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Impact of Assay Conditions on Activity Estimate and Kinetics Comparison of *Aspergillus niger* PhyA and *Escherichia coli* AppA2 Phytases

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Aspergillus niger PhyA and *Escherichia coli* AppA2 are increasingly used in animal feed for phosphorus nutrition and environmental protection. The objective of this study was to determine the impacts of assay conditions on activity estimates of these two phytases and to compare their biochemical characteristics at a pH similar to the stomach environment. The activities of the unpurified AppA2 were more variable than those of PhyA with three commonly used phytase activity assays. The variations associated with AppA2 were accounted for by buffer, pH, and the inclusion of Triton X-100 and BSA by approximately one-third each. At the commonly observed stomach pH of 3.5, the purified AppA2 had a lower affinity to phytate (a higher K_m), but greater V_{max}, k_{cat}, and k_{cat}/K_m than those of PhyA. In summary, differences between AppA2 and PhyA in responses to activity assay conditions and in inherent kinetic properties should be considered in interpreting their feeding efficacy.

KEYWORDS: AppA2; assay method; comparison; kinetics; PhyA; phytase

INTRODUCTION

Phytases are added in animal feed to improve their phosphorus nutrition and to reduce their manure phosphorus excretion. Currently, *Aspergillus niger* NRRL 3135 PhyA and *Escherichia coli* AppA2 are two phytases widely used by the animal industry. Although both enzymes belong to the histidine acid phosphatase family (*1*), reported feeding efficacies of PhyA and AppA2 on the activity unit basis in swine and poultry diets are rather different (*2*−*7*). Several potential impacts of phytase feeding efficacy, such as the physiological state of the animal, feeding system, and diet composition, have been identified (*8*−*10*). However, the relative contributions of the inherent enzymatic properties of individual phytases and their particular activity assay conditions to their reported feeding efficacy differences are unclear.

Despite recent progress in establishing a common method to measure phytase activity (*11*, *12*), three major assay methods have been used: the molybdenum blue method (method 1) (*6*, *13*, *14*), the molybdovanadate method (method 2) (*3*, *15*, *16*), and the acetone phosphomolybdate method (method 3) (*17*, *18*). With the same chemical principle, all three methods should allow a respectively consistent estimate of phytase activity. However, each of these assays differs in choice of buffer, pH, substrate concentration, and the use of ancillary chemicals such as bovine serum albumen (BSA) and Triton X-100. Along with different methods to extract phytase from solid preparations (*11*, *19*), these methods could give intriguing activity values of any given preparation of AppA2 and PhyA for functional comparisons.

Although enzymatic properties of PhyA and AppA2 have been previously characterized (*20*−*22*), the results were confounded with different protein expression systems or purities. Furthermore, the nutritional relevance of the data was also limited because of the lack of consideration of gastric conditions such as acidic pH instead of the optimal pH of the enzymes. Therefore, we conducted two experiments to determine the impact of assay conditions on the activity estimate of unpurified (resembling the feed status of the enzymes) PhyA and AppA2 (experiment 1) and to compare their kinetic characteristics at the commonly observed gastric pH 3.5 after being expressed in the same *Pichia pastoris* X-33 host and purified to >95% homogeneity (experiment 2).

MATERIALS AND METHODS

Source and Preparation of Phytase. Unless otherwise indicated, all reagents were purchased from Sigma-Aldrich or Fisher Scientific, and all assays were performed at 37 °C, using the substrate sodium phytate (dodecasodium salt of phytic acid from rice, P-3168). Phytase activity assay method 3 (*17*, *18*) was used in experiment 2. One unit (U) of phytase activity is defined as the release of 1 μmol of orthophosphate per minute.

PhyA and AppA2 were constitutively expressed in *P. pastoris* X-33 using the pGAPZαA vector (Invitrogen Corp., Carlsbad, CA). PhyA was expressed in shake-flask culture (*23*), and AppA2 was expressed in large-scale fermentation for the commercial production of OptiPhos (JBS United, Sheridan, IN). Preliminary experiments in our laboratory indicated the enzyme properties of AppA2 from large-scale production were comparable to those produced in shake-flask culture (*24*, *25*). In experiment 1, the PhyA culture supernatant was used, so units were expressed

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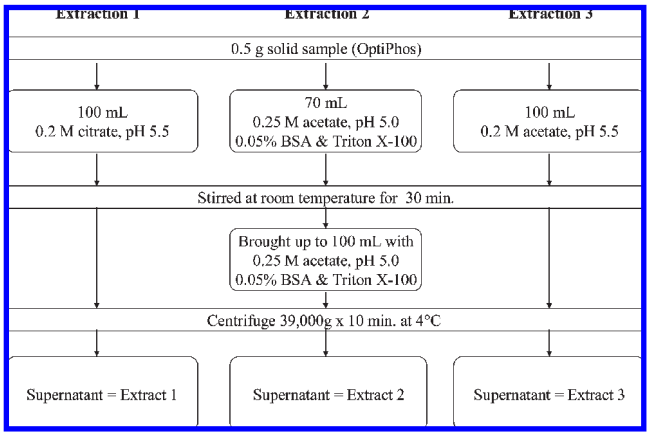

Figure 1. Steps of the three phytase-extraction methods. AppA2 was extracted from the supplement OptiPhos (JBS United, Sheridan, IN). Stirring was performed in an Erlenmeyer flask with a stir bar slowly rotating so as not to create bubbles on the liquid surface.

per gram of unpurified total supernatant protein (11.6 mg/mL) as assessed by Lowry assay (*26*). For AppA2, three extracts (**Figure 1**) of OptiPhos were used, so units were expressed per gram of OptiPhos. In experiment 2, the PhyA culture supernatant was concentrated by ultrafiltration (YM-10, Amicon Bioseparations, Millipore, Bedford, MA) and was subjected to two rounds of sequential ion-exchange chromatography (Macro-Prep High Q and Macro-Prep High S, Bio-Rad Laboratories, Hercules, CA) to achieve >95% homogeneity (confirmed by SDS-PAGE, data not shown). Purified AppA2 was prepared from the fermentation supernatant similarly as for PhyA. Units for PhyA and AppA2 were expressed per milligram of pure phytase protein.

Experiment 1: Impacts of Assay Conditions on Activity Estimate of Unpurified Preparation. *Activity Variation with Three Methods*. The three assays of AppA2 were all performed using extract 2 (**Figure 1**). Method 1 was performed as described (*13*, *14*) using a 5.4 mM (final) sodium phytate solution (**Figure 2**). Method 2 was performed as described (*15*) with a slight modification using 5.0 mM sodium phytate in the presence of BSA and Triton X-100 (1.7×10^{-3}%). Method 3 was performed as described (*17*, *18*) with a slight modification using a 0.75 mM final sodium phytate concentration. Each assay was performed in triplicate with individual blanks. All enzyme dilutions were performed with pipetted volumes of at least 50 μL to ensure accuracy. The blanks for the three methods were performed by adding the respective stop reagents to the enzyme before addition of the substrate.

All assays used standards diluted from the same 50 mM KH_2PO_4 stock (in deionized water). The standards for method 1 were prepared by making a 90 μM (0.24 U/mL) KH_2PO_4 solution in deionized water and then diluting it serially to 45, 22.5, 11.25, and 5.625 μM (0.12, 0.06, 0.03, and 0.015 U/mL, respectively). To 2 mL of KH_2PO_4 standard or deionized water (standard blank) was added 2 mL of fresh reagent C (freshly made 3 parts of 1 M sulfuric acid, 1 part of 2.5% ammonium heptamolybdate, and 1 part of 10% ascorbic acid), the mixture was incubated at 50 °C for 20 min, and the absorbance was read at 820 nm. The standards for method 2 were prepared by making a 25 mM (0.833 U/mL) KH_2PO_4 solution in 0.25 M acetate, pH 5.0, with 0.05% BSA and 0.05% Triton X-100 and then diluting it serially to 12.5, 6.25, 3.125, and 1.563 mM (0.417, 0.208, 0.104, and 0.052 U/mL, respectively). KH_2PO_4 standard (0.2 mL) or deionized water (standard blank) was added to 1.8 mL of 0.25 M acetate, pH 5.0, to which 4 mL of 7.5 mM sodium phytate, 0.25 M acetate, pH 5.0, and 4 mL of stop reagent (freshly made 1 part of ammonium vanadate solution (20 mM ammonium vanadate, 0.43% nitric acid), 1 part of ammonium heptamolybdate solution (10% ammonium heptamolybdate, 0.25% ammonia), and 2 parts of 21.7% nitric acid) were added. After centrifugation at 3500*g* for 10 min, the absorbance was read at 415 nm. The standards for method 3 were prepared by making a 390.6 μM (3.91 U/mL) KH_2PO_4 solution in deionized water and then serially diluting it to 195.3, 97.65, 48.825, 24.413, and 12.206 μM (1.95, 0.98, 0.49, 0.24, and 0.12 U/mL, respectively). To 1 mL of KH_2PO_4 standard or deionized water (standard blank) was added 2 mL of acetone

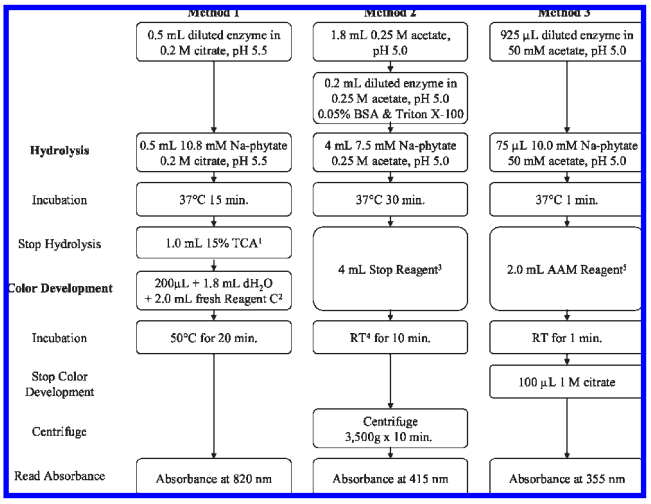

Figure 2. Steps of the three phytase activity assay methods. Method 1 is the molybdenum blue method (*13*, *14*), method 2 the molybdovanadate method (*15*, *16*), and method 3 the acetone phosphomolybdate method (*17*, *18*). The blanks for the three methods were performed by adding the stop reagent (15% TCA for method 1, Stop Reagent for method 2, and AAM Reagent for method 3) to the enzyme prior to adding the substrate. 1, trichloroacetic acid; 2, freshly made 3 parts of 1 M sulfuric acid, 1 part of 2.5% ammonium heptamolybdate, and 1 part of 10% ascorbic acid; 3, Stop Reagent is freshly made 1 part of ammonium vanadate solution (20 mM ammonium vanadate, 0.43% nitric acid), 1 part of ammonium heptamolybdate solution (10% ammonium heptamolybdate, 0.25% ammonia), and 2 parts of 21.7% nitric acid; 4, room temperature; 5, Acetone Ammonium Molybdate Reagent is freshly made 2 parts of acetone, 1 part of 10 mM ammonium heptamolybdate, and 1 part of 5 N sulfuric acid.

ammonium molybdate (AAM; freshly made 2 parts of acetone, 1 part of 10 mM ammonium heptamolybdate, and 1 part of 5 N sulfuric acid), and then after exactly 1 min, 100 μL of 1 M citrate was added, and the absorbance was read at 355 nm.

The absorption of BSA and Triton X-100 in 0.25 M acetate buffer was measured at 415 nm against a reference of 0.25 M acetate buffer. This was done because the phosphate standards for method 2 contained 0.05% BSA and Triton X-100, whereas the reactions contained 1.7×10^{-3}% BSA and Triton X-100. We needed to determine whether this difference had any effect on the absorbance readings.

Relative Effects of Buffer, pH, BSA, and Triton X-100 on Activity Estimate. To test their effect on the outcome of method 1, buffer, pH, and the presence of BSA and Triton X-100 (**Table 1**, top) were singly and additively changed from the conditions of method 1 to the conditions of method 2. The relative contribution of each assay condition factor to the enzyme activity differences between the two methods was calculated as follows: The total difference between the outcomes of methods 1 and 2 was set equal to 100%. The fraction of this difference made up after each factor change was defined as the relative contribution of the respective factor. Only factors contributing to differences between methods 1 and 2 were studied because these two methods showed the greatest difference in activity outcome.

Effect of Extraction Condition on Activity Estimate. Because extract 2 of AppA2 was used for all of the assay comparisons, the effect of extraction method (**Figure 1**) on activity estimate was tested separately (**Table 1**, bottom) by assaying the three extracts of AppA2 with method 1.

Experiment 2: Comparisons of Biochemical Characteristics of Purified Enzymes. *Kinetics*. The kinetic parameters (K_m, V_{max}, k_{cat}, k_{cat}/K_m) of PhyA and AppA2 were determined in 50 mM glycine−HCl, pH 3.5 at 37 °C, with 11 substrate concentrations ranging from 30 μM to 5 mM in a 30 s assay ($n = 3$). Protein concentrations were determined by the absorbance at 280 nm and extinction coefficients, 49423 M^{-1} cm^{-1} for PhyA and 50450 M^{-1} cm^{-1} for AppA2, as calculated using ProtParam (*27*), and then corrected for level of glycosylation as determined by SDS-PAGE. Glycosylated molecular weights were used for the calculation

Table 1. Factors Affecting Enzyme Activity in Method 1[a]

	PhyA		AppA2	
	U × 10/g	% rel activity	U × 10^2/g	% rel activity
Effect of Assay Conditions (All AppA2 Assays Performed with Extract 2)				
buffer (pH 5.5, no Triton or BSA)[b]				
citrate†	275 ± 9.5	100	423 ± 0.93b	100
succinate	291 ± 6.9	106	189 ± 7.0a	45
acetate	284 ± 1.7	103	711 ± 38c	168
pH (0.25 M acetate, no Triton or BSA)				
5.5†	284 ± 1.7a	100	711 ± 38a	100
5.0	293 ± 1.7b	103	930 ± 18b	131
Triton and BSA (0.25 M acetate, pH 5.0)[c]				
no†	293 ± 5.7	100	930 ± 18a	100
yes	300 ± 5.4	102	1165 ± 12b	125
Effect of Extraction Conditions				
extract (0.2 M citrate, pH 5.5, no Triton or BSA)[d]				
1 (extracted without Triton or BSA)			360 ± 19[f]	85
2 (extracted with Triton and BSA)[e]†			423 ± 0.93	100
extract (0.2 M acetate, pH 5.5, no Triton or BSA)				
2 (extracted with Triton and BSA)[g]†			711 ± 38	100
3 (extracted without Triton or BSA)			610 ± 21	86

[a] In all assays, the final reaction contained approximately 0.1 U of PhyA or AppA2, with 5.4 mM sodium phytate as substrate. Assay conditions are shown in bold for each factor tested. Values are presented as the mean ($n = 3-6$) ± SE. Activities are for unpurified proteins. Means respective to enzyme under the same condition not sharing the same letter are different ($P < 0.05$). Within each condition tested, the variables are compared to the control (†) and presented as percentages of the control activity, respective to enzyme (% relative activity). [b] Triton and BSA represent Triton X-100 and bovine serum albumin. Even though no Triton or BSA was added to the assay, because extraction 2 was performed in the presence of Triton and BSA, the final concentration of Triton and BSA in the assay of extract 2 in citrate buffer was 2×10^{-5}%, and in acetate or succinate buffer was 8×10^{-6}%. Note that extraction only applies to AppA2, and that extract 2 exclusively was used to test all factors. [c] When Triton X-100 and BSA were added to the assay, the final reaction concentration was 1.7×10^{-3}%. [d] Extract 1 of AppA2 was extracted in 0.2 M citrate, pH 5.5, for 30 min at room temperature. Extract 2 of AppA2 was extracted in 0.25 M acetate, pH 5.0, with 0.05% BSA and Triton X-100 for 30 min at room temperature. Extract 3 of AppA2 was extracted in 0.2 M acetate, pH 5.5, for 30 min at room temperature. After extraction, the mixture was centrifuged at 39000*g* 4 °C for 10 min and the supernatant used for assays. [e] Final reaction concentration of Triton X-100 and BSA was 2×10^{-5}%. [f] $n = 16$. [g] Final reaction concentration of Triton X-100 and BSA was 8×10^{-6} %.

of V_{max}. For PhyA, the reaction glycosylated protein concentration was 78.8 μg/mL, whereas that of AppA2 was 27.2 μg/mL.

Competitive Inhibition. Competitive inhibition by *myo*-inositol hexasulfate (MIHS, I6005, Sigma-Aldrich, St. Louis, MO) was determined in 50 mM acetate buffer, pH 5.0, at 58 °C in a 1 min assay. The relative activity of the enzymes was determined in nine concentrations of MIHS ranging from 1 to 50 μM, and the K_i for the enzymes was determined using nine concentrations of sodium phytate ranging from 30 to 1000 μM in the absence and presence of 30 μM MIHS (PhyA) or 20 μM MIHS (AppA2).

Guanidine Hydrochloride (GuCl) Inactivation. GuCl inactivation was performed in a total volume of 200 μL of 50 mM acetate pH 5.0, with 9 GuCl concentrations ranging from 0 to 2 M at 25 °C for 20 min. The final concentration of AppA2 was 3.06 μg/μL, and that of PhyA was 2.1 μg/μL. The enzymes were then diluted in their respective acetate and GuCl buffers and assayed at 58 °C in a 30 s assay.

Optimal Temperature and pH Profiles. The optimal temperature was determined in 50 mM acetate, pH 4.75, at 22, 37, 42, 51, 58, 65, 70, 75, and 80 °C in a 1 min assay. The pH profile was determined in 0.2 M glycine−HCl, pH 2−4; in 0.2 M acetate, pH 4.25−5.5; in 0.2 M 2-(*N*-morpholino)ethanesulfonic acid (MES), pH 6−6.5; and in 0.2 M imidazole, pH 7−9, at 37 °C in a 1 min assay.

Statistical Analysis. Data were analyzed by JMP Statistical Discovery Software (release 6.0.0; SAS Institute Inc., Cary, NC). For the impacts of three methods on phytase activity (**Figure 3**), we used one-way ANOVA to focus on the difference of a given phytase to various assay conditions, followed by the Student's *t* test to compare means. For the impacts of different factors in a given method (**Table 1**), Student's *t* test was used to compare means of different conditions. For the kinetic parameters (**Table 2**), Student's *t* test was used to compare mean differences. Significance was set at a *P* value of 0.05. Data are presented as mean ± SE.

RESULTS

Experiment 1. *Activity Variations with Three Methods*. PhyA showed no activity differences between methods 1 and 3 (**Figure 3**). However, the activity by method 2 was different ($P < 0.05$) from that of both of these methods. In comparison, AppA2 showed differences ($P < 0.05$) among all three assays. There was nearly a 3-fold difference between methods 1 and 2, whereas method 3 was approximately halfway between the other two methods. The BSA and Triton X-100 in 0.25 M acetate buffer were found to have no greater absorbance at 415 nm than 0.25 M acetate buffer alone (data not shown), so this difference did not affect the outcomes of the assays.

Impacts of Assay Conditions on Activity Estimate. PhyA displayed no activity differences among the three buffers (**Table 1**). However, AppA2 showed differences ($P < 0.05$) between any two of the three buffers, with a range of > 3.5-fold in acetate buffer versus in succinate buffer. Both PhyA and AppA2 had activity differences ($P < 0.05$) between pH 5.0 and 5.5 (**Table 1**). PhyA showed a 3% increase in activity at pH 5.0 as compared to pH 5.5, whereas AppA2 showed a 31% increase in activity at pH 5.0 as compared to pH 5.5. PhyA showed no differences in activity whether in the presence or absence of BSA and Triton X-100 (**Table 1**). However, AppA2 showed a 25% increase in activity ($P < 0.05$) in the presence of BSA and Triton X-100 when in 0.25 M acetate buffer, pH 5.0.

Effect of Extraction Condition on Activity Estimate. When extracted into solution in the presence of different buffers, AppA2 showed no different activities ($P = 0.17$ for method 1 citrate assay and $P = 0.12$ for method 1 acetate assay) given constant assay conditions (**Table 1**, bottom). However, a strong trend was apparent, showing an increased activity when extracted in the presence of BSA and Triton X-100. The ratio of AppA2 activity of extract 1 (extracted in the absence of BSA and Triton X-100) over extract 2 (extracted in buffer containing 0.05% BSA and Triton X-100) was 0.85. Likewise, the ratio of AppA2 activity of extract 3 (extracted in the absence of BSA and Triton X-100) over extract 2 was 0.86. These represented numerical activity increases

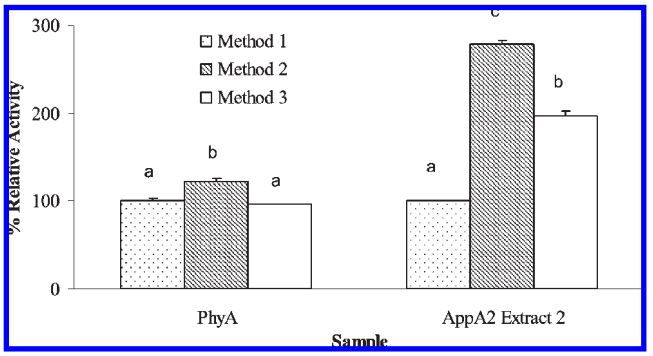

Figure 3. Variations of PhyA and AppA2 activities with three different phytase assays. Results are expressed relative to method 1 (100% of PhyA = 2.75×10^3 U/g of unpurified protein and 100% of AppA2 = 4.23×10^4 U/g of OptiPhos). Values are presented as the mean (n = 3, except AppA2 extract 2 method 2, n = 16) ± SE. Method 1 was performed in 0.2 M citrate, pH 5.5, method 2 was performed in 0.25 M acetate, pH 5.0, in the presence of BSA and Triton X-100, and method 3 was performed in 0.05 M acetate, pH 5.0. Means for the respective enzyme not sharing the same letter are significantly different ($P < 0.05$).

Table 2. Kinetics Comparisons of Purified PhyA and AppA2[a]

	PhyA	AppA2
K_m (μM)	34 ± 3	74 ± 3
V_{max} (U mg^{-1})	120 ± 0	1070 ± 20
k_{cat} (s^{-1})	170 ± 0	840 ± 20
k_{cat}/K_m ($\times 10^6$ M^{-1} s^{-1})	5 ± 0.3	11.4 ± 0.4

[a] All values were different ($P < 0.01$) between the two enzymes. Values are mean ± SE. The kinetic parameters were determined in 50 mM glycine−HCl, pH 3.5, at 37 °C with 11 sodium phytate concentrations ranging from 30 μM to 5 mM in a 30 s assay (n = 3). For PhyA, the reaction glycosylated protein concentration was 78.8 μg/mL and the glycosylated molecular mass was 84 kDa (via SDS-PAGE, data not shown), whereas for AppA2 the reaction glycosylated protein concentration was 27.2 μg/mL and the glycosylated molecular mass was 47 kDa (via SDS-PAGE, data not shown).

of 18 and 16%, respectively, by extraction in the presence of BSA and Triton X-100 versus extraction in the absence of BSA and Triton X-100.

Experiment 2. Compared to PhyA (**Table 2**), AppA2 had greater ($P < 0.01$) K_m (2-fold), V_{max} (9-fold), k_{cat} (5-fold), and k_{cat}/K_m (2-fold). The glycosylated molecular masses were 84 and 47 kDa for PhyA and AppA2, respectively (data not shown). PhyA was nearly 20 times more resistant to competitive inhibition by MIHS than AppA2 (K_i = 3.9 vs 0.2 μM) and retained a greater percent of activity than AppA2 between 1 and 50 μM MIHS. Both enzymes showed a rise in activity in the presence of low concentrations of GuCl as compared to the absence of GuCl, with PhyA peaking at 122% activity at 250 mM GuCl and AppA2 peaking at 161% activity at 63 mM GuCl. PhyA was more resistant to GuCl inactivation, requiring >4-fold more GuCl (1.4 vs 0.3 M) to produce the same 50% inhibition of enzyme activity. Both enzymes had two pH optima: PhyA with a peak at 2.0 and a greater peak from 5.0 to 5.5, whereas AppA2 had peaks at 3.4 and 5.0. PhyA and AppA2 had optimal temperatures of 65 °C (pH 5.0) and 58 °C (pH 3.5), respectively.

DISCUSSION

The activity values of AppA2 were more variable with the three assay methods than those of PhyA. Nearly identical activity of PhyA was determined from methods 1 and 3. Whereas methods 1 and 2 gave only 22% difference in PhyA activity, these two methods produced nearly a 3-fold disparity for AppA2. Overall,

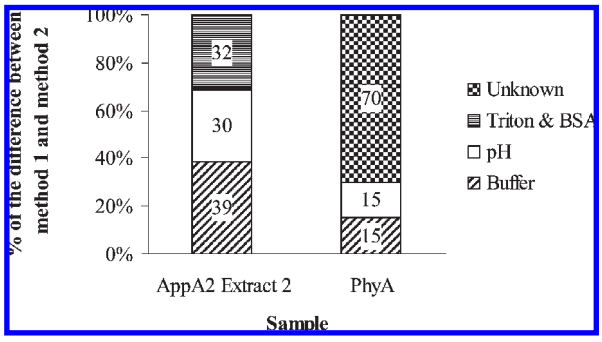

Figure 4. Relative contributions of the assay condition factors to enzyme activity variations between methods 1 and 2. The effect of each factor is presented as the portion of the total difference between the two methods accounted for by that factor of 100%. Results are for unpurified proteins.

the pH (*22*), the type of buffers (*28*), and the inclusion of ancillary chemicals such as the detergents Triton X-100 and BSA (*29*, *30*) each accounted for approximately one-third of the variations of AppA2 (**Figure 4**). The salt ions in solution can interact with the ionized basic and acidic amino acid side chains of the enzyme because of different ion sizes and electromagnetic strengths (*31*, *32*). Different salts have been shown to have a great effect on the biochemical characteristics of both PhyA and AppA2 (*33*). The pH of the buffers may affect the phytase activity by altering ionization states of the side chains in the acidic and basic amino acids of the phytase protein, as well of the substrate. The 25% increase in activity of AppA2 when assayed in the presence of 1.7×10^{-3}% BSA and Triton X-100 may be due to the nonionic detergent and/or BSA that has polar and hydrophobic portions to interact with the protein and affect its activity. Similarly, extraction of AppA2 in the presence of BSA and Triton X-100 tended to increase activity by 18 and 16%, respectively, when assayed in either citrate or acetate buffer in the absence of BSA and Triton X-100. This was likely due to the remaining small concentrations (2×10^{-5}% in citrate and 8×10^{-6}% in acetate buffer) of the compounds carried over into the assay from the extraction. Because we used extract 2 (containing BSA and Triton X-100) of AppA2 for all assay comparisons, activities may be numerically higher than if extract 1 or 3 had been used.

Because purified proteins were not used in experiment 1, we cannot completely rule out other confounding factors, although AppA2 was the extract of a commercial product and is therefore practically applicable. It should also be noted that the variability was due to the specific reaction conditions associated with the method rather than the method itself and that none of the conditions generally used by the three methods strive to simulate the gastric conditions under which the phytases function in the digestive system. Nevertheless, the variations in phytase activity as a result of assay method may affect the interpretation of feeding efficacy of phytase based on units supplemented. Thus, it is important to clearly state actual assay conditions in enabling field users to compare phytase products and researchers to improve expression levels (*34*−*36*) and to identify new phytases (*16*, *37*).

The molecular mass for the glycosylated PhyA produced in *P. pastoris* was 84 kDa, as compared to 85 kDa expressed natively in *A. niger* (*20*) and 66 kDa overexpressed in *A. niger* (*38*). The molecular mass for the glycosylated AppA2 produced in *P. pastoris* was 47 kDa, as compared to 51−56 kDa overexpressed in *P. pastoris* X-33 under an inducible promoter and 47 kDa overexpressed in *E. coli* (*38*). These molecular mass differences were due to variable extents of glycosylation and were supposed to exert no effect on kinetic parameters (*21*, *38*). Under the

commonly observed gastric pH in weanling pigs (*39*, *40*), PhyA possessed a better affinity for sodium phytate as seen by its lower K_m than AppA2. However, the latter had a superior V_{max}, turnover number (k_{cat}), and catalytic efficiency (k_{cat}/K_m) at pH 3.5. The Michaelis−Menten constant (K_m) is generally regarded as being inversely related to the enzyme's affinity for substrate. In a corn−soybean meal based diet, the concentration of phytic acid in the digesta (12% DM) of duodenum of swine was found to be 0.11% (1.7 mM) (*41*). This concentration of phytic acid is >10-fold greater than the K_m of either PhyA or AppA2. Thus, substrate concentrations do not likely limit the catalytic efficiency of phytases in the stomach (*42*). Therefore, the lower K_m of PhyA, although beneficial from a biochemical perspective, is unlikely to be of much advantage under gastric conditions. The maximum velocity (V_{max}) is the maximum catalytic rate of an enzyme under substrate saturation conditions. The turnover number (k_{cat}) is the number of molecules of product (orthophosphate for phytases) released per unit time (per second as presented here) by one molecule of enzyme under conditions of substrate saturation. The k_{cat}/K_m, or catalytic efficiency, is a measure of how efficiently an enzyme catalyzes a reaction when it comes into contact with a molecule of substrate. Because the concentration of phytic acid in the stomach is relatively high compared to the K_m, these parameters are more likely to have a greater effect on the overall efficiency of catalysis of phytic acid in the stomach. In addition, the pH optimum and overall pH-activity profile of AppA2 is better suited for its function in the stomach than PhyA, as weanling pigs showed stomach pH around 3.5 (*39*, *40*), at which the PhyA pH-activity profile has a trough and AppA2 a peak. Along with greater resistance to pepsin degradation (*43*, *44*), AppA2 is an apparently preferred feed additive compared to PhyA.

The better resistance to GuCl by PhyA implies a greater stability than AppA2, as protein denaturation by GuCl can be used to estimate protein conformational stability (*45*). By equating inactivation and denaturation, PhyA is more stable than AppA2, which agrees with previously published data (*24*, *46*). This greater stability correlates with PhyA's lesser activity variation than AppA2 among the three different assay methods seen in experiment 1. This greater stability will be of benefit to storage or transportation of the phytase before application. PhyA also has a better resistance than AppA2 to MIHS. However, as MIHS, to our best knowledge, is not present in the diet in any appreciable amount, this confers little practical benefit in the digestive system. Interestingly, AppA2 in particular showed increased activity in the presence of low concentrations of GuCl. This phenomenon has previously been observed with other enzymes and may be due to a conformational change in the active site (*47*, *48*). If this improved activity could be added into the enzyme, it would be of great advantage.

In conclusion, AppA2 activity displayed much greater variation with various assay conditions than that of PhyA, suggesting a higher sensitivity to environmental factors or changes. Meanwhile, AppA2 possessed better kinetic characteristics than PhyA at the commonly observed gastric pH of 3.5. Both comparative aspects of AppA2 and PhyA may explain the observed feeding efficacy of these two enzymes in swine and poultry.

ABBREVIATIONS USED

AAM, acetone ammonium molybdate; AppA2, *Escherichia coli* AppA2 phytase; BSA, bovine serum albumen; GuCl, guanidine hydrochloride; MES, [2-(*N*-morpholino)ethanesulfonic acid]; MIHS, *myo*-inositol hexasulfate; PhyA, *Aspergillus niger* NRRL 3135 PhyA phytase; TCA, trichloroacetic acid.

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