JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

A Simple in Vitro Test Tube Method for Estimating the Bioavailability of Phosphorus in Feed Ingredients for Swine

DAVID W. BOLLINGER,[†] ATSUSHI TSUNODA,[†,§] DAVID R. LEDOUX,[†] MARK R. ELLERSIECK,[‡] AND TRYGVE L. VEUM*,[†]

Department of Animal Science and Agricultural Experiment Station, University of Missouri, Columbia, Missouri 65211-5300

A simplified in vitro test tube (TT) method was developed to estimate the percentage of available P in feed ingredients for swine. The entire digestion procedure with the TT method consists of three consecutive enzymatic digestions carried out in a 50-mL conical test tube: (1) Pre-digestion with endo-xylanase and beta-glucanase for 1 h, (2) peptic digestion for 2 h, and (3) pancreatic digestion for 2 or 4 h. The TT method is simpler and much easier to perform compared to the dialysis tubing (DT) method, because dialysis tubing is not used. Reducing sample size from 1.0 to 0.25 g for the TT method improved results. In conclusion, the accuracy and validity of the TT method is equal to that of our more complicated DT method ($r = 0.97$, $P < 0.001$), designed to mimic the digestive system of swine, for estimating the availability of P in plant-origin feed ingredients.

KEYWORDS: In Vitro; swine; feed ingredients; phosphorus; bioavailability

INTRODUCTION

The quick and accurate measurement of phosphorus (P) bioavailability from ingredients used to formulate animal feeds is of great importance to the livestock industry, not only for maximizing growth but also for minimizing water pollution from unutilized nutrient excretion in animal manure (*1*, *2*). In cereal grains and oilseed meals, the main ingredients used in monogastric animal feed, 60−80% of the P is stored as phytate (*3*). The phytate P in cereal grains and oilseeds is poorly utilized because the digestive systems of monogastric animals produce little to no phytase (*4*), the enzyme required to hydrolyze P from the inositol ring (*5*). There are two different approaches to measuring nutrient availability, either in vivo or in vitro testing. It is obvious that in vivo testing is the most valid way to determine P bioavailability of feed ingredients. However, P availability determined in vivo is limited by the time and expense required for animal experiments. Therefore, in vitro testing is a faster and lower cost approach to obtain a valid estimate of available P (aP) in feed ingredients (*6*, *7*). In vitro approaches have also been used to estimate the digestibility of protein (*8*−*10*), Zn (*11*, *12*), Fe (*13*), and P based on P solubility in dilute acid (*14*, *15*).

In vitro procedures were designed in our laboratories to mimic the digestive system of pigs and poultry by using dialysis tubing to estimate P availability in plant-origin ingredients for swine (*6*, *7*) and poultry (*16*). Unfortunately, these dialysis tubing (DT) methods were not effective in estimating the P availability of animal-origin ingredients (*7*). In addition, the DT method requires time for preparation of the dialysis tubing, the dialysis buffer, and the complicated quantitative transfer of digesta into the dialysis tubing. An in vitro method that is simple, easy to use, inexpensive, and equally accurate for both plant and animal origin feed ingredients is desired. Therefore, the two objectives for this study were to evaluate a simplified and faster (reduced incubation time) in vitro method developed in our laboratory that used conical centrifuge tubes (TT method) to estimate the availability of P in plant feed ingredients compared with our DT method, and to compare 1.0-, 0.50-, and 0.25-g sample sizes for the TT method.

MATERIALS AND METHODS

Enzymes. Natugrain containing Endo-xylanase (8250 units/g EC 3.2.1.8) and Endo-B-glucanase (6000 units/g EC 3.2.1.6) was provided by BASF Corporation Mount Olive, NJ. Porcine pepsin (P-6887; EC 3.4.23.1) and pancreatin (P-7545; activity = 8 × USP) were purchased from Sigma Chemical Co., St Louis, MO.

Chemicals. All chemicals used were reagent quality or better. Water was 18 MΩ or equivalent.

Samples and Sample Preparation. A normal Harrington Check (HC) barley and the near isogenic low phytic acid 422 (LPA 422) barley grains were obtained from the USDA-ARS National Small Grains Germplasm Research Facility, Aberdeen, ID. Soybean meal (SBM) and corn were obtained from the University of Missouri-Columbia (UMC) feed mill. Whole soybeans (WSB) were obtained from the UMC Agronomy Department The SBM and WSB were extruded (extSBM and extWSB, respectively) in a single screw extruder (Insta-Pro Model 2000R, Triple F, Des Moines, IA) with a 0.95-cm die opening, at 132 ± 2 °C for 25 ± 2 s. Prior to extrusion, the WSB were cracked in a hammer mill, and the SBM was "preconditioned" by mixing 15% water

* To whom correspondence should be addressed. Tel.: (573)882−4331. Fax: (573)882−6827. E-mail veumt@missouri.edu.
[†] Department of Animal Science.
[‡] Agricultural Experiment Station.
[§] Requirement for MS degree In Animal Science.

10.1021/jf0345907 CCC: $27.50
Published on Web 03/05/2004

Table 1. Total Phosphorus, Phytic Acid Phosphorus, and Available Phosphorus Concentrations of Corn, Barleys, and Soybean Products

	analyzed values, %[a]			published values, %		
ingredient	total P (%)	phytic acid P (%)	anal. estimate of P availability (%)[b]	total P (%)[c]	phytic acid P (%)[d]	in vivo P availability (%)[c]
corn	0.274	0.237	13.5	0.28	0.18–0.24	12–14
Barley Grains						
Harrington check	0.354	0.242	31.6	0.36	0.19–0.27	30–31
low phytic acid 422	0.338	0.134	60.4			
Soy Products						
48% soybean meal	0.715	0.512	28.4	0.69	0.39	23–25
extruded soybean meal	0.549	0.387	29.5			
whole soybeans	0.514	0.369	28.2	0.59	0.33	
extruded whole soybeans	0.561	0.397	29.2	0.59		

[a] Air dry basis. [b] Analytical estimate of P availability = ((total P − phytate P)/total P) × 100. [c] Values from Cromwell and Coffey (*1*) and NRC (*4*). [d] Values from Viveros et al. (*19*) and Ravindran et al. (*20*, *21*).

and 15% crude soy oil with 70% SBM, air-dry basis. All sample ingredients were ground in a Wiley laboratory mill to pass a 1-mm mesh screen. Then, sub-samples were reground in a Tecator 1093 sample mill to pass a 0.5 mm mesh screen. All samples were kept in sealed plastic bags at 4 °C until analyzed.

Modified Dialysis Tubing in Vitro Method (DT method). The DT method used in this experiment is a modified version of our original in vitro DT method designed to mimic the digestive system of the pig (*6*), which consisted of two consecutive enzymatic digestions: a pepsin digestion followed by a pancreatin digestion, concurrent with dialysis. The modifications made to our original DT method are as follows: (a) the addition of a predigestion step, containing endo-β-glucanase and endo-xylanase, which increased carbohydrate digestibility and reduced sample viscosity; (b) the amounts of pepsin and pancreatin added to the digesta were increased to increase protein digestion and the release of P from the feedstuff matrix; and (c) the amount of $NaHCO_3$ added with the pancreatin was reduced to optimize digesta pH.

Testing in our laboratory found that the addition of beta-glucanase and endo-xylanase in a predigestion step increased carbohydrate digestion and liquefied the sample, especially for feedstuffs such as soy products and barley. This decreased the difficulty in transferring the digesta into the dialysis tubing and allowed the required mixing of the digestive enzymes with the substrate. In a preliminary experiment with HC barley, we tested $NaHCO_3$ concentrations of 0.2, 0.4, 0.6, 0.8, and 1.0 M in the pancreatic digestion step and measured digesta pHs of 1.93, 2.83, 5.50, 6.83, and 7.43, respectively. Hydrolyzed P values were 17.1, 17.5, 23.7, 28.6, and 26.6%, respectively. Our results found that adjusting the $NaHCO_3$ level to 0.8 M in the pancreatic digestion step lowered digesta pH below 7.0 and increased P hydrolysis in barley, providing in vitro aP values that were similar to the in vivo aP values obtained in studies with swine (*1*, *4*). An increase in the concentrations of pepsin and pancreatin in the digesta samples improved the in vitro estimate of P availability in some plant ingredients, as described in our results below. Thus, the modified DT method in the present experiment consisted of three consecutive enzyme digestion steps: (1) predigestion with beta-glucanase and endo-xylanase, (2) pepsin digestion, and (3) pancreatic digestion concurrent with dialysis. These steps are described as follows:

(*1*) *Predigestion*. Finely ground sample (1.0, 0.5, or 0.25 g) (0.5-mm screen) was mixed with 3 mL of 0.04% sodium azide solution containing 5.3 mg of Natugrain (Endo-xylanase 8.250 units/g and Endo-β-glucanase 6000 units/g, BASF Corporation Mount Olive, NJ) /mL in a 10-mL plastic syringe. Due to time constraints of the digesta transfer to the dialysis tubing, sample start times require at least a 3 min interval. The sample and enzyme solution were vortexed and incubated in a water bath with shaking at 120 rpm and 39 °C for 60 m.

(*2*) *Pepsin Digestion*. Following predigestion, the digesta sample was mixed with 1.0-mL of a 0.85 N-HCl solution containing 6000, 12 000, or 24 000 U of porcine pepsin, giving a final level of 1500, 3000, or 6000 U/mL as indicated. After the digesta and peptic enzyme solution was vortexed, the digesta was incubated in the same water bath at 39 °C for 2 h.

(*3*) *Pancreatin Digestion*. At the end of the pepsin digestion phase, samples were quantitatively transferred to dialysis tubing segments (18 cm long) and 1.3 mL of a 0.8 M $NaHCO_3$ solution containing 5.65, 11.30, or 22.60 mg porcine pancreatin/mL (8× United States Pharmacopoeia (USP)) was added to the pepsin digesta as indicated. After the digesta and enzyme solution were well mixed, dialysis tubing was sealed on each end with clamps. The dialysis tubing (molecular weight cut off of 12 000−14 000, diameter 1.6 cm, Sigma Chemical Co., St Louis, MO) was placed in a 250-mL flask containing 100 mL of 0.05 M succinate buffer. Samples were incubated at 39 °C with shaking at 120 cycles per min for 2 or 4 h as indicated. After the pancreatic incubation period, hydrolyzed P dialyzed into the succinate buffer was determined colorimetrically on a spectrophotometer at 415 nm as described by Engelen et al. (*17*). In the DT method, each ingredient was analyzed in triplicate.

Test Tube in Vitro Method (TT Method). (*1*) *Predigestion*. In the TT method, predigestion is the same as that for the DT method except that the sample for the TT method is placed in a 50-mL plastic, conical centrifuge tube. Two sample sizes, 1.0 and 0.25 g, were evaluated with the barley, corn, and soy product samples. With the TT method, samples can be started at 1 min intervals.

(*2*) *Peptic Digestion*. After predigestion was completed, the digesta was mixed with 1.0 mL of a 0.85 N HCl solution containing 24 000 U of porcine pepsin, giving a final concentration of 6000 U/mL and incubated with shaking at 120 cycles per min for 120 min at 39 °C.

(*3*) *Pancreatic Digestion*. Immediately following the pepsin digestion, 1.3 mL of a 0.8 M $NaHCO_3$ solution containing 22.6 mg of porcine pancreatin/mL (8× USP) was added to the peptic digesta. After mixing, the digesta was incubated at 39 °C with shaking at 120 cycles per min for 2 or 4 h. Approximate pH of digesta after addition of pancreatin was 7.06 in blanks. After the pancreatic digestion, test tubes were placed at 0 °C, to halt enzymatic activity in the digesta. Samples 10−40 mL of a 2 N HCl solution was added to the digesta and mixed thoroughly to stop enzyme activity and dilute the digesta. The volume of 2 N HCl added was dependent on the estimated amount of hydrolyzed P in digesta. After 2 N HCl additions, tubes were centrifuged at 1000*g* for 20 min to remove particulates from supernatants. Hydrolyzed P in the clear sample supernatant was determined colorimetrically on a spectrophotometer at 415 nm (*17*). Each ingredient was analyzed in six replicates. Hydrolyzed P in the DT and TT methods was calculated by subtracting a blank, with the same enzyme additions, from gross hydrolyzed *P* values obtained from the samples.

Total P and Phytic Acid P Determinations. Sample total P (tP) was determined by wet ashing (using nitric + perchloric acid digestion) and then running the Molybdate−Vanadate method for inorganic P (*18*). Phytic acid P was determined by the AOAC method (*18*). **Table 1** shows our analyzed values for tP, phytic acid P content, and the calculated percentage of tP that is available for swine in HC barley, LPA 422 barley, and the soy products. The published values for tP content, phytic acid P content (*19*−*21*), and the published in vivo percentages of tP in these ingredients that are available for swine (*1*, *4*) are also shown in **Table 1**.

Table 2. Relationship between Enzyme Concentration and P Hydrolysis in Barley Determined with the Dialysis Tubing Method

	enzyme concentration		P availability (%)[a]	
pepsin × pancreatin	pepsin (U)	pancreatin (mg)	Harrington check[b]	low phytic acid 422[c]
(1 × 1)[d]	6000	5.65	17.5[e]	32.6[e]
(1 × 2)	6000	11.30	17.6[e]	33.8[e]
(1 × 4)	6000	22.60	17.8[e]	32.5[e]
(2 × 1)	12 000	5.65	27.5[f]	46.0[f]
(2 × 2)	12 000	11.30	33.3[gh]	50.2[g]
(2 × 4)	12 000	22.6	28.1[f]	52.5[g]
(4 × 1)	24 000	5.65	30.3[fg]	43.2[f]
(4 × 2)	24 000	11.30	34.7[h]	49.9[g]
(4 × 4)	24 000	22.60	29.5[f]	56.3[h]

[a] P availability (%) = (hydrolyzed P/total P) × 100. [b] SEM for Harrington check barley was 0.827. [c] SEM for low phytic acid barley was 0.873. [d] The ratio of 1 × 1 refers to the enzyme concentrations of 6000 U of pepsin and 5.65 mg of pancreatin. [e–h] Means with no common superscripts in columns differ ($P < 0.01$).

Table 3. Relationship between Sample Size, P Hydrolysis, and pH for Individual Soy Products Determined with the Test Tube Method

		P availability, %			pH		
	sample	hydrolysis time			hydrolysis time		
item	size (g)	2 h	4 h	SEM	2 h	4 h	SEM
soybean meal[b]	1.00	0.7[b]	1.2[b]	0.3	6.48[b]	6.66[c]	0.01
	0.50	5.2[d]	5.6[e]	0.3	6.67[c]	6.75[d]	0.01
	0.25	18.8[d]	21.5[e]	0.3	6.96[e]	6.97[e]	0.01
extruded soybean meal[c]	1.00	2.8[b]	6.3[c]	0.5	6.70[c]	6.65[b]	0.01
	0.50	13.2[d]	15.5[d]	0.5	6.89[e]	6.84[d]	0.01
	0.25	25.3[e]	30.8[f]	0.5	6.94[f]	6.95[f]	0.01
whole soybean[d]	1.00	0.0[b]	0.0[b]	0.4	6.61[b]	6.61[b]	0.01
	0.50	4.1[c]	4.3[c]	0.4	6.79[c]	6.79[c]	0.01
	0.25	29.7[e]	23.3[d]	0.4	6.92[e]	6.88[d]	0.01
extruded whole soybean	1.00	2.4[b]	2.3[b]	0.9	6.63[b]	6.64[b]	0.01
	0.50	14.4[c]	13.8[c]	0.9	6.77[d]	6.72[c]	0.01
	0.25	31.1[d]	27.4[d]	0.9	6.85[e]	6.82[e]	0.01

[a] Available P (%) = (hydrolyzed P/total P) × 100. [b–f] Means within soy product samples with no common superscript differ ($P < 0.01$).

Statistical Analysis. The enzyme and soy product data were analyzed by analysis of variance (ANOVA) as a completely random design (*22*) using SAS (SAS Institute Inc., Cary, NC). The enzyme concentration data were arranged as a 3 × 3 factorial (3 levels of pepsin and 3 levels of pancreatin) (**Table 2**), and the soy products data were arranged as a 3 × 2 factorial (3 sample sizes and 2 incubation times, **Table 3**). Each soy product was analyzed individually. The main effects of in vitro method (DT method with 1.0 g samples, and TT method with 1.0 and 0.25 g samples) and pancreatic incubation (hydrolysis) time (2 or 4 h for both methods) were analyzed as a completely random design ANOVA with the treatments arranged as a 3 × 2 factorial (**Table 4**) according to the following model; Xijk = μijk + ai + βj + a βij + eijk, where μijk = overall mean, ai = method, βj = time, and e = error contribution with average 0 and variance d2, $i = 1...$ a; $j = 1...$ b; and $k = 1...$n. Significance was reported at $P < 0.05$, with a trend between $P \geq 0.06$ and $P \leq 0.10$.

RESULTS AND DISCUSSION

The Preincubation Step. The swine DT method as originally developed by Liu et al. (*6*) did not include a predigestion step. Because some feed ingredients, especially soy products and barley grains, tended to produce a thick gellike paste after the addition of HCl in the tryptic phase, it was desirable to modify the original DT method to correct this problem. Thus, a preincubation step was added to hydrolyze xylans and glucans, which liquefied the sample required for the proper mixing of enzyme and substrate. Endo-xylanase and endo-β-glucanase addition to the digesta samples as a preincubation step resulted in the complete liquefaction of samples in a few minutes (data not shown). Endo-xylanase degrades arabinoxylan, a nonstarch polysaccharide, which is known to increase digesta viscosity and reduce nutrient availability (*23*). Endo-β-glucanase degrades beta-glucans, which also increase digesta viscosity (*24*). The predigestion step allows proper mixing of the digesta samples and sample transfer to the dialysis tubing after the peptic digestion phase.

Optimization of Peptic and Pancreatic Enzyme Levels. Normal physiological digestion of protein in a feedstuff also releases P from the matrix of the feedstuff. This allows the percentage of aP to be determined relative to the tP in the sample. Preliminary tests conducted in our laboratory indicated that the amount of pepsin (3000 units) and pancreatin (2.4 mg) added per gram of sample in our original DT method (*6*, *7*) were not adequate for optimal protein hydrolysis and P release for some feed ingredients. Enzyme concentrations of 1, 2, and 4-fold that of the original DT method with pepsin and pancreatin were examined using two barleys, HC and LPA 422. **Table 2** shows that an increase in the concentration of both pepsin and pancreatin using the DT method resulted in an increase in the amount of P hydrolyzed in normal (HC) and low phytic acid (LPA 422) barley. In HC barley, the highest percentage of hydrolyzed P occurred ($P < 0.01$) using 2 or 4× pepsin with 2× pancreatin. For LPA 422 barley, the highest percentage of P hydrolyzed ($P < 0.01$) was observed using 4× pepsin with 4× pancreatin. However, the percentage of P hydrolyzed from HC barley using 4× pepsin with 4× pancreatin was 29.5%, which was similar to that found in in vivo studies for swine (*4*) and chickens (*25*) at 30 and 28%, respectively. Therefore, both pepsin and pancreatin concentrations were increased to 4× of the concentrations used in our original DT method (*6*, *7*) for use with both the TT and DT in vitro methods in the present study.

Reduction of Sample Size for Soy Product Digestions. In our preliminary runs, we were not able to obtain a reasonable amount of hydrolyzed P with the TT method from any of the soy products when a 1.0 g sample was used (**Table 3**) with either a 2 or 4 h pancreatic digestion. As pepsin, and especially pancreatin, contain significant levels of P, it was not desirable to increase enzyme levels beyond 4× for pancreatin compared with our original DT method (*6*, *7*). Therefore, we reduced the sample size from 1.0 to 0.5 g and ultimately to 0.25 g to increase the enzyme/sample ratio. For the 0.5 and 0.25 g sample groups, the enzyme/sample ratio of pepsin and pancreatin would be 8 and 16 times higher than the original method, respectively. We found that the percentages of hydrolyzed P for our soy product samples measured after 2 or 4 h of peptic digestion increased ($P < 0.01$) as sample size was reduced from 1.0 to 0.5 to 0.25 g. The 0.25-g samples had the highest hydrolyzed P values, ranging from 18.8 to 31.1% (**Table 3**). These estimated aP values are close to the in vivo percentages of tP in soybean products that are available to swine (23.0% in soybean meal without hulls) (*4*). The reduction in sample size raised the digesta pH of SBM, extSBM, and WSB from about pH 6.6 with 1.0 g samples to 6.9 with 0.25 g samples, which is close to the pH that optimized hydrolyzed P released in our preliminary experiments with 0.8 M $NaCO_3$. The increase in digesta pH may allow for greater pancreatic enzyme activity. The extWSB samples had a smaller increase in pH from about 6.6 to 6.8.

Comparison of the DT and TT Methods. The DT method with 1.0-g samples and the TT method with 1.0- and 0.25-g samples were compared at pancreatic hydrolysis times of 2 and

Table 4. Comparison of the Dialysis Tubing and Test Tube in Vitro Methods for Estimating Available P as a Percentage of Total P in Barley Grains, Corn, and Soy Products[a]

	dialysis tubing method			test tube method								
	1.0 g sample			1.0 g sample			0.25 g sample			P Values		
	hydrolysis time			hydrolysis time			hydrolysis time			main effects		interaction
ingredients	2 h	4 h	SEM	2 h	4 h	SEM	2 h	4 h	SEM	time	method	time × method
					barley							
Harrington check	25.7^b	32.0^c	1.4	27.0^b	28.1^b	1.0	45.2^d	48.1^d	1.0	0.01	0.01	0.10
low phytic acid 422	41.8^b	50.6^c	1.1	42.9^b	43.9^b	0.8	65.3^d	65.4^d	0.8	0.01	0.01	0.01
corn	7.7^b	9.2^c	0.4	14.8^d	16.8^e	0.3	9.4^c	10.1^c	0.3	0.01	0.01	0.09
					soy products							
soybean meal	12.4^c	17.6^d	0.5	0.7^b	1.2^b	0.3	22.0^f	19.6^e	0.3	0.01	0.01	0.01
extruded soybean meal	13.4^d	17.4^e	0.5	2.8^b	6.3^c	0.3	30.4^g	26.1^f	0.3	0.01	0.01	0.01
whole soybeans	13.4^c	16.8^d	1.0	0.0^b	0.0^b	0.5	29.4^f	22.7^e	0.5	0.04	0.01	0.01
extruded whole soybeans	14.8^c	20.2^d	0.4	2.3^b	2.3^b	0.3	30.6^f	27.2^e	0.3	0.02	0.01	0.01

[a] The DT method with 1.0-g samples and the TT method with 0.25 g samples (2 and 4 h data combined for both methods) were equal in estimating available P as a percentage of total P ($r = 0.97$, $P < 0.001$). The SEM for the DT method is about 1.5 times that of the TT method because ingredient replication number is 3 for the DT method and 6 for the TT method. $^{b-g}$ Means in a row with no common supercript differ ($P < 0.05$).

4 h in a 3 × 2 factorial arrangement of the treatments as shown in **Table 4**. The method by hydrolysis time interactions were significant ($P < 0.01$) for LPA422 barley and all the soy products, with a trend ($P \leq 0.10$ for HC barley and corn. These interactions occurred because of the differences between the DT and TT methods in response to pancreatic hydrolysis time. For the DT method, the hydrolyzed P values were higher ($P < 0.05$) and more valid at 4 h compared with 2 h for all the ingredients tested. For the DT method with a 4-h pancreatic hydrolysis time, the hydrolyzed P values for the barley grains and corn are within the range of published in vivo values for swine, although all the values for the soy products are slightly below published in vivo data (*4*).

For the TT method with 1.0-g samples, the hydrolyzed P values at both 2 and 4 h are within the published range of in vivo swine data for barley grains and corn (*4*); although the values for the soy products are clearly not valid (**Table 4**). When sample size was reduced to 0.25 g for the TT method, the hydrolyzed P values increased for all ingredients, with similar hydrolyzed P values at both 2 and 4 h for barley grains and corn. However, for soy products, the 2-h incubation gave higher ($P < 0.05$) and more valid hydrolyzed P values than the 4-h hydrolysis, compared with in vivo swine data (*4*). Therefore, the TT method with a 0.25-g sample and a 2-h pancreatic hydrolysis provided the most valid in vitro estimate of hydrolyzed P for all the ingredients tested.

A comparison of our modified DT in vitro method (modifications as described in the Materials and Methods of this paper) and our in vitro TT method found that our simplified TT method with 0.25-g ingredient samples and a 2-h pancreatic incubation was at least equal or superior to our DT method that used 1.0-g samples and a 4-h pancreatic incubation to estimate aP as a percentage of total P in feed ingredients, with published in vivo data from swine experiments as the standard (*1*, *4*). Our original DT in vitro method was validated with in vivo data obtained using growing swine (*6*). Correlation coefficients between our original DT method and the in vivo criteria (P digestibility, daily gain, daily feed consumption, and feed efficiency) averaged 0.999 ($P \leq 0.04$) (*6*). Therefore, in the present experiment, we validated our TT in vitro method (0.25-g sample and 2-h incubation) by comparing that data with the data from our validated DT method (1-g sample and a 4-h incubation) as well as published in vivo P availability (*1*, *4*). A significant correlation ($r = 0.97$, $P < 0.001$) confirms the positive relationship between these two in vitro methods in the present study. The availability of P for swine, expressed as a percentage of tP, was determined by in vivo studies with growing pigs to range from 12 to 14% for corn and averaged 30−31% for barley grains (*1*, *4*). These values are similar to the P availablilty values estimated by our DT and TT methods. The LPA 422 barley, with a reduction in phytic acid content but no reduction in tP content, would be expected to have a higher P availability than HC barley. The availablilty of P reported for in vivo studies with SBM is 23−25% (*1*, *4*), which is similar to the TT method value obtained with the 0.25-g sample hydrolyzed for 2 h (**Table 4**). There are no reported in vivo P availablilty values for WSB, extSBM, or extWSB. The lower hydrolyzed P values obtained from soy product samples using the DT method may be due to nutrient components in the digesta blocking the pores in the dialysis tubing and interfering with diffusion of hydrolyzed P into the dialysis buffer. This cannot occur with the TT method because dialysis tubing is not used.

Soybean products are economical sources of plant protein for animal diets globally. Thus, it was important to adapt the TT method to give estimates of P availability that were comparable to those reported for soybean products in vivo, which our original DM method failed to accomplish (*6*, *7*). Our initial tests on all soy products using 1.0 g samples with the TT method gave unsatisfactory results with P availabilities ranging from 0 to 6%. However, reducing the sample size for our TT method from 1.0 to 0.25 g gave soy product hydrolyzed *P* values ranging from 19.6 to 30.6%, which are similar to published in vivo results (*4*). Reevaluation of the sample size used in the DT method (*6*) was not an objective of the current experiment. However, we expect that reducing the sample size in the DT method from 1.0 to 0.5 or 0.25 g while keeping the liquid (plus enzyme) volume constant will also improve the consistency of the DT method. The reduction in sample size may allow the pancreatic hydrolysis to be completed in 2 h compared with the 4 h currently required for the DT method. Also, the smaller sample size will reduce the possibility of the substrate blocking the pores of the dialysis tubing used in the DT method. Therefore, the sample size used in the DT method deserves further consideration in a future in vitro method study.

Phytate solubility after peptic and pancreatic digestion is negatively affected by an increase in Ca concentration in the sample (*27*). Compared with cereal grains, soybeans and SBM have a relatively high Ca content which may reduce phytic acid hydrolysis. A high Ca level may also interfere with the peptic digestion phase. Additional factors in soy products that lower

peptic digestion and reduce P hydrolysis are the high pH and plant protein content compared with grains. Soy products also have very low native phytase activities and relatively high amounts of phytate that is known to inhibit enzymatic activity (*28*, *29*). The major effect of reducing the soy product sample size from 1.0 to 0.25 g was to increase the fluid volume relative to the ingredient sample dry matter to allow adequate mixing of the enzymes with the sample during the incubation steps. Another beneficial effect was the "dilution" of potential enzymatic inhibitors in soy product samples during the digestion steps.

To optimize the hydrolysis of P from digesta, the original DT method of Liu et al., (*6*, *7*) that used a 1-g sample with a 2-mL peptic solution addition, was modified as described in Materials and Methods. First, the addition of a predigestion step that added endo-xylanase and β-glucanase before the peptic step was effective in reducing sample viscosity and "liquefied" the sample, especially the soy products and barley grains. This facilitated the breakdown of plant cell walls and allowed greater access of the digestive enzymes to the substrate. The addition of xylanase to wheat-based feeds has reduced digesta viscosity and increased P availability for broiler chickens (*23*). The reduction of sample viscosity in the present experiment greatly reduced the difficulty of transferring the digesta slurry from the syringe to the dialysis membrane in the DT method and allowed proper mixing of the digesta and the peptic and pancreatic enzyme solutions in both methods. Second, the peptic volume was increased to 4 mL, which eliminated the uneven wetting encountered for some sample types when only 2 mL of peptic solutions was added. Third, our original in vitro method for estimating the bioavailability of P for swine (Liu et al., *6*, *7*) was adapted from a poultry in vitro method (*16*). We examined the effects of pancreatic enzyme concentrations on P hydrolysis in diets and feed ingredients in our original DT method (*6*, *7*), but not the peptic enzyme effects. On reexamination of the peptic enzyme effects, we found that the concentration of pepsin in our original DT method (*6*) was not optimal for hydrolysis of P in some ingredients, and that 4× pepsin (four times the concentration used in our original DT method) maximized P hydrolysis in both HC and LPA 422 barleys (**Table 2**). For pancreatin, a concentration of 2× maximized P hydrolysis in HC barley, whereas a concentration of 4× was required for LPA 422 barley. The combination of 4× pepsin followed by 4 × pancreatin gives values for hydrolyzed P (**Table 2**) that are close to in vivo (*1*, *4*, *25*) and in vitro (*7*) values of aP for swine reported for HC and LPA 422 barley. Fourth, digesta pH may not have been optimized during the pancreatic digestion phase in our original in vitro DT method (*6*, *7*). Gastrointestinal enzymatic activities and the formation of mineral-protein complexes are significantly affected by the digesta pH (*28*). A high gastric pH may lead to the formation of insoluble Ca−Zn-phytate complexes in the stomach (*27*). Wolter et al. (*12*) reported that increasing the pH from 6.2 to 7.4 during pancreatic digestion reduced the dialyzability of Ca, Mg, Fe, and Cu. Reducing $NaHCO_3$ from 1.0 to 0.8 M in the pancreatin step in both the DT and TT methods in the present study kept the pH below 7.0 and increased P hydrolysis. These results confirm the importance of pH on mineral solubility in the pancreatic step of in vitro digestion models (*12*, 30).

Veum et al. (*26*) suggested that the analytical values (*18*) for phytic acid P and tP could be used to estimate the percentage of tP that is available in feed ingredients. Subtracting phytic acid P from tP provided a good analytical estimate of P availability in normal barley compared with the in vitro estimate by our original DT method (*26*). In the present experiment, analytical estimates (phytic acid P subtracted from tP) of P availability for all the ingredients in **Table 1** (barley grains, corn and soy products) were highly correlated with the in vitro P availability estimates by the DT method (1.0-g samples and 4-h incubation, $r = 0.95$, $P = 0.001$) and the TT method (0.25-g samples and 2-h incubation, $r = 0.94$, $P = 0.001$). Even though the analyzed values for phytic acid P and tP may be used to estimate P availability, the laboratory work required to analyze for phytic acid P is very laborious, time-consuming, and expensive. An HPLC method for phytic acid analysis will reduce the analytical time required; although sample cost will increase unless large numbers of samples are run on a regular basis.

Both of our DT and TT in vitro methods are easier, faster, and less expensive than the phytic acid P analysis. However, the TT method is simpler and faster than the DT method, while maintaining the same level of validity for estimating P availability.

In conclusion, the results of this experiment indicate that we accomplished our objective of developing a simple, valid, in vitro TT method to estimate hydrolyzed P that will be much easier to use than our in vitro DT method. The TT method will eliminate the preparation of dialysis tubing, succinate buffer, syringes, and dialysis tubing clips used for dialysis. The TT method also avoids the difficult step of quantitatively transferring the digesta from the syringe to the dialysis tubing and reduces the handling time for each sample. Approximately 20 samples can be run at one time with the DT method, whereas double to triple that number (40−60) samples can be run with the TT method. The analytical value for phytic acid P may also be used to estimate P availability in feed ingredients by subtracting phytic acid P from total P.

Our simplified in vitro test tube procedure is comparable to our in vitro dialysis tubing method for measuring hydrolyzed P (estimated available P) and is a valid alternative to conducting in vivo studies to evaluate P availability in feed ingredients fed to swine. Compared to the dialysis tubing method, this simple in vitro test tube procedure reduces preparation time, eliminates the complicated and time-consuming manipulation of digesta during transfer to dialysis tubing and allows a greater number of samples to be run simultaneously.

ACKNOWLEDGMENT

We thank the USDA-ARS National Small Grains Germplasm Research Facility, Aberdeen, ID, for providing the Harrington check and low phytic acid 422 barleys, and the University of Missouri-Columbia Agronomy Department, for providing the whole soybeans.

LITERATURE CITED

(1) Cromwell, G. L.; Coffey, R. D. Phosphorus − a key essential nutrient, yet a possible major pollutant − its role in animal nutrition. *Biotechnology in the Feed Industry, Proceedings of Alltech's Seventh Annual Symposium;* Alltech Technol. Publication, 3031; Lyons, T. P., Ed.; Alltech, Inc.: Nicholasville, KY, 1991; pp 133−145.

(2) Browner, C. M.; Fox, J. C.; Frace, S. E.; Anderson, D. F.; Goodwin, J.; Shriner, P. H. Development document for the proposed revisions to the national pollutant discharge elimination system regulation and the effluent guidelines for concentrated animal feeding operations. EPA-821-R-01−003, U.S. Environmental Protection Agency, Office of Water (4303): Washington, D.C., 2001; pp 1−155.

(3) Maga, J. A. Phytate: Its chemistry, occurrence, food interactions, nutritional significance, and methods of analysis. *J. Agric. Food Chem.* **1982**, *30*, 1−9.
(4) NRC. National Research Council. In *Nutrient Requirements of Swine*, 10th ed.; National Academy of Sciences: Washington, D.C., 1998.
(5) Pointillart, A.; Fontaine, N.; Thomasset, M. Phytate phosphorus utilization and intestinal phosphatases in pigs fed low phosphorus: wheat or corn diets. *Nutr. Rep. Int.* **1984**, *29*, 473−483.
(6) Liu, J.; Ledoux, D. R.; Veum, T. L. In vitro procedure for predicting the enzymatic dephosphorylation of phytate in corn-soybean meal diets for growing swine. *J. Agric. Food Chem.* **1997**, *45*, 2612−2617.
(7) Liu, J.; Ledoux, D. R.; Veum, T. L. In vitro prediction of phosphorus availability in feed ingredients for swine. *J. Agric. Food Chem.* **1998**, *46*, 2678−2681.
(8) Babinszky, L.; van der Meer, J. M.; Boer, H.; den Hartog, L. A. An in vitro method for prediction of the digestible crude protein content in pig feeds. *J. Sci. Food Agric.*. **1989**, *50*, 173−178.
(9) Drake, A. P.; Fuller, M. F.; Chesson, A. Simultaneous estimations of precaecal protein and carbohydrate digestion in the pig. In *In Vitro Digestion for Pigs and Poultry;* Fuller, M. F., Ed.; CAB International: Wallingford, U.K., 1991; pp 162−176.
(10) van der Meer, J. M.; Perez, J. M. In vitro evaluation of European diets for pigs. Prediction of the organic matter digestibility by an enzymic method or by chemical analysis. *J. Sci. Food Agric.* **1992**, *59*, 359−363.
(11) Lease, J. G. Availability to the chick of zinc-phytate complexes isolated from oil seed meals by an in vitro digestion method. *J. Nutr.* **1967**, *93*, 523−532.
(12) Wolters, M. G. E.; Schreuder, H. A. W.; van den Heuvel, G.; van Lonkhuijsen, H. J.; Hermus, R. J. J.; Voragen, A. G. J. A continuous in vitro method for estimation of the bioavailability of minerals and trace elements in foods: application to breads varying in phytic acid content. *Br. J. Nutr.* **1993**, *69*, 849−861.
(13) Miller, D. D.; Schricker, B. R.; Rasmussen, R. R.; Van Campen, D. An in vitro method for estimation of iron availability from meals. *Am. J. Clin. Nutr.* **1981**, *34*, 2248−2256.
(14) Day, E. J.; McNaughton, J.; Dilworth, B. C. Chemical versus chick bioassay for phosphorus availability of feed grade sources. *Poult. Sci.* **1973**, *52*, 393−395.
(15) Yoshida, M.; Ishikawa, M.; Nakajima, H.; Hotta, S. Solubility of phosphorus in citric acid solution as an index of biological availability. *Ghana Farmer* **1979**, *16*, 290−292.
(16) Zyla, K.; Ledoux, D. R.; Veum, T. L. Complete enzymic dephosphorylation of corn-soybean meal feed under simulated intestinal conditions of the turkey. *J. Agric. Food Chem.* **1995**, *43*, 288−294.
(17) Engelen, A. J.; van der Heeft, F. C.; Randsdorp, P. H. G.; Smit, E. L. C. Simple and rapid determination of phytase activity. *J. AOAC Int.* **1994**, *77*, 760−764.
(18) AOAC. *Official Methods of Analysis*, 15th ed.; Helrich, K., Ed. Association of Official Analytical Chemists: Arlington, VA, 1990.
(19) Viveros, A.; Centeno, C.; Brenes, A.; Canales, R.; Lozano, A. Phytase and acid phosphatase activities in plant feedstuffs. *J. Agric. Food Chem.* **2000**, *48*, 4009−4013.
(20) Ravindran, V.; Ravindran, G.; Sivalogan S. Total and phytate phosphorus contents of various feeds and feedstuffs of plant origin. *Food Chem.* **1994**, *50*, 133−136.
(21) Ravindran, V.; Bryden, W. L.; Kornegay, E. T. Phytates: Occurrence, bioavailability, and implications in poultry nutrition. *Poult. Avian Biol. Rev.* **1996**, *6*, 125−143.
(22) Snedecor, G. W.; Cochran, W. G. *Statistical Methods*, 8th ed.*;* Iowa State University Press: Ames, IA, 1989.
(23) Zyla, K.; Gogol, D.; Koreleski, J.; Swiatkiewicz, S.; Ledoux, D. R. Simultaneous application of phytase and xylanase to broiler feeds based on wheat: in vitro measurements of phosphorus and pentose release from wheats and wheat-based feeds. *J. Sci. Food Agric.* **1999**, *79*, 1832−1840.
(24) Fengler, A. I.; Marquardt, R. R. Water-soluble pentosans from rye: I. Isolation, partial purification, and characterization. *Cereal Chem.* **1988**, *65*, 291−297.
(25) Li, Y. C.; Ledoux, D. R.; Veum, T. L.; Raboy, V.; Zyla, K.; Wikiera, A. Bioavailability of phosphorus in low phytic acid barley. *J. Appl. Poult. Res.* **2001**, *10*, 86−91.
(26) Veum. T. L.; Ledoux, D. R.; Bollinger, D. W.; Raboy, V.; Cook, A. Low-phytic acid barley improves calcium and phosphorus utilization and growth performance in growing pigs. *J. Anim. Sci.* **2002**, *80*, 2663−2670.
(27) Champagne, E. T.; Phillippy, B. Q. Effects of pH on calcium, zinc, and phytate solubilities and complexes following in vitro digestions of soy protein isolate. *J. Food Sci.* **1989**, *54*, 587−592.
(28) Reddy, N. R.; Pierson, M. D.; Sathe, S. K.; Salunkhe, D. K. *Phytates in Cereals and Legumes*; CRC Press: Boca Raton, FL, 1989.
(29) Caldwell, R. A. Effect of calcium and phytic acid on the activation of trypsinogen and the stability of trypsin. *J. Agric. Food Chem.* **1992**, *40*, 43−46.
(30) Rao, B. S. N.; Prabhavathi, T. An in vitro method for predicting the bioavailability of iron from foods. *Am. J. Clin. Nutr.* **1978**, *31*, 169−175.

Received for review June 4, 2003. Revised manuscript received January 15, 2004. Accepted February 1, 2004. Partial support provided by Agromedic Co. LTD, Tokyo, Japan.

JF0345907