Asian-Aust. J. Anim. Sci.
Vol. 24, No. 12 : 1718 - 1728
December 2011

www.ajas.info
http://dx.doi.org/10.5713/ajas.2011.11132

Effects of Keratinase on Performance, Nutrient Utilization, Intestinal Morphology, Intestinal Ecology and Inflammatory Response of Weaned Piglets Fed Diets with Different Levels of Crude Protein

D. Wang, X. S. Piao*, Z. K. Zeng, T. Lu, Q. Zhang, P. F. Li, L. F. Xue and S. W. Kim[1]

Ministry of Agriculture Feed Industry Centre, State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing 100193, China

ABSTRACT : Two experiments were conducted to investigate the *in vitro* ability of keratinase to hydrolyze soybean glycinin and β-conglycinin and to evaluate the *in vivo* effects of keratinase when included in corn-soybean diets with different levels of crude protein and fed to nursery pigs. In experiment 1, a saturated keratinase solution (1 ml) was added to two blank controls of either glycinin or β-conglycinin resulting in the hydrolysis of 94.74% glycinin and 88.89% β-conglycinin. In experiment 2, 190 pigs (8.3±0.63 kg BW) were allotted to one of four treatments in a 2×2 factorial arrangement on the basis of body weight, and sex was balanced among the pens. The effects of crude protein (19 vs. 22%) and keratinase (0 vs. 0.05%) were studied. Each treatment was applied to six pens with seven (two pens) or eight pigs per pen. Pigs were fed the experimental diets for 21 d. Weight gain and feed conversion ratio were improved ($p<0.05$) with keratinase supplementation while feed intake was reduced ($p<0.05$). Keratinase supplementation increased ($p<0.05$) the apparent total tract digestibility of dry matter, energy, crude protein and phosphorus. Keratinase supplementation also increased n-butyric acid in the cecum and colon, lactobacilli and total anaerobe counts in the colon as well as the ratio of villus height to crypt depth in the ileum. Additionally, fecal score, ammonia nitrogen and branch chain volatile fatty acids in the colon, *E. coli* and total aerobe counts in the colon, crypt depth in the jejunum and ileum as well as serum interleukin-1 and interleukin-6 concentrations were also decreased ($p<0.05$) by keratinase supplementation. A reduction in dietary crude protein decreased ($p<0.05$) colon ammonia nitrogen concentration and cecal propionic acid and branch chain volatile fatty acid concentrations. In addition, cecal *E. coli* counts, colon total anaerobe counts, ileal crypt depth, and serum interleukin-1 and interleukin-6 concentrations were also decreased ($p<0.05$) with the reduction of dietary crude protein. With the exception of fecal scores, there were no significant interactions between crude protein and keratinase. This study provides evidence that dietary keratinase supplementation improved nursery pig performance by improving intestinal morphology and ecology, thus improving nutrient digestibility and alleviating the inflammatory response. (**Key Words :** Keratinase, Crude Protein, Digestibility, Performance, Weaned Piglets)

INTRODUCTION

Soybean meal is one of the most commonly used protein source for pigs (Swick, 2007). Although most of the proteins in soybean meal are easily digested (McDonnell et al., 2010), some are difficult to digest especially for newly weaned piglets. Problematic proteins include glycinin (about 40% of the total soybean globulin proteins), β-conglycinin (about 30%), and other minor proteins (Brandom and Friedman, 2002; Sun et al., 2007). Previous studies have shown that these proteins can damage intestinal morphology and impair immune function in newly-weaned pigs (Qiao et al., 2003; Sun et al., 2007; Yoo et al., 2009). When such dietary factors are combined with

* Corresponding Author : Xiangshu Piao. Tel: +86-10-62733588, Fax: +86-10-62733688, E-mail: piaoxsh@mafic.ac.cn
[1] Department of Animal Science, North Carolina State University, Raleigh, NC, 27695, USA.
Received May 6, 2011; Accepted June 10, 2011

the stress of weaning, both nutrient metabolism and the immune function of weaned piglets can be seriously impaired (Kong et al., 2009).

Keratinase is a broad range protease which hydrolyzes different protein substrates including casein, collagen, elastin and keratin as well as other proteins containing cystine disulfide bonds (Yu et al., 1972; Lin et al., 1996; Tu et al., 1998; Gradišar et al., 2005). Keratinase has been used to improve digestibility of feather meal for livestock (Kim and Patterson, 2000). It has also been shown that keratinase supplementation in corn-soybean meal-based broiler diets improved performance (Odetallah et al., 2003, 2005), amino acid utilization (Wang et al., 2006) and gut villus structure (Wang et al., 2008).

The cystine disulfide bonds in glycinin and β-conglycinin can impair digestion (Hou and Chang, 2004; Golubovic et al., 2005) and thus it is reasonable to hypothesize that dietary supplementation with keratinase could improve the amino acid utilization and performance of weaned piglets. Therefore, the present study was conducted to investigate the *in vitro* ability of keratinase to hydrolyze soybean glycinin and β-conglycinin and to evaluate the *in vivo* effects of keratinase on performance, nutrient utilization, intestinal morphology, intestinal ecology and the inflammatory response of nursery pigs fed corn-soybean diets with different crude protein concentrations.

MATERIALS AND METHODS

Keratinase preparation

The keratinase used in the present experiments was produced by *Bacillus licheniformis* PWD-1 after 48 h fermentation at 50°C, followed by concentrating and spray-drying (Wang and Shih, 1999). It is marketed under the brand name Cibenza DP100™ by Novus International (Shanghai, China). The keratinase activity in the product was 612,000 U/g where a unit is defined as an increase of 0.1 in absorbance at a wavelength of 280 nm under the conditions described by Gradišar et al. (2000).

In vitro glycinin and β-conglycinin hydrolysis

The ability of keratinase to hydrolyze soybean glycinin and β-conglycinin was determined according to the method described by Böckle et al. (1995) with some modifications. Purified glycinin and β-conglycinin were obtained from the Food Institute at China Agricultural University (Patent Number 200410029589.4, Beijing, China).

Approximately 0.4 g of glycinin or β-conglycinin was incubated in 11 ml potassium phosphate buffer (50 mM) for four hours at 37°C to serve as a blank control (Table 1). In addition, a saturated keratinase solution was prepared by incubating keratinase (4 mg) in 50 ml potassium phosphate buffer (50 mM) for 45 minutes (pH 7.8, 37°C). For these treatments, 1 ml of the potassium phosphate buffer of the glycinin and β-conglycinin control solutions was replaced by 1 ml of the keratinase solution. Loss of dry weight for glycinin and β-conglycinin was determined after they were filtered through membrane (pore size, 0.2 μm; Linghang Equipment Ltd., Tianjin, China), washed with deionized water and freeze-dried.

In vivo animals and experimental design

All the procedures used in this study were approved by the China Agricultural University Animal Care and Use Committee (Beijing, China). A total of 190 crossbred pigs [Duroc×(Landrace×Large White)], comprising an equal number of barrows and gilts, with an average body weight of 8.3±0.63 kg and weaned at 30±2 days of age were allotted to one of four treatments in a 2×2 factorial arrangement with two crude protein concentrations (19 vs. 22%) and two levels of supplementation with keratinase (0 vs. 0.05%) on the basis of body weight, and sex was balanced among the pens. Each treatment was applied to six pens, with seven (two pens) or eight pigs per pen. Wherever possible, an equal number of barrows and gilts were housed in each pen.

The experimental diets were formulated using the ideal protein concept with the ratio of threonine, total sulfur containing amino acids and tryptophan set at 65, 60, and 19% on a true ileal digestible (TID) amino acid basis

Table 1. Hydrolyzing ability of keratinase on soybean glycinin and β-conglycinin

Treatment	Glycinin control	β-Conglycinin control	Glycinin+ keratinase	β-Conglycinin+ keratinase
Potassium phosphate buffer (ml)	10.00	10.00	10.00	10.00
Substrate	Glycinin	β-Conglycinin	Glycinin	β-Conglycinin
Keratinase solution (ml)	0.00	0.00	1.00	1.00
Potassium phosphate buffer (ml)	1.00	1.00	0.00	0.00
Mass of substrate (g)	0.36	0.59	0.38	0.54
Non-hydrolyzable dry matter (g)	0.32	0.44	0.02	0.06
Hydrolysis rate[1] (%)	11.81	24.20	94.74	88.89

[1] Hydrolysis rate was calculated according to the equation: Hydrolysis rate = (Mass of substrate-Non-hydrolyzable dry matter)/Mass of substrate×100.

relative to lysine (Yi et al., 2006). The dietary concentrations of TID lysine were 1.30% and 1.10% in the high and low protein diets, respectively (Table 2). TID of the amino acids in the different ingredients were calculated according to NRC (1998). Methionine was provided as methionine hydroxy analogue (Novus International, Shanghai, China). All diets were formulated to provide 3.40 Mcal/kg of digestible energy and meet the nutrient requirements suggested by NRC (1998) for 10 to 20 kg pigs with the exception of the protein concentration in the low protein diets which were 10% lower than the NRC recommendations (NRC, 1998).

The pigs were housed in 2.5×2.5 m^2 raised pens with a slatted floor. The temperature of pig barn was maintained at 25°C. Feed and water were available *ad libitum*. Pigs and feeders were weighed at the beginning and the end of the experiment to determine average daily gain, average daily feed intake and feed conversion. Feed samples were collected at the beginning of the experiment. The pigs were fed the experimental diets for 21 d.

Pigs were observed for clinical signs of diarrhea and a scoring system was applied to indicate the presence and severity of diarrhea according to the method of Pierce et al. (2005). Scores for individual pens were recorded daily each morning by three technicians unaware of the dietary treatment that the pigs were being fed. Fresh excreta were ranked using the following scale: 1 = normal hard feces; 2 = slightly soft feces; 3 = soft, partially formed feces; 4 = loose, semi-liquid feces; and 5 = watery, mucous-like feces.

On days 19, 20, and 21 of the experiment, fresh fecal

Table 2. Ingredient composition and nutrient content of experimental diets

Item	Dietary treatment			
	High protein	High protein+keratinase	Low protein	Low protein+keratinase
Ingredient (% as fed)				
Corn	52.98	52.93	56.57	56.52
Soybean meal	24.90	24.90	23.35	23.35
Whey powder	9.80	9.80	10.00	10.00
Fish meal	6.60	6.60	4.30	4.30
Soy oil	2.01	2.01	1.98	1.98
Dicalcium phosphate	0.86	0.86	1.06	1.06
Limestone	0.51	0.51	0.65	0.65
Salt	0.25	0.25	0.25	0.25
Vitamin and mineral premix[1]	1.00	1.00	1.00	1.00
L-lysine-HCL, 98%	0.36	0.36	0.27	0.27
Methionine hydroxy analogue [2]	0.22	0.22	0.14	0.14
L-threonine	0.20	0.20	0.14	0.14
Tryptophan	0.06	0.06	0.04	0.04
Chromic oxide	0.25	0.25	0.25	0.25
Keratinase (Cibenza DP100^{TM})	0.00	0.05	0.00	0.05
Nutrient concentration (% analyzed [3])				
Digestible energy, Mcal/kg	3.40	3.40	3.40	3.40
Crude protein	22.34	21.84	18.96	19.02
Calcium	0.81	0.79	0.79	0.80
Phosphorus	0.60	0.60	0.60	0.60
Lysine	1.61	1.61	1.31	1.35
Methionine and cystine	0.81	0.76	0.70	0.67
Threonine	0.98	0.99	0.81	0.82
Tryptophan	0.27	0.27	0.20	0.23

[1] Vitamin and mineral premix provided the following per kilogram of diet: vitamin A, 12,000 IU as vitamin A acetate; vitamin D, 2,500 IU as vitamin D_3; vitamin E, 30 IU as DL-α-tocopheryl acetate; 12 μg of vitamin B_{12}; vitamin K, 3 mg as menadione sodium bisulfate; D-pantothenic acid, 15 mg as calcium pantothenate; 40 mg of nicotinic acid; choline, 400 mg choline as choline chloride; Mn, 40 mg as manganous oxide; Zn, 100 mg as zinc oxide; Fe, 90 mg as iron sulfate; Cu, 8.8 mg as copper oxide; I, 0.35 mg as ethylenediamine dihydroiodide; and Se, 0.3 mg as sodium selenite.

[2] The efficiency for methionine hydroxy analogue to provide methionine was 84% (Novus International, Shanghai, China).

[3] All nutrient concentrations are analyzed values except digestible energy.

samples from each pen were collected immediately after excretion to determine the apparent total tract digestibility of nutrients. After collecting, approximately 100 g fecal sample was immediately stored at -20°C. Before analysis, the 3-d composite sample was oven dried at 65°C for 72 h. Apparent digestibility of nutrients was measured using the indicator method according to the procedures of Fan and Sauer (2002).

On day 21 of the experiment, a 7-8 ml blood sample was collected by jugular vein puncture from one randomly selected barrow in each pen using a 10 ml plain blood collection tube (Greiner Bio-One GmbH, Kremsmunster, Australia). Serum samples were obtained by centrifugation at 1,342×g at 4°C for 10 min and stored at -80°C until analysis for interleukin-1, interleukin-6 and tumor necrosis factor-α.

At the end of the three-week study period, the 24 pigs which provided blood samples were euthanized by exsanguination after electrical stunning. Tissue samples from the small intestine were immediately collected. Segments of duodenum, jejunum, and ileum, about 10 cm in length, were removed and flushed with ice-cold 0.9% saline solution to remove any excess blood and digesta. Then, the tissues were fixed with a 10% formaldehyde-phosphate buffer and kept at 4°C to preserve the tissue for microscopic assessments of mucosa morphology.

Samples of digesta (20 ml) from the ileum, cecum, and colon were obtained and stored at -80°C until analysis for ammonia nitrogen concentration, volatile fatty acid concentrations, and bacteria measurements.

Chemical analysis

Feed and fecal samples were ground to pass through a 1 mm sieve and then analyzed for dry matter, crude protein, gross energy, calcium, and phosphorus (Association of Official Analytical Chemists, 2000). Dry matter was measured using method 930.15 (AOAC, 2000), crude protein (nitrogen×6.25) using method 988.05 of AOAC (2000), calcium and phosphorus using official method 935.13 and method 965.17 of AOAC (2000), respectively. Gross energy was determined by an automatic adiabatic oxygen bomb calorimeter (Parr 1281 Automatic Energy Analyzer, Moline, IL). Chromium content was analyzed using an atomic absorption spectrophotometer (Hitachi Z-5000 Automatic Absorption Spectrophotometer, Tokyo, Japan) according to Williams et al. (1962).

The amino acid content of the feed samples was assayed using ion-exchange chromatography with an automatic amino acid analyzer (L-8800 Hitachi Automatic Amino Acid Analyzer, Tokyo, Japan) after hydrolyzing with 6 N HCl at 110°C for 24 hours. Cystine was determined as cysteic acid and methionine as methionine sulfone after preoxidation with performic acid and pre-column derivation using phenylisothiocyanate (L-8800 Hitachi Automatic Amino Acid Analyzer, Tokyo, Japan). Tryptophan was determined after hydrolyzing with 4 M NaOH at 110°C for 22 hours using phenylisothiocyanate (Model 76337, Agilent Technologics, Waldbronn, Germany).

Intestinal digesta and microbial counting

Samples of digesta were placed in a 2.5 mL test tube. The pH of the digesta was taken on site, immediately after collection, using a pH Meter (HI8424 pH Meter, Hanna, Romania), which was standardized with certified pH 4.01 and pH 7.01 buffer solutions. Some very viscous samples were diluted with distilled water to enable their pH to be determined. The ammonia nitrogen concentration in the digesta samples was determined using the methods described by Htoo et al. (2007). Concentrations of volatile fatty acids in digesta obtained from the ileum, cecum, and colon were determined by gas chromatography (Model 5890 Hewlett Packard, Avondale, Pennsylvania) following the procedures described by Shen et al. (2009). Microbial analysis was carried out according to the procedures introduced by Shen et al. (2009).

Histological measurements

Histological measurements were carried out according to the procedures described by Shen et al. (2009). Briefly, fixed intestinal samples were prepared by using conventional paraffin embedding techniques. Samples were sectioned at a 5 μm thickness and stained with hematoxylin and eosin. Villus height and crypt depth were then measured at 40× magnification with a microscope (Olympus CK40, Olympus Optical Company, Shenzhen, China). A minimum of 10 well-oriented and intact villi were selected from each pig to measure crypt depth and villus height.

Cytokine analyses

Serum concentrations of interleukin-1, interleukin-6, and tumor necrosis factor-α were quantified according to the manufacturer's instructions using enzyme-linked immunosorbent assay (ELISA) kits designed specifically for pigs (R&D Systems, Xiamen, China). The sensitivity of the kits was 30, 2, and 20 ng/L, respectively. The intra-assay coefficient of variation for each index was less than 10%. All samples were assayed in duplicate using a TEACAN Plate Reader (TEACAN Asia, Shanghai, China).

Statistical analyses

Data were analyzed as a 2×2 factorial arrangement of treatments with two protein concentrations and two keratinase concentrations. Pen served as the experiment unit. Analyses were performed using the General Linear Models procedure of SAS (SAS Inst., Inc., Cary, NC) and included the main effects of crude protein concentration, keratinase

Table 3. Effects of keratinase and protein level on the performance and faecal scores of weaned piglets[1]

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
Feed intake (g/d)	742	715	776	709	19	0.30	<0.01	0.15
Weight gain (g/d)	466	509	481	514	14	0.38	<0.01	0.65
Feed conversion ratio (g/g)	1.60	1.41	1.62	1.36	0.03	0.78	<0.01	0.58
Fecal score [3]								
d 1-7	2.16	1.78	2.19	1.93	0.09	0.10	<0.01	0.23
d 8-14	1.96	1.52	1.84	1.76	0.08	0.39	<0.01	<0.01
d 15-21	1.91	1.53	1.68	1.57	0.07	0.02	<0.01	<0.01

[1] Values represent mean of six pens. [2] Pooled standard error.
[3] Fecal score: 1 = Normal hard feces; 2 = Slightly soft feces; 3 = Soft, partially formed feces; 4 = Loose, semi-liquid feces; and 5 = Watery, mucous-like feces.

supplementation and their interaction, body weight served as a blocking factor. p values less than 0.05 were considered significant, and 0.05<p≤0.1 were defined as tendencies.

RESULTS

In vitro glycinin and β-conglycinin hydrolysis

Keratinase had considerable ability to hydrolyze soybean glycinin and β-conglycinin (Table 1). While 11.81% of the glycinin and 24.20% of the β-conglycinin were hydrolyzed in the control, 94.74% of the glycinin and 88.89% of the β-conglycinin were hydrolyzed by the added keratinase.

In vivo weaned piglets trial

Performance : Average daily gain and feed conversion ratio were significantly improved (p<0.01) by keratinase supplementation (Table 3). However, average daily feed intake of pigs fed the keratinase supplemented diets was reduced (p<0.01) compared with those fed non-supplemented diets. Crude protein concentration had no effect on pig performance in this experiment.

Fecal scores : Keratinase supplementation reduced (p<0.01) fecal scores in all phases of the experiment in comparison with non-supplemented pigs (Table 3). The lower protein concentration reduced (p<0.05) fecal scores of piglets during the last week of the trial. The interaction between crude protein concentration and keratinase supplementation was significant (p<0.05) during the last two weeks of the study.

Apparent total tract digestibility : Keratinase supplementation increased (p<0.05) the apparent total tract digestibility of dry matter, gross energy, crude protein, and phosphorus while calcium digestibility was not affected (Table 4). Crude protein concentration had no effect on apparent digestibility of nutrients. No interactions were observed between keratinase and crude protein level regarding nutrient digestibility.

Digesta pH, ammonia nitrogen, and volatile fatty acid concentration : The pH at various locations in the intestine were unaffected by crude protein concentration or keratinase supplementation (Table 5). Keratinase supplementation tended to decrease the pH values, especially in the ileum (p = 0.06). Ammonia nitrogen concentration at various locations along the intestinal tract was also reduced (p<0.05) by keratinase supplementation. A reduction in protein concentration led to lower (p<0.05) ammonia nitrogen concentrations in the cecum and colon but not in the ileum.

No treatment related effects were detected regarding

Table 4. Effects of keratinase and crude protein level on the apparent total tract digestibility (%) of weaned piglets fed diets with different levels of crude protein[1]

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
Dry matter	85.54	88.14	86.67	87.85	0.72	0.50	<0.01	0.26
Crude protein	83.94	87.13	84.58	87.32	0.91	0.56	<0.01	0.74
Gross energy	85.97	88.55	87.10	88.38	0.72	0.45	<0.01	0.30
Calcium	63.83	65.10	64.30	68.53	1.74	0.26	0.12	0.39
Phosphorus	48.12	51.65	49.15	52.91	1.76	0.50	0.04	0.94

[1] Values represent mean of six pens. [2] Pooled standard error.

Table 5. Effects of keratinase on pH values and ammonia nitrogen concentrations from different parts of the intestine of weaned piglets fed diets with different levels of crude protein[1]

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
pH								
Ileum	7.13	6.80	7.00	6.77	0.15	0.61	0.06	0.74
Cecum	6.70	6.34	6.58	6.26	0.24	0.69	0.17	0.93
Colon	6.80	6.40	6.74	6.50	0.21	0.92	0.14	0.71
Ammonia nitrogen (mg/L)								
Ileum	91	61	75	42	15	0.25	0.04	0.94
Cecum	290	230	245	192	19	<0.01	<0.01	0.81
Colon	313	275	278	221	24	0.04	0.04	0.66

[1] Values represent mean of six pens. [2] Pooled standard error.

acetic acid concentrations measured in samples obtained from the ileum, cecum or colon (Table 6). Keratinase supplementation reduced ($p<0.05$) the concentrations of propionic acid, n-pentanoic acid and total volatile fatty acids in the colon as well as total tract iso-valeric acid. However, keratinase supplementation increased ($p<0.05$) n-butyric acid concentration in both the cecum and colon. Reducing the level of crude protein also reduced ($p<0.05$)

Table 6. Effects of keratinase on volatile fatty acid concentration (mmol/L) on various locations along the intestinal tract of weaned piglets fed diets with different levels of crude protein[1]

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
Acetic acid								
Ileum	0.54	0.77	0.70	0.84	0.05	0.44	0.24	0.77
Cecum	76	73	76	73	0.72	0.98	0.72	0.98
Colon	57	65	71	69	2.57	0.09	0.56	0.35
Propionic acid								
Ileum	0.24	0.31	0.37	0.38	0.03	0.56	0.80	0.88
Cecum	46	46	36	28	3.58	<0.01	0.29	0.33
Colon	30	24	36	26	2.18	0.32	0.04	0.49
Iso-butyric acid								
Ileum	2.03	1.15	1.35	0.48	0.26	0.17	0.08	0.99
Cecum	4.07	2.93	1.93	0.68	0.59	0.01	0.15	0.95
Colon	1.44	0.61	1.56	1.24	0.17	0.22	0.07	0.41
n-Butyric acid								
Ileum	1.94	3.57	0.74	1.28	0.50	0.07	0.24	0.55
Cecum	8.89	17.40	6.40	14.29	2.04	0.39	0.02	0.92
Colon	9.34	18.83	2.79	14.88	2.85	0.40	0.02	0.80
Iso-valeric acid								
Ileum	0.53	0.13	0.34	0.09	0.08	0.05	<0.01	0.17
Cecum	2.08	1.02	1.95	0.98	0.24	0.82	0.01	0.90
Colon	2.57	1.01	2.34	0.58	0.40	0.50	<0.01	0.84
n-Pentanoic acid								
Ileum	0.52	-	0.06	-	0.10	0.18	0.10	0.18
Cecum	4.38	2.89	3.74	2.50	0.35	0.57	0.14	0.89
Colon	4.25	2.53	3.90	1.71	0.49	0.39	<0.01	0.72
Total volatile fatty acid								
Ileum	7.43	4.30	4.10	2.54	0.84	0.04	0.06	0.51
Cecum	150	135	134	112	6.48	0.12	0.13	0.76
Colon	114	103	130	101	5.41	0.39	0.03	0.29

[1] Values represent mean of six pens. [2] Pooled standard error.

Table 7. Effects of keratinase on microbial concentration (log_{10} cfu/g of digesta) at various locations along the intestinal tract of weaned piglets fed diets with different levels of crude protein[1]

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
Ileum								
E. coli	6.05	6.00	6.02	5.98	0.20	0.76	0.58	0.99
Lactobacilli	5.88	5.84	5.82	6.02	0.21	0.45	0.33	0.14
Total anaerobes	6.11	6.08	6.05	6.07	0.18	0.69	0.93	0.72
Total aerobes	6.13	6.11	6.08	6.09	0.22	0.71	0.94	0.90
Cecum								
E. coli	7.02	6.85	6.93	6.74	0.15	0.03	<0.01	0.81
Lactobacilli	6.86	7.08	7.05	7.13	0.18	0.08	0.03	0.30
Total anaerobes	7.18	7.23	7.08	7.16	0.16	0.20	0.35	0.82
Total aerobes	7.10	7.08	7.07	7.07	0.14	0.77	0.88	0.88
Colon								
E. coli	7.16	6.80	7.06	6.79	0.22	0.38	<0.01	0.46
Lactobacilli	6.95	7.17	7.07	7.23	0.18	0.18	0.01	0.61
Total anaerobes	7.22	7.32	7.00	7.29	0.18	0.03	<0.01	0.09
Total aerobes	7.33	7.09	7.29	7.04	0.17	0.43	<0.01	0.96

[1] Values represent mean of six pens. [2] Pooled standard error.

concentrations of cecal propionic acid, cecal iso-butyric acid and ileal iso-valeric acid as well as the total volatile fatty acid concentration in the ileum.

Intestinal microflora : Microbial composition of the digesta from the ileum was not affected by dietary treatment (Table 7). Keratinase supplementation reduced ($p<0.05$) *E. coli* counts in the cecum and colon and total aerobes counts in the colon. However, the lactobacilli counts in both the cecum and colon and total anaerobes counts in colon were increased ($p<0.05$). The reduction of dietary crude protein concentration led to a lower counts ($p<0.05$) of both *E. coli* in the cecum and total anaerobes in the colon.

Histological measurements : There was no effect of keratinase supplementation, crude protein concentration, or their interaction on villus height at various locations of the small intestine (Table 8). No dietary effects were detected in villus height, crypt depth and the villus height to crypt depth ratio detected in the duodenum. However, crypt depth in the jejunum and ileum decreased ($p<0.05$) with keratinase supplementation and crypt depth in the ileum was reduced ($p<0.05$) with the reduction in dietary crude protein concentration. Keratinase supplementation increased ($p<0.05$) the villus height to crypt depth ratio in the ileum.

Table 8. Effects of keratinase on small intestinal morphology of weaned piglets fed diets with different levels of crude protein[1]

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
Villus height (μm)								
Duodenum	378	376	375	381	5.43	0.84	0.77	0.48
Jejunum	336	341	339	344	6.51	0.06	0.15	0.59
Ileum	312	324	317	327	3.89	0.07	0.08	0.69
Crypt depth (μm)								
Duodenum	127	123	121	119	2.29	0.06	0.15	0.59
Jejunum	116	109	111	105	3.11	0.15	0.04	0.95
Ileum	112	109	109	106	2.65	0.04	<0.01	0.83
Villus height to crypt depth ratio								
Duodenum	2.98	3.07	3.09	3.20	0.07	0.09	0.18	0.85
Jejunum	2.91	3.12	3.05	3.29	0.09	0.32	0.24	0.93
Ileum	2.79	3.00	2.92	3.09	0.08	0.15	0.02	0.78

[1] Values represent mean of six pens. [2] Pooled standard error.

Serum pro-inflammatory cytokines concentrations : Serum analysis showed that interleukin-1 and tumor necrosis factor-α concentrations were reduced ($p<0.05$) by reducing the dietary crude protein concentration (Table 9). Keratinase supplementation also reduced ($p<0.05$) serum interleukin-1 along with interleukin-6. The concentration of serum tumor necrosis factor-α also tended to be reduced ($p = 0.07$) by keratinase supplementation.

DISCUSSION

The hydrolyzing ability of keratinase on casein, collagen, elastin, keratin and other proteins of animal or plant origin has been widely shown *in vitro* (Yu et al., 1972; Lin et al., 1996; Gradišar et al., 2005). One of the possible biochemical explanations allowing keratinase to perform this function was proposed by Tu et al. (1998), who specified that keratinase was composed of protein-disulfide reductase and peptidohydrolase. Protein-disulfide reductase breaks down the cystine disulfide bonds present in some indigestible proteins and then peptidohydrolase hydrolyzes the denatured protein into peptides and amino acids (Tu et al., 1998; Gupta and Ramnani, 2006).

Given the chemical structure of glycinin and β-conglycinin, whose main features exhibit disulfide bonds (Hou and Chang, 2004; Golubovic et al., 2005), we hypothesized that keratinase should be able to hydrolyze glycinin and β-conglycinin. This hypothesis was tested in the *in vitro* study which clearly demonstrated the ability of keratinase to hydrolyze glycinin and β-conglycinin. While only 11.81% of the glycinin and 24.20% of the β-conglycinin were hydrolyzed in the control, 94.74% of the glycinin and 88.89% of the β-conglycinin were hydrolyzed in the incubations with added keratinase.

In the present study, supplementation with keratinase improved weight gain and feed conversion but reduced the feed intake of weaned piglets. Similar results have been obtained by Odetallah et al. (2003, 2005) who reported that supplementation of 1 g/kg keratinase improved body weight gain and feed efficiency but reduced feed intake for broiler chickens fed high or low crude protein corn soybean based meal diets. More recently, Wang et al. (2008) also reported a positive effect of supplementation of keratinase for broiler chickens fed diets containing soybean and cottonseed meals.

While the optimum pH for keratinase produced by Bacillus licheniformis PWD-1 is about 7.5 (Lin et al., 1996), keratinase can in fact perform actively within a pH range between 4 and 13 (Gupta and Ramnani, 2006). The pH conditions normally found in the lower jejunum and ileum provide a similar environment (Braude et al., 1976), which probably aids keratinase function in this part of the digestive tract. Wang (2007) also reported that keratinase could perform its hydrolyzing function without being denatured or broken down by the gastrointestinal tract in broiler chickens.

Microflora, which consists of commensal and pathogenic bacteria in the digestive system of pig, can be affected by differences in the nutrient composition of diets (Gaskins, 2000). Indigestible nutrients in the gut lumen, such as protein and carbohydrates, provide a substrate for microbes to ferment and proliferate. Branch chain volatile fatty acids and ammonia are mainly products of protein fermentation while acetic, propionic and butyric acids are products of carbohydrate fermentation (Le et al., 2007). One of the important changes in the intestinal ecology of piglets fed keratinase observed in the present study was that less ammonia was produced in the gut compared with piglets fed un-supplemented diets. Excessive ammonia negatively affects growth and differentiation of intestinal epithelial cells and leads to a higher pH value, and increase the incidence of diarrhea (Gaskins, 2000). In addition, the finding of a reduction in branch chain volatile fatty acid is consistent with the fact that keratinase supplementation increased the apparent total tract digestibility of crude protein, which would result in less protein being fermented in the gut.

Other key changes in piglets fed diets supplemented with keratinase were lower numbers of *E. coli* and higher numbers of lactobacilli in the hindgut. These results correspond with less ammonia nitrogen, less branch chain volatile fatty acids in the digesta and a numerically lower pH value in the gut. The lower pH might favor the development of beneficial bacteria and inhibit the development of harmful bacteria while an abnormally high

Table 9. Effects of keratinase on serum pro-inflammatory cytokines concentrations of weaned piglets fed diets with different levels of crude protein 1

	High protein		Low protein		PSE[2]	p-value		
	0	0.05% keratinase	0	0.05% keratinase		Protein level	Keratinase	Interaction
IL-1 (ng/L)	319	255	271	217	17	0.02	<0.01	0.76
IL-6 (ng/L)	54	48	50	48	1.69	0.16	<0.01	0.11
TNF-α (ng/L)	160	134	131	115	7.51	0.04	0.07	0.61

[1] Values represent mean of six pens. [2] Pooled standard error.

intestinal pH would provide a better environment for *E. coli* to colonize on the villi, thus resulting in diarrhea (Gaskins, 2000). Changes regarding the reduction in harmful bacteria and the improved biochemical condition of the intestine could possibly enhance the health of the gut ecology as beneficial bacteria would be more likely to thrive. This might be the explanation for the higher n-butyric acid concentration which benefits the growth of intestinal epithelial cells (Gaskins, 2000; Panda et al., 2009) in piglets fed keratinase.

Diarrhea caused by infectious disease is a serious problem in weaning animals and usually leads to an increased mortality (Gaskins, 2000). The results of the present study indicate that supplementation with keratinase reduced diarrhea of piglets as shown by the lower fecal scores. This effect might be due to the hydrolysis of soybean glycinin and β-conglycinin which increased the digestibility of nutrients and decreased numbers of *E. coli*.

The marked changes that occur in gut structure and function after weaning, such as villous atrophy and crypt hyperplasia, are generally associated with poor performance as they can cause a temporary decrease in the digestive and absorptive capacity of the small intestine (Gaskins, 2000). In fact, an increase in the villus to crypt ratio is known to associate with a better nutrient absorption (Montagne et al., 2003). In this study, decreased crypt depth in both the jejunum and ileum and a higher villus to crypt ratio in the ileum were observed in pigs supplemented with keratinase after weaning. The improvement in apparent total tract digestibility of dry matter, gross energy, crude protein, and phosphorus in piglets fed keratinase supplemented diets is likely a consequence of this improvement in intestinal morphology.

Intestinal inflammation causes villus atrophy and thus reduces nutrient digestibility (Spurlock, 1997). The hypothesis that the immune response to dietary antigens, some of which are derived from soy protein (such as glycinin and β-conglycinin), leads to local inflammation is one of the most plausible reasons for the nutritional weaning-associated morphological changes in the pig intestine (Li et al., 1991; Qiao et al., 2003). Pro-inflammatory cytokines (interleukin-1, interleukin-6, and tumor necrosis factor-α) produced during the immune response to infection might alter protein and lipid metabolism and as a result, influence growth and efficiency of gain (Spurlock, 1997).

In the present study, weaned piglets performance was not affected by crude protein level. This indicates that the balance of amino acids is actually more important than the level of crude protein. This result is consistent with some previous studies (Le Bellego and Noblet, 2002; Li et al., 2011). The reduction of crude protein also caused less production of ammonia nitrogen and *E. coli* in the cecum and fewer total anaerobes in the colon. The result is consistent with the findings of Nyachoti et al. (2006) and Htoo et al. (2007). Diets with high crude protein levels have a high buffering capacity and should increase gastric pH (Nyachoti et al., 2006). The pH, however, was not affected by crude protein concentration in the present study, and, beside the fecal scores, no interaction between crude protein concentration and keratinase supplementation was observed. Possible explanations for the lack of effect might be due to the small gradient between the two diets, similar to what Htoo et al. (2007) reported.

CONCLUSION

It can be concluded from this study that supplementation with keratinase at the concentration of 0.05% had a positive effect on the performance of weanling piglets fed corn-soybean diets. Keratinase supplementation also reduced the incidence of diarrhea and the release of pro-inflammatory cytokines while improving nutrient digestibility, intestinal morphology and intestinal ecology.

ACKNOWLEDGMENTS

This study was completed at the State Key Laboratory of Animal Nutrition (2004DA125184-0810) of China. Financial support was provided by Novus International Trading Company (Shanghai, China), the NSFC (No. 31072040), SKLAN (No. 2004DA125184-0810), Guangdong (No. 2009B090300110) and National Program of China (No. nyhyzx07-034).

REFERENCES

AOAC. 2000. Official methods of analysis, 17th ed. Association of Official Analytical Chemists, Arlington, VA.

Böckle, B., B. Galunsky and R. Müller. 1995. Characterization of a keratinolytic serine proteinase from *Streptomyces pactum* DSM 40530. Appl. Environ. Microbiol. 61:3705-3710.

Brandom, D. L. and M. Friedman. 2002. Immunoassays of soy proteins. J. Agric. Food Chem. 50:6635-6642.

Braude, R., R. J. Fulford and A. G. Low. 1976. Studies on digestion and absorption in the intestines of growing pigs. Br. J. Nutr. 36:497-510.

Fan, M. Z. and W. C. Sauer. 2002. Determination of true ileal amino acid digestibility and the endogenous amino acid outputs associated with barley samples for growing-finishing pigs by the regression analysis technique. J. Anim. Sci. 80:1593-1605.

Gaskins, H. R. 2000. Intestinal bacteria and their influence on swine growth. In: Swine Nutrition (Ed. A. J. Lewis and L. L. Southern). CRC Press, New York, pp. 585-608.

Golubovic, M., S. H. van Hateren, M. Ottens, G. J. Witkamp and L. A. M. van der Wielen. 2005. Novel method for the production of pure glycinin from soybeans. J. Agric. Food Chem.

53:5265-5269.

Gradišar, H., S. Kern and J. Friedrich. 2000. Keratinase of *Doratomyces microspores*. Appl. Microbiol. Biotechnol. 53:196-200.

Gradišar, H., J. Friedrich, I. Križaj and R. Jerala. 2005. Similarities and specificities of fungal keratinolytic proteases: Comparison of keratinases of *Paecilomyces marquandii* and *Doratomyces microsporus* to some known proteases. Appl. Microbiol. Biotechnol. 71:3420-3426.

Gupta, R. and P. Ramnani. 2006. Microbial keratinases and their prospective applications: An overview. Appl. Microbiol. Biotechnol. 70:21-33.

Hou, D. H. and S. K. C. Chang. 2004. Structural characteristics of purified glycinin from soybeans stored under various conditions. J. Agric. Food Chem. 52:3792-3800.

Htoo, J. K., B. A. Araiza, W. C. Sauer, M. Rademacher, Y. Zhang, M. Cervantes and R. T. Zijlstra. 2007. Effect of dietary protein content on ileal amino acid digestibility, growth performance, and formation of microbial metabolites in ileal and cecal digesta of early-weaned pigs. J. Anim. Sci. 85:3303-3312.

Kim, W. K. and P. H. Patterson. 2000. Nutritional value of enzyme or sodium hydroxide treated feathers from dead hens. Poult. Sci. 79:528-534.

Kong, X. F., Y. L. Yin, Q. H. He, F. G. Yin, H. J. Liu, T. J. Li, R. L. Huang, M. M. Geng, Z. Ruan, Z. Y. Deng, M. Y. Xie and G. Wu. 2009. Dietary supplementation with Chinese herbal powder enhances ileal digestibilities and serum concentrations of amino acids in young pigs. Amino Acids 37:573-582.

Le Bellego, L. and J. Noblet. 2002. Performance and utilization of dietary energy and amino acids in piglets fed low protein diets. Livest. Prod. Sci. 76:45-58.

Le, P. D., A. J. A. Aarnink, A. W. Jongbloed, C. M. C. van der Peet-Schwering, N. W. M. Ogink and M. W. A. Verstegen. 2007. Interactive effects of dietary crude protein and fermentable carbohydrate levels on odour from pig manure. Livest. Sci. 114:48-61.

Li, D. F., J. L. Nelssen, P. G. Reddy, F. Blecha, R. D. Klemm, D. W. Giesting, J. D. Hancock, G. L. Allee and R. D. Goodband. 1991. Measuring suitability of soybean products for early-weaned pigs with immunological criteria. J. Anim. Sci. 69:3299-3307.

Li, P. F., X. S. Piao, Z. K. Zeng, D. Wang, L. F. Xue, R. F. Zhang, B. Dong and S. W. Kim. 2011. Effects of the standard ileal digestible lysine to metabolizable energy ratio on performance, nutrient digestibility, and plasma parameters of 10 to 28 kg pigs. J. Anim. Sci. Biotech. 2:35-43.

Lin, X., J. C. H. Shih and H. E. Swaisgood. 1996. Hydrolysis of feather keratin by immobilized keratinase. Appl. Environ. Microbiol. 62:4273-4275.

McDonnell, P., C. O'Shea, S. Figat and J. V. O'Doherty. 2010. Influence of incrementally substituting dietary soya bean meal for rapeseed meal on nutrient digestibility, nitrogen excretion, growth performance and ammonia emissions from growing-finishing pigs. Arch. Anim. Nutr. 64:412-424.

Montagne, L., J. R. Pluske and D. J. Hampson. 2003. A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. Anim. Feed Sci. Technol. 108:95-117.

NRC. 1998. Nutrient requirements of swine 10th rev. ed. Natl. Acad. Press, Washington, DC, USA.

Nyachoti, C. M., F. O. Omogbenigun, M. Rademacher and G. Blank. 2006. Performance responses and indicators of gastrointestinal health in early-weaned pigs fed low-protein amino acid-supplemented diets. J. Anim. Sci. 84:125-134.

Odetallah, N. H., J. J. Wang, J. D. Garlich and J. C. H. Shih. 2003. Ketatinase in starter diets improves growth of broiler chicks. Poult. Sci. 82:664-670.

Odetallah, N. H., J. J. Wang, J. D. Garlich and J. C. H. Shih. 2005. Versazyme supplementation of broiler diets improves market growth performance. Poult. Sci. 84:858-864.

Panda, A. K., S. V. Rama Rao, M. V. L. N. Raju and G. Shyam Sunder. 2009. Effect of butyric acid on performance, gastrointestinal tract health and carcass characteristics in broiler chickens. Asian-Aust. J. Anim. Sci. 22:1026-1031.

Pierce, K. M., J. J. Callan, P. McCarthy and J. V. O'Doherty. 2005. Performance of weanling pigs offered low or high lactose diets supplemented with avilamycin or inulin. Anim. Sci. 80:313-318.

Qiao, S. Y., D. F. Li, J. Y. Jiang, H. J. Zhou, J. S. Li and P. A. Thacker. 2003. Effect of moist extruded full-fat soybeans on gut morphology and mucosal cell turnover rate (Time) of weanling pigs. Asian-Aust. J. Anim. Sci. 16:63-69.

Shen, Y. B., X. S. Piao, S. W. Kim, L. Wang, P. Liu, I. Yoon and Y. G. Zhen. 2009. Effects of yeast culture supplementation on growth performance, intestinal health, and immune response of nursery pigs. J. Anim. Sci. 87:2614-2624.

Spurlock, M. E. 1997. Regulation of metabolism and growth during immune challenge: An overview of cytokine function. J. Anim. Sci. 75:1773-1783.

Sun, P., D. F. Li, Z. J. Li, B. Dong and F. L. Wang. 2007. Effects of glycinin on IgE-mediated increase of mast cell numbers and histamine release in the small intestine. J. Nutr. Biochem. 19:627-633.

Swick, R. A. 2007. Selecting soy protein for animal feed. 15th Annual ASAIM Southeast Asian Feed Technology and Nutrition Workshop, Bali, Indonesia.

Tu, G. Q., Y. J. Ye, B. Zhang, M. J. Guo and Y. H. Sun. 1998. Studies on the biomechanism of keratin decomposed by streptomyces. Acta Agriculturae Universitatis Jiangxiensis 20:1-5.

Wang, H. Y. 2007. Effects of enzyme supplementation on broiler chicks fed different type diets and the underlying mechanism. Ph.D. Thesis, China Agricultural University, Beijing, China.

Wang, H. Y., Y. M. Guo and J. C. H. Shih. 2008. Effects of dietary supplementation of keratinase on growth performance, nitrogen retention and intestinal morphology of broiler chickens fed diets with soybean and cottonseed meals. Anim. Feed Sci. Technol. 140:376-384.

Wang, J. J. and J. Shih. 1999. Fermentation production of keratinase from *Bacillus licheniformis* PWD-l and a recombinant *B. subtillis* FDB-29. J. Ind. Microbiol. Biothnol. 22:608-616.

Wang, J. J., J. D. Garlich and J. C. H. Shih. 2006. Beneficial effects of versazyme, a keratinase feed additive, on body weight, feed conversion, and breast yield of broiler chickens. J. Appl. Poult. Res. 15:544-550.

Williams, C. H., D. J. David and O. Iismaa. 1962. The determination of chromic oxide in faeces sample by atomic

absorption spectrophotometry. J. Agric. Sci. 59:381-385.

Yi, G. F., A. M. Gaines, B. W. Ratliff, P. Srichana, G. L. Allee, K. R. Perryman and C. D. Knight. 2006. Estimation of the true ileal digestible lysine and sulfur amino acid requirement and comparison of the bioefficacy of 2-hydroxy-4-(methylthio) butanoic acid and DL-methionine in eleven to twenty six-kilogram nursery pigs. J. Anim. Sci. 84:1709-1721.

Yoo, J. S., H. D. Jang, J. H. Lee and I. H. Kim. 2009. Effect of fermented soy bean protein on nitrogen balance and apparent fecal and ileal digestibility in weaned pigs. Asian-Aust. J. Anim. Sci. 22:1167-1173.

Yu, R. J., J. Ragot and F. Blank. 1972. Keratinases: Hydrolysis of keratinous substrates by three enzymes of *Trichophyton mentagrophytes*. Experimentia 28:1512-1513.