Effect of exogenous phytase on feed inositol phosphate hydrolysis in an in vitro rumen fluid buffer system

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ABSTRACT

Three in vitro experiments using a rumen fluid buffer system were performed to investigate the effect of addition of 4 experimental phytases (Phy1, Phy2, Phy3, and Phy4) compared with no addition of phytase on feed inositol phosphate hydrolysis in wheat and rapeseed cake to determine which of the 4 phytases was most suitable under rumen-like conditions. The feedstuffs were incubated with a mixture of physiological buffer, ruminal fluid, and exogenous phytase at pH 6.2, after which the samples were incubated for different periods. Incubations were stopped using HCl, and the samples were analyzed for inositol phosphates via high performance ion chromatography. Addition of phytase (Phy1) resulted in enhanced degradation of *myo*-inositol hexakisphosphate (InsP₆) in rapeseed cake, whereas addition of exogenous phytase did not improve the degradation of InsP₆ in wheat. Only rapeseed cake was therefore used subsequently. All 4 phytases increased degradation of InsP₆ in rapeseed cake in the in vitro system, and degradability of InsP₆ increased with higher incubation time and higher phytase dosages, independent of phytase. Addition of 2 units of phytase per gram of substrate of the phytases Phy1, Phy2, Phy3, and Phy4 led to an undegraded InsP₆ content of 56, 49, 70, and 18%, respectively, when incubated with rapeseed cake for 6 h, indicating that Phy2 and Phy4 were the most effective phytases. However, Phy2 had a higher specific activity than Phy4, as 60% of the original InsP₆ content was remaining after 3 h when 5 mg of enzyme protein per gram of substrate of Phy2 was added to rapeseed cake, whereas 150 mg of enzyme protein per gram of substrate of Phy4 was necessary to achieve a similar result. Therefore, Phy2 appeared to be most applicable under rumen-like conditions.

Key words: phosphorus, inositol phosphate, phytase, rumen

INTRODUCTION

Phosphorus (P) is an essential mineral nutrient for cattle, but environmental concerns are often linked to P (Sharpley and Winthers, 1994). Phosphorus intake and excretion in cattle are tightly linked (Morse et al., 1992a; Wu et al., 2000; Dou et al., 2003), and therefore even small improvements in the availability of feed P will improve whole-farm P balance and decrease potential P runoff from farms (Knowlton et al., 2004).

Phytate (*myo*-inositol hexakisphosphate, **InsP₆**) is the main storage form of P in cereals, legumes, and oilseeds (Nelson et al., 1968; Viveros et al., 2000), and about 60 to 80% of total P in cereals is found as InsP₆, whereas about 35 to 50% of total P in by-products such as soybean meal and rapeseed cake is found as InsP₆ (Eeckhout and De Paepe, 1994; Viveros et al., 2000). Roughages have a much lower content of InsP₆, and only traces have been found in alfalfa and different grasses (Nelson et al., 1976), as most roughage consists of stems and leaves. Phytase catalyzes the hydrolysis of InsP₆, producing *myo*-inositol pentakis-, tetrakis-, tris-, bis-, and monophosphates (**InsP₅** to **InsP₁**, respectively). Inorganic phosphate is released during the hydrolysis of inositol phosphate (Irving, 1980; Wodzinski and Ullah, 1996). Phytases can be categorized into 3 major classes: (1) histidine acid phosphatases, (2) β -propeller phytases, and (3) purple acid phosphatases (Mullaney and Ullah, 2003). The protein tyrosine phosphatase-like phytases found in bacteria (e.g., *Selenomonas ruminantium*) in the rumen constitute a fourth class of phytases (Nakashima et al. 2007).

Until very recently inositol phosphate was considered to be totally available to ruminants because of the above-mentioned phytase activity in the rumen (Clark et al., 1986; Morse et al., 1992b). However, studies have indicated that degradability of InsP₆ in the rumen can be negatively affected by processing of feedstuffs by heat or formaldehyde treatment (Konishi et al., 1999; Park et al., 1999; Bravo et al., 2000). Other studies have shown that the rumen degradation of InsP₆ is not complete (Kincaid et al., 2005; Sehested and Lund, 2007), and a recent experiment showed that the mi-

Received June 4, 2010.

Accepted November 1, 2010.

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crobial phytase activity in the rumen of lactating cows was limiting for degradation of feed inositol phosphate (Sehested and Lund, 2007). The experiment showed that the ruminal degradation of inositol phosphate was increased by supplementation of exogenous phytase. This agrees with the findings of Knowlton et al. (2007), who found that exogenous phytase reduced P excretion in dairy cows.

Exogenous phytase is extensively used as a feed additive for monogastric animals (Poulsen et al., 2007). The efficiency of phytase depends on pH in the digestive tract, among other factors, and because monogastric animals and cattle have different pH in the digestive tract, the types of phytase used as feed additive in monogastric animals may not necessarily be the most effective phytases in cattle. Different phytases may vary in their effect on hydrolysis of inositol phosphate under rumen-like conditions.

The aim of the present experiment was to investigate the effect of 4 different experimental phytases on *in vitro* ruminal degradation of inositol phosphate of feedstuffs.

MATERIALS AND METHODS

Feed type, incubation time, and concentration of phytase were used to evaluate 4 phytases, to determine which of the 4 phytases was most efficient under rumen-like conditions.

Phytases

The 4 experimental phytases (**Phy1** to **Phy4**) used were supplied by Novozymes A/S (Bagsvaerd, Denmark). Three of the phytases (Phy1, Phy2, and Phy3) were microbial histidine acid phosphatase phytases, and Phy4 was a β -propeller phytase. The activity of phytase is expressed in 2 ways: (1) phytase units (FTU) or (2) specific activity (FTU per amount of enzyme protein). One FTU is the amount of enzyme that liberates 1 μ mol of inorganic phosphate per minute from a 0.0051 M Na-phytate solution at pH 5.5 and 37°C (Engelen et al., 1994). Although the specific activities of Phy1, Phy2, and Phy3 were comparable, the specific activity of Phy4 was approximately 100-fold lower.

Phytase activity was measured at 37°C in the pH range of 2.0 to 7.5. A buffer cocktail (50 mM glycine, 50 mM acetic acid, and 50 mM Bis-Tris), with pH adjusted to the appropriate value with 4 M HCl or 4 M NaOH, was used for all pH values. In brief, 75 μ L of phytase-containing solution was mixed with 75 μ L of substrate [100 mg of Na-phytate from rice (cat. no. P0109, Sigma-Aldrich, St. Louis, MO) in 10 mL of buffer]. Samples were incubated with shaking in an Eppen-

Table 1. Content of DM, total inositol phosphate P (InsP-P), and *myo*-inositol hexakisphosphate P (InsP₆-P) as well as phytase activity in the wheat and rapeseed cake used in the present experiment

Item	Wheat	Rapeseed cake
DM (%)	90.4	91.3
InsP-P (mg/g of DM)	2.7	8.3
InsP ₆ -P (mg/g of DM)	2.4	7.3
Phytase (FTU/g of feed) ¹	403	0

¹One FTU is the amount of enzyme that liberates 1 μ mol of inorganic phosphate per min from 0.0051 M Na-phytate solution at pH 5.5 and 37°C (Engelen et al., 1994).

dorf thermomixer for 15 min at 37°C. Subsequently, 75 μ L of stop reagent [mix of 10 mL of molybdate solution (10% wt/vol ammonium hepta-molybdate in 0.25% wt/vol ammonia solution), 10 mL of ammonium vanadate (0.24% commercial product from Bie & Berntsen, cat. no. LAB17650; Bie & Berntsen, Herlev, Denmark), and 20 mL of 21.7% (wt/vol) nitric acid] was added to terminate the enzymatic reaction and to form a colored phosphovanadomolybdate complex. Absorbance at 405 nm was measured in a Molecular Devices (Berks., UK) micro-titer plate reader.

Feedstuffs

Wheat (not heat treated) and rapeseed cake (13.6% fat) were used as examples of grain and protein feeds, respectively. Content of DM, total inositol phosphate P, and InsP₆ P, as well as endogenous phytase activity, in the 2 feedstuffs are presented in Table 1. The feed samples were ground through a 1-mm screen by using a hammer mill. Feedstuffs were extracted in aqueous solution and analyzed for phytase activity (FTU) according to Engelen et al. (1994).

Experiment 1: Effect of Phytase Addition, Feedstuff, and Incubation Time

The experiment was a $2 \times 2 \times 4$ factorial design with 2 feedstuffs (wheat or rapeseed cake) incubated with or without phytase (Phy1 at 2 FTU/g of feed). Four incubation times (0, 3, 6, and 24 h) were evaluated and each combination was replicated 3 times.

Experiment 2: Effect of Phytase and Phytase Concentration

The experiment was a $5 \times 2 \times 3$ factorial design with 1 feedstuff (rapeseed cake) incubated without or with 1 of 4 experimental phytases (control, Phy1, Phy2, Phy3, or Phy4) at 2 concentrations of each phytase (1 or 2 FTU/g of feed). Based on experiment 1, 3 incubation

times (0, 3, or 6 h) were evaluated. Each combination was replicated 5 times.

Experiment 3: Effect of Phytase Type and Enzyme Protein Amount

The 2 best-performing phytases from experiment 2 were used in experiment 3. The experiment was a $2 \times 4 \times 3$ factorial design with 1 feedstuff (rapeseed cake) incubated without or with 1 of 2 phytases (control, Phy2, or Phy4) at 4 amounts of enzyme protein (5, 25, 50, or 150 mg of enzyme/g of feed). Three incubation times (0, 3, or 6 h) were evaluated. Each combination was replicated 5 times.

In Vitro Incubation

The in vitro incubation used was a modification of the 2-stage technique for the in vitro digestion of forage crops that, in the original version, determines the potential OM or DM digestibility of feedstuffs for cattle (Tilley and Terry, 1963). In this study, only stage 1 was followed. The feedstuff was incubated with a mixture of ruminal fluid and physiological buffer solution modified after Tilley and Terry (1963): Bis-Tris (0.036 M; Sigma-Aldrich B4429), NaHCO_3 (0.12 M), NaCl (0.008 M), KCl (0.008 M), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.0006 M), and $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.0004 M). All chemicals were of analytical grade.

Ruminal fluid was collected from 3 rumen-cannulated heifers maintained on a diet based on hay (66%) and concentrate (33%; DM basis). The ruminal fluid was strained through 2 layers of cheese cloth into preheated thermos bottles and kept at 38 to 39°C. The ruminal fluid was always collected immediately before use and the ruminal fluids from the 3 heifers were mixed in the proportion 1:1:1. The ruminal fluid was then mixed with a premade CO_2 -gassed physiological buffer solution in the proportion 1:5, and pH was kept at 6.2 at 40°C by addition of 2 M HCl. The pH (6.2) was lower than that used by Tilley and Terry (1963; pH 6.7 to 6.9), because the original phosphate buffer was replaced with a Bis-Tris buffer to avoid buffer P interference and to achieve a pH close to the ruminal pH found in production animals. Approximately 0.5 g of feed was placed in each tube and 50 mL of the mixture of ruminal fluid and buffer solution was added along with phytase.

Phytases dissolved in demineralized water were added to the tubes (0.5 mL in experiment 1 and 1.0 mL in experiments 2 and 3). Corresponding amounts of water were added to the control tubes. To obtain anaerobic conditions the tubes were gassed with CO_2 , sealed with a rubber cork, and incubated in a water bath at 40°C for 0, 3, 6, or 24 h. During incubation, samples were gently

shaken after 1.5, 4.5, and 7 h. Inositol phosphates were extracted from the in vitro samples following a slight modification of the method by Carlsson et al. (2001). In brief, 6 mL of 2 M HCl was added to each sample (50.5 mL in experiment 1 or 51 mL in experiments 2 and 3) and the resulting samples were mixed (500 rpm) for 3 h at 22°C interrupted by a freezing step. Subsequently, the samples were centrifuged ($1,800 \times g$, 4°C, 5 min) and the supernatants were recovered. The addition of 2 M HCl reduced pH to <1 and the extractant thus also served to inactivate the phytase.

Inositol Phosphate Analysis

Inositol phosphates were analyzed using a modification of the method by Skoglund et al. (1997) described in detail by Pontoppidan et al. (2007). Before analysis, 500 μL of the extract supernatant was placed in ultra-centrifugal filter devices (Microcon YM-30, Millipore, Billerica, MA) and filtered by centrifugation ($12,000 \times g$, 0°C, 30 min). The filtered sample supernatants were analyzed undiluted by high performance ion chromatography (Dionex Corp., Sunnyvale, CA), and inositol phosphates (InsP_6 to InsP_3) were detected after postcolumn reaction with 0.1% $\text{Fe}(\text{NO}_3)_3 \cdot 9\text{H}_2\text{O}$ in a 20 mL/L solution of HClO_4 , by UV absorbance at 290 nm. A reference sample for the identification of peaks was prepared by dissolving 0.5 g of phytic acid dodecasodium salt hydrate in 50 mL of 0.5 M HCl. The solution was autoclaved for 1 h at 124°C. Before analysis, the hydrolysate was diluted 3 times in water. Peaks were quantified according to an InsP_6 standard curve (0.01 to 1 mM InsP_6). The reference and standard samples were not extracted, being readily soluble, and also not filtered, as the 30-kDa membrane is not expected to retain any of the inositol phosphates. The detector response factors of the lower inositol phosphates are lower than that of InsP_6 because the number of attached phosphate groups that act with Fe^{3+} to give a UV response is lower. Theoretically, the response of InsP_X should be $X/6$ of that of the response of InsP_6 and a correction factor of $6/X$ should be used. This principle was confirmed experimentally by Skoglund et al. (1997). Therefore, the amounts of InsP_3 , InsP_4 , and InsP_5 were estimated in the current study using the theoretical correction factors of 2 ($= 6/3$), 1.5 ($= 6/4$), and 1.2 ($= 6/5$).

Statistical Analysis

Experiment 1. Data for inositol phosphates were analyzed using PROC MIXED (version 9.2, SAS Institute Inc., Cary, NC) and the response variables were expressed by a linear model. The variables in the analy-

Figure 1. Relative activities as a function of pH for the 4 experimental phytases used in the present study. Activity at optimum pH = 100.

ses were as follows: Y = dependent variable, μ = overall mean, R_i = replicate number ($i = 1, 2, 3$), F_j = feed (j = wheat, rapeseed cake), P_k = phytase addition (k = none, Phy1), T_l = incubation time ($l = 0, 3, 6, 24$), and ε_{ijkl} = residual error; interaction terms are represented as combinations of uppercase and subscript letters used to define the variables.

Effects of feed, phytase addition, and incubation time on the amount of InsP_6 , InsP_5 , InsP_4 , and InsP_3 and percentage of undegraded InsP_6 were evaluated with the model

$$Y_{ijkl} = \mu + \text{FPT}_{jkl} + \text{FP}_{jk} + \text{FT}_{jl} + \text{PT}_{kl} \\ + F_j + P_k + T_l + \varepsilon_{ijkl},$$

with the term R_i included in the random statement. All variables were treated as class variables. Significance of difference was declared when $P < 0.05$; Tukey's test was used to separate the means when effect of treatment was declared significant. Data are presented as least squares of means and associated standard error of the means.

Experiments 2 and 3. The statistical model used in experiment 1 was also used to analyze data in experiment 2 and 3, except that the class variables were different and the number of replications was increased from 3 to 5. The class variables used in experiment 2 were phytase (none, Phy1, Phy2, Phy3, and Phy4), concentration (1 and 2 FTU), and time (0, 3, and 6 h), and the class variables used in experiment 3 were phytase (none, Phy2, and Phy4), amount of enzyme protein (5, 25, 50, and 150 mg of enzyme protein), and time (0, 3, and 6 h).

RESULTS AND DISCUSSION

Phytases

The relative activity of the 4 phytases (Phy1, Phy2, Phy3, and Phy4) as a function of pH is shown in Figure 1. The activity values for each pH are expressed as a percentage of the value at optimum. Phytase 3 had the lowest optimum pH whereas Phy4 had the highest optimum pH (3.5 vs. 6.5). Phytase 4 seemed to have a less well defined optimum pH than the other phytases. Phytase 1 and Phy2 had more than 80% of their relative activity in a narrow pH range (3.7 to 4.8 and 4.2 to 6.0, respectively), whereas Phy3 and Phy4 had more than 80% of their relative activity in a broader pH range (2.7 to 5.0, and 5.0 to 7.5, respectively), indicating that Phy3 and Phy4 were less sensitive for pH changes than were Phy1 and Phy2.

Experiment 1

The first experiment investigated the effect of adding phytase to 2 different feedstuffs and the effect of incubation time. The content of inositol phosphate P in wheat and rapeseed cake with or without phytase (Phy1) incubated at different times is shown in Figure 2. Rapeseed cake had a higher content of InsP_6 compared with wheat, similar to the observation of Eeckhout and De Paepe (1994). A significant effect of the interaction between the factors feed type, incubation time, and phytase addition on the content of InsP_6 ($P < 0.0001$), InsP_5 ($P = 0.0003$), InsP_4 ($P < 0.0001$), and InsP_3 ($P < 0.0001$) was found. For both feedstuffs, degradation of InsP_6 depended on incubation time. The longer the incubation times the more InsP_6 was hydrolyzed; after 24 h of incubation, nearly all InsP_6 was found to be hydrolyzed in both feedstuffs irrespective of phytase addition. However, after 6 h of incubation all InsP_6 was already hydrolyzed in wheat independent of phytase addition, and degradation of inositol phosphate seemed to be much faster in wheat compared with rapeseed cake.

Addition of phytase increased the degradation of InsP_6 in rapeseed cake irrespective of incubation time, whereas addition of phytase did not have any effect in wheat (Figure 3). The interaction between the factors feed type, incubation time, and phytase addition was found to affect the percentage of undegraded InsP_6 ($P < 0.001$). The wheat used in the present study contained a much larger amount of natural phytase compared with rapeseed cake (403 vs. 0 FTU/g of feed), which agrees with other studies (Eeckhout and De Paepe, 1994), and this may explain why addition of an exogenous phytase had no additional effect on

Figure 2. Undegraded inositol P (InsP-P) content shown for the individual inositol phosphates (*myo*-inositol hexakis-, pentakis-, tetrakis-, and trisphosphates, InsP₆ to InsP₃) in wheat and rapeseed cake with or without phytase (Phy1) and incubation for 0, 3, 6, or 24 h. Least squares means and SEM of 3 determinations are shown.

degradation of InsP₆ in wheat. The rapid degradation of inositol phosphate in the wheat control sample compared with the rapeseed cake control sample can also be explained by the endogenous phytase activity in the wheat. Results indicate that a positive effect of adding exogenous phytase to a feed ration depends on which feedstuff the ration contains.

Degradation of inositol phosphates in the control samples could be caused by endogenous phytase activ-

ity in feeds and the ruminal fluid. Only wheat contained phytase activity (Table 1), whereas inositol phosphate degradation in the rapeseed cake control samples was also significant. Therefore, endogenous phytase activity must also have been present in the ruminal fluid. Ruminal phytase activity mainly originates from bacteria associated with the solid rumen fraction (Yanke et al., 1998). Ruminal fluid for this experiment was obtained by straining of ruminal samples through cheese cloth,

Figure 3. Percentage of undegraded *myo*-inositol hexakisphosphate (InsP₆) as a function of incubation time (h) in wheat (A) and rapeseed cake (B) with no phytase added (control) or with phytase (Phy1). Least squares means and SEM of 3 determinations are shown.

Figure 4. Percentage of undegraded *myo*-inositol hexakisphosphate (InsP₆) as a function of incubation time (h) in rapeseed cake with 1 FTU (phytase unit)/g of feed (A) or 2 FTU/g of feed (B) of 1 of 4 phytases (Phy1, Phy2, Phy3, Phy4) or none (control). Least squares means and SEM for 5 determinations are shown.

and it is not clear to what extent the observed inositol phosphate degradation in the rapeseed cake control samples reflect the *in vivo* condition.

It is clear from the present study (Figure 2) that degradation of InsP₅, InsP₄, and InsP₃ starts before InsP₆ is depleted. The lower inositol phosphates seemed to accumulate when InsP₆ was degraded (Figure 2), indicating competition for different inositol phosphate substrates by the phytase. Earlier studies have shown that InsP₆ is the preferred substrate over InsP₅ and InsP₄ (Frias et al., 2003; Pontoppidan et al., 2007), and the present study indicates that degradation of InsP₆ is the limiting step for complete degradation of inositol phosphate. It seems that when InsP₆ was hydrolyzed, the rest of the inositol phosphates (InsP₅ to InsP₃) followed soon after. Therefore, only data presenting the degradation of InsP₆ will be shown for experiments 2 and 3.

Based on the results from the first experiment, incubation times of 3 and 6 h were chosen for experiments 2 and 3. Rapeseed cake was chosen to be the only feedstuff in the subsequent experiments because it contained a higher amount of InsP₆ and because addition of phytase in non-heat-treated wheat did not have an additional effect on the degradability of InsP₆.

Experiment 2

The second experiment compared 4 phytases and investigated the effect of the concentration (FTU/g of feed) of these phytases on degradation of InsP₆ in rapeseed cake incubated *in vitro*. A significant interaction was observed between the factors incubation time, phytase addition, and phytase concentration on InsP₆ content ($P = 0.027$) and percentage of undegrad-

ed InsP₆ ($P = 0.027$). The percentage of undegraded InsP₆ as a function of incubation time in rapeseed cake when 1 of 4 phytases was added is presented in Figure 4. Results showed that higher incubation time leads to higher degradation of InsP₆ in rapeseed cake, which confirms the findings in the first experiment. The addition and concentration of phytase also had an effect on the degradation of InsP₆. The different phytases degraded rapeseed cake inositol phosphate to different extents. At similar phytase unit dosages (FTU/g of feed), Phy4 most efficiently reduced the level of InsP₆ and Phy2 was found to be second best, and Phy3 was least efficient. Addition of 2 FTU/g of feed of the phytases Phy1, Phy2, Phy3, and Phy4 after 6 h of incubation led to an undegraded InsP₆ content of 56, 49, 70, and 18%, respectively. These results are not surprising when the pH curves (Figure 1) for the 4 phytases are taken into consideration. Phytases Phy2 and Phy4 had their optimum of activity at a higher pH than the other 2 phytases, and this higher pH is more similar to that found in the forestomachs of cattle. Using Ca-phytate and *in vitro* rumen fermentation, Raun et al. (1956) found that the optimal pH was 5.5 for phytate hydrolysis by ruminal microbes.

Moreover, addition of 2 FTU/g of feed led to greater hydrolysis of InsP₆ compared with addition of 1 FTU/g of feed, regardless of which phytase was added ($P < 0.0001$). When 2 FTU/g of feed of each phytase was added, the InsP₆ contents were 4.1, 3.6, 5.1, and 1.3 mg/g of DM after 6 h of incubation compared with 4.6, 3.9, 5.5, and 2.0 mg/g of DM when 1 FTU/g of feed of the 4 phytases (Phy1, Phy2, Phy3, and Phy4) was added.

Phytases Phy2 and Phy4 were chosen to be further examined in a subsequent experiment because they

Figure 5. Percentage of undegraded *myo*-inositol hexakisphosphate (InsP₆) as a function of incubation time (h) in rapeseed cake with no phytase, phytase 2 (Phy2), or phytase 4 (Phy4) at 1 of 4 concentrations: 5 (A), 25 (B), 50 (C), or 150 (D) mg of enzyme protein/g of feed. Least squares means and SEM for 5 determinations are shown.

were found to be most effective on degradation of InsP₆ at given concentrations of phytase units.

Experiment 3

In experiment 2, the phytases were compared at given concentrations of phytase units. In experiment 3, Phy2 and Phy4 were dosed based on amount of enzyme protein per gram of feed, because the specific activity for the phytases was different (i.e., the amount of enzyme needed to obtain a phytase unit). The amount of enzyme protein varied (5, 25, 50, or 150 mg of enzyme protein/g of feed), and Figure 5 shows undegraded InsP₆ content when the 2 phytases were added. Significant effects of the interaction between the factors incubation time, amount of enzyme protein, and phytase on InsP₆ content ($P = 0.0041$) and on percentage of undegraded InsP₆ ($P = 0.0041$) were found. Figure 5 confirms that hydrolysis of InsP₆ depended on incubation time independently of choice of phytase (4.5 vs. 2.6 for Phy2

and 6.3 vs. 5.3 for Phy4 at 3 and 6 h of incubation and concentration 5 mg of enzyme protein/g of feed). Figure 5 also shows that a higher concentration of enzyme protein led to higher hydrolysis of InsP₆ regardless of which of the 2 phytases was added to rapeseed cake. After 6 h of incubation, the undegraded InsP₆ contents were 36 and 73% at 5 mg of enzyme protein/g of feed versus 14 and 31% at 150 mg of enzyme protein for Phy2 and Phy4, respectively. However, it seems that the marginal degradation of InsP₆ declined when the concentration of enzyme increased. Figure 6 presents the percentage of undegraded InsP₆ as a function of concentration (mg of enzyme protein/g of feed), and it is clear that addition of higher concentrations of Phy2 did not lead to a higher degradation of InsP₆ (17 vs. 14% after 6 h for the concentrations 50 and 150 mg of enzyme protein).

Addition of Phy2 increased the degradation of InsP₆ relative to addition of Phy4 on a milligram to milligram basis (Figure 5). This trial clearly revealed that a higher

Figure 6. Percentage of undegraded *myo*-inositol hexakisphosphate (InsP₆) in rapeseed cake as a function of concentration (mg of enzyme protein/g of feed) of exogenous phytase (Phy2 or Phy4) at 3 h (A) or 6 h (B) of incubation. Least squares means and SEM for 5 determinations are shown.

amount of Phy4 enzyme protein is necessary to obtain the same effect as Phy2. Figure 5 shows that approximately 60% of the original InsP₆ was left after 3 h when 5 mg of enzyme protein of Phy2 was added to rapeseed cake, whereas 150 mg of enzyme protein was necessary to achieve a similar result for Phy4. This means that the amount of Phy4 must be 30 times higher than Phy2 to obtain the same effect, and therefore Phy4 may not be suitable for practical application. This leads to the recommendation that Phy2 is the most promising phytase used in the present *in vitro* experiment.

Use of exogenous phytase in dairy production is at present very limited. Our study indicates that exogenous phytase can enhance ruminal inositol phosphate degradation and thereby increase P availability and permit a lower allocation of P in the diet. However, it is not clear to what extent the observed inositol phosphate degradation in the present *in vitro* study reflects the *in vivo* situation, and further investigations regarding the effect of exogenous phytase allocated *in vivo* are needed.

CONCLUSIONS

The effect of adding exogenous phytase on inositol phosphate hydrolysis in feedstuffs *in vitro* depends on the type of feed, incubation time, concentration of phytase, and type of phytase. In non-heat-treated wheat, inositol phosphate was rapidly degraded in the control sample and no additional effect of phytase (Phy1) was observed in this case. Addition of each of the 4 experimental phytases used in the experiment increased degradation of inositol phosphates in rapeseed cake, but Phy2 and Phy4 were shown to be the most

effective. However, much less enzyme protein of Phy2 was needed to obtain the same effect as achieved with Phy4 and thus Phy2 appears most promising under rumen-like conditions.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Inger Østergaard (Aarhus University, Tjele, Denmark) and Daisy Hilbrands (Novozymes A/S, Bagsvaerd, Denmark) for their skillful technical assistance. Funding for the present study was provided by the Danish Food Industry Agency (no. 3412-08-02257), Novozymes A/S (Bagsvaerd, Denmark) and Faculty of Agricultural Sciences, Aarhus University (Tjele, Denmark).

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