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Degradation of Phytates in Distillers' Grains and Corn Gluten Feed by *Aspergillus niger* Phytase

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Abstract Distillers' dried grains with solubles (DDGS) and corn gluten feed (CGF) are major coproducts of ethanol production from corn dry grind and wet milling facilities, respectively. These coproducts contain important nutrients and high levels of phytates. The phytates in these products cannot be digested by nonruminant animals; consequently, large quantities of phytate phosphorus (P) are deposited into the soil with the animal wastes which potentially could cause P pollution in soil and underground water resources. To reduce phytates in DDGS and CGF, a phytase from *Aspergillus niger*, PhyA, was investigated regarding its capability to catalyze the hydrolysis of phytates in light steep water (LSW) and whole stillage (WS). LSW and WS streams are the intermediate streams in the production of CGF and DDGS, respectively, and contribute to most of the P in these streams. Enzyme loadings with activity of 0.1, 1, 2, and 4 FTU/g substrate and temperatures of 35 and 45 °C were investigated regarding their influences on the degree of hydrolysis. The analysis of the hydrolyzate suggested to a sequentially degradation of phytates to lower order myo-inositol phosphate isomers. Approximately 90% phytate P of LSW and 66% phytate P of WS were released, suggesting myo-inositol monophosphate as the end product. The maximum amount of released P was 4.52±0.03 mg/g LSW and 0.86±0.01 mg/g WS.

Keywords Distillers' grains · Light steep water · Whole stillage · *Aspergillus niger* phytase · Hydrolysis · Phytates · Myo-insotitol phosphate isomers · Inorganic phosphate

Introduction

Nonpoint pollution of surface waters from agricultural phosphorus (P) and its effect on the environment is a focused issue for USEPA, USDA, and US agricultural and environmental leaders alike. Eutrophication of surface waters occurs, in part, due to nonpoint pollution from agricultural and livestock production practices [1]. One such practice occurs in ethanol

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production where large quantities of DDGS and CGF coproducts are produced. DDGS and CGF are major coproducts of ethanol production from corn dry grind and wet milling facilities, respectively. These coproducts are relatively high in P content and are predominantly marketed as feed coproducts. For example, DDGS P content is reported as 7–10 g/kg on dry base (db) by Rausch and coworkers [2]. Livestock feeding with high levels of P feedstuff results in high P manure which could pollute surface waters and create environmental concerns [3–5]. McDowell and Sharpley [6] investigated the release of P from soil surface to surface runoff and subsurface drainages. In this study, P concentrations that cause eutrophication was given at 0.01 to 0.03 mg/L. P concentration was correlated to release of P into surface runoff and subsurface drainage which resulted in guidelines for agricultural and environmental management.

In the production of ethanol, for every bushel of corn 2.4–2.7 gal of ethanol and 16–18 lbs of DDGS or 12–13 lbs of CGF are produced. Approximately 82% of the ethanol produced from corn is by the dry grind processes as compared to about 18% from the wet milling processes [7]. In the wet milling process, most of the phytates are extracted into the light steep water stream, while, some of the phytates are left in the germ. Light steep water is concentrated and added to the bran to form CGF which accounts for most of the P content of the corn. Rausch and coworkers [8] measured concentration and flow of P in three wet milling plants and concluded that 86% of the P entering the steeping process was removed in the light steep water. However, only 66% of the total P entering the process was accounted for in the CGF, corn gluten meal (CGM), and dried germ streams. They blamed the discrepancy on the sampling errors and simplifications in the process simulation. The production of CGF, CGM and dried germ was approximately six million metric tons in 2006 [7].

In the dry grind process, after the separation of ethanol from the beer stream, a slurry stream of all the processing remains called whole stillage (WS) is formed. This stream, which is further processed to form DDGS, contains the whole of the P content of the starting corn. In 2006, more than 15 million metric tons of DDGS were produced by the corn dry grind industries which were predominantly marketed as feed coproducts [7]. Due to an increase in the demand for ethanol, the production of the coproducts is expected to double within the next few years which further will increase the quantity of the available DDGS and CGF. Proper marketing of these coproducts is critical to the sustainability of the ethanol industry.

DDGS and CGF have important nutrient contents that add value to animal diets. However, the P content of DDGS and CGF is higher than what is needed in the diets. The P content of DDGS and CGF ranges from 7 to 10 g P/kg (db), which is much higher than common grains and the requirements of most ruminants [2]. Excess diet P is excreted resulting in high P manure [9]. A recent study of 16 Nebraska cattle feeding operations showed these coproducts to have P input to output ratios doubled that of those not using such coproducts [10]. This means for land application purposes P is more likely to be an environmental problem for animal feeding operations using ethanol coproducts as a feedstuff than for those not using the coproducts.

Phytate or inositol hexakisphosphate ($InsP_6$) is found in most cereal seeds. This organic complex is the main storage form of P in grains and seeds [11]. Phytates serve several physiological functions, especially in seed germination. Historically, phytates have been considered solely as antinutrients because they are known as strong chelators of divalent minerals such as Ca^{2+}, Mg^{2+}, $Zn^{2+}$and Fe^{2+}. Binding of phytates with these minerals diminishes their bioavailability [12]. Moreover, phytates are also capable of binding with starch and proteins while preventing their assimilation through the digestive system. Ninety

percent of the phytates in corn is found in the germ portion of the kernel which accounts for approximately 50% to 80% of the P in corn [13]. Under normal physiological conditions, phytate–mineral complexes are unavailable for assimilation in nonruminant animals but can be readily utilized by ruminants [14]. In the swine industry, this problem is addressed through the use of the enzyme phytase as a feed ingredient [15].

Phytases (inositol hexaphosphate hydrolase) have been reported to hydrolyze phytate phosphorus and improve phosphorus utilization in animal feeds on various diets [16, 17]. Phytases are a special class of phosphatases that catalyze the sequential hydrolysis of phytate to various lower order inositol esters (IP_5–IP_1) and in some cases, free inositol and at the same time release free inorganic phosphates, bound minerals, starch, and proteins. Phytases are presents in plants, microorganisms, and certain animal tissues [18]. Depending on their origin, phytases behave differently in terms of specificity toward the position of hydrolysis on the inositol ring, formation of intermediates and final product, biochemical characteristics (e.g., pH optima and susceptibility to inhibitors and activators), and biophysical characteristics (e.g., thermostability limits) [18–21]. Three distinct classes of phytase, EC 3.1.3.8, EC 3.1.3.26, and EC 3.1.3.72, have been identified, which initiate the dephosphorylation of phytate at the 3, 6, and 5 positions of the inositol ring, respectively [22, 23]. The effect of microbial phytases (EC 3.1.3.8) as feed supplement is well documented in the literature. *Aspergillus niger*, which has been most extensively studied, has shown the highest activity yield when compared to 14 other *Aspergillus* spp. in screening for phytase production organisms [24]. *A. niger* is known to produce two phytases, phytase A (PhyA) with an optimal pH at 2.0 and 5.5 and phytase B (PhyB) with an optimal pH at 2.5 [25, 26]. PhyA is the first commercialized phytase (Natuphos™) and currently holds a large share of the world market [27]. Its comparatively low price ($1.6~2.4/lb) makes it ideal for industrial applications. The catalytic efficacy of PhyA as an animal feed additive has been testified [28, 29], and thoroughly reviewed by Wodzinski and Ullah [30].

A previous study has shown that the WS stream contains the whole P which is introduced into the dry grind process through corn, and the light steep water (LSW) stream contains most of the P that enters the wet milling process [31]. The objective of this study was to explore the feasibility of reducing phytates in CGF and DDGS via hydrolyzing the LSW and WS with PhyA. The extent of hydrolysis was then evaluated by the inorganic phosphates (Pi) released during the process. Influences caused by variables such as enzyme loading and reaction temperature were also investigated. Determination of the distribution and form of P compounds in the corn milling operations and facilitation of the degradation of the P in these compounds to Pi form is fundamental to the approaches that need to be taken to properly separate them from these streams and balance the amount of P compounds in the DDGS and CGF coproducts.

Material and Methods

Materials

Substrates LSW samples used in this study were from Cargill, a wet milling corn facility in Blair, NE, USA. WS samples were from Abengoa Bioenergy, a dry grind corn ethanol facility in York, NE, USA. The WS samples were finely grounded for experimental use with a laboratory blender (blender 7010HS, Waring Commercial, Vernon Hills, IL, USA). All samples were kept in a refrigerator (4 °C) prior to use. When samples contained a

mixture of solid and liquid, care was entailed to ensure the homogeneity of the samples taken before experiments.

Enzyme Natuphos 10,000 liquid enzyme (3-phytase produced from *A. niger*) was purchased from BASF (Florham Park, NJ, USA). The nominal activity of this enzyme was 10,000 FTU/g. This enzyme was diluted with deionized (DI) water into 10, 100, 200, and 400 FTU/mL before use.

Chemicals Sulfuric acid (95–98%), sodium molybdate dehydrate (99.5%), ascorbic acid, hydrochloric acid (37%), potassium dihydrogen phosphate (1 M), zinc oxide (99.9%), potassium hydroxide, phytic acid (50% *w*/*w*), myo-inositol (99%), hydrochloric acid (37%), perchloric acid (70%), and iron (III) nitrate nonahydrate (98%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). DI water was further purified by a Simplicity Ultra Pure Water System from Millipore (Billerica, MA, USA). Hydrochloric acid (0.5 M) and a solution of 1 g/L $Fe(NO_3)_3$ in 0.33 M $HClO_4$ were filtered by 0.22-μm membrane filter from Millipore before use.

Enzymatic Hydrolysis

Hydrolysis reactions were carried out in capped 125-mL Erlenmeyer flasks. Flasks were initially charged with 100 g of LSW or WS and a prespecified amount of enzyme. The reaction mixture was incubated in a temperature-controlled incubator (Imperial III, Lab-Line Instruments, Inc., Melrose, IL, USA) in which a shaker (C2 classic platform shaker, New Brunswick Scientific Co., Ltd., Edison, NJ, USA) was placed to mix the reaction flasks at 200 rpm. At 0, 0.5, 1, 2, 4, 6, 8, and 24 h, aliquots of about 10 g each were taken from the reaction mixture. The reaction was terminated by heating the samples in a water bath at 100 °C for 10 min. Precautions were taken to avoid water vapor entering the samples. All hydrolysis experiments were performed in duplicates and the presented data are the mean values for the replicates.

Analysis

Enzyme Activity One phytase unit of activity is defined as the amount of the enzyme, which, at 37 °C and pH 5.5, liberates 1.00 μmol/min of inorganic P from 0.0051 mol/L sodium phytate. The analysis was based on the method developed by Engelen and coworkers [32]. In this procedure, the phytase source was subjected to incubation with sodium phytate in order to liberate inorganic phosphate from the substrate. Molybdate–vanadate reagent was added to the reaction mixture to stop the reaction. Moreover, this reagent results in the formation of a colored complex with the freed inorganic phosphates. The absorbance of the yellow P complex was measured at a wavelength of 415 nm. A standard calibration curve which was developed by BASF for the activity of Natuphos 10,000 phytase was used.

Total Phosphorus The photometric dry-ashing procedure for the analysis of P was based on the standard method as adopted by the Nordic Committee on Food Analysis/AOAC International for determination of P in a variety of food samples [33]. Measurements were based on a colorimetric method where the color of the treated sample reflected the concentration of P. The samples were ashed to remove the organic (C, H, O) materials. Hydrochloric acid was added to the remaining inorganic ash residue to convert the P

residues to a dissolved P form. This solution was used for color reaction based on the formation of a blue complex between phosphate and sodium molybdate in the presence of ascorbic acid as the reducing agent. The blue color of the complex was directly proportional to the amount of P.

Phosphorus in Phosphate Form (Pi) Experiments were carried out to determine the phosphate content in LSW and the liquid fraction of the WS samples during the course of dephosphorylation experiments. These procedures were based on a previous study [31] which recognizes the fact that P in the form of phosphate is totally soluble in water, whereas P in the form of phytates could be found in both the solid and the liquid fractions of samples. Consequently, the ashing step and the subsequent addition of HCl were eliminated from the color reaction procedure, and the rest of the procedure was followed as stated above for the determination of the total phosphorous.

For the determination of P in the liquid fraction of WS, samples were first centrifuged in a centrifuge (8464 Thermo Electron Co., Milford, MA, USA), at 11,000×*g* for 10 min. The supernatant was then passed through a polytetrafluoroethylene (PTFE) 25 mm, 0.20 μm porosity syringe filter (Agilent Technologies, Wilmington, DE, USA) to ensure the removal of all residual suspended solids. The collected supernatants were then subjected to the drying procedure as mentioned above.

Reagent Preparation All reagents were prepared as described by Pulliainen and Wallin [33] with some modifications. The procedures are as follows: (a) Sodium molybdate solution was prepared by mixing 140 mL of sulfuric acid (18 M) with 300 mL DI water and 12.5 g of sodium molybdate in a 500-mL volumetric flask. (b) Ascorbic acid solution was prepared daily by adding 5 g ascorbic acid to DI water in 100-mL volumetric flask. (c) Molybdate-ascorbic acid solution was prepared immediately before use by adding 50 mL molybdate solution and 20 mL ascorbic acid in a 200-mL volumetric flask. (d) A 2-mg P/mL P stock solution was prepared by diluting 8.788 g potassium dihydrogen phosphate (KH_2PO_4) in a 1-L volumetric flask. (e) P working solution was prepared by diluting the P stock solution to 0.1 mg P/mL and was used to prepare the calibration curve.

Color Reaction Dried samples (0.5–1.5 g) along were initially ashed with zinc oxide in a muffle furnace (McMaster-Carr 31605k55, Chicago, IL, USA). Sample preparation followed the procedure outlined by Pulliainen and Wallin [33]. A quantity of the P solution so prepared (1.0–10.0 mL) was taken for the color reaction with molybdate–ascorbic acid solution. Samples were taken in 1 mL cuvettes for analysis. The percent P content of the liquid samples was calculated using the calibration curve.

The experimental procedures for the determination of phosphate content of the supernatant fraction of the samples bypassed the ashing step and the subsequent addition of HCl. The amount of sample taken for these experiments was 1–5 mL in a 200-mL volumetric flask which depended on the expected P concentration. This was then neutralized with 50% KOH, and the rest of the procedure followed Pulliainen and Wallin [33]. All experiments were repeated twice. The mean values for the replicates and the standard deviations are presented in the "Results and Discussion" section.

Calibration Quantitative determination of P in the samples was determined based on a calibration curve using different P concentrations. The P working solution was used in the calibration. Known volumes of this solution (1.00, 2.00, 3.00, 4.00, 5.00, and 6.00 mL) were charged into a 50-mL volumetric flask and diluted with DI water to 15 mL. This was

followed by addition of 20 mL molybdate–ascorbic acid solution. The rest of the color reaction procedure was followed.

Analysis The concentration of inositol phosphates in the LSW and WS was analyzed by high performance liquid chromatography (HPLC) equipped with a post column derivatizer. A post column derivatizer provides for the derivatization of the material after they are separated in the column. This is performed in the reactor compartment of this device with an appropriate derivatization reagent.

A UV spectrophotometer (Thermo Electron Co. 335906 GENESYS 10S, Milford, MA, USA) was used to measure the P content by relating the intensity of the blue colored samples to the amount of P in them. The instrument was first zeroed with a blank reference sample. Sample absorbance was measured at 823 nm.

The HPLC system used for the analysis of inositol phosphates (P in phytate form) was a Waters Alliance system (Milford, MA, USA) and consisted of a separation module (Waters 2695) and a dual λ absorbance detector (Waters 2487). The HPLC was equipped with a post column derivatization instrument (Pickering Laboratories PCX5200, Mountain View, CA, USA). The analytical column used for the separation was a CarboPac PA-100 and a CarboPac PA-100 guard column manufactured by Dionex (Sunnyvale, CA, USA). The mobile phase was provided by gradient elution of a (0.5 M) HCl (A) and DI water solution (B) at 1.0 mL/min. The following describes the gradient elution profile which was maintained during the run; 0–16 min, 8–20% A, 92–80% B; 16–33 min, 20–37% A, 80–63% B; 33–49 min, 37–100% A, 63–0% B; 49–50 min, 100% A, 0% B; and 50–50.1 min, 100–8% A, 0–92% B. Total run time for this method was 60 min. Samples were filtered with a PTFE 25 mm, 0.20 μm porosity syringe filter (Agilent Technologies, Wilmington, DE, USA) prior to the injection. Samples (25 μL) were injected via an injection port at an initial column temperature of 25 °C. The separated samples were mixed in the post column derivatizer reactor with a mixture of 0.1% $Fe(NO_3)_3$ $9H_2O$ and 2.0% $HClO_4$ at 0.8 mL/min. The HPLC system pressure range was from 2,250 to 2,400 psi, and post column pressure was held constant at 200 psi. The dual λ absorbance detector was set at 295 nm. Chen et al. [34] have reported the detection of 27 peaks representing IP_2–IP_6 isomers during the acidic hydrolysis (2 M HCl) of commercial phytic acid. This was the basis for identifying the peaks of the phytates in this study.

Results and Discussion

Phosphorus Content

Experiments were performed to determine the total P and the Pi content of WS and LSW. The Pi content of the samples established the baseline for the hydrolysis studies. Experimental procedures outlined earlier in the "Material and Methods" section were followed. The results are summarized in Table 1. The total P and the Pi content for LSW were 5.1±0.04 and 1.2±0.01 mg/g LSW, respectively. The phytate P, quantified by HPLC, was 4.0±0.01 mg/g LSW and was mainly in the form of $InsP_6$, accompanied by small amounts of $InsP_5$. About 0.07 mg/g LSW in Pi was attributed to the phytates which was due to the hydrolysis of $InsP_6$ to $InsP_5$ during the steeping process. The total P content in WS was determined to be 1.3±0.02 mg/g WS. The total P content of the liquid and the solid fractions of the WS were determined to be 1.0±0.06 and 0.3±0.04 mg/g WS, respectively. The Pi in the liquid fraction of the WS was 0.7±0.01 mg/g WS, accounting for 70% of the

Table 1 The distribution of phosphate P and phytate P in the liquid and solid fractions of the LSW and WS samples

Sample description	Total P [mg P/g sample]	Phytate P [mg P/g sample]	Phosphate P [mg P/g sample]
LSW	5.1±0.04	4.0±0.01	1.2±0.01
WS	1.3±0.02	NA	0.7±0.01
Liquid fraction of WS	1.0±0.06	NA	0.7±0.01
Solid fraction of WS	0.3±0.04	NA	NA

The data are mean value of two independent experiments. All values are based on wet biomass

WS whole stillage, *NA* not available

total P in the liquid fraction or 54% of the total P in WS. The remainder of phosphorous in the liquid fraction of the WS was mainly attributed to phytate P, mostly in form of $InsP_1$ and with trace amounts of $InsP_3$ and $InsP_2$ isomers.

Enzymatic Hydrolysis of Phytates

Hydrolysis experiments were carried out to examine the effect of PhyA enzyme on phytates in LSW as described earlier in the "Material and Methods" section. The time dependency of phytate degradation was analyzed by HPLC, and the formation of the inositol phosphate isomers was identified based on a reference chromatogram by Chen et al. [34]. The chromatograms for the hydrolysis of phytates in LSW, subjected to 1 FTU/g LSW, 35 °C, and pH 4.3, is presented in Fig. 1. As is shown by the chromatogram marked 0 h in this figure, $InsP_6$ was the main component of phytates in LSW, accompanied by a small amount of DL-Ins(1,2,4,5,6)P_5 and trace amount of DL-Ins(1,2,3,4,5)P_5. The analysis of the formed intermediate components in this figure suggests to a stepwise mechanism for the dephosphorylation of the phytates in LSW ($InsP_6$) via the formation of $InsP_5$, $InsP_4$, $InsP_3$ and $InsP_2$. During the first 1 h of the incubation, a marked decrease of $InsP_6$ content of the reaction mixture was observed, with concomitant increases of DL-Ins(1,2,4,5,6)P_5 and DL-Ins(1,2,5,6)P_4 content. Small amounts of DL-Ins(1,2,3,4,5)P_5, DL-Ins(1,3,4,5)P_4, DL-Ins (1,2,4,5)P_4, and trace amount of DL-Ins(2,4,5,6)P_4 were also observed at this time. The formation of DL-Ins(1,2,4,5,6)P_5 as the major $InsP_5$ isomer confirmed that *A. niger* initiates its attack on phytate at the 3 position [22]. As the incubation time was increased, the quantities of DL-Ins(1,2,4,5,6)P_5, DL-Ins(1,2,3,4,5)P_5, DL-Ins(1,2,5,6)P_4, DL-Ins(1,3,4,5) P_4, DL-Ins(1,2,4,5)P_4, and DL-Ins(2,4,5,6)P_4 decreased and eventually disappeared, while DL-Ins(1,2,6)P_3, DL-Ins(1,4,5)P_3, and DL-Ins(2,4,5)P_3 appeared as the major degradation products in the mixture. After 6 h incubation, $InsP_2$ isomers appeared as the main phytate components. These isomers include DL-Ins(1,2)P_2, DL-Ins(1,4)P_2, DL-Ins(2,4)P_2, DL-Ins (2,5)P_2, and DL-Ins(4,5)P_2 and are identified by peaks 2–4 in Fig. 1. Their further degradation was observed with prolonged incubation. After 8 h incubation, no isomers of phytates were observed in the chromatogram; however, the presence of Ins(2)P_1 is suspect as documented by other researchers [21, 32]. $InsP_1$ isomers were not separated in the analytical procedure which was followed in this study [34].

Effect of Enzyme Loading

Experiments were performed to examine the effect of enzyme loading on the hydrolysis of phytates in LSW and WS samples. In these experiments P in the form of phosphates (Pi) was quantified according to the method described earlier in the method section and was recognized

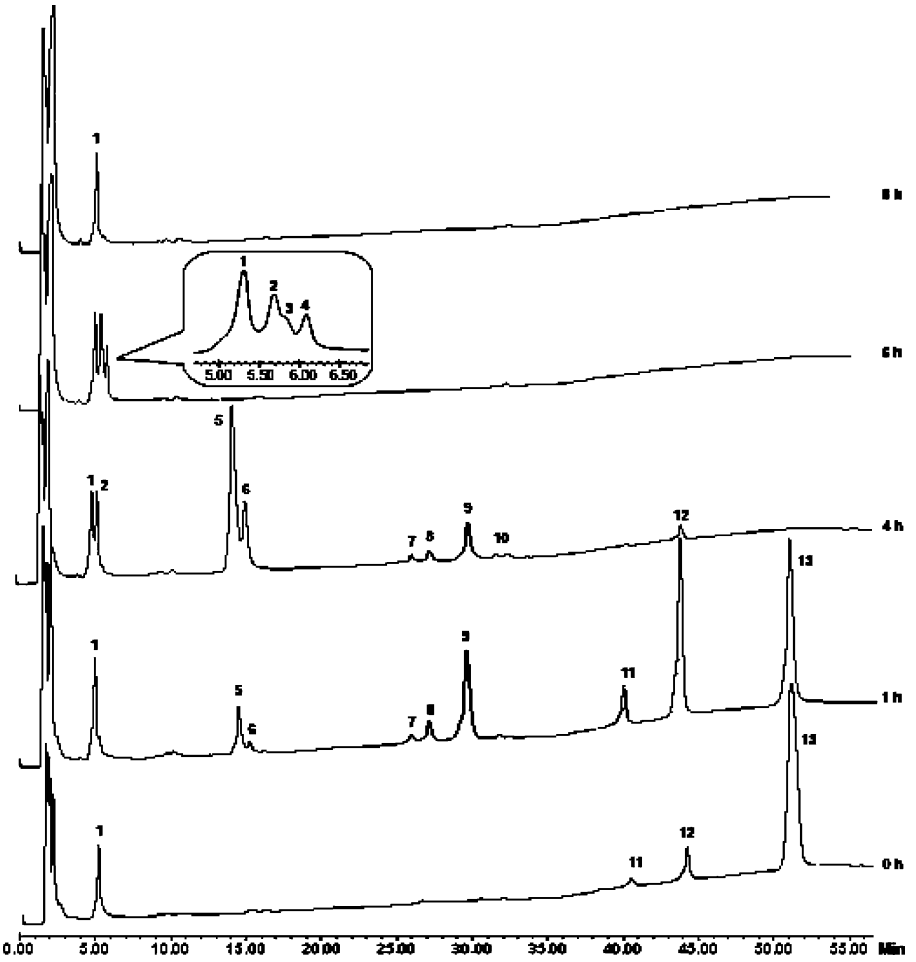

Fig. 1 Chromatogram of the hydrolysis of phytate in LSW at 35 °C, pH 4.33 and 200 rpm of shaker speed. Peaks: (*1*) unknown peak; (*2*) DL-Ins(1,2)P_2; (*3*) DL-Ins(2,4)P_2, DL-Ins(1,4)P_2; (*4*) DL-Ins(2,5)P_2, DL-Ins (4,5)P_2; (*5*) DL-Ins(1,2,6)P_3; (*6*) DL-Ins(1,4,5)P_3, DL-Ins(2,4,5)P_3; (*7*) DL-Ins(1,2,4,5)P_4; (*8*) DL-Ins (1,3,4,5)P_4; (*9*) DL-Ins(1,2,5,6)P_4; (*10*) DL-Ins(2,4,5,6)P_4; (*11*) DL-Ins(1,2,3,4,5)P_5; (*12*) DL-Ins(1,2,4,5,6) P_5; and (*13*) $InsP_6$

as an indicator of the extent of the hydrolysis of phytates [12, 35]. For LSW, the extent of hydrolysis was quantified by the increase in the amount of Pi compared to the initial amount of Pi. The amount of Pi in LSW, which was attributed to slight hydrolysis during the steeping process, was also considered in the determination of the extent of hydrolysis reaction.

Enzyme loadings at 0.1, 1, and 2 FTU/g substrates were investigated for the hydrolysis of phytates in LSW. The experimental results quantifying the amount of released Pi from phytates as a function of time and otherwise identical conditions of 35 °C and pH 4.3 are presented in Fig. 2. Under the performed hydrolysis conditions, a monotonic increase in the amount of released Pi was observed during the first 6 h of incubation. After this initial period, the amount of released Pi leveled off and additional incubation time appeared to have very little effect on the amount of formed Pi. Figure 2 reveals for the fastest rate of hydrolysis to correlate with the highest enzyme loading. At 2 FTU/g LSW, the plateau in

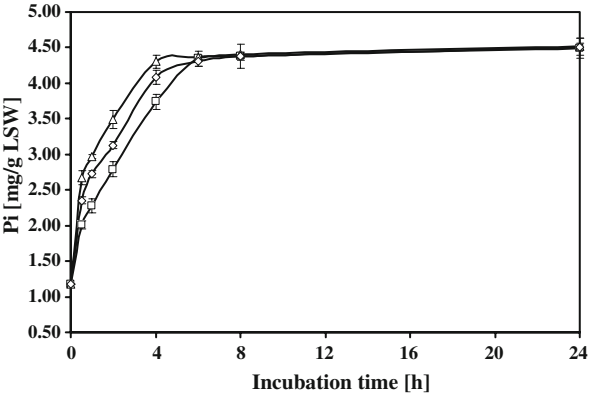

Fig. 2 The effect of enzyme loading on the extent hydrolysis of LSW at 35 °C, pH 4.33 and 200 rpm of shaker speed (*unfilled square*) 0.1 FTU/g LSW; (*unfilled diamond*) 1 FTU/g LSW; (*unfilled triangle*) 2 FTU/g LSW

the formation of Pi was reached after about 4 h, whereas it took more than 6 h for this point to be reached at 0.1 FTU/g LSW. The maximum amount of Pi was 4.52±0.03 mg/g LSW after 8 h incubation. This was reflective of an increase in the amount of Pi from 24% to 90 wt.% based on the total amount of P in the LSW. This level of Pi formation also was indicative of the release of about 83.5% of phytate in Pi form during this process. As was discussed in the previous section, the remaining phytates are believed to be mostly in form of $InsP_1$ [21, 36]. This limitation is suggested to be due to the incapability of the enzyme to degradate $Ins(2)P_1$. $Ins(2)P_1$ as the end product is blamed on the structure of phytic acid. In phytic acid, five of the six phosphate groups are in equatorial position which can be released from the inositol ring easily with the PhyA enzyme, while the 2-phosphate group is in an axial position which is refractory to the hydrolysis with this enzyme [21, 36].

Enzyme loadings with activities of 0.1, 1, 2, and 4 FTU/g WS were investigated for the hydrolysis of phytates in WS. Figure 3 summarizes the results which show an increasing trend similar to that of LSW for the formation of Pi from phytates in WS. The most effective period of hydrolysis was during the first 2 h where a monotonic increase in the amount of released Pi was observed. After this initial period, the amount of released Pi leveled off, and very little hydrolysis was detected afterward. Under hydrolysis conditions of 35 °C and at pH 4.85, the maximum amount of released Pi was detected at 0.86±0.01 mg/g WS at enzyme loadings of 1, 2, and 4 FTU/g WS. During the process, the Pi in the WS increased from 0.7±0.01 mg/g WS (54% of total P in WS) to 0.86±0.01 mg/g WS (66% of total P in WS). The amount of Pi

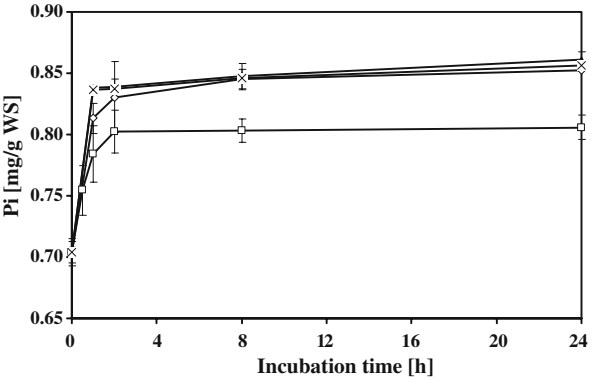

Fig. 3 The effect of enzyme loading on the extent hydrolysis of WS at 35 °C; pH 4.85 and 200 rpm of shaker speed. (*unfilled square*) 0.1 FTU/g WS; (*unfilled diamond*) 1 FTU/g WS; (*unfilled triangle*) 2 FTU/g WS; (*x mark*) 4 FTU/g WS

Table 2 Time course of phosphate release from liquid and solid fraction of WS

Incubation time [h]	Pi released from WS [mg/g WS]	Pi released from liquid fraction [mg/g WS]	Pi released from solid fraction [mg/g WS]
0	0.70±0.01	0.70±0.01	0.00±0.01
0.5	0.80±0.01	0.76±0.02	0.04±0.01
1	0.83±0.02	0.75±0.03	0.07±0.01
2	0.84±0.01	0.76±0.01	0.08±0.01
8	0.85±0.02	0.76±0.01	0.09±0.01
24	0.85±0.01	0.78±0.01	0.07±0.01

Temperature 45 °C; pH 4.85; enzyme loading: 4 FTU/g substrate. The data are mean value of two independent experiments

released in the hydrolysis mixture with the smallest enzyme loading of 0.1 FTU/g WS was at 0.81±0.02 mg/g WS. A comparison between the hydrolysis of phytates in LSW and WS showed a much higher overall conversion of phytates in LSW which is suggestive of a different behavior between the two substrates. The higher conversion of phytates to Pi in LSW may be attributed to the fact that more than 75% of P in this substrate was in the form of soluble phytates (Table 1), which were readily available and were hydrolyzed by the PhyA enzyme (Fig. 2). The distribution of P in the WS samples was considerably different from LSW. As shown in Table 1, more than 75% of the total amount of P was in the liquid fraction of WS substrate with 70% of it in form of Pi. This suggested to an extensive hydrolysis of phytates in this substrate during the course of the corn dry grind process. P in the liquid fraction of WS was predominantly in Pi form (70%) with the balance containing mostly $InsP_1$ and traces of $InsP_2$ and $InsP_3$ isomers with no appreciable amount of $InsP_4$–$InsP_6$ isomers. As the distribution of the Pi released from the liquid and solid fractions of WS indicates (Table 2), there was equal contribution from the liquid and solid fractions of the WS to the hydrolysis reaction. This contribution was at about 25% of the unhydrolyzed P in the two fractions after 24-h reaction. The low level of hydrolysis in the liquid fraction is mainly because of the low level of phytates in this stream. As was indicated earlier, most of the P in the liquid fraction was in Pi form and very little of $InsP_2$ and $InsP_3$ were available to be hydrolyzed. The slow rate of hydrolysis of phytates in the solid fraction of the WS may be attributed to the diffusional limitations and lack of their availability to the active sites of the PhyA enzyme. The deactivation of PhyA enzyme due to its adsorption to the solid particles may also have played a role in lower conversion of phytates in the WS samples. To

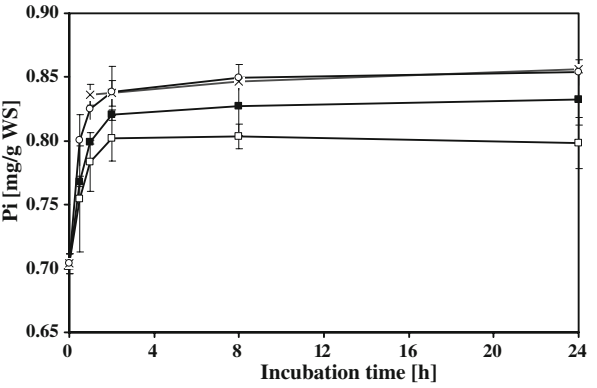

Fig. 4 The effect of incubation temperature on the extent of hydrolysis of WS at pH 4.85 and 200 rpm of shaker speed; (*unfilled square*) 0.1 FTU/g WS at 35 °C; (*filled square*) 0.1 FTU/g WS at 45 °C; (*x mark*) 4 FTU/g WS at 35 °C; (*unfilled circle*) 4 FTU/g WS at 45 °C

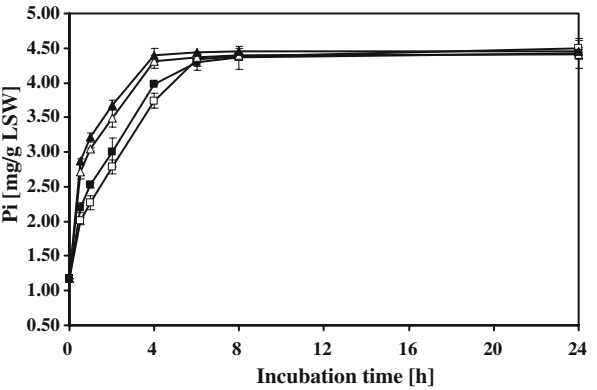

Fig. 5 The effect of incubation temperature on the extent of hydrolysis of LSW at pH 4.33 and 200 rpm of shaker speed; (*unfilled square*) 0.1 FTU/g LSW at 35 °C; (*filled square*) 0.1 FTU/g LSW at 45 °C; (*unfilled triangle*) 2 FTU/g LSW at 35 °C; (*filled triangle*) 4 FTU/gWS at 45 °C

investigate potential loss of enzyme activity in the WS samples, fresh enzyme at 0.1 and 1 FTU/g WS were added to WS sample which was initially subjected to hydrolyzed with 0.1 FTU/g WS enzyme for 2 h. As is shown in Fig. 6, a significant increase in the amount of Pi was observed which was indicative of enzyme deactivation possibly by adsorption to the solid surfaces during the course of the hydrolysis of WS. However, examination of this procedure at higher enzyme loadings did not result in the formation of any additional Pi, which suggests that potential deactivations are compensated for by the higher presence of enzyme at the 1, 2, and 4 FTU/g WS levels of enzyme loadings.

Effect of Temperature

Experiments were performed to examine the effect of temperature on the extent of the hydrolysis of WS and LSW with PhyA and the formation of Pi. Reactions were carried out at 35±1.0 and 45±1.0 °C. As expected and shown in Figs. 4 and 5, the experimental results confirmed an increasing trend in the formation of Pi as a function of time. The intensity of this trend correlated well with the temperature and enzyme loading. Examination of the results for the hydrolysis of WS (Fig. 4) reveals a monotonic increase in the formation of Pi from the inception of the reaction to 2 h into the reaction which then started to slow done and eventually leveled off at longer reaction times. The results show a significant fraction of P in the Pi form at the end of the experiment. For example, at the conclusion of the

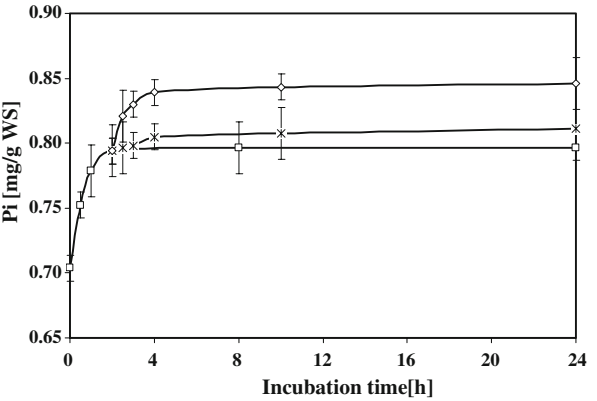

Fig. 6 Addition of PhyA after 2 h hydrolysis of WS at 35 °C, pH 4.85, and 200 rpm of shaker speed; (*unfilled square*) 0.1 FTU/g WS; (*x mark*) addition of 0.1 FTU/g WS; (*unfilled diamond*) addition of 1 FTU/g WS

hydrolysis reaction at 45 °C and 4 FTU/g WS of enzyme loading about 66% of the P was determined to be in the Pi form. Considering the initial form of the Pi in the WS of 54%, there was a 12% increase in the concentration of Pi. Experimental results for the hydrolysis of LSW revealed a similar trend as for the WS and are presented in Fig. 5. Nevertheless, a more significant part of the phytate P was released in the Pi form and a much longer reaction time was required for the reaction to complete. For example, at the conclusion of the hydrolysis of LSW at 45 °C and 4 FTU/g LSW of enzyme loading about 90% of the P was determined to be in the Pi form after 8-h reaction. This shows an increase of about 66% due to the hydrolysis reaction. When compared with WS, LSW showed a much higher level of phytate hydrolysis and higher levels of Pi formation which may be attributed to a much larger initial concentration of P in phytate form in LSW compared with the WS stream. The initial form of Pi in the WS was measured at 54% of the total P compared to about 24% for the LSW. Also the availability of the phytates in the liquid phase in LSW makes their hydrolysis more favorable compared to the WS for which about 20% of the P is in the solid fraction at the beginning of the reaction (Fig. 6).

Conclusions

The dephosphorylation of phytates in LSW and WS with PhyA from *A. niger* was investigated, which proceeded via the formation of $InsP_5$, $InsP_4$, $InsP_3$, and $InsP_2$ intermediates with Pi and $InsP_1$ as the end products. During the process, the amount of phosphate P in the substrates was increased from 54% to 66% in the WS, and from 20% to 90% in the LS, suggesting to a substantial dephosphorylation of the phytates in the LSW and WS via PhyA catalyzed hydrolysis. Furthermore, this finding also suggests that the phytates level in the CGF and DDGS could be largely reduced, since LSW and WS contribute to a significant portion of phytates in these products. The majority of the phytate P in the LSW was in the form of soluble $InsP_6$ and $InsP_5$, which were readily hydrolyzed by the PhyA enzyme. However, the phytate P in the WS exists as both soluble phytates in the liquid fraction (mainly in the form of $InsP_3$ and $InsP_2$) and insoluble phytates in the solid fraction. The phytates in the liquid fraction were readily hydrolyzed by the enzyme, while the phytates in the solid fraction were partly degraded. Diffusional limitations and lack of their availability to the active sites of the PhyA enzyme were blamed for the slow rate of degradation of phytates in the solid fraction of WS.

Variations in enzyme loading and temperature on the hydrolysis rate and extent of the dephosphorylation were also examined. The time dependency of Pi release showed similar trends for both substrates with an increase in the trend of degradation with increases in temperature and enzyme loading. The most effective period of degradation was during the first 2 h for WS and 6 h for LSW, and further increase of the reaction time had little or no effect on the total amount of the Pi released. Under the experimental conditions which were investigated, the optimum hydrolysis for both LSW and WS was at 1 FTU/g substrate and at 45 °C.

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