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Methodological Aspects of Measuring Phytase Activity and Phytate Phosphorus Content in Selected Cereal Grains and Digesta and Feces of Pigs

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This study was to examine the time course of sample-specific linearity of intrinsic phytase hydrolysis in major cereal grains and in ileal digesta and fecal samples and to determine the time course of the microbial phytase-catalyzed hydrolysis of various sources of phytate for estimating phytate phosphorus (P) content. The intrinsic phytase activity in barley, corn, oat, and wheat samples was measured over multiple time points from 0 to 120 min at 1.5 mmol·L^{-1} of sodium phytate at pH 5.5 and 37 °C. Time courses of hydrolysis of purified phytate and phytate associated with the cereal grain samples and the pig digesta and fecal samples were examined with the Natuphos microbial phytase over multiple time points from 0 to 48 h of incubation. The intrinsic phytase hydrolysis was linear ($P < 0.05$) for up to 120 min for the barley, corn, and wheat samples, whereas in the oat sample the hydrolysis was linear ($P < 0.05$) for only up to 30 min of incubation. The intrinsic phytase activities (phytase unit: μmol·kg^{-1} of dry matter·min^{-1}) for the barley, corn, and wheat samples were estimated to be 693, 86, and 1189 by linear regression analysis. Intrinsic phytase activity (412 phytase units) for the oat sample based on a 30-min incubation was considerably higher than the value (103 phytase units) determined from the 120-min incubation for the same oat sample. There were quadratic with plateau relationships ($P < 0.05$) between the hydrolytic release of inorganic P from various sources of phytate and the incubation time. The minimal incubation times required for the complete hydrolysis of phytate were estimated to be 4, 3, and 11 h for the purified phytate, the cereal grain samples, and the pig digesta and feces, respectively. It was concluded that multiple time point experiments need to be conducted to determine valid intrinsic phytase activity and phytate P content in samples through intrinsic and microbial phytase hydrolysis incubations.

KEYWORDS: Intrinsic phytase activity; phytate phosphorus; cereal grains; pigs

INTRODUCTION

Vegetal ingredients are one of the major sources of dietary phosphorus (P), and a large proportion of the total P in plant ingredients is phytate P (*1*). Phytase hydrolysis of phytate is the rate-limiting step in the digestive utilization of vegetal sources of P in pigs, as demonstrated by Golovan et al. (*2*). Phytase is found in many sources including vegetal ingredients (*1*), feed additives (*3*), microbes in the lumen of the gastrointestinal tract of animals (*4*−*6*), and gut mucosa of some animal species (*7*).

Methodological aspects of the phytase activity assay are an essential issue in P nutrition (*7*, *8*). Two major approaches are used in phytase activity assays including the one time point method (*8*) and the multiple time point linearity analysis method (*7*). The one time point method measures phytase activity over one period of time, for exampoe, 1 h of incubation, by assuming a constant velocity of inorganic P hydrolysis under a given assay condition (*9*, *10*). The linearity analysis of multiple time point incubations is also used for measuring phytase activity (*7*). How sources of phytase affect the suitability of the two phytase activity assay methods is unclear.

Several methods have also been developed to measure phytate P content in samples including the precipitation method (*11*−*13*), the high-performance liquid chromatography method (*14*−*16*), the ion-exchange chromatography method (*17*), and the phytase incubation method (*18*). The precipitation and the high-performance liquid chromatography methods analyze total phytate P content by measuring total phytic acid content and

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assuming the inositol phosphate to be associated with six phosphate groups (*17*). The ion-exchange chromatography method allows the analysis of various isomers of the inositol phosphates with one to six phosphate groups (*17*). Nevertheless, an application of the ion-exchange chromatography method may be limited by its relatively complicated procedures and the commercial supply of purified phytate isomers serving as standards. Although intrinsic plant phytase may be limited in complete hydrolysis of phytate P, microbial phytase is shown to completely dephosphorylate phytate with prolonged incubations (*19*, *20*). Thus, microbial phytase incubation can be a convenient and reliable method for measuring total phytate P content. However, there are fewer literature reports on how sources of phytate affect the time course and the minimal incubation time required for the complete microbial phytase hydrolysis of phytate.

Therefore, the objectives of this study were to examine the time course of sample-specific linearity of intrinsic phytase hydrolysis in major cereal grains and to investigate the time course of the microbial Natuphos phytase-catalyzed hydrolysis of various sources of phytate for estimating the phytate P content in biological samples.

MATERIALS AND METHODS

Principles of Determination. The method of Eeckhout and De Paepe (*9*) was adopted for the phytase activity assay. Phytase activities in samples were determined by linear analysis of the amount of inorganic phosphate released from sodium phytate over multiple time points during a 2-h incubation period. The total amount of P released at each time point was calibrated for nonspecific P contribution that was not due to phytase hydrolysis. The phytase activity unit (FTU) was then defined as the net release of 1 μmol of phosphate from 1.5 mmol·L^{-1} sodium phytate per minute and per unit of solid (kg) and/or liquid (L) samples under in vitro incubation conditions of pH 5.5 and 37 °C.

The analysis of phytate P was based on the direct hydrolysis of phytate in samples by microbial phytase. Under the provision of abundant phytase, inorganic phosphate groups bound to the inositol ring in the sample will be sequentially released (*21*). Microbial phytase is very potent in this hydrolysis (*22*). The ample supply of microbial phytase with prolonged incubations will render the complete release of P from phytate moieties (22). The effectiveness and complete hydrolysis can be examined by monitoring the time course of phytate hydrolysis and recovery from sodium phytate purified from rice (Sigma-Aldrich, St. Louis, MO). The total amount of phosphate released was calibrated for the nonspecific P contributions from both test samples and the phytase enzyme preparation. The amount of phytate P is therefore measured.

Sources and Preparation of Samples. Feed grade grain samples of barley, corn, oats, and wheat used in the present study were obtained from a local commercial feed-ingredient supplier with one sample for each grain. Distal ileal digesta and fecal samples were collected from grower pigs fed corn-based semipurified diets that contained 180 g·kg^{-1} of the selected corn sample. Digesta sampling and drying procedures had been previously described (*23*). All samples were finely ground through a 0.5-mm mesh screen.

Assay Solution Preparations. Sodium acetate buffer (0.1 mol·L^{-1}) was prepared by dissolving 8.203 g sodium acetate ($NaC_2H_3O_2$, anhydrous, Sigma-Aldrich) into 900 mL of distilled and deionized water. It was then brought to a volume of 1 L and adjusted to pH 5.5 with acetic acid (100%) and 0.1 mol·L^{-1} sodium hydroxide solution.

Sodium phytate substrate solution at the concentration of 1.5 mmol·L^{-1} was prepared by dissolving 1.428 g sodium phytate (dodecasodium salt extracted from rice, $C_6H_6O_{24}P_6Na_{12}$, 97% purity, Sigma-Aldrich) into 900 mL of 0.1 mol·L^{-1} sodium acetate buffer. The solution was then brought up to the volume of 1 L and adjusted to pH 5.5 with acetic acid (100%) and 0.1 mol·L^{-1} sodium hydroxide solution.

A liquid microbial phytase preparation (Natuphos 10,000L, BASF Canada, Georgetown, ON) that contained 1075 FTU·mL^{-1} was used in the phytate P assay. Proposed phytate degradation recovery tests were conducted with two sodium phytate buffers (0.066 and 0.126 mmol·L^{-1} sodium phytate at pH 5.5). The phytate buffers were prepared by dissolving 63 and 120 mg of the sodium phytate into 900 mL of 0.1 mol·L^{-1} sodium acetate solutions, respectively. The solutions were then brought up to the volume of 1 L and adjusted to pH 5.5 with acetic acid (100%) and 0.1 mol·L^{-1} sodium hydroxide solution. The TCA (trichloroacetic acid at 0.92 mol·L^{-1}) solution was prepared by dissolving 75.0 g of crystalline TCA into 425 mL of distilled and deionized water.

Enzyme Activity Assay. Samples of ~0.4−0.6 g were weighed into 250-mL Erlenmeyer flasks. The amount of samples used was dependent on their potential phytase activity, with more for those of lower phytase activity such as corn and oats and less for those of higher activity such as wheat and barley, to measure a significant amount of inorganic P released from phytase hydrolysis. All tests were conducted in triplicate.

The TCA stop solution (15 mL at 0.92 mol·L^{-1}) was added to a set of flasks with samples and followed by adding 85 mL of the 0.1 mol·L^{-1} sodium acetate buffer at pH 5.5 to calibrate background P contributions in the test samples. The flasks were swirled well, and 1 mL of the mixture from each of the flasks was transferred for the measurement of the nonspecific P contributions at time zero.

The sodium phytate substrate solution (100 mL of the 1.5 mmol·L^{-1} at pH 5.5) was added to each of the incubation flasks to initiate the intrinsic phytase activity assays. The flasks were then incubated in a water bath (SW-20D, Julabo USA Inc., Kutztown, PA) at 37.0 ± 0.1 °C with a shaking speed at 120 rpm. Aliquots (1 mL) of the incubated mixture from the incubation flasks were transferred into a culture tube (16 × 100 mm) at 10, 30, 60, 90, and 120 min of incubations, respectively. The TCA stop solution (1 mL at 0.92 mol·L^{-1}) was immediately added into the culture tubes containing the sampled incubation mixtures. These culture tubes were shaken well and were then centrifuged (IEC Centra CL3R with rotor 243, IEC, MA) at 2000*g* for 15 min. Inorganic P contents in the supernatant were measured with a spectrophotometer (Pharmacia LKB Biotechnology, Uppsala, Sweden) according to an established method (*24*).

The net release of P was calculated after calibration of the nonspecific P contributions at time zero. The net release of P was plotted against incubation time points by using the Figure P program (Figure P, 1993, Biosoft, Cambridge, U.K.). The slope of a linear response was defined to be the intrinsic FTU of the samples expressed as micromoles of inorganic phosphate released per minute from 1 kg of dry matter (DM) samples.

Phytate P Recovery from Purified Sodium Phytate. The two sodium phytate buffers (100 mL of the 0.066 and 0.126 mmol·L^{-1} sodium phytate) were added to each of the 250-mL Erlenmeyer flasks that contained 0.4 mL of the Natuphos 10,000L enzyme preparation. The flasks were then incubated in a water bath at 37.0 °C with a shaking speed at 120 rpm. Aliquots of the incubated mixture (1 mL) were removed for measurement of the total amount of inorganic P released at 0.5, 1, 2, 4, 8, 16, and 24 h of incubations, respectively. The net release of inorganic P from the purified sodium phytate upon hydrolysis by the microbial phytase was calculated with the correction of background P contribution from the enzyme preparation. Recovery rates were calculated as the percentage of inorganic P recovered from the total amount of P added as sodium phytate.

Phytate P Assay for Samples. Ingredient samples of ~0.5 g were precisely weighed (0.4 g for digesta and 0.35 g for feces) into 50-mL centrifugation tubes. An ice-cold incubation buffer (8 mL at 0.1 mol·L^{-1} sodium acetate at pH 5.5) was added to each tube. The sample mixtures were homogenized (PowerGen 700 D homogenizer, model ES-115, Fisher Scientific, Pittsburgh, PA) at 10000 rpm for 2 min in an ice-cold environment. The homogenizer blade was rinsed with 7 mL of the 0.1 mol·L^{-1} sodium acetate buffer (pH 5.5). The homogenized sample was quantitatively transferred into a 250-mL Erlenmeyer flask, and 85 mL of the ice-cold incubation buffer (0.1 mol·L^{-1} sodium acetate at pH 5.5) was added to the flask.

The content in the flask was well mixed, and 1 mL of the mixture was transferred into a centrifugation tube that contained 1 mL of the

0.92 mol·L^{-1} TCA stop solution. The P content measured in the sample was regarded as the amount of nonspecific P content at time zero of incubations.

After sampling for the measurement of nonspecific P content, the Natuphos 10,000L enzyme preparation (0.4 mL per flask) was added into each of the flasks that contained the samples and the buffer. The flasks were covered with aluminum foil and incubated in a water bath at 37 °C with a shaking speed at 120 rpm. Aliquots (1 mL) of the incubated mixture were transferred for measuring the total hydrolytic release of inorganic phosphate at the time points of 1, 2, 3, 5, 8, 16, 24, and 36 h of incubations for the cereal grain samples, respectively. The incubation time points were 0, 8, 16, 24, 36, and 48 h, respectively, for digesta and fecal samples. Similar incubations were conducted to measure the amount of inorganic P released from the phytase enzyme preparation samples.

Calculations and Statistical Analyses. The amount of phytate P released at each incubation time point was calculated as the net release of P from samples due to the phytase-specific hydrolysis. This net release was the difference between the total amount of P released after incubation from the sample flasks and the nonspecific P released at time zero of incubations, corrected for the P released from the phytase enzyme preparation at the same incubation time point. For the calculation of phytate P content in samples, the maximum plateau value of net P released was used.

In the case of phytate P content analysis, the segmented quadratic with plateau model and related SAS procedures (SAS Institute Inc., Cary, NC) were used to demonstrate the phosphate release pattern and to obtain the minimal incubation time required for the maximal phytate hydrolysis for different samples.

$$Y = a + bx + cx^2 \qquad \text{if } x < x_0 \tag{1}$$

$$Y = \text{PI} \qquad \text{if } x > x_0 \tag{2}$$

In eqs 1 and 2, Y is the amount of P released, a is the intercept of the quadratic equation, b is the slope of the linear effect, x is the incubation time, c is the slope of the quadratic effect, x_0 is the breakpoint on the x-coordinate between the quadratic and the plateau sections and represents the minimal incubation time required for the maximal phytate hydrolysis, and PI is the initial plateau value of P released.

For values of $x < x_0$, the equation relating Y and x is quadratic; for values of $x > x_0$, the equation is a constant, that is, the plateau value. The two sections must meet at x_0 on the x-coordinate, and the curve must be continuous and smooth. Thus, x_0 and PI can be obtained from eqs 3 and 4 according to Fan et al. (*25*).

$$x_0 = 0.5b/c \tag{3}$$

$$\text{PI} = a + bx_0 + cx_0^2 = a - b^2/(4c) \tag{4}$$

The Figure P program (Biosoft) was used for the analyses of linear and curvilinear regressions between the released P and incubation time points for phytase activity assay. The presence of linear or quadratic relationships between the release of P and incubation time points was considered to be significant when $P < 0.05$ for associated parameter estimates.

RESULTS AND DISCUSSION

Phytase Activity. Time courses of phytate hydrolysis by intrinsic phytase in the selected cereal grains are compared in **Figure 1**. There were linear relationships ($P < 0.05$) between the amount of P released and incubation time for barley, corn, and wheat samples, and these linear relationships lasted for up to 2 h (**Figure 1A**). In these cases, the slopes of these linear relationships represent the initial velocity over the whole incubation period. Because the initial velocity of an enzyme reaction often represents the catalytic potential of the enzyme (*26*), this slope is equal to the enzyme activity or the intrinsic phytase activity of the ingredient sample (*27*).

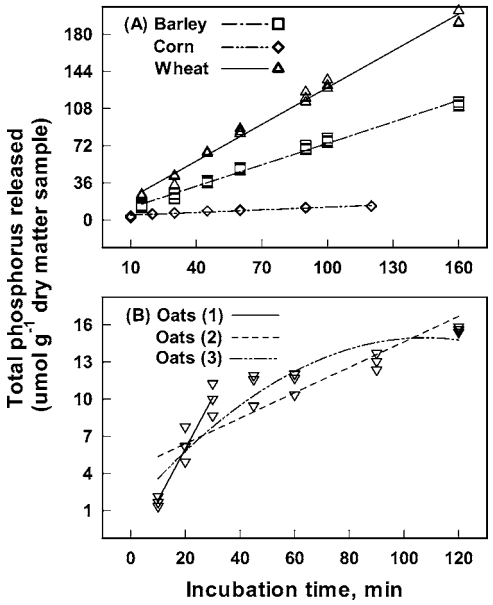

Figure 1. Time course of monitoring the amount of phosphorus (P) released from selected cereal grain samples by intrinsic phytase hydrolysis [y, released P, μmol·g^{-1} of dry matter ingredient, mean ± SE, $n = 3$ for each time point; x, incubation time, h]. (**A**) Linear relationship between the amount of P released and incubation time. Wheat, $Y = 9.547 + 1.189x$, $r^2 = 0.99$; barley, $Y = 5.006 + 0.693x$, $r^2 = 0.99$; and corn, $Y = 3.926 + 0.086x$, $r^2 = 0.91$; $P < 0.05$ for all parameter estimates. (**B**) Curvilinear and linear relationships between the amounts of P released from the same oat sample and incubation time: (1) analyzed linear relationship in the oat sample for up to 30 min of incubation, $Y = -2.362 + 0.412x$, $r^2 = 0.99$, $P < 0.05$; (2) analyzed linear relationship in the oat sample for up to 2 h of incubation, $Y = 4.348 + 0.103x$, $r^2 = 0.77$, $P < 0.05$; and (3) analyzed quadratic relationship in the oat sample for up to 2 h of incubation, $Y = 1.130 + 0.257x - 0.001x^2$, $r^2 = 0.87$, $P < 0.05$ for all parameter estimates.

Intrinsic phytase activities for the selected barley, corn, and wheat samples are obtained in the present study and are compared with the literature values (**Table 1**). It should be emphasized that those literature values of intrinsic phytase activities, as compiled in **Table 1**, were all measured by using the one time point (1−2 h of incubation) method (*9*, *28*, *29*). As compared in **Table 1**, intrinsic phytase activities measured in the barley, corn, and wheat samples of this study were within the range of values reported in the literature. The intrinsic phytase activity was relatively low in corn and high in barley and wheat samples (**Table 1**). The variability in the intrinsic phytase activities among different studies within the same cereal grains of corn, barley, and wheat was likely due to intrinsic differences in genetics and growing conditions associated with specific cereal grain samples. Thus, for these ingredients, either the one time point approach or the multiple time point method can lead to very similar results.

However, in the case of the oat sample (**Figure 1B**), the linearity between the amount of inorganic P released and incubation time was affected ($P < 0.05$) by the incubation time. As shown in **Figure 1B-oats (1)**, the linearity for the oat sample lasted ($P < 0.05$) for only up to 30 min of incubation. There was a quadratic relationship ($P < 0.05$) between the amount of inorganic P released and incubation time when the incubation time was extended to 120 min [**Figure 1B-oats (3)**]. Therefore, intrinsic phytase activity in the oat sample (412 ± 18 FTU kg^{-1} of DM) was obtained through the linearity analysis for the incubation up to 30 min [**Figure 1B-oats (1)**]. On the contrary,

Table 1. Comparison of the Analyzed Contents of Total and Phytate Phosphorus (P) and Intrinsic Phytase Activity in Selected Cereal Grain Samples of This Study with the Literature Data

ingredient	content or activity	present study[a]	lit. value	refs cited
barley	total P, g kg^{-1}	4.12	3.1–3.7	*9*, *28*, *29*
	phytate P, g kg^{-1}	2.73 ± 0.03	1.9–2.2	*9*, *28*, *29*
	phytate P, % of total P	63.3 ± 0.8	56–61	*9*, *28*, *29*
	phytase activity, units kg^{-1}	693 ± 14	582–1016	*9*, *28*, *29*
corn	total P, g kg^{-1}	4.24	2.3–2.8	*9*, *28*, *29*, *37*, *38*
	phytate P, g kg^{-1}	2.98 ± 0.05	1.7–2.2	*9*, *28*, *29*, *37*
	phytate P, % of total P	70.4 ± 1.3	66–84.6	*9*, *28*, *29*, *37*
	phytase activity, units kg^{-1}	86 ± 5	15–70	*9*, *28*, *29*
oats	total P, g kg^{-1}	3.62	–3.6	*9*, *29*
	phytate P, g kg^{-1}	2.22 ± 0.01	1.7–2.1	*9*, *29*
	phytate P, % of total P	61.1 ± 0.2	59	*9*, *29*
	phytase activity, units kg^{-1}	412 ± 18	42–84	*9*, *28*, *29*
wheat	total P, g kg^{-1}	4.54	2.9–3.5	*9*, *28*, *29*
	phytate P, g kg^{-1}	2.89 ± 0.01	2.0–2.3	*9*, *28*, *29*
	phytate P, % of total P	63.8 ± 0.2	67–79	*9*, *28*, *29*
	phytase activity, units kg^{-1}	1189 ± 22	1193–1637	*9*, *28*, *29*

[a] All values (mean ± SE, $n = 3$) were on DM basis. The values of phytate P content were the amount of P released from phytase-specific hydrolysis after 8 h of incubation.

arbitrarily fitting a linear relationship for up to 120 min or using the one time point incubation of 120 min, as shown in **Figure 1B-oats (2)**, resulted in an underestimated intrinsic phytase activity (103 ± 21 FTU·kg^{-1} of DM) in the oat sample. This value was close to the values (42−84 FTU·kg^{-1} DM sample) reported in the literature (*9*, *28*, *29*). The duration of the linearity of intrinsic phytase hydrolysis depends on many factors, including the equilibrium constants, the fractional saturation of enzyme with substrate(s) and product(s), the buffering capacity of the incubation medium, and the concentration ratio of substrates relative to the enzyme (*27*). Therefore, the multiple time point rather than the one time point method should be used to obtain reliable intrinsic phytase activities in biological samples. Clearly, the one time point method substantially underestimated the intrinsic phytase activity value of the same oat sample in the present study.

As determined by the linearity analysis of multiple time points of the results, phytase activities in distal ileal digesta and feces of grower pigs fed corn-based diets were 52 and 410 FTU·kg^{-1} of DM sample, respectively. The ileal digesta phytase activity of this study was lower than the values (80−1440 FTU·kg^{-1} of DM samples) reported by Rapp et al. (*30*) but close to those (29−34 FTU·kg^{-1} of DM sample) reported by Yi and Kornegay (*31*). In contrast, Bruce and Sundstøl (*32*) found no phytase activity in ileal digesta of pigs. Microbial fermentation in the gastrointestinal tract could be the main source of phytase activity in digesta and feces. Rapp et al. (*30*) suggested that the phytase activity in ileal digesta was independent of source and amount of dietary phytase activity but was affected by retention time of the digesta in the digestive tract. Longer sampling time after feeding leads to higher phytase activity (*30*). It was reported that lactic acid bacteria, yeast, and nonpathogenic *Escherichia coli* were detected in the digesta of the small intestine of pigs (*33*). Most of these microbes are phytase producers (*3*−*6*). Furthermore, more active microbial fermentation in the large intestine could be expected, which may explain the higher phytase activity detected in feces from the present study. Despite the existence of high phytase activity in the large intestine and in feces, its contribution to phosphate absorption in the large intestine could be of little importance. It has been shown that a large proportion of P in feces is in the form of soluble inorganic phosphates, and the large intestine is not likely to be capable of absorbing phosphates in pigs (*23*, *34*).

Table 2. Time Course of Recovery of Phosphorus Released from Purified Sodium Phytate at Low and High Concentrations during Enzymatic Hydrolysis by Microbial Phytase[a,b]

incubation time, h	recovery,[c] %, at substrate concentration of	
	0.066 mmol·L^{-1}	0.126 mmol·L^{-1}
0	0	0
0.5	82.6 ± 1.0	82.6 ± 0.8
1	85.5 ± 1.4	85.2 ± 1.1
2	88.8 ± 0.9	84.6 ± 0.5
4	101.0 ± 2.4	96.4 ± 0.9
8	100.3 ± 1.7	99.2 ± 0.7
16	101.4 ± 1.5	100.2 ± 1.7
24	101.3 ± 1.8	100.5 ± 1.8

[a] Incubation conditions: temperature, 37 ± 0.1 °C; volume of incubation medium, 100 mL of the substrate solution + 0.4 mL of the liquid microbial phytase preparation. [b] Microbial phytase (Natuphos 10,000L) preparation was provided by BASF Canada, Georgetown, ON, Canada. This phytase preparation contains 1075 phytase units·mL^{-1} measured at the condition of 1.5 mmol·L^{-1} sodium phytate, 37 ± 0.1 °C, and pH 5.5. [c] Inorganic P released as a percentage of the total P associated with sodium phytate used in the incubation. Values are mean ± SE, $n = 4$.

Phytate P Content. Our second objective was to examine methodological aspects of measuring phytate P content in biological samples by using the microbial phytase incubation. The application of microbial phytase hydrolysis for the analysis of total phytate P content depends on the complete hydrolysis of all the phosphate groups bound to the *myo*-inositol ring. Up to five inorganic phosphates bound to each *myo*-inositol molecule in the phytate moiety are readily and sequentially released by phytase action with *myo*-inositol-2-phosphate being the final product (*20*, *21*, *35*). Complete hydrolysis of the remaining phosphate group could be further achieved in the presence of *myo*-inositol monophosphatase (*21*). On the other hand, complete hydrolysis of phytate was also demonstrated with the addition of microbial phytase along with prolonged hydrolytic incubations (*19*, *20*, *36*). Therefore, we chose a microbial phytase preparation of Natuphos for this hydrolysis incubation analysis.

To validate the microbial phytase incubation as an assay method and to verify the complete hydrolysis of phytate P by microbial phytase, recovery experiments were conducted with purified phytate extracted from rice. As shown in **Table 2**, the time course of microbial hydrolysis of phytate P complex was

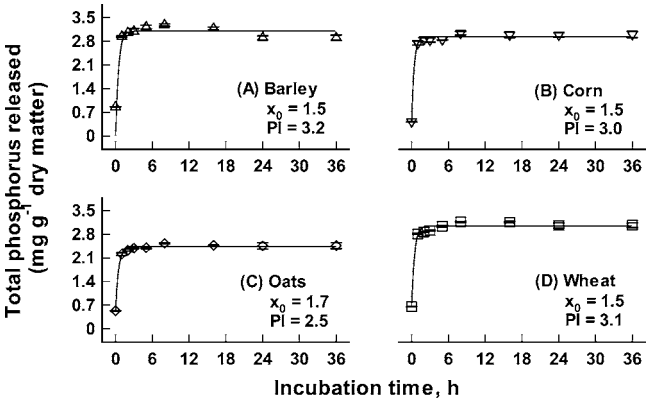

Figure 2. Quadratic with plateau relationships between the amount of phosphorus (P) released and incubation time from selected cereal grain samples after enzymatic hydrolysis by microbial phytase [*Y*, total P released, mg·g^{-1} of dry matter sample, mean ± SE, $n = 3$ for each time point; *x*, incubation time, h; x_0, breakpoint between the quadratic and the plateau sections; PI, initial plateau value]. (**A**) Barley, $Y = 0.98 + 3.64x - 1.26x^2$, $r^2 = 0.99$; (**B**) corn, $Y = 0.48 + 4.05x - 1.38x^2$, $r^2 = 0.99$; (**C**) oats, $Y = 0.60 + 2.71x - 0.82x^2$, $r^2 = 0.99$; (**D**) wheat, $Y = 0.75 + 3.82x - 1.31x^2$, $r^2 = 0.99$, $P < 0.05$ for all parameter estimates.

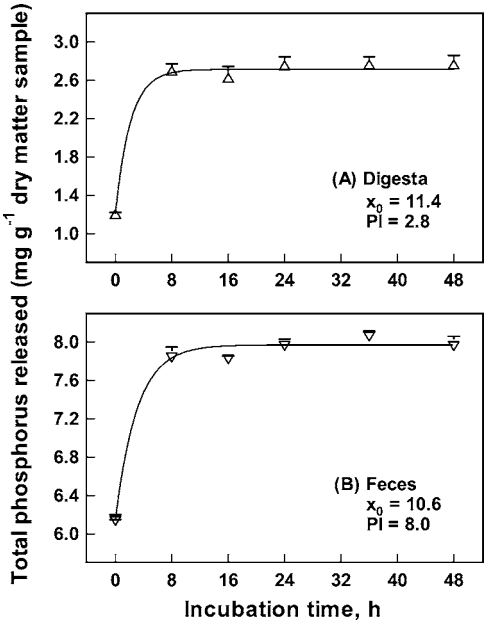

Figure 3. Quadratic with plateau relationships between the amount of phosphorus (P) released and incubation time from digesta and fecal samples collected from pigs fed corn-based diets during enzymatic hydrolysis by microbial phytase [*Y*, total amount of P released, mg·g^{-1} of dry matter sample, mean ± SE, $n = 3$ for each time point; *x*, incubation time, h; x_0, breakpoint between the quadratic and the plateau sections; PI, initial plateau value]. (**A**) Digesta, $Y = 1.16 + 0.28x - 0.01x^2$, $r^2 = 0.96$; (**B**) feces, $Y = 6.15 + 0.34x - 0.02x^2$, $r^2 = 0.94$, $P < 0.05$ for all parameter estimates.

examined at low (0.066) and high (0.126 mmol·L^{-1}) phytate concentrations. About 83% of the phytate P was readily released by the microbial phytase hydrolysis with <1 h of incubation, representing about five of the six inorganic phosphate groups bound to the inositol molecule in the phytate structure. With prolonged hydrolytic incubations, the remaining inorganic P was completely hydrolyzed with 100% recovery. The amount of purified phytate added in the recovery experiments was ~1−2 times the amount of phytate present in 0.5 g of selected cereal grain samples, or 0.35 g of the digesta and fecal samples per incubation. The complete hydrolysis of the amount of phytate was achieved with the addition of ~430 FTU microbial phytase activity units per incubation.

Furthermore, it was interesting to observe that the complete hydrolysis of the purified phytate P complex at 0.066 and 0.126 mmol·L^{-1} occurred at about 4 and 8 h of incubation, respectively (**Table 2**). Thus, the larger the amount of phytate substrate present, the longer it took to achieve complete hydrolysis. Therefore, multiple time point incubation experiments need to be conducted to monitor and reach the plateau level of phytate hydrolysis.

The time courses of the microbial phytase hydrolysis of phytate associated with the selected barley, corn, oat, and wheat grain samples are compared in **Figure 2**. There were quadratic with plateau relationships ($P < 0.05$) between the hydrolytic release of inorganic P from the phytate associated with the cereal grain samples and the incubation time (**Figure 2**). The minimal amounts of time required to reach the initial plateau were very similar (1.5 h) for the barley, corn, and wheat grain samples (**Figure 2A**,**B**,**D**). However, it took a relatively longer time (1.7 h) in the oat sample (**Figure 2C**). By definition, the amount of P released by the specific microbial phytase hydrolysis was determined to be phytate P. As compared in **Table 1**, phytate P content in the selected cereal grain samples, as grams per kilogram of DM sample or as a percentage of the total P, was close to and/or within the range of values reported in the literature (*9*, *28*, *29*, *37*, *38*).

There were also quadratic with plateau relationships ($P < 0.05$) between the hydrolytic release of inorganic P from phytate in the distal ileal digesta and fecal samples and the incubation time (**Figure 3**). The minimal amounts of time required to reach the initial plateau hydrolysis of phytate in the ileal digesta and fecal samples were similar, that is, 11 h. The content of phytate P in the digesta and fecal samples was determined to be 1.56 and 1.92 g·kg^{-1} of DM sample, and this accounted for 30 and 18% of the total P contents in these two samples, respectively. In addition, it was also worthwhile pointing out that the minimal amount of incubation time required was much longer for the ileal digesta and fecal samples than for the cereal grain samples. Two major factors were likely to be responsible for the minimal amount of time required for the complete hydrolysis of phytate in any biological samples. First, differences in sample background and matrix, including ion strength, might have potentially affected this. Second, it is known that phytase can readily hydrolyze up to five of the six inorganic phosphates bound to the inositol ring (*4*, *35*). However, the last phosphate in the inositol phosphate moiety can be further cleaved with microbial *myo*-inositol monophosphatase and prolonged incubations (*19*−*21*, *36*). Under this context, it is also perceivable that the majority of the phytate P in the distal ileal digesta and feces is the *myo*-inositol monophosphate remnant, thus taking a much longer time for its complete hydrolysis.

As in any other laboratory analyses, repeatable phytate P analysis requires delicate laboratory skills. Procedures such as grinding of samples, flask volume, and shaking speed during incubation may affect the results obtained. The present method requires maximum contact areas between the enzyme and the phytate from the samples. This will not be a problem for purified phytate as it is easily soluble in aqueous solution. However, solid samples must be finely ground and kept in flat-bottom flasks during incubation with a proper speed of shaking.

On the other hand, the multiple time point incubation may not be needed for the analysis of phytate P in similar types of samples. As long as a minimum incubation time for the complete

hydrolytic release of phytate in the sample is determined in a previous time course study, similar time points could also be applied to other samples of similar origin.

The potential advantages of using this microbial phytase hydrolysis method over other conventional methods in the analysis of phytate P content are, first, fewer steps in the whole procedure, and second, no demand for sophisticated equipment. Furthermore, there is no need to assume different ratios between bound phosphates and inositol molecules in phytate from different sample origins. For example, assumed ratios between bound phosphates and inositol molecules in phytate from different sample origins are critical to the indirect ferric precipitation procedure (*36*). Furthermore, the release of phytate P could be complete with prolonged incubation, which could render relatively reliable results in comparison with other methods. Uppström and Svensson (*36*) observed higher phytic acid content in rapeseed meal by using a wheat phytase-based incubation method than by the ferric precipitation method as reported by Wheeler and Ferrel (*18*). Results of the present study suggest that microbial phytase incubation can be a reliable and convenient method in phytate P analysis for cereal grains and digesta and feces of pigs fed cereal grain-based diets.

In conclusion, as demonstrated with the selected cereal grain samples from barley, corn, oats, and wheat, linearity of intrinsic phytase hydrolysis of phytate is affected by sources of phytase. Thus, intrinsic phytase activity should be determined by linear analysis over multiple time points rather than by a single time point of incubation. The present literature data of intrinsic phytase activity in oat samples are likely underestimated for this reason. Furthermore, there are quadratic with plateau relationships between the microbial phytase hydrolytic release of inorganic P from various sources of phytate and incubation time. Phytate P content in biological samples can be reliably and conveniently determined through in vitro microbial phytase incubations. However, the minimal incubation time required for reaching the plateau level of phytate hydrolysis is affected by sources of phytate. It is concluded that multiple time point experiments need to be conducted to determine valid intrinsic phytase activity and phytate P content in samples through the intrinsic and microbial phytase incubation analyses.

ABBREVIATIONS USED

DM, dry matter; P, phosphorus; FTU, phytase activity unit; TCA, trichloroacetic acid.

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