Factors affecting storage stability of various commercial phytase sources[1,2]

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ABSTRACT: A 360-d study was performed to evaluate the effects of different environmental conditions on storage stability of exogenous phytases. Coated and uncoated products from 3 phytase sources [Ronozyme P (DSM Nutritional Products, Basel, Switzerland), OptiPhos (Phytex LLC, Sheridan, IN), and Phyzyme (Danisco Animal Nutrition, Marlborough, UK)] were stored as pure forms, in a vitamin premix, or in a vitamin and trace mineral (VTM) premix. Pure products were stored at −18, 5, 23, and 37°C (75% humidity). Premixes were stored at 23 and 37°C. Sampling was performed on d 0, 30, 60, 90, 120, 180, 270, and 360. Sampling of the pure products stored at −18 (lack of sample) and 5°C (because of mold growth) was discontinued after d 120. Stability was reported as the residual phytase activity (% of initial) at each sampling point. For the stability of the pure forms, all interactive and main effects of the phytase product, coating, time, and storage temperature were significant ($P < 0.01$), except for the time × coating interaction. When stored at 23°C or less, pure phytases retained at least 91, 85, 78, and 71% of their initial phytase activity at 30, 60, 90, and 120 d of storage, respectively. However, storing pure products at 37°C reduced ($P < 0.01$) phytase stability, with OptiPhos retaining the most ($P < 0.01$) activity. Coating mitigated ($P < 0.01$) the negative effects of high storage temperature for Ronozyme and OptiPhos (from d 90 onward), but not for Phyzyme. For the stability of phytase in different forms of storage, all interactive and main effects of phytase product, form, coating, time, and temperature of storage were significant ($P < 0.01$). When stored at room temperature (23°C), retained phytase activities for most the phytase sources were more than 85, 73, and 60% of the initial activity up to 180 d when stored as pure products, vitamin premixes, or VTM premixes, respectively. When stored at 37°C, pure phytase products had greater ($P < 0.01$) retention of initial phytase activity than when phytases were mixed with the vitamin or VTM premixes. Coated phytases stored in any form had greater ($P < 0.01$) activity retention than the uncoated phytases at all sampling periods. Results indicate that storage stability of commercially available phytases is affected by duration of storage, temperature, product form, coating, and phytase source. Pure products held at 23°C or less were the most stable. In premixes, longer storage times and higher temperatures reduced phytase activity, but coating mitigated some of these negative effects.

Key words: enzyme, phytase, stability, storage

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INTRODUCTION

The rapidly growing global market for animal feed enzymes is largely attributed to increased use of exogenous phytases by the feed industry (van Beilen and Li, 2002; Iyer and Ananthanarayan, 2008). In fact, phytases are now used routinely in swine and poultry diets as an economical aid in the digestibility of P and an important measure for environmental protection (Selle and Ravindran, 2007). Consequently, a growing number of commercial phytases are available. These phytases are produced from various microbial sources that may have varying efficacies (Adeola et al., 2006; Kerr et al., 2010) and physicochemical characteristics (Lei and Stahl, 2001; Boyce and Walsh, 2006).

The ultimate value of any phytase product is dependent on its efficacy and stability. As with any catalytic

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protein, phytases lose a significant amount of activity when subjected to feed processing treatments (Jongbloed and Kemme, 1990; Spring et al., 1996); thus, research has focused on optimizing the thermostability of exogenous phytases in industrial settings. Nutritionists most often consider the minimum guaranteed phytase after feed processing in their diet formulations; however, the stability of phytases during storage receives little attention. In some circumstances, because of poor inventory management, we have observed classical signs of rickets in pigs because of extended phytase storage of 6 to 12 mo. No independent study has been conducted to evaluate the effects of various factors such as coating, time, or storage temperature on the stability of commercial phytases. Some suggest that potential interactions may occur between phytase and some components of the premixes and that such interaction may affect phytase activity; however, no data support this assumption. Therefore, the objective of this study was to determine the effects of coating, storage form, storage temperature, and duration of storage on the stability of 6 commercially available phytases.

MATERIALS AND METHODS

This study was conducted at the Animal Nutrition Laboratory and at the Bioprocessing and Industrial Value Added Program Building at Kansas State University (Manhattan, KS). Animal Care and Use Committee approval was not obtained for this experiment because no animals were used.

Phytase Sources

This experiment used 6 commercially available phytases: OptiPhos 2000-M [uncoated, declared potency of 2,000,000 phytase units (**FTU**)/kg; Phytex LLC, Sheridan, IN], OptiPhos 2000-PF (coated, declared potency of 2,000,000 FTU/kg; Phytex LLC), Phyzyme XP 5000 G (uncoated, declared potency of 5,000,000 FTU/kg; Danisco Animal Nutrition, Marlborough, UK), Phyzyme XP 10000 TPT (coated, declared potency of 10,000,000 FTU/kg; Danisco Animal Nutrition), Ronozyme P-M [uncoated, declared potency of 50,000,000 phytase units (**FYT**)/kg; DSM Nutritional Products, Basel, Switzerland], and Ronozyme P-CT (coated, declared potency of 10,000,000 FYT/kg; DSM Nutritional Products). One phytase unit (FTU or FYT) was defined as the amount of enzyme that catalyzes the release of 1 μmol of inorganic P per minute from 5.1 m*M* sodium phytate in pH 5.5 buffer at 37°C. Pure cornstarch was used as a negative control because of its minimal inherent phytase activity. The coated and uncoated phytases were obtained from a third-party distributor. For coated sources, a lipid or carbohydrate coating encapsulates the phytase to minimize oxidation at high temperatures, particularly during pelleting conditions. The type of coating varies by manufacturer, and its components are typically guarded as intellectual property. The manufacturing dates of all products were obtained from the original suppliers to ensure that the products were within 6 mo of manufacture and were not expired.

Pure Products

On d 0, 1.36 kg of each of the pure phytase products and cornstarch were individually placed into 12 open, single-lined paper bags. Three bags (observations) of each product were stored in a freezer (−18°C), in a refrigerator (5°C), at room temperature (23°C), and in a controlled environment chamber set at 37°C and 75% humidity. At sampling, the bag was mixed and a sample of approximately 50 g was taken. Each bag was blind sampled at d 30, 60, 90, 120, 180, 270, and 360, and samples were sent immediately after collection to Technical Marketing Analytical Services of DSM Nutritional Products Inc. (Belvidere, NJ) for phytase analysis using a slight modification of AOAC official method 2000.12 (Engelen et al., 1994, 2001; AOAC, 2000). Each bag of the cornstarch control, OptiPhos 2000-M, and OptiPhos 2000-PF was blind sampled a second time, and samples were sent to Phytex LLC (Portland, ME) for phytase analysis using the Phytex method (Han et al., 1999). Sampling of the pure products stored at −18 and 5°C was discontinued after d 120 because not enough material was available for sampling at −18°C and because of mold growth from the high humidity in the retained 5°C samples. Thus, only pure products stored at 23 and 37°C were sampled for all time points.

Premixes

Each phytase product and the cornstarch control were added and mixed with either the Kansas State University vitamin premix or the Kansas State University vitamin and trace mineral (**VTM**) premix (Table 1). The amount added for each phytase product was determined such that including 0.30% premix in the diet would provide the activity of phytase recommended by their respective manufacturers (250 FTU/kg, OptiPhos 2000-M and OptiPhos 2000-PF; 500 FTU/kg, Phyzyme XP 5000 G and Phyzyme XP 10000 TPT; 1,850 FYT/kg, Ronozyme P-M and Ronozyme P-CT).

A total of 2.46, 2.46, 1.36, 0.68, 2.52, and 0.50 kg of pure OptiPhos 2000-M, OptiPhos 2000-PF, Phyzyme XP 5000 G, Phyzyme XP 10000 TPT, Ronozyme P-M, and Ronozyme P-CT, respectively, were weighed. Cornstarch was added to the pure phytase products to create 9.83 kg (OptiPhos) or 6.80 kg (Phyzyme XP and Ronozyme P) batches, which were mixed using a paddle mixer for 5 min. A total of 49.1 kg (OptiPhos) or 34.0 kg (Phyzyme XP and Ronozyme P) of vitamin or VTM premix was added to each batch and mixed using a paddle mixer for an additional 12 min to create premix batches of 59.0 kg (OptiPhos) or 40.8 kg

Table 1. Composition of the vitamin and vitamin and trace mineral (VTM) premixes used in the study[1]

Item	Added per kilogram of vitamin premix	Added per kilogram of VTM premix
Vitamin		
Vitamin A, IU	3,674,371	1,837,186
Vitamin D, IU	459,296	229,648
Vitamin E, IU	14,697	7,349
Vitamin K, mg	1,470	735
Riboflavin, mg	2,756	1,378
Niacin, mg	16,535	8,267
Pantothenic acid, mg	9,186	4,593
Cobalamin, mg	13	6
Trace mineral		
Copper ($CuSO_4$), mg	—	4,593
Iodine [$Ca(IO_3)_2$], mg	—	83
Iron ($FeSO_4$), mg	—	45,930
Manganese ($MnSO_4$), mg	—	11,023
Selenium ($NaSeO_3$), mg	—	83
Zinc ($ZnSO_4$), mg	—	45,930

[1]The amount added for each phytase product was determined such that including 0.30% premix in the diet would provide the phytase recommended by their respective manufacturers [250 phytase units (FTU)/kg, OptiPhos 2000-M and OptiPhos 2000-PF (Phytex LLC, Sheridan, IN); 500 FTU/kg, Phyzyme XP 5000 G and Phyzyme XP 10000 TPT (Danisco Animal Nutrition, Marlborough, UK); 1,850 phytase units (FYT)/kg, Ronozyme P-M and Ronozyme P-CT (DSM Nutritional Products, Basel, Switzerland)].

(Phyzyme XP and Ronozyme P). Additionally, 59.0 kg of cornstarch made up the control batch.

The 7 batches each were divided equally into 6 open, single-lined paper bags. Three bags of each batch were stored either at room temperature (approximately 23°C and <40% humidity) or in the environmentally controlled chamber set at 37°C and 75% humidity. Before sampling, each bag was mixed to ensure that a representative sample was collected. A sample from each bag was taken every 30 d until d 180, with 2 remaining samples taken at d 270 and 360. Each sample was blinded and sent immediately after collection to the same laboratories as used for the pure samples for phytase analysis.

Statistical Analyses

Data were analyzed using a mixed model (MIXED procedure; SAS Inst. Inc., Cary, NC) to determine the interactive and main effects of coating, storage form, storage temperature, and time on the activity of 6 commercially available phytase sources. Because the vitamin and VTM premixes were stored only at room temperature and in the environmentally controlled heat chamber, 2 analyses were performed. The first was with the pure forms only, and the second was for the pure forms, vitamin premixes, and VTM premixes at 23 and 37°C. Least squares means were calculated for each independent variable. When treatment effect was a significant source of variation, differences were determined by using the preplanned pairwise comparisons (PDIFF option of SAS). Statistical significance and tendencies were set at $P \leq 0.05$ and $P < 0.10$, respectively, for all statistical tests.

RESULTS

Initial Phytase Activity

The calculated and analyzed initial (d 0) phytase activities of the samples are shown in Table 2. On the basis of the AOAC assay, the control samples for the pure product, the vitamin premix, and the VTM premix contained 4,967 to 10,500 phytase units/kg. However, the phytase activities of the control samples analyzed using the Phytex assay were much greater than those analyzed using the AOAC assay. For all 3 products, the AOAC-analyzed phytase activities of OptiPhos, Phyzyme XP, and Ronozyme P were 1.96 to 2.95, 0.97 to 1.57, and 1.03 to 1.42 times greater than their calculated phytase activities, respectively. On the basis of the Phytex assay, samples of the pure OptiPhos 2000-M and OptiPhos 2000-PF had similar (1.01 to 1.02 times) phytase activities compared with their calculated activities. In contrast, the phytase activities of both OptiPhos products added to the vitamin and the VTM premix were less, ranging from 0.30 to 0.68 of their calculated activities.

Pure Products

All interactive and main effects of phytase product, coating, time, and storage temperature were significant ($P < 0.01$; Table 3), except for the time × coating interaction.

When the phytases were stored at 23°C or less, their retained activity when stored in pure form decreased ($P < 0.01$) as the duration of storage increased regardless of the phytase source or coating (Figures 1, 2, and

Table 2. Calculated and analyzed phytase composition of samples at d 0[1]

Item	Phytase composition				
	Calculated, PU[2]/kg	AOAC analysis, PU/kg	AOAC ratio[3]	Phytex analysis, PU/kg	Phytex ratio[4]
Pure product					
Control[5]	0	10,500	—	3,343,000	—
OptiPhos 2000-M[6]	2,000,000	3,932,000	1.96	2,046,000	1.02
OptiPhos 2000-PF[6,7]	2,000,000	5,179,000	2.58	2,022,000	1.01
Phyzyme XP 5000 G[8]	5,000,000	5,144,000	1.03	—	—
Phyzyme XP 10000 TPT[7,8]	10,000,000	10,587,000	1.06	—	—
Ronozyme P-M[9]	50,000,000	52,148,500	1.04	—	—
Ronozyme P-CT[7,9]	10,000,000	12,057,500	1.20	—	—
Vitamin premix					
Control[5]	0	4,967	—	37,000	—
OptiPhos 2000-M[6]	83,333	214,425	2.51	41,000	0.49
OptiPhos 2000-PF[6,7]	83,333	250,853	2.95	57,000	0.68
Phyzyme XP 5000 G[8]	166,666	266,339	1.57	—	—
Phyzyme XP 10000 TPT[7,8]	166,666	266,116	1.57	—	—
Ronozyme P-M[9]	616,666	738,388	1.19	—	—
Ronozyme P-CT[7,9]	616,666	637,467	1.42	—	—
Vitamin and trace mineral premix					
Control[5]	0	4,948	—	77,000	—
OptiPhos 2000-M[6]	83,333	209,424	2.45	25,000	0.30
OptiPhos 2000-PF[6,7]	83,333	244,067	2.87	55,000	0.66
Phyzyme XP 5000 G[8]	166,666	209,437	1.23	—	—
Phyzyme XP 10000 TPT[7,8]	166,666	166,239	0.97	—	—
Ronozyme P-M[9]	616,666	699,542	1.13	—	—
Ronozyme P-CT[7,9]	616,666	877,884	1.03	—	—

[1]Values represent means of 3 replicates sampled in duplicate. The AOAC analysis was performed at the DSM Nutritional Products laboratory (Belvidere, NJ); the Phytex analysis was performed at Phytex LLC (Sheridan, IN).

[2]PU = phytase units.

[3]Ratio of average AOAC analyzed values to calculated values.

[4]Ratio of Phytex analyzed values to calculated values.

[5]Cornstarch used as the negative control.

[6]Phytex LLC.

[7]Coated phytase.

[8]Danisco Animal Nutrition, Marlborough, UK.

[9]DSM Nutritional Products, Basel, Switzerland.

Table 3. Probabilities of interactive and main effects of storage time, temperature, coating, and phytase product on stability (as defined by percentage of initial phytase activity) of commercially available phytase products in pure forms

Item	*P*-value
Interactive effect	
Time × temperature × coating × product	<0.001
Time × temperature × product	<0.001
Time × temperature × coating	<0.001
Time × coating × product	<0.001
Temperature × coating × product	<0.001
Temperature × coating	<0.001
Temperature × product	<0.001
Time × temperature	<0.001
Time × coating	0.43
Time × product	<0.001
Coating × product	<0.001
Main effect	
Time	<0.001
Temperature	<0.001
Coating	<0.001
Product	<0.001

3). At d 30, 60, and 90, pure phytases retained at least 91, 85, and 78% of their initial phytase activity, respectively. Until d 120, the pure forms retained 71 to 102% of their initial phytase activity, except for uncoated Ronozyme P-M, which retained 59% at 5°C. However, storing pure products at 37°C had greater ($P < 0.01$) effects on phytase stability. At d 30, both coated and uncoated OptiPhos products stored in pure form retained 91 to 93% of their initial activity when stored at 37°C, whereas the coated and uncoated Phyzyme phytases retained 69 to 74%. Coated Ronozyme P-CT retained 69% of its initial phytase activity at d 30, but uncoated Ronozyme P-M retained only 36%. Afterward, phytases stored in pure forms retained at least 44, 39, and 33% of their initial phytase activities at d 60, 90, and 120, respectively, except for uncoated Ronozyme P-M, which retained only 5% of its initial phytase activity at d 120. At d 180, 270, and 360, phytases stored in pure forms at 37°C had retained phytase activities ranging from 1 to 53%, compared with 50 to 109% when stored at 23°C.

Coated OptiPhos 2000-PF had similar retention rates compared with uncoated OptiPhos 2000-M at d

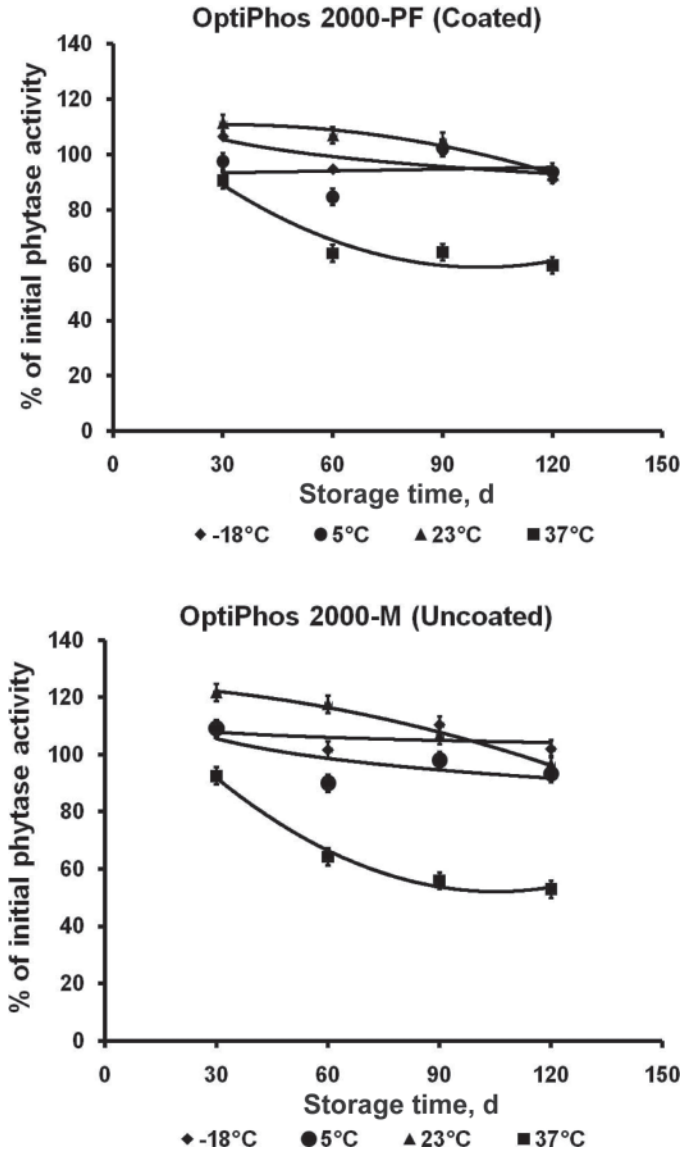

Figure 1. Residual phytase activity (% of initial) for OptiPhos 2000-PF (coated; Phytex LLC, Sheridan, IN) and OptiPhos 2000-M (uncoated; Phytex LLC) as affected by storage temperature [freezer (−18°C), refrigerator (5°C), room temperature (23°C), and controlled environment chamber (37°C and 75% humidity)] and time (30 to 120 d). Each data point (least squares means ± 2.32) is the mean of 3 observations.

30 and 60 when stored at 37°C, but coating improved ($P < 0.01$) retention rates from d 90 onward. Coating also improved ($P < 0.01$) the retained phytase activities of Ronozyme phytase throughout the duration of the study; however, coated Phyzyme had less ($P < 0.01$) phytase activity than uncoated Phyzyme until d 360. Among the coated phytases, the retention rates of Ronozyme P-CT were less ($P < 0.01$) than those of OptiPhos 2000-PF until d 120, whereas the retention rates of Ronozyme P-CT were similar to those of Phyzyme 10000 TPT until d 90. Among the uncoated phytases, OptiPhos 2000-M had greater ($P < 0.01$) phytase activity than both Phyzyme 5000 G and Ronozyme P-M at d 30, but Phyzyme 5000 G retained more ($P < 0.01$) phytase activity than the other 2 uncoated phytases from d 90 onward.

Premixes

All interactive and main effects of phytase product, form, coating, time, and storage temperature were significant ($P < 0.01$; Table 4), except for the time × form × coating and coating × temperature interactions ($P < 0.08$).

When stored at 23°C, pure uncoated forms of OptiPhos 2000-M and Phyzyme 5000 G retained more ($P < 0.01$) phytase activity with an increasing duration of storage than did the phytase-supplemented vitamin or VTM premixes (Figures 4, 5, and 6). However, the phytase activity of pure, uncoated Ronozyme P-M was similar among the pure, vitamin, and VTM samples. Pure phytase products retained at least 85 and 72% of

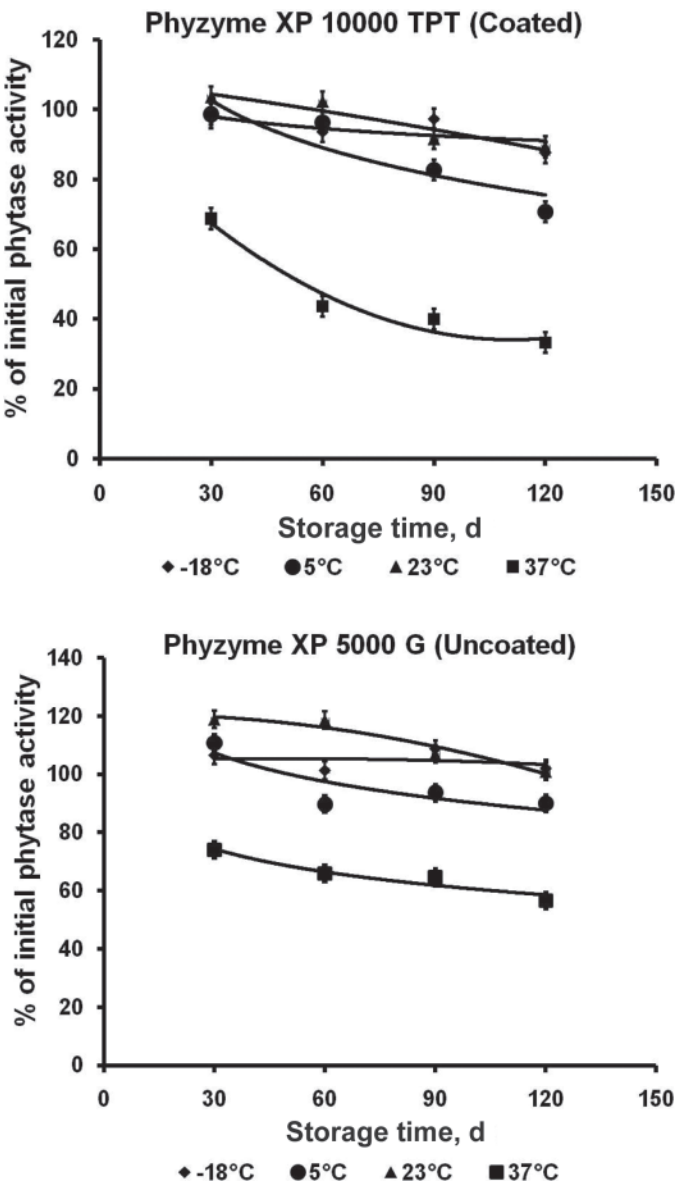

Figure 2. Residual phytase activity (% of initial) for Phyzyme XP 10000 TPT (coated; Danisco Animal Nutrition, Marlborough, UK) and Phyzyme XP 5000 G (uncoated; Danisco Animal Nutrition) as affected by storage temperature [freezer (−18°C), refrigerator (5°C), room temperature (23°C), and controlled environment chamber (37°C and 75% humidity)] and time (30 to 120 d). Each data point (least squares means ± 2.32) is the mean of 3 observations.

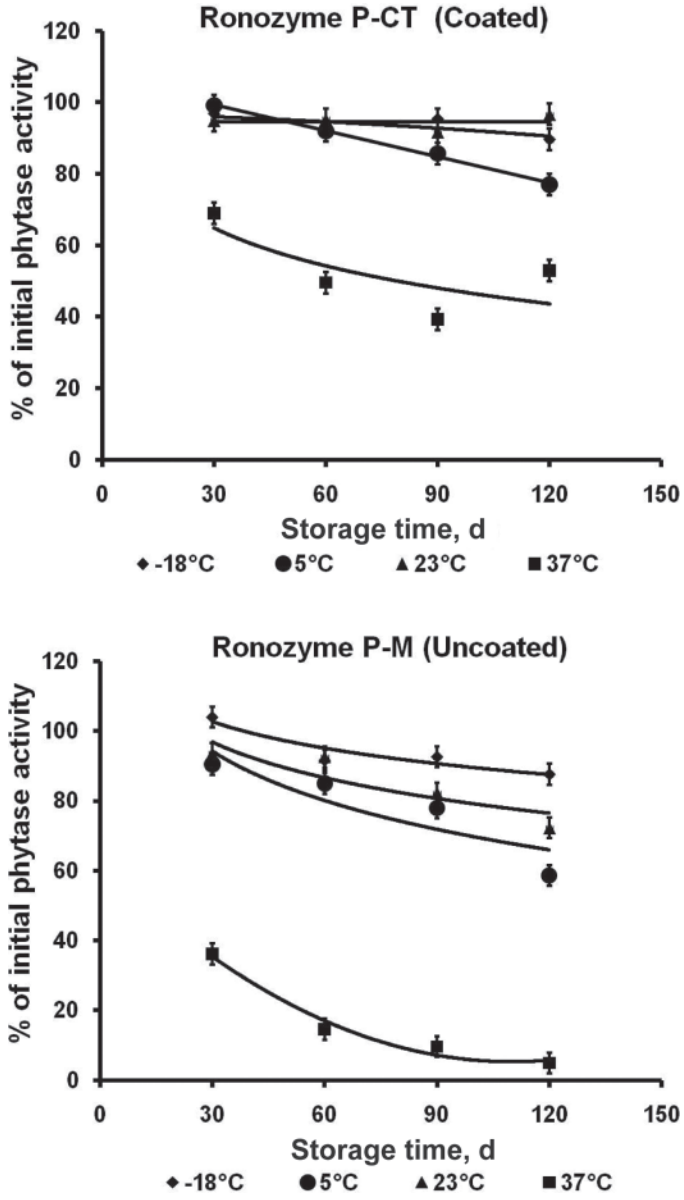

Figure 3. Residual phytase activity (% of initial) for Ronozyme P-CT (coated; DSM Nutritional Products, Basel, Switzerland) and Ronozyme P-M (uncoated; DSM Nutritional Products) as affected by storage temperature [freezer (−18°C), refrigerator (5°C), room temperature (23°C), and controlled environment chamber (37°C and 75% humidity)] and time (30 to 120 d). Each data point (least squares means ± 2.32) is the mean of 3 observations.

their initial phytase activities until d 180 and 360, respectively, except for uncoated Ronozyme P-M (50%). In contrast, phytase-supplemented vitamin premixes retained at least 73% of their activity until d 180, except for uncoated Phyzyme 5000 G (67%). At d 270 and 360, uncoated Phyzyme 5000 G and Ronozyme P-M retained 56 to 59% of their initial phytase activities, whereas the rest of the phytases retained at least 68%. Among all the phytases, coated OptiPhos 2000-PF retained the most activity (>92%; $P < 0.01$) until d 360 when mixed with the vitamin premixes. In comparison, coated Ronozyme P-CT retained at least 83% of its initial phytase activity, whereas coated Phyzyme 10000 TPT retained at least 73% of its activity until d 360. For the phytase-supplemented VTM premixes, retained phytase activities were at least 60% until d 180, except for uncoated OptiPhos 2000-M (43%). At d 270 and 360, uncoated OptiPhos 2000-M had only 28% of its initial phytase activity, compared with at least 52% for the rest of phytases when mixed into the VTM premixes. As in the vitamin premixes, coated OptiPhos 2000-PF retained the most activity ($P < 0.01$) among all the phytases when mixed into the VTM premixes; however, its retention rates were less ($P < 0.01$) than the rates obtained in the vitamin premixes. At d 360, coated OptiPhos 2000-PF, Ronozyme P-CT, and Phyzyme 10000 TPT retained at least 83, 75, and 63% of their initial phytase activities, respectively.

When stored at 37°C, retained phytase activities were much less ($P < 0.01$) than the retention rates observed in samples stored at 23°C regardless of the phytase source, coating, or form of storage. For the phytase-supplemented vitamin and VTM premixes, retained phytase activities after only 30 d of storage were 59 and 62% on average, which were less ($P < 0.01$) than the 72% for the pure phytase products. Uncoated Ronozyme P-M was the least stable when mixed into

Table 4. Probabilities of interactive and main effects of storage time, form, temperature, coating, and phytase product on stability (as defined by percentage of initial phytase activity) of commercially available phytase products

Item	*P*-value
Interactive effect	
Time × form × coating × product × temperature	<0.001
Time × form × coating × product	<0.001
Time × form × coating × temperature	<0.001
Time × form × product × temperature	<0.001
Time × coating × product × temperature	<0.001
Form × coating × product × temperature	<0.001
Time × form × coating	0.07
Time × form × product	<0.001
Time × form × temperature	<0.001
Time × coating × product	<0.001
Time × coating × temperature	<0.001
Time × product × temperature	0.003
Form × coating × product	<0.001
Form × coating × temperature	0.004
Form × product × temperature	<0.001
Coating × product × temperature	<0.001
Time × form	<0.001
Time × coating	0.003
Time × product	<0.001
Time × temperature	<0.001
Form × coating	<0.001
Form × product	<0.001
Form × temperature	<0.001
Coating × product	<0.001
Coating × temperature	0.08
Product × temperature	<0.001
Main effect	
Time	<0.001
Form	<0.001
Coating	<0.001
Product	<0.001
Temperature	<0.001

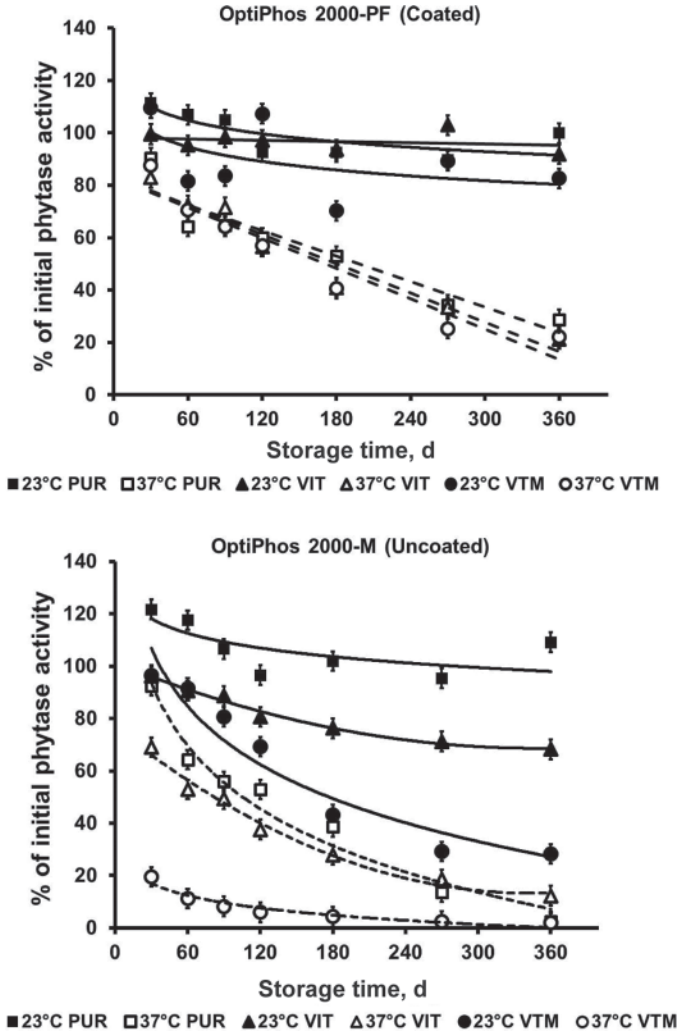

Figure 4. Residual phytase activity (% of initial) for OptiPhos 2000-PF (coated; Phytex LLC, Sheridan, IN) and OptiPhos 2000-M (uncoated; Phytex LLC) as affected by form of storage [as pure product (PUR), in a vitamin premix (VIT), or in a vitamin and trace mineral premix (VTM)], storage temperature [room temperature (23°C) and controlled environment chamber (37°C and 75% humidity)], and time (30 to 360 d). Each data point (least squares means ± 3.75) is the mean of 3 observations.

vitamin premixes, retaining only 31% of its initial phytase activity at d 30. For the VTM premixes, uncoated OptiPhos 2000-M was the most affected; it retained only 20% of its initial phytase activity after a month of storage. At d 180, the phytase treatments had 3 to 53% of their initial phytase activities. At the end of the study (d 360), all the phytases had less than 28% of their initial phytase activities.

The coated phytases stored in pure form or as phytase-supplemented vitamin or VTM premixes had greater ($P < 0.01$) phytase activities than the uncoated phytases at all sampling periods. However, the differences in phytase activity between the coated and uncoated phytases were smaller ($P < 0.01$) when they were stored in pure forms than when the phytases were in the vitamin and VTM premixes. At d 30, 60, and 90, the differences in retained phytase activities between the coated and uncoated phytases ranged from 4.2 to 4.5, 11.5 to 28.6, and 33.4 to 44% when the phytases were in their pure form or as vitamin VTM premixes. At d 30, coated phytases had similar phytase activities among the 3 sources when stored at 37°C; however, uncoated OptiPhos 2000-M and Phyzyme 5,000 XP phytases stored in pure form had greater ($P < 0.01$) phytase activities than those mixed with the vitamin and VTM premixes. Likewise, uncoated OptiPhos 2000-M retained greater ($P < 0.01$) phytase activity in vitamin premixes than in VTM premixes.

DISCUSSION

Phytase Assays

Jones et al. (2010) previously demonstrated that accuracy of the analysis for phytase activity depended on the phytase product and the assay method. On the

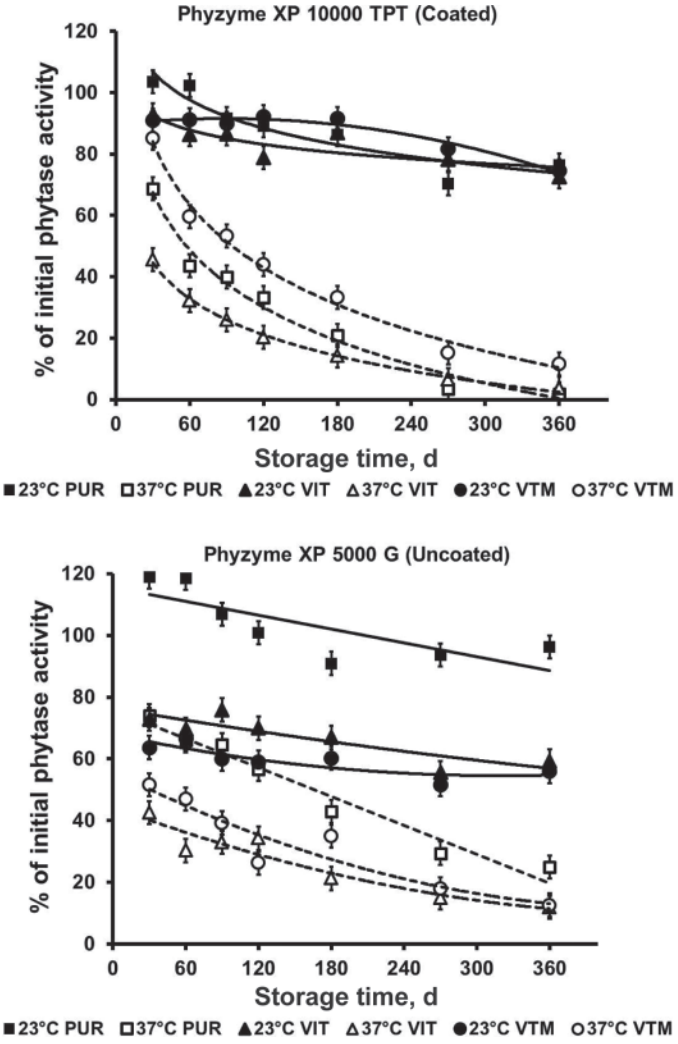

Figure 5. Residual phytase activity (% of initial) for Phyzyme XP 10000 TPT (coated; Danisco Animal Nutrition, Marlborough, UK) and Phyzyme XP 5000 G (uncoated; Danisco Animal Nutrition) as affected by form of storage [as pure product (PUR), in a vitamin premix (VIT), or in a vitamin and trace mineral premix (VTM)], storage temperature [room temperature (23°C) and controlled environment chamber (37°C and 75% humidity)], and time (30 to 360 d). Each data point (least squares means ± 3.75) is the mean of 3 observations.

basis of the AOAC method, the initial phytase activity of OptiPhos was 2 to 3 times greater than the calculated activity, which is similar (2.5 times) to the difference observed by Jones et al. (2010). The analyzed initial phytase activities for Phyzyme and Ronozyme were closer (1 to 1.6 times greater) to the calculated activities, which was expected because the AOAC assay is the recommended method of analysis for these products. For OptiPhos, the analyzed initial phytase activity was similar to the calculated activity when the Phytex assay (the recommended assay for this product) was used. Therefore, these results confirmed our earlier observations.

Phytases in Pure Forms

As with all catalytic proteins, phytases are sensitive to denaturation reactions. Denaturation is the unfolding of the enzyme tertiary structure to a disordered polypeptide, which may lead to an irreversible loss of activity or inactivation (Iyer and Ananthanarayan, 2008). Exposure of phytases to heat, added moisture, and mechanical pressure are factors that lead to denaturation of the enzyme (Ward, 2002). For animal feeds, the largest potential for denaturation in feed processing is pelleting, where temperatures may reach 60 to 90°C. *Aspergillus niger* phytase, which was the first commercially available phytase, yielded the most activity compared with other microorganisms tested (Shieh and Ware, 1968); however, it was stable only to 63°C (Ullah and Gibson, 1987). Potentially greater losses in phytase activity may be incurred if expanders or extruders are used to produce the diets because feed is subjected to temperatures as high as 115 to 140°C (Fancher, 1996). Therefore, optimizing the thermostability of exogenous phytases, especially during feed processing, has received much attention in the past decade and has contributed significantly to the expanded use of phytases in swine and poultry diets.

It is common practice for phytase manufacturers to provide overages in phytase activity to account for potential losses during feed processing treatments and storage. However, data are limited regarding the storage stability (defined as percentage of initial phytase activity) of commercial phytases, except for those reported by manufacturers in product registrations (European Food Safety Authority, 2006, 2008, 2009). Although temperatures and conditions from manufacture, transport, and storage of phytases may not approximate conditions during feed processing, enough variation occurs in storage conditions and time among phytase users to expect further losses in phytase activity. Most nutritionists do not measure phytase activity at the time of use; therefore, understanding that the stability of different commercial phytases during storage is affected by temperature and time is important.

The results of this study demonstrated that when phytase is stored at room temperature (23°C) or lower,

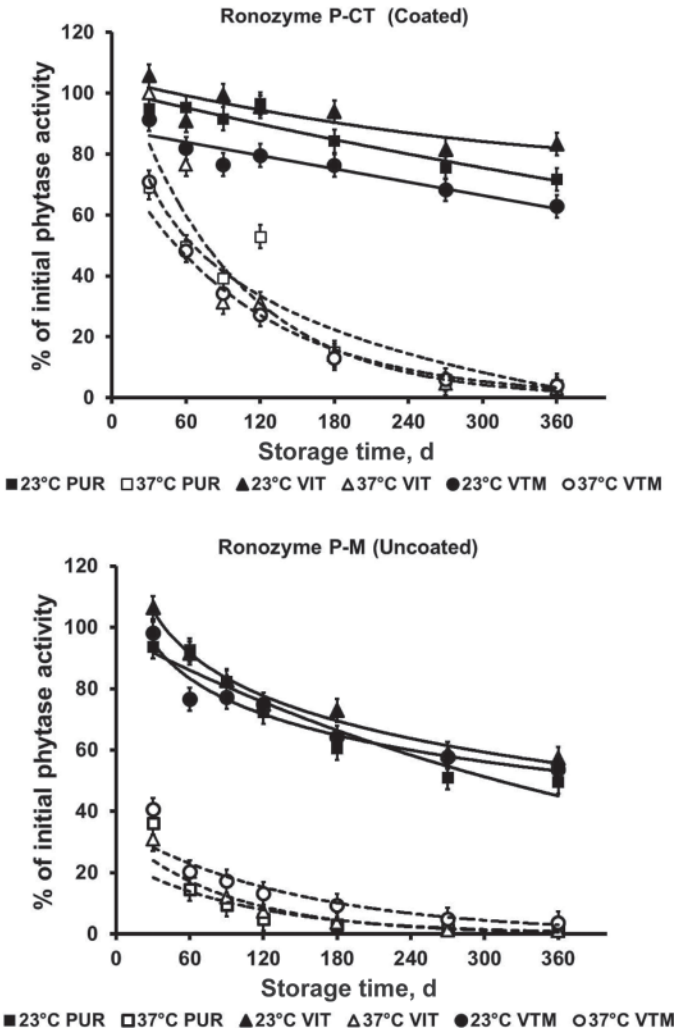

Figure 6. Residual phytase activity (% of initial) for Ronozyme P-CT (coated; DSM Nutritional Products, Basel, Switzerland) and Ronozyme P-M (uncoated; DSM Nutritional Products) as affected by form of storage [as pure product (PUR), in a vitamin premix (VIT), or in a vitamin and trace mineral premix (VTM)], storage temperature [room temperature (23°C) and controlled environment chamber (37°C and 75% humidity)], and time (30 to 360 d). Each data point (least squares means ± 3.75) is the mean of 3 observations.

the pure product retained most (approximately 85%) of its activity up to 60 d of storage regardless of the phytase source or coating. However, phytase source influenced stability when storing the product for more than 60 d at 23°C or lower, with OptiPhos and Phyzyme retaining more activity than Ronozyme. In our study, coated Phyzyme XP 5000 G and Phyzyme XP 10000 TPT retained 90.9 and 86.3% of their initial activity, respectively, when stored at 23°C for 180 d; these retention rates are similar to those reported to the European Food Safety Authority (2006, 2008). In these reports, the product had 87 and 80% of its initial activity after 365 d of storage at 20°C. However, our results did not confirm the retention rates reported for uncoated Ronozyme P-M (European Food Safety Authority, 2009). It was reported that after 180 d, uncoated Ronozyme P-M retained 99 and 90% of its initial phytase activity when stored at 10 and 25°C, respectively, which was

greater than our observations (58.7% for 120 d at 5°C and 60.6% after 180 d at 23°C).

Storing phytase in ambient temperatures greater than 23°C with high humidity proved detrimental to the stability of the pure product. More important, phytase source affected retention rates with increasing storage time, with the greatest rates recovered from OptiPhos, followed by Phyzyme and, finally, by Ronozyme. This ranking of the 3 phytase sources persisted throughout the study. The difference in retained phytase activities was extremely large between OptiPhos and Ronozyme (91.5 vs. 52.6% after 30 d, 45.8 vs. 8.9% after 180 d). In the European Food Safety Authority (2009) report, uncoated Ronozyme P-M kept at 40°C and 60% relative humidity retained only 50% of its initial phytase activity after 30 d, which is similar to the rate retained in our study. The stability limit of *Escherichia coli* phytases, such as OptiPhos and Phyzyme, is 60°C (Golovan et al., 2000), whereas the stability limit for *Peniophora lycii* phytases is 80°C (Phillippy, 2002). Both temperatures are higher than the heat treatment used in this study; however, one major difference is that these thermal stability rates were determined by incubating the enzyme at low pH for a short duration (approximately 30 min), whereas the enzyme was subjected to lower but sustained heat for a longer duration (up to 180 d) in this study. Another factor may be the high humidity (75%) in the chambers in our study. Yang et al. (2007) evaluated the effects of increasing ambient humidity (from 53 to 90%) on the stability of commercial phytases stored at high ambient temperatures (40°C for 70 d) and observed that phytase activity decreased significantly with increasing ambient humidity. This suggests that regardless of the phytase source, the environmental conditions set in the current study were sufficient to denature the enzyme and reduce activity. These conditions did not attempt to mimic real conditions during transport or storage of the product, when temperatures and humidities may be more variable, but the results demonstrate the importance of maintaining good conditions (e.g., 23°C or lower and low ambient humidity) during storage to achieve greater stability from phytase products.

Fat or carbohydrate coating of phytases is one widely adopted technology used to improve phytase thermostability during feed processing. Numerous studies on the effects of coating have focused more on retention of phytase activity after feed processing (Eeckhout, 2002; Sorbara et al., 2007) or on effects on subsequent growth performance (Emiola et al., 2007; Timmons et al., 2008). No study has evaluated the effects of coating on the storage stability of phytase products. Overall, coated pure products had greater phytase activities than uncoated pure products when exposed to 37°C and increased storage time, but this differed between phytase sources. Coating was beneficial for Ronozyme and OptiPhos from d 90 onward, but not for Phyzyme, for which the uncoated product retained more activity than the coated product throughout the study. This suggests that the type of coating may differ between phytase manufacturers and that some coated phytase products may provide better protection during storage than others.

Phytases in Premixes

For most of the commercial phytase products tested, retained phytase activities were more than 85, 73, and 60% of their initial activity up to 180 d when stored as pure products, vitamin premixes, or VTM premixes, respectively, and when storage temperatures were 23°C. The exceptions were uncoated Ronozyme P-M for the pure phytase products, uncoated Phyzyme XP 5000 G for the vitamin premixes, and uncoated OptiPhos 2000-M for the VTM premixes. Previously, the stability of coated Phyzyme XP 10000 TPT was tested in a VTM premix that was stored at 20°C for 180 d (European Food Safety Authority, 2008). The product retained 76% if its initial phytase activity, which is less than the 92% observed in our study. The stability of uncoated Ronozyme P-M was also evaluated previously when mixed with VTM premixes and stored at 25°C (European Food Safety Authority, 2009). After 90 d, 89% of the initial activity was retained, which was greater than the 77% observed in this study. In general, greater retention was observed with increasing storage time when phytases were stored as pure products than when they were mixed into either of the premixes. This suggests that storing phytases in pure forms may have advantages in retaining the original phytase activity compared with including it in premixes when stored at room (23°C) temperature or lower.

When phytase was mixed into vitamin or VTM premixes and exposed to heat treatment (37°C), coated phytases retained greater activities than uncoated phytases, especially when stored for more than 90 d. However, some differences surfaced among phytase sources in that coating had the greatest benefits for OptiPhos. Results also showed that uncoated phytases had very poor stability when stored as premixes for as little as 30 d. The loss of phytase activity was greater when phytase was mixed with VTM premixes than when it was mixed with vitamin premixes, suggesting that high heat and humidity as well as potential interactions with some components of the premixes increased the rate of phytase denaturation. Previous work has shown that mixing inorganic trace minerals with vitamins leads to significant losses in vitamin activity, which is thought to be due to the presence of ionic charges in mineral salts that can act as oxidizing agents (Shurson et al., 1996). The objective of this study was not to identify specific vitamins or trace minerals that may have contributed to greater losses in phytase activity, but results indicate that that coating mitigated the negative effects of heat and humidity for Ronozyme and OptiPhos, but not Phyzyme. This study also demonstrated the differing abilities of coating technologies to protect phytases against not only environmental degradation

but also the negative effects of certain components in vitamin and VTM premixes.

The stability of commercially available phytases during storage is affected by numerous factors, such as storage time, temperature, product form, coating, and source. Pure phytase products stored at 23°C or lower were the most stable. In premixes, longer storage times and higher temperatures reduced phytase activity, but coating mitigated some of these negative effects.

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