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RESEARCH ARTICLE

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Suitable condition of enzymatic reactivity for determining the digestibility of feedstuffs phosphorus *in vitro* by dialysis tube method

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Abstract In order to develop a new method for determining the phosphorus (P) digestibility *in vitro* in feedstuffs by dialysis tube, a $L_{32}(4^9)$ orthogonal experiment with eight factors (4 levels for each factor) and a single factor experiment on the enzymatic reactivity were carried out. The sequence of significance of the eight factors on sample-P dialyzability was as follows: trypsin digestion for 6 h, dialyzing solution at 100 mL, pH of pepsin solution at 2.5, pepsin concentration at 2000 $U \cdot mL^{-1}$ pepsin digestion for 100 min, at temperature of 35°C, trypsin concentration at 1625 $U \cdot mL^{-1}$, and pH of trypsin solution at 6.5, respectively. And *in vitro* dialyzabilities of P in soybean meal, barley, sorghum, peanut meal, and rapeseed meal were $(36.91 \pm 0.58)\%$, $(27.28 \pm 0.94)\%$, $(26.95 \pm 0.58)\%$, $(30.51 \pm 0.83)\%$, and $(20.82 \pm 1.09)\%$, respectively.

Keywords phosphorus, *in vitro*, digestibility, suitable conditions of enzymatic reactivity

1 Introduction

Phosphorus (P) is an essential element for animal nutrition. As a limited and nonrenewable natural resource, P is the third most expensive nutrient after energy and protein in swine nutrition and feeding. Furthermore, excessive P output in swine manure is one of the key elements contributing to environmental pollution (Fan et al., 2001; Huo, 2002). Available P in feedstuffs determined by *in vivo* or *in vitro* method is the key parameter of feed formulation to reduce excretion of P. The parameter determined by *in vivo* method is the most impersonal and reliable, but the method is time-consuming, cockamamie and costly. And the *in vitro* method with the advantages of double-quickness and high efficiency is worth popularizing. Today, most studies on digestible nutrients *in vitro* were about proteins in feed, and the only study on available P *in vitro* was reported by Liu et al. in 1997.

The *in vitro* gastrointestinal model method of pigs was short of systemic studies. The objective of our research was to modify the enzymatic reactivity conditions of the *in vitro* method, and to determine P digestibility in soybean meal, barley, sorghum, peanut meal and rapeseed meal by dialysis tube.

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2 Materials and methods

2.1 Materials

Dialysis tubing (molecular weight cut-off 12000, diameter 21 mm), pepsin (activity 3000–3500 $U \cdot mg^{-1}$) and trypsin (activity $\geqslant 250$ $U \cdot mg^{-1}$) were purchased from Sigma Chemical Co., Ameresco Chemical Co. and Ameresco Chemical Co., respectively. The other chemicals of analytical grade were obtained from Chinese Chemical Co..

2.2 Samples

Samples of soybean meal, barley, sorghum, peanut meal and rapeseed meal were collected from feedstuff market in Guangzhou city.

2.3 Experimental design

In order to develop a new method for determining the digestibility of feedstuffs phosphorus *in vitro* by dialysis tube, the effects of eight factors (4 levels for each factor) in a $L_{32}(4^9)$ orthogonal experiment and a single factor experiment on the enzymatic reactivity were discussed (Table 1).

Table 1 Factors and their levels of orthogonal table $L_{32}(4^9)$

factors	levels			
	1	2	3	4
pepsin concentration/U·mL^{-1}	1000	1500	2000	2500
time of pepsin digestion/min	50	75	100	125
pH of pepsin solution	2.0	2.5	3.0	3.5
trypsin concentration/U·mL^{-1}	875	1125	1375	1625
time of trypsin digestion/h	2	4	6	8
pH of trypsin solution	6.0	6.5	7.0	7.5
volumes of dialyzing solution/mL	100	200	300	400
temperature/°C	35	37	39	41

2.4 Protocol for *in vitro* digestion procedures

An *in vitro* procedure of pepsin and trypsin digestion was adopted. Fourfold samples (1 ± 0.001 g) of feedstuffs were weighed and stored in 10-mL glass test tubes. The samples were hydrolyzed with 2 mL pepsin and HCl solution whose pH values were 2.0, 2.5, 3.0 and 3.5, respectively. Then the tubes were vortexed, sealed with parafilm and incubated in a water bath at 35°C, 37°C, 39°C or 40°C at different time duration showed in Table 1. In this period, it was designed to simulate conditions in stomach. Some doses of 1 mol·L^{-1} $NaHCO_3$ containing 0.5 mL trypsin solution at a pH value showed in Table 1 were added dropwise with constant stirring into each tube. The slurry was transferred quantitatively to 10 cm segments of dialysis tubing. Segments were placed in a conical flask containing some volumes of 8.5% NaCl and 0.1 N hydrochloric acid in barbiturate buffer (at pH values of 6.0, 6.5, 7.0 and 7.5) and incubated in a shaking water bath at 35°C, 37°C, 39°C and 41°C, respectively, in a way that the small intestine phase of digestion was simulated.

Ten mL samples were used to determine the inorganic phosphate in the dialysate after 2 h, 4 h, 6 h and 8 h.

Based on the results of orthogonal experiment, the more accurate time of trypsin digestion and the uppermost factor were selected by a single-factor test at eight levels of 2 h, 3 h, 4 h, 5 h, 6 h, 8 h, 10 h and 12 h, respectively.

2.5 Calculation

Dialyzability of P in feedstuff $= [D/(m \times A)] \times 100\%$, where D, m, A represent dialysis P in buffer (mg), the mass of sample (mg) and mass rate between P and sample (%).

2.6 Index measurement and analytical method

Total phosphorus in feed and buffer was determined colorimetrically by the molybdovanadate method (Zhang, 2003), and calcium in feed was determined by EDTA titration (Zhang, 2003).

2.7 Statistical analysis

Experimental data were analyzed with SAS (6.12), and given in mean value $\pm$ standard error.

3 Results

Total phosphorus and calcium in soybean meal, barley, sorghum, peanut meal, and rapeseed meal were 0.69% and 0.23%, 0.28% and 0.04%, 0.21% and 0.01%, 0.83% and 0.20%, 1.19% and 0.75%, respectively.

3.1 Effect of conditions of enzymatic reactivity on dialyzability of P in feedstuff

Based on R value in orthogonal experiment, the effect of time of trypsin digestion in eight factors on dialyzability of P was the most significant, followed by the volume of dialyzing solution, pH value of pepsin solution, pepsin concentration, time of pepsin digestion, temperature, trypsin concentration and pH value of trypsin solution (Table 2).

Dialyzability of P increased by $y = 3.3x + 3.08$ ($R^2 = 0.99$) during the time of trypsin digestion from 2 h to 8 h. And response curves of volumes of dialyzing solution from 100 mL to 400 mL, pH of pepsin solution from 2.0 to 3.5, pepsin concentration from 1000 U·mL^{-1} to 2000 U·mL^{-1}, time of pepsin digestion from 50 min to 125 min, temperature from 35°C to 39°C and trypsin concentration from 875 U·mL^{-1} to 1625 mg·mL^{-1} changed by $y = 0.0002x^2 - 0.1611x + 40.723$ ($R^2 = 0.99$), $y = -10.78x^2 + 53.366x - 42.284$ ($R^2 = 0.97$), $y = 0.0099x + 5.7667$ ($R^2 = 0.99$), $y = -0.004x^2 + 7.897x - 15.726$ ($R^2 = 0.99$), $y = -0.7875x^2 + 56.665x - 997.39$ ($R^2 = 1$) and $y = 0.00001x^2 - 0.0363x + 37.577$ ($R^2 = 0.92$), respectively. Besides, response of pH values of trypsin solution from 6.0 to 7.5 was not significant. Based on maximal dialyzability of P, the most suitable conditions of time of trypsin digestion, volumes of dialyzing solution, pH value of pepsin solution, pepsin concentration, time of pepsin digestion, temperature and trypsin concentration were 8 h, 100 mL, 2.5, 2000 U·mL^{-1}, 100 min, 35°C, 1625 U·mL^{-1} and 6.5, respectively.

3.2 More accurate time of trypsin digestion by a single-factor test

The P dialyzability of soybean meal during the time of trypsin digestion from 2 h to 12 h was $(9.24 \pm 0.34)\%$, $(13.25 \pm 0.68)\%$, $(15.93 \pm 0.73)\%$, $(24.78 \pm 0.45)\%$, $(35.65 \pm 0.98)\%$, $(41.40 \pm 0.45)\%$, $(52.17 \pm 1.55)\%$ and $(61.46 \pm 1.48)\%$, respectively, and increased by $y = 5.44x - 2.315$ ($R^2 = 0.98$). And an inflexion was observed at the time of trypsin digestion for 6 h. The

Table 2 Analysis of orthogonal experiment

No.	factor								dialyzability of P/%
	I/U·mL^{-1}	II/min	III	IV/U·mL^{-1}	V/h	VI	VII/mL	VIII/°C	
1	1000	50	2.0	875	2	6.0	100	35	11.59 ± 0.20
2	1000	75	2.5	1125	4	6.5	200	37	16.28 ± 0.44
3	1000	100	3.0	1375	6	7.0	300	39	13.31 ± 0.33
4	1000	125	3.5	1625	8	7.5	400	41	11.56 ± 0.62
5	1500	50	2.0	1125	4	7.0	300	41	8.26 ± 0.73
6	1500	75	2.5	875	2	7.5	400	39	9.08 ± 0.28
7	1500	100	3.0	1625	8	6.0	100	37	58.69 ± 1.02
8	1500	125	3.5	1375	6	6.5	200	35	15.59 ± 0.33
9	2000	50	2.5	1375	8	6.0	200	39	18.60 ± 0.56
10	2000	75	2.0	1625	6	6.5	100	41	49.75 ± 2.07
11	2000	100	3.5	875	4	7.0	400	35	12.27 ± 0.33
12	2000	125	3.0	1125	2	7.5	300	37	9.66 ± 0.27
13	2500	50	2.5	1625	6	7.0	400	37	16.82 ± 0.50
14	2500	75	2.0	1375	8	7.5	300	35	29.25 ± 1.15
15	2500	100	3.5	1125	2	6.0	200	41	5.67 ± 0.36
16	2500	125	3.0	875	4	6.5	100	39	24.52 ± 0.41
17	1000	50	3.5	875	8	6.5	300	37	15.29 ± 0.35
18	1000	75	3.0	1125	6	6.0	400	35	16.52 ± 0.60
19	1000	100	2.5	1375	4	7.5	100	41	34.61 ± 0.93
20	1000	125	2.0	1625	2	7.0	200	39	9.39 ± 0.08
21	1500	50	3.5	1125	6	7.5	100	39	14.24 ± 0.39
22	1500	75	3.0	875	8	7.0	200	41	27.99 ± 0.47
23	1500	100	2.5	1625	2	6.5	300	35	10.92 ± 0.25
24	1500	125	2.0	1375	4	6	400	37	15.41 ± 0.58
25	2000	50	3.0	1375	2	6.5	400	41	10.85 ± 0.32
26	2000	75	3.5	1625	4	6.0	300	39	11.71 ± 0.18
27	2000	100	2.0	875	6	7.5	200	37	32.42 ± 1.51
28	2000	125	2.5	1125	8	7.0	100	35	61.70 ± 1.11
29	2500	50	3.0	1625	4	7.5	200	35	12.76 ± 0.30
30	2500	75	3.5	1375	2	7.0	100	37	10.44 ± 0.33
31	2500	100	2.0	1125	8	6.5	400	39	17.22 ± 0.60
32	2500	125	2.5	875	6	6.0	300	41	13.57 ± 0.44
K_1	127.55	108.42	173.34	146.84	77.61	152.25	217.03	169.61	
K_2	161.66	170.02	181.58	148.55	135.82	160.42	138.80	169.01	
K_3	207.06	180.39	174.78	148.06	171.32	160.18	111.97	118.07	
K_4	130.25	161.40	96.71	183.08	241.78	153.68	108.23	162.26	
$K_1/4$	15.94	13.55	21.68	18.36	9.70	19.03	27.13	21.20	
$K_2/4$	20.21	21.25	22.70	18.57	16.98	20.05	17.35	21.13	
$K_3/4$	25.88	22.55	21.85	18.51	21.42	20.02	14.00	14.76	
$K_4/4$	16.28	20.18	12.09	22.89	30.22	19.21	13.59	20.28	
R	9.94	9.00	10.61	4.53	20.52	1.02	13.54	6.37	

Note: I, II, III, IV, V, VI, VII and VIII represent pepsin concentration, time of pepsin digestion, pH value of pepsin solution, trypsin concentration, time of trypsin digestion, pH value of trypsin solution, volume of dialyzing solution and temperature, respectively.

increasing ratio of P dialyzability at the time of trypsin digestion from 2 h to 6 h was higher than that at the time of trypsin digestion from 6 h to 12 h. Therefore, trypsin digestion for 6 h was selected as the more accurate parameter.

3.3 P dialyzability of feedstuff

The P dialyzability of soybean meal, barley, sorghum, peanut meal, and rapeseed meal measured by this method was (36.91 ± 0.5)%, (27.28 ± 0.94)%, (26.95 ± 0.58)%, (30.51 ± 0.83)%, and (20.82 ± 1.09)%, respectively.

4 Discussion

Firstly, a key of *in vitro* measurement of available phosphorus is to select suitable conditions of enzymatic reactivity mainly including enzyme concentration, temperature, time of pepsin digestion, volume of dialyzing solution, pH value of trypsin solution and so on. Secondly, quantity and granularity of samples, separating techniques of inorganic P were also important. In these factors, digestive enzymes, such as pepsin, trypsin and small intestine liquid, etc. showed the most importance. Enzymes in *in vitro* digestion experiment should have the

characteristics of endogenous enzymes in theory (Ji et al., 1994). Therefore, pepsin and trypsin were preferred. Furthermore, the maximal parameter value of *in vitro* digestibility was commonly selected as the suitable condition (Savioe and Gauthier, 1986; Huang et al., 1999).

The suitable parameter of enzymatic reactivity in this experiment was 4000 U trypsin per 1 g sample, which was in accordance with that recommended by Liu et al. (1997). When the pH value of pepsin solution was at 2.0 to 3.5, the P dialyzability of feedstuff decreased, which accorded with the report by Ji et al. (1994). Why? It was likely that the activity of pepsin decreased with the increasing of pH. Based on these results, the suitable pH value of pepsin solution in the experiment was 2.5, approximately consistent with 2.5 and 2.75 reported by Liu et al. (1997) and Zyla et al. (1999), respectively, indicating that P and CP dialyzabilities could increase with the prolonging of pepsin digestion. Furthermore, an inflexion at 100 min was observed in our study, which was 25 min longer than the time reported by Liu et al. (1997). When samples were treated with trypsin solution, the pH 6.5 approached pH 6.0 of Liu et al. (1997) and pH 6.1 of Zyla et al. (1999). As for temperature of enzymatic reactivity, the parameter of P in the *in vitro* dialysis method was 35°C, the same to that of CP in the experiment by (Huang et al., 1999), but lower than 39°C of Liu et al. (1997) and 40°C of Zyla et al. (1999). In the *in vitro* dialysis tube method established by Mauron et al. (1955) and successively modified by Steinhart and Kirchgessner (1973), and Gauthier et al. (1982), the volume of dialyzing solution was very important. The dialyzability of CP in feedstuff showed an increase with the change of dialyzing solution volume in many reports (Liu et al., 1997; Xi et al., 2003), however our dialyzability of P as well as that of Liu et al. (1997) and Zyla et al. (1999) were all just reverse, and the suitable volume of dialyzing solution was 100 mL among these tests.

Correlation analysis indicated that the dialyzability of P in the five feedstuffs had no close correlation with that reported by Liu et al. (1997) but a partly correlation with that by Fang (2003).

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