS
$T \times R_t$			NS	NS	NS	NS	NS
$M \times R_t$			NS	NS	NS	<0.0001	NS
$T \times M \times R_t$			NS	NS	NS	NS	NS

[a] Mixture of ground wheat and ground defatted soybeans (1:2, w/w). Each value is the mean of two observations ($n = 2$) ± SEM. nd = not detectable.

(**Figure 1**). The same tendency was seen in the sham treatments (**Table 2**), but because only one experimental run was conducted, firm conclusions cannot be drawn. Affrifah et al. (*32*) reported that cowpea flour incubated at 95 °C had lower endogenous phytase activities after incubation at 35% moisture as compared to incubation at 10% moisture. Thus, the result of the latter study coincides with our observations for the 95 °C treatment. The reason for this is that water is needed to promote the unfolding of proteins and enzymes during thermal denaturation (*33*). However, the literature available on this subject is very limited, and more work needs to be done to understand the combined effects of moisture and temperature on the activity of phytase, and thus the hydrolysis of IP6.

The major degradation of IP6 already after 2 min (**Figure 1B**) seems reasonable because the velocity in enzymatic reactions generally decreases as the substrate concentration is reduced (*34*). According to Greiner et al. (*35*), reduced rate of phytate hydrolysis may also be due to the inhibition caused by free phosphate groups or due to a slower rate of hydrolysis for lower groups of inositol phosphates. In experiment 1, the treatment lasted for 45 min only and did not allow us to determine if groups of IP3 or IP4 were accumulated, or if these groups were intermediate stages toward the complete dephosphorylation of IP6. Formation of the Ins(1,2,5,6)P_4 isomer agrees well with the observation by Greiner et al. (*35*), where the same isomer of IP4 was formed during a 90 min incubation with an

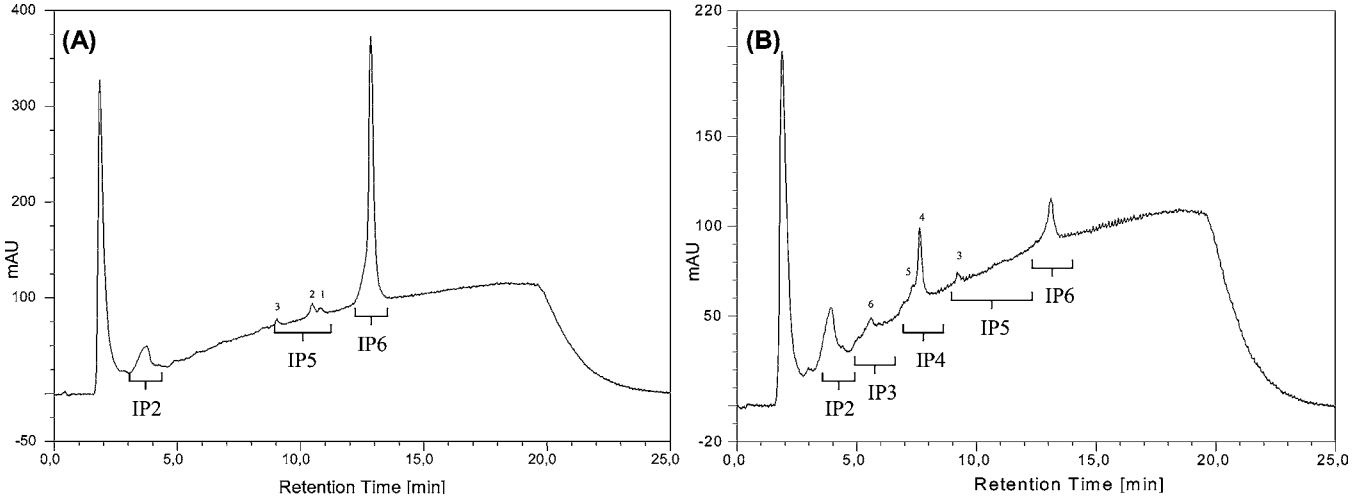

Figure 2. Chromatographic profiles of raw material (ground wheat and ground defatted soybeans, 1:2, w/w) (**A**), and raw material incubated with phytase for 45 min at 45 °C and 45% moisture in experiment 1 (**B**). Groups of inositol phosphates (IP6–IP2) are displayed in the chromatograms, along with specific peaks: 1, Ins(1,3,4,5,6)P_5; 2, Ins(1,2,4,5,6)P_5; 3, Ins(1,2,3,4,5)P_5; 4, Ins(1,2,5,6)P_4; 5, unidentified peaks; 6, Ins(1,2,6)P_3 and Ins(1,2,3)P_3.

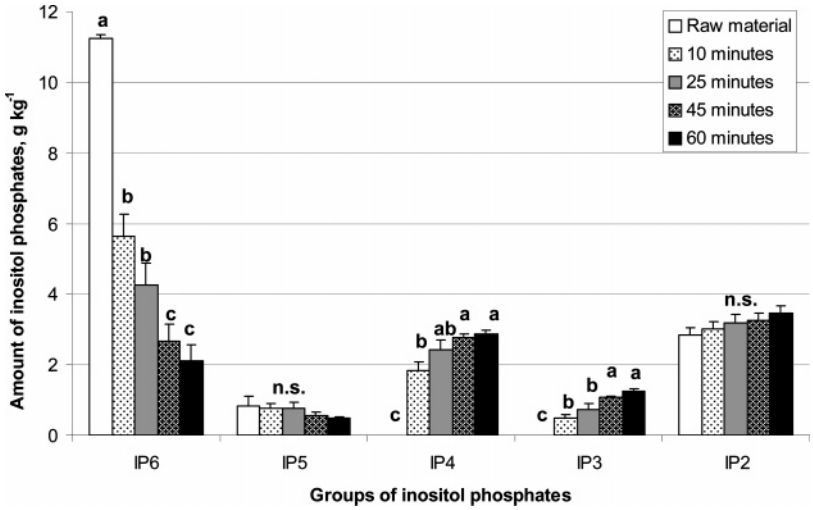

Figure 3. Content of inositol phosphates (g IP6–IP2 kg^{-1}) in a mixture of ground wheat and ground defatted soybeans (1:2, w/w) processed at 45 °C and 45% moisture at varying retention times (10, 25, 45, and 60 min) in a conventional large-scale mixer conditioner from experiment 2. Each bar is the mean of three observations ($n = 3$), error bars indicate SEM, and the different letters indicate significant difference ($P < 0.05$) between treatments (min).

E. coli phytase. It should be noted, however, that in the latter experiment sodium phytate was used as a substrate, and the incubation temperature was 35 °C. Because we did not analyze for IP1, free *myo*-inositol, or free phosphate in our samples, we were not able to complement the picture with regard to products derived from hydrolysis of IP6. However, because the most efficient treatment (45 °C × 45% moisture × 45 min) caused a degradation of IP6 of about 86% (from 11.3 to 1.6 g IP6 kg^{-1}), it seems reasonable that the hydrolysis yielded substantial amounts of IP1 or free phosphate. In a study by Bergman et al. (*36*), hydrothermal processing of barley resulted in a 95−96% reduction of IP6 and increased the level of free *myo*-inositol. In the latter study, the process consisted of several treatment steps, and it is unlikely that the method used could be transformed to an online feed production. The treatment in experiment 1 seemed to degrade IP6 more efficiently, because the content of IP6 was reduced by 71% after 10 min incubation in experiment 1 as compared to 50% degradation in experiment 2. However, after incubation for 45 min, the content of IP6 was further reduced to 86% in experiment 1 as compared to 76% in experiment 2. A slower response in degradation of IP6 in experiment 2 could be a result of using equipment with different size (6 L vs 400 L) and construction. The two nozzles used in the large mixer-conditioner were placed in the middle of the conditioner chamber. A more careful distribution of the nozzles could have enhanced the efficiency of the application of phytase-containing water. Thus, because neither of the two conditioners had been designed for the present purpose, there probably would be a lot to gain when it comes to optimization and further modifications of a mixer-conditioner ideal for the present purpose.

As our design did not cover moisture levels above 45%, there still is a chance that additional water would improve the outcome of the incubation. However, increasing the moisture levels above 45% would complicate the extrusion process and increase the use of process water. In a conventional process, the moisture level in most feed mixtures is 25−30% when leaving the preconditioner and entering the extruder (*19*).

The accumulation of IP3 and IP4 indicated that the *E. coli* phytase used was insufficient with regard to completing the dephosphorylation of inositol phosphates in the raw material. In particular, the Ins(1,2,5,6)P_4 seemed to accumulate. However, the antinutritive potential of groups of IP3 and IP4 is not as significant as that of IP6 (*5*). Moreover, in past years, attention

has been devoted to the potential positive effect of lower inositol phosphates such as IP3 and IP4. Several reports indicate that derivatives of IP6 (mainly IP3) could have anticarcinogenic effects (*37*−*39*) and the function of Ins(1,4,5)P_3 and several other IPs as second messengers in cells (*40*, *41*) may indicate certain beneficial effects associated with these derivatives. The principle of the method used in the present study may be applied for a number of feed enzymes. For example, xylanase would probably degrade nonstarch polysaccharides quite efficiently at 45 °C at pH ≈ 4.5−5.0, because the optimal conditions for xylanase coincide well with the most efficient treatment used in the present study (*42*). Use of other enzymes such as proteases, amylases, and galactosidases could also apply to the present method, and thus increase the nutritional value of the feed.

In conclusion, the present study shows that IP6 from a ground mixture of wheat and soybean (1:2, w/w) was successfully hydrolyzed by a method suitable to be an integral part of the online processing of compound feed. The degradation of IP6 in a feed mixture with added exogenous *E. coli* phytase (2500 FTU kg^{-1}) was most efficient when incubated at 45 °C and 45% moisture. The latter combination reduced the content of IP6 by 86% after 45 min, together with a simultaneous formation of groups of IP3 and IP4, mainly represented by the Ins(1,2,5,6)-P_4 isomer.

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LITERATURE CITED

(1) Reddy, N. R. Occurrence, distribution, content, and dietary intake of phytate. In *Food Phytates*; Reddy, N. R., Sathe, S. K., Eds.; CRC Press: New York, 2002; pp 25−51.

(2) Clark, W. D., Jr.; Wohlt, J. E.; Gilbreath, R. L.; Zajac, P. K. Phytate phosphorous intake and disappearance in the gastrointestinal tract of high producing dairy cows. *J. Anim. Sci.* **1986**, *42*, 1509−1512.

(3) Selle, P. H.; Ravidran, V.; Caldwell, R. A.; Bryden, W. L. Phytate and phytase: consequences for protein utilization. *Nutr. Res. Rev.* **2000**, *13*, 255−278.

(4) Nolan, K. B.; Duffin, P. A.; Mcweeny, D. J. Effects of phytate on mineral bioavailability − in vitro studies on Mg^{2+}, Ca^{2+}, Fe^{3+}, Cu^{2+} and Zn^{2+} (also Cd^{2+}) solubilities in the presence of phytate. *J. Sci. Food Agric.* **1987**, *40*, 79−85.

(5) Sandberg, A.-S.; Brune, M.; Carlsson, N.-G.; Hallberg, L.; Skoglund, E.; Rossander-Hulthèn, L. Inositol phosphates with different numbers of phosphate groups influence on iron absorption in humans. *Am. J. Clin. Nutr.* **1999**, *70*, 240−246.

(6) Thacker, P. A.; Rossnagel, B. G.; Raboy, V. Phosphorus digestibility in low-phytate barley fed to finishing pigs. *Can. J. Anim. Sci.* **2003**, *83*, 101−104.

(7) Atwal, A. S.; Eskin, N. A. M.; McDonald, B. E.; Vaisey-Genser, M. The effects of phytate on nitrogen utilization and zinc metabolism in young rats. *Nutr. Rep. Int.* **1980**, *21*, 257−267.

(8) Pallauf, J.; Pietsch, M. Dietary phytate reduces magnesium bioavailability in growing rats. *Nutr. Res.* **1998**, *18*, 1029−1037.

(9) Cowieson, A. J.; Acamovic, T.; Bedford, M. R. The effects of phytase and phytic acid on the loss of endogenous amino acids and minerals from broiler chickens. *Br. Poult. Sci.* **2004**, *45*, 101−108.

(10) Denstadli, V.; Skrede, A.; Krogdahl, Å.; Sahlstrøm, S.; Storebakken, T. Feed intake, growth, feed conversion, digestibility, enzyme activities and intestinal structure in Atlantic salmon (*Salmo salar* L.) fed graded levels of phytic acid. *Aquaculture* **2006**, *256*, 365−376.

(11) Richardson, N. L.; Higgs, D. A.; Beames, R. M.; McBride, J. R. Influence of dietary calcium, phosphorus, zinc and sodium phytate level on cataract incidence, growth and histopathology in juvenile chinook salmon (*Oncorhynchus tshawytscha*). *J. Nutr.* **1985**, *115*, 553−567.

(12) Sathe, S. K.; Venkatachalam, M. Influence of processing technologies on phytate and its removal. In *Food Phytates*; Reddy, N. R., Sathe, S. K., Eds.; CRC Press: New York, 2002; pp 157−188.

(13) Kemme, P. A.; Jongbloed, A. W.; Mroz, Z.; Kogut, J.; Beynen, A. C. Digestibility of nutrients in growing-finising pigs is affected by *Asperigillus niger* phytase, phytate and lactic acid levels. 2. Apparent total tract digestibility of phosphorus, calcium and magnesium and ileal degradation of phytic acid. *Livest. Prod. Sci.* **1999**, *58*, 119−127.

(14) Brenes, A.; Viveros, A.; Arija, I.; Centeno, C.; Pizarro, M.; Bravo, C. The effect of citric acid and microbial phytase on mineral utilization in broiler chicks. *Anim. Feed Sci. Technol.* **2003**, *110*, 201−219.

(15) Snow, J. L.; Baker, D. H.; Parsons, C. M. Phytase, citric acid, and 1α-hydroxycholeclaciferol improve phytate phopsphorus utilization in chicks fed a corn-soybean meal diet. *Poult. Sci.* **2004**, *83*, 1187−1192.

(16) Edwards, H. M.; Carlos, A. B.; Kasim, A. B.; Toledo, R. T. Effects of steam pelleting and extrusion of feed on phytate phosphorus utilization in broiler chickens. *Poult. Sci.* **1999**, *78*, 96−101.

(17) Cain, K. D.; Garling, D. L. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. *Prog. Fish-Cult.* **1995**, *57*, 114−119.

(18) Finley, J. W. Effects of processing on proteins: An overview. In *Protein Quality and the Effects of Processing*; Phillips, R. D., Finley, J. W., Eds.; Marcel Dekker, Inc.: New York, 1989; pp 1−7.

(19) Rokey, G. Petfood and fishfood extrusion. In *The Technology of Extrusion Cooking*; Frame, N. D., Ed.; Blackie Academic & Professional: London, UK, 1994; pp 144−189.

(20) AOAC. *Official methods of analysis of AOAC International*, 17 ed.; AOAC International: Gaithersburg, MD, 2000.

(21) Carlsson, N.-G.; Bergman, E.-L.; Skoglund, E.; Hasselblad, K.; Sandberg, A.-S. Rapid analysis of inositol phosphates. *J. Agric. Food Chem.* **2001**, *49*, 1695−1701.

(22) Skoglund, E.; Carlsson, N.-G.; Sandberg, A.-S. Determination of isomers of inositol mono- to hexaphosphates in selected foods and intestinal contents using high-performance ion chromatography. *J. Agric. Food Chem.* **1997**, *45*, 431−436.

(23) *SAS Institute SAS User's Guide: Statistics*, 5th ed.; SAS Institute: Cary, NC, 1985.

(24) Vats, P.; Banerjee, U. C. Production studies and catalytic properties of phytases (*myo*-inositolhexakisphosphate phosphohydrolases): an overview. *Enzyme Microb. Technol.* **2004**, *35*, 3−14.

(25) Greiner, R.; Konietzny, U.; Jany, K. D. Purification and characterization of two phytases from *Escherichia coli*. *Arch. Biochem. Biophys.* **1993**, *303*, 107−113.

(26) Igbasan, F. A.; Manner, K.; Miksch, G.; Borriss, R.; Farouk, A.; Simon, O. Comparative studies on the in vitro properties of phytases from various microbial origins. *Arch. Anim. Nutr.* **2000**, *53*, 353−373.

(27) Phillippy, B. Q.; Bland, J. M. Gradient ion chromatography of inositol phosphates. *Anal. Biochem.* **1988**, *175*, 162−166.

(28) Ravindran, V.; Bryden, W. L.; Kornegay, E. T. Phytates: Occurrence, bioavailibility and implications in poultry nutrition. *Poult. Avian Biol. Rev.* **1995**, *6*, 125−143.

(29) Carlson, D.; Poulsen, H. D. Phytate degradation in soaked and fermented liquid feed-effect of diet, time of soaking, heat treatment, phytase activity, pH and temperature. *Anim. Feed Sci. Technol.* **2003**, *103*, 141−154.

(30) Jongbloed, A. W.; Kemme, P. A. Effect of pelleting mixed feeds on phytase activity and the apparent absorbability of phosphorus and calcium in pigs. *Anim. Feed Sci. Technol.* **1990**, *28*, 233−242.

(31) Wang, X.-Y.; Meng, F.-G.; Zhou, H.-M. Unfolding and inactivation during thermal denaturation of an enzyme that exhibits phytase and acid phosphatase activities. *Int. J. Biochem. Cell Biol.* **2003**, *36*, 447−459.

(32) Affrifah, N. S.; Chinnan, M. S.; Dixon Phillips, R. Heat-moisture treatments of cowpea flour and their effects on phytase inactivation. *Food Eng. Phys. Prop.* **2005**, *70*, 98−103.

(33) Parkin, K. L. Environmental effects on enzyme activity. In *Enzymes in Food Processing*; Nagodawithana, T., Reed, G., Eds.; Academic Press: San Diego, CA, 1993; pp 39−69.

(34) Parkin, K. L. General characteristics of enzymes. In *Enzymes in Food Processing*; Nagodawithana, T., Reed, G., Eds.; Academic Press, Inc.: San Diego, CA, 1993; pp 7−37.

(35) Greiner, R.; Carlsson, N. G.; Alminger, M. L. Stereospecificity of myo-inositol hexakisphosphate dephosphorylation by a phytate-degrading enzyme of *Escherichia coli*. *J. Biotechnol.* **2000**, *84*, 53−62.

(36) Bergman, E.-L.; Fredlund, K.; Reinikainen, P.; Sandberg, A.-S. Hydrothermal processing of barley (cv. Blenheim): Optimization of phytate degradation and increase of free myo-inositol. *J. Cereal Sci.* **1999**, *29*, 261−272.

(37) Fox, C. H.; Eberl, M. Phytic acid (IP6), novel broad spectrum anti-neoplastic agent: a systematic review. *Compl. Ther. Med.* **2002**, *10*, 229−234.

(38) Shamsuddin, A. M. Anti-cancer function of phytic acid. *Int. J. Food Sci. Technol.* **2002**, *37*, 769−782.

(39) Vucenik, I.; Shamsuddin, A. M. Cancer inhibition by inositol hexaphosphate (IP6) and inositol: From laboratory to clinic. *J. Nutr.* **2003**, *133*, 3778S−3784S.

(40) Shears, S. B. The versatility of inositol phosphates as cellular signals. *Biochim. Biophys. Acta* **1998**, *1436*, 49−67.

(41) Joseph, S. K. The inositol triphosphate receptor family. *Cell. Signalling* **1996**, *8*, 1−7.

(42) Weng, X. Y.; Sun, J. Y. Construction, expression, and characterization of a thermostable xylanase. *Curr. Microbiol.* **2005**, *51*, 188−192.

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