Research Note: Delay in sampling influences the profile of phytate in gizzard digesta and ileal digestibility of phosphorus in broilers

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ABSTRACT The objective of this study was to investigate the effect of different durations of time delay when sampling digesta from the gizzard and ileum of broilers on the degradation of *myo*-inositol hexakisphosphate (**InsP$_6$**) and digestibility of phosphorus (**P**). There was 1 experimental diet with a supplemental phytase activity of 1,212 phytase units/kg feed, which was provided to birds from day 13 to 18 after hatching. The diet was formulated to provide 6.6 g/kg Ca and 1.9 g/kg nonphytate P and fed to 24 cages of 6 birds. The 24 cages of birds were further randomly divided into 6 subgroups of 4 cages from which the digesta samples in the gizzard and ileum were collected at 0, 5, 10, or 20 min postmortem. The results showed that the concentration of $InsP_6$ decreased linearly ($P = 0.002$), $InsP_5$ decreased quadratically ($P = 0.038$), and the summation of concentrations of P in $InsP_{6-4}$ decreased linearly ($P = 0.028$) in the gizzard digesta with the increasing delay of sampling. In the ileum, the digestibility of phytate P tended to decrease linearly ($P = 0.087$), and the digestibility of total P decreased linearly ($P = 0.026$) with prolonged delay. In conclusion, delay in sampling could alter the measured profile of InsP esters in gizzard digesta probably because of a continued effect of supplemental phytase, while the ileal digestibility of total P could diminish. Therefore, standard sampling procedures should be implemented to minimize variance.

Key words: broiler, digestibility, phosphorus, phytase, phytate, sampling

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INTRODUCTION

Phytate is ubiquitous in plant feedstuff, and dietary supplementation with phytase as a countermeasure to release phosphorus (**P**) is common. Phytate-P content as a percentage of total P in common cereals ranges from 59% for oats to 70% for sorghum and from 36 to 54% for oil meals of rapeseed, sunflower, and soybean (Eeckhout and De Paepe, 1994). The degradation of phytate by broiler chickens was 30.8, 30.7, 32.2, and 34.9% for corn, wheat, barley, and soybean meal, respectively, which increased to 59.0, 46.8, 71.3, and 72.4% accordingly with the addition of phytase, respectively (Leske and Coon, 1999). In addition, phytate/phytic acid can bind dietary proteins (Yu et al., 2012) and cations such as Zn^{2+}, Cu^{2+}, Mn^{2+}, Fe^{2+} and Ca^{2+} and thus rendering them less available (Angel et al., 2002) and increases loss of endogenous minerals, amino acids (Cowieson et al., 2004), and mucin (Onyango et al., 2009). These antinutritional effects can be mitigated by supplementing diets with exogenous phytases. For example, removal of the antinutritional effects of phytic acid can improve ileal amino acid digestibility in broilers with a mean response of around 4% (Cowieson et al., 2016).

The degradation of phytate by phytase can continue in samples we collected from animals if not speedily and properly processed. This could pose a challenge to the representativeness of the samples and thus our confounding the interpretation of the results about the samples beyond the reality of processes in animals. As shown by Laird et al. (2019), significant phytate hydrolysis occurs in the gastric chyme of pigs during postsampling times at room temperature, irrespective of the supplementation of phytase. In preliminary experiments with phytase supplementation in our laboratory (unpublished), we observed almost complete "disappearance" of *myo*-inositol hexakisphosphate (**InsP$_6$**) in the gizzard with phytase supplementation and then "reappearance" of $InsP_6$ in the ileum. In the same vein, the literature has shown that, in the presence of supplemental phytase,

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the degradation of $InsP_6$ ranged from 85 to 96% (Truong et al., 2017) and even reached 100% in the gizzard (Walk et al., 2014), whereas it ranged from 36 to 68% at the distal ileum (Truong et al., 2014, 2017). These contradictions in the extent of the cumulative degradation of $InsP_6$ along the gastrointestinal tract could be partly because of variance in the extent of phytate extraction from digesta during analysis (Truong et al., 2017) and partly to more retained solid markers relative to $InsP_6$ in the gizzard owing to the selective pylorus (Vergara et al., 1989). To further explain the high degradation of phytate in gizzard digesta, we essayed an assumption that the exogenous phytase could continue to degrade phytate in gizzard digesta, whereas this continuous effect of phytase is limited in ileal digesta sample owing to the higher pH.

The aim of the present study was to investigate the effect of different duration of delays in sampling digesta from the gizzard and ileum on the degradation of phytate and digestibility of P in broilers.

MATERIALS AND METHODS

The animal protocol for this research was approved by the Animal Welfare Committee of DSM (China) Animal Nutrition Research Center. The research complied with the guidelines in European Union council directive 2010/63/EU for animal experiments. The experiment was conducted at DSM Animal Nutrition Research Center Co., Ltd., Bazhou, P. R. China.

Animals

Three hundred male birds (Cobb 500) were fed a standard broiler starter diet from day 1 to 12 after hatching. The standard starter diet was based on corn and soybean meal and met the requirements of chickens for energy and all nutrients. On day 13 after hatching, 144 birds (277 ± 1.3 g; mean ± SEM) were sorted by BW and assigned to 24 cages (95 cm × 80 cm × 80 cm) with 6 birds per cage in a way that the average initial BW was similar across cages. Birds were provided ad libitum access to water and the experimental diet from day 13 to 18 after hatching. Titanium was included at 3 g/kg feed as an indigestible marker.

Room temperature and ventilation were controlled by a computer system to provide an optimal environment for the birds. The room temperature was set at 32°C at the outset of the trial and reduced by 2°C per week thereafter. The lighting cycle was 20L:4D.

Experimental Diets

There was 1 experimental diet including 100 mg phytase/kg feed (10,000 phytase units (**FYT**)/g; RONOZYME HiPhos; DSM Nutritional Products, Switzerland). The phytase activity was analyzed as 1,212 FYT/kg feed. The diet was formulated using corn (53.5%), soybean meal (35.4%), rice bran (5.0%), soybean oil (2.8%), salt (0.3%), limestone (1.2%), and dicalcium phosphate (0.3%) as the main ingredients to provide 6.6 g/kg Ca and 1.9 g/kg nonphytate P, which were inadequate relative to the recommendations prescribed by the NRC (1994). The diet was pelleted at 75°C.

Sampling

On day 18 after hatching, all the birds were slaughtered by cervical dislocation for collection of digesta in the gizzard and ileum. The ileum was defined as the section from Meckel's diverticulum to 2 cm proximal to the ileocecal junction. Digesta from birds within a cage were pooled.

The 24 cages were divided into 6 groups for sacrifice. The 4 cages in the same group were sacrificed in a total of 5 min, and each cage was timed with an electronic timer that was set to start with the completion of cervical dislocation of all the birds in the same cage. The timer ensured that the collection of samples from the birds of the same cage was completed within 1.5 min either immediately after the completion of cervical dislocation, which represented 0 min delay for 1 cage or after a delay of either 5, 10, or 20 min for the other 3 cages within the same slaughter group by placing the intact birds at room temperature. The collection was carried out by a designated group of people with prior training, and one person was tasked to collect from one bird in each cage. The collected samples were immediately immersed in liquid nitrogen for snap-freezing and entered a freeze-drying process on the same d of sample collection.

Chemical Analyses

The digesta samples were freeze-dried to a constant weight and ground to pass through a 0.5-mm screen before analysis. The samples were dried at 105°C in an oven for 4 h for DM determination (method 934.01; AOAC International, 2006). Titanium and Ca were determined by inductively coupled plasma optical emission spectrometry (Optima TM 8000, PerkinElmer, Shelton, CT; method 985.01; AOAC International, 2006) after microwave digestion.

Instead of using conventional method based on microwave digestion and inductively coupled plasma optical emission spectrometry, P was measured with a colorimetric method at 655 nm with ammonium molybdate as the colorant to align with the enzymatic method for phytate P analysis. Total P was determined after treating the dietary and digesta samples with megadose of phytase to release the P bound by phytate. For this phytase reaction, the pure form of the phytase tested in the current animal trial was used to release the P bound by phytate. The free P, not bound by phytate, was determined after overnight extraction in 0.66 M HCl. Phytate P was calculated as the difference between the total P and free P.

Inositol phosphates were analyzed using the method described by Pontoppidan et al. (2012). Duplicate

samples (0.5 g) were extracted in 5 mL 0.5 M HCl (500 rpm, 20°C) for 3 h. Supernatants were recovered and centrifuged at 12,000 g and 0°C in an ultracentrifugal filter device (Microcon YM-30, Millipore, Bedford, MA). Filtered samples were analyzed by high-performance ion chromatography (GP50-2, Dionex Corp., Sunnyvale, CA). Inositol phosphates were detected by UV absorbance at 290 nm after postcolumn reaction with 1 g/L $Fe(NO_3)_3 \cdot 9H_2O$ in a 20 ml/L solution of $HClO_4$. A reference sample for identification of the individual InsP isomers were prepared by dissolving 0.5 g of sodium phytate (Sigma-Aldrich, St. Louis, MO) in 50 mL of 0.5 M HCl. Peaks were assigned according to Skoglund et al. (1997).

Calculations and Statistical Analyses

Each cage was an experimental unit. The data were analyzed by GLM procedure of SAS (SAS Institute, 2008). Orthogonal contrasts were constructed to test the linear and quadratic effects of sampling time. The statistical significance was defined at $P < 0.05$. A tendency was declared for $0.05 < P < 0.10$. The least square means are presented.

Degradation coefficients of InsP esters and digestibility of total P and phytate P based on index method were calculated using the following equations:

$$D_i = 1 - (T_i / T_o) \times (N_o / N_i)$$

where D_i is the degradation or digestibility coefficient; T_i and T_o are the titanium concentrations of diet and digesta, respectively (mg/kg of DM); N_i and N_o are the concentrations of InsP esters, total P or phytate P in diet and digesta, respectively (mg/kg of DM).

RESULTS AND DISCUSSION

In the presence of supplemental phytase, the degradation of $InsP_6$ varied from 85 to 100% (Walk et al., 2014; Truong et al., 2017) in the gizzard of broilers, whereas it ranged from 36 to 68% at the level of the ileum (Tamim et al., 2003; Truong et al., 2014; 2017). The variance in degradation of $InsP_6$ could be attributable to phytase type and dose, phytate source (Leske and Coon, 1999), dietary Ca level (Plumstead et al., 2008), dietary cholecalciferol level (Mohammed et al., 1991), age of birds (Olukosi et al., 2007), choice of indigestible marker (Vergara et al., 1989), the extent of phytate extraction from digesta samples (Truong et al., 2017), and different analytical methods (Wu et al., 2009). Rarely reported is the sampling procedure controlling the time for sampling before storage, which may be part of the reason for the variance. The only relevant article to our knowledge is by Laired et al. (2019) who clearly showed that phytate degradation continues with time in collected gastric chyme from pigs at room temperature, and the samples should be snap-frozen on dry ice (−79°C) rather than at −20°C. In the present study, we kept the birds intact during different delays in collecting the samples, and the samples were snap-frozen by liquid nitrogen (−196°C). Despite the differences, it is clear that the time from sacrifice of the animals to the collection of samples and from collection of samples to their proper storage should be both minimized in a concerted manner in phytase studies.

The present study clearly showed that the concentration of $InsP_6$ decreased linearly ($P = 0.002$), $InsP_5$ decreased quadratically ($P = 0.038$), and the summation of concentrations of P in $InsP_{6–4}$ decreased linearly ($P = 0.028$) in the gizzard digesta with the increasing delay of sampling (Table 1). This suggested that the degradation of phytate by added phytase continued in the gizzard of broilers after slaughter considering that the pH in gizzard contents is low enough to allow the continued action of the supplemental phytase. The optimum pH (3–4.5) of this phytase matches the pH in the crop (4.3–5.1, Kierończyk et al., 2016) and gizzard (1.9–4.5, Svihus, 2014), and thereby crop and gizzard should be the primary site for phytate degradation considering the neutral pH (6.5–7.5) environment in the small intestine. This is consistent with the generalization that phytate hydrolysis should mainly take place in the proximal intestinal tract where the pH is more conducive to phytase activity (Selle and Ravindra, 2007) and phytate solubility. In addition, the gradual fermentation of digesta in the gastrointestinal tract after the sacrifice of birds could have brought about some phytase of microbial origin. The present study, however, does not have a control diet that was not supplemented with phytase to allow us to compare the response with delay in sampling at each of the sampling times.

It is of note that considerable differences in pH activity profile were observed for some mainstream commercial acid phytases, and thus, their phytate-degrading activities varied at different pH conditions (Menezes-Blackburn et al., 2015). Therefore, delaying digesta collection from the gizzard might mean less or even negligible continued degradation of phytate for some phytases with pH optima not entirely fitting the gizzard pH conditions. In addition, the effect of sampling time on postmortem phytate degradation in the gizzard may be dose dependent and have a greater relevance when "super" doses of phytase are added to feed. In such situations, timing of sampling is critical, and the degradation of phytate in the gizzard may be influenced by a very short delay in sampling considering the overwhelming existence of supplemental phytase in digesta for a relatively small amount of phytate as substrate, which posed a challenge to collect physiologically representative samples to study phytate degradation in the gizzard. In light of the findings in the present study, standardized sampling procedures should be implemented to minimize variance in profiling InsP esters in the digesta of the gizzard. Formulation of a standard sampling procedure is beyond the scope of this study but should take into account the following principles: the sacrifice of animals in the same block should be synchronized or at least controlled in a very short period of

Table 1. Concentration of *myo*-inositol phosphate (InsP) esters and P in $InsP_{6–4}$ (μmol/g DM) in the digesta of the gizzard and ileum.[1,2]

	Delay in sampling for phytase diet, min					Significance level	
Items	0	5	10	20	SEM	Linear	Quadratic
Gizzard							
$InsP_6$	4.69	4.37	4.03	3.10	0.336	0.002	0.744
$InsP_5$	2.92	2.03	2.14	2.38	0.258	0.335	0.038
$InsP_4$	2.36	2.15	2.75	2.66	0.226	0.183	0.791
$InsP_{6–4}$	9.98	8.55	8.92	8.14	0.557	0.058	0.478
P in $InsP_{6–4}$	52.21	44.97	45.88	41.17	2.885	0.028	0.432
Ileum							
$InsP_6$	26.11	25.68	27.36	27.73	1.899	0.455	0.993
$InsP_5$	5.78	5.99	6.58	6.05	0.453	0.642	0.311
$InsP_4$	0.42	0.82	1.38	0.39	0.517	0.934	0.155
$InsP_{6–4}$	32.31	32.50	35.32	34.17	2.173	0.466	0.571
P in $InsP_{6–4}$	187.26	187.34	202.60	198.18	12.622	0.457	0.667

Abbreviation: FYT, phytase units.
[1]The diet were formulated to provide 6.6 g/kg Ca and 1.9 g/kg nonphytate P; the phytase activity was analyzed as 1,212 FYT/kg feed.
[2]1 cage is an experimental unit; 6 replicates (cages) per treatment; 6 birds per replicate.

time; the sampling should initiate immediately after the sacrifice and proceed with all the treatments simultaneously; and the collected samples should be snap-frozen with dry ice or liquid nitrogen without delay. A caveat here is that due attention should be paid to randomizing the euthanasia process without inducing an inadvertent bias by killing all birds and then leave them queuing up for sampling or killing all birds in one treatment after another.

There was a linear decrease in ileal digestibility of total P ($P = 0.026$) and a tendency for a less degradation of phytate P ($P = 0.087$) with more delay in ileal sampling (Table 2), which implies the "reappearance" of phytate P or phytate P and free P in ileum with the longer delay. The "reappearance" is unlikely to be associated with de novo synthesis of phytate. In rats, it has been found that endogenous synthesis of $InsP_6$ is not determinative, and most $InsP_6$ present in the organism is of dietary origin (Grases et al., 2001). The reason for "reappearance" of $InsP_6$ could be related to the continued peristaltic contractions of the intestinal tract after cervical dislocation, which might have resulted in movement of digesta toward the aboral end of the digestive tract, and thus, the digesta from the upper part of the intestinal tract containing more phytate P and free P could be propelled into the ileum where the samples were taken. This would have resulted in seemingly decreased digestibility of total P and "reappearance" of phytate P with longer delay in sampling. It is also noteworthy that the samples collected in the ileum may not be exactly representative of what was previously collected in the gizzard owing to the postsacrifice movement of digesta. In the same principle, Summers and Robblee (1985) mentioned that contamination of the terminal region of the small intestine by less-digested contents could have occurred in sacrificed birds by cervical dislocation but not at all in anesthetized birds, which did not show any peristaltic contractions of the intestinal tract. After the termination of the active peristalsis, which should not last very long, the passive diffusion of digesta toward the direction of the distal end of the gut could also have contributed to the aforementioned progression of digesta. Another explanation of minor importance could be the reflux of P and some InsP esters from the basal side of the gut into the lumen after the sacrifice of the birds. Paracellular P fluxes were bidirectional, and the contribution of paracellular absorption of P dominates under normal dietary conditions (Knöpfel et al., 2019). Although it has long been assumed that phytate cannot cross the lipid bilayer of plasma membranes because of inadequate carriers and thus its absorption is rather improbable (Schlemmer et al., 2009), $InsP_3$ could traverse through cellular membrane and become the main form of inositol phosphate in epithelial cells of the digestive tract (Duliński et al., 2016). With longer delay in sampling, the epithelial cells could have become more vulnerable to sloughing and thereby be taken as part of ileal digesta inflating the endogenous loss of P. In pigs, it has been proven that shedding and autolysis of epithelial tissues developed progressively in the gastrointestinal tract over the 24-h period postmortem (Thorpe and Thomlinson, 1967). More research is warranted to investigate the shedding and

Table 2. Digestibility or degradation coefficients of phytate P, total P, $InsP_6$, $InsP_{6–5}$, and $InsP_{6–4}$ at the ileum.[1,2]

	Delay in sampling for phytase diet, min					Significance level	
Items	0	5	10	20	SEM	Linear	Quadratic
$InsP_6$	0.62	0.62	0.58	0.56	0.030	0.118	0.931
$InsP_{6–5}$	0.60	0.60	0.55	0.54	0.029	0.101	0.906
$InsP_{6–4}$	0.59	0.59	0.53	0.53	0.029	0.104	0.654
P in $InsP_{6–4}$	0.59	0.60	0.54	0.54	0.029	0.102	0.758
Phytate P	0.54	0.55	0.49	0.47	0.032	0.087	0.977
Total P	0.62	0.62	0.57	0.55	0.025	0.026	0.959

Abbreviation: FYT, phytase units.
[1]The diet was formulated to provide 6.6 g/kg Ca and 1.9 g/kg nonphytate P; the phytase activity was analyzed as 1,212 FYT/kg feed.
[2]1 cage is an experimental unit; 6 replicates (cages) per treatment; 6 birds per replicate.

autolysis of epithelial tissues in a time frame more relevant to sampling in broilers.

CONCLUSION

The present study showed that delay in sampling could alter the measured profile of phytate in gizzard digesta probably owing to continued effects of supplemental phytase, while the ileal digestibility of total P could diminish. Therefore, standard sampling procedures should be implemented to minimize variance.

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