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Effect of Feeding Enzymolytic Soybean Meal on Performance, Digestion and Immunity of Weaned Pigs*

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ABSTRACT : The objective of this study was to investigate the effect of enzymolytic products of soybean meal (ESBM), as one of the protein sources in the diet, on the growth performance, nutrient digestibility and immune function of weaned piglets. Soybean meal produced by bioprocessing with fermentation and enzymolysis contains reduced anti-nutritional factors and improved protein utilization. A total of 240 weaned piglets (Duroc×Landrace×Yorkshire, 9.01±0.22 kg body weight) were randomly allocated to 4 treatments with 6 pens per treatment and 10 piglets per pen. The diets were based on corn-soybean meal and ESBM partially replaced soybean meal and soybean protein isolate at the inclusion level of 5, 10 or 15% in the basal diet. Feed intake and body weight were measured weekly. On days 24 to 27, faeces of each replicate were proportionally collected to determine the nutrient digestibility. On day 28 of the experiment, one piglet from each replicate was slaughtered humanely to collect immune organs. The results showed that inclusion of ESBM increased ($p<0.05$) the final weight, daily feed intake and daily gain of weaned pigs compared with the control diet, and ESBM at the inclusion levels of 10 and 15% improved ($p<0.05$) the feed/gain compared with the control diet. There were no differences ($p>0.05$) in daily feed intake among the levels of ESBM, but increasing the levels of ESBM from 5 to 15% improved ($p<0.05$) the final weight, average daily gain of pigs and feed/gain. The inclusion of ESBM at 5 to 15% increased ($p<0.05$) the digestibility of crude protein (CP) by 5 to 16%, and ESBM at 15% increased ($p<0.05$) the digestibility of digestible energy (DE), Ca and P compared with the control diet. ESBM increased ($p<0.05$) the relative weights of thymus and mandibular lymph nodes by 57.7 and 29.6%, respectively. The percentages of T lymphocytes, CD4+ and CD8+ in peripheral blood of weaned piglets were also increased ($p<0.05$) by feeding ESBM. The results suggest that ESBM can be a better protein source in improving growth performance, nutrient digestibility and immune function of weaned piglets. (**Key Words :** Enzymolytic Soybean Meal, Digestion, Immunity, Performance, Weaned Pig)

INTRODUCTION

A main reason for the low soybean meal tolerance in piglets is that even adequately heated soybean meals contain high levels of several heat stable antinutritional factors (Li et al., 1990). Glycinin and beta-conglycinin are the main antigen proteins in soybean meals, which can cause a transient increase in crypt cell production, and subsequent malabsorption and villus atrophy in weaned piglets (Stokes et al., 1986; Dierick et al., 2004; Jezierny et al., 2010). Soybean trypsin inhibitors are also important factors to depress the growth performance and health status of piglets (Yen et al., 1974), depending on the inclusion level of soybean meal and the content of these anti-nutritional factors (Garthoff et al., 2002).

It is reported that the nutritional values of soybean protein and the growth performance of piglets can be improved when soybean meal was treated with either protease hydrolyses (Caine et al., 1997; 1998; Dierick et al., 2004) or fermentation in pigs (Feng et al., 2007; Jones et al., 2010; Kim et al., 2010a,b). Enzymolitic soybean meal treated with both fermentation and protease hydrolyses increases the proportions of soluble proteins and small peptides besides the depression in most antinutritional factors in soybean meal, which may be more potential to improve health status of weaned piglets. However, the knowledge is very limited in the immune function of weaned pigs.

The objective of this study was to investigate the effect of enzymolytic soybean meal (ESBM) partially replacing soybean meal and soy protein isolate on the growth

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performance, nutrient digestibility and immune function of weaned crossbred piglets in a 28-day feeding trial.

MATERIALS AND METHODS

Enzymolytic soybean meal

Two soybean meal products, extracted dehulled soybean meal (SBM) and its hydrolysates with microbial protease, enzymolytic soybean meal (ESBM), were obtained from Continent Biotech (Qingdao, China). The process of ESBM product included SBM fermented (1:2 wt/vol in distilled water) at pH 4.5 for 12 h, then incubated with derivatives thereof, microbial protease, for 24 h at 50°C and dried at 65°C.

Diets and animals

The control diet was based on corn-soybean meal and experimental diets contained ESBM at 5, 10 or 15% inclusions in the basal diet. During the trial, no antibiotics were offered to weaned pigs via either feed or water. The formulation of the diets is given in Table 1.

A total of 240 weaned piglets (Duroc×Landrace×Yorkshire, 28 days old) with similar body weight (9.01±0.22 kg body weight) were randomly allocated to four treatments with 6 pens per treatment and 10 piglets per pen. The temperature of the pig shed was 20 to 22°C and the relative humidity was 50 to 70%. The pigs were fed *ad lib*. and had free access to water. The body weight and feed intake of pigs were measured weekly on pen basis. Diarrhea was monitored daily throughout the experiment. This trial was conducted for 28 days in accordance with laws and regulations that control experiments and procedures in live animals in China, as overseen by the Chinese Animal Research Authority.

Sampling

On day 24 to 27, faeces per pen replicate were collected, pooled and proportionally dried at 65°C for the determination of the coefficient of total tract apparent digestibility of nutrients using the method of TiO_2 indicator. Care was taken during the collection of faeces samples to avoid contamination from hairs and other foreign materials.

On day 28 of trial, following 8 hours of fasting, 6 pigs with average body weights from each treatment (1 pig per

Table 1. Formulation and chemical compositions of diets (as fed basis)

Item	Control	ESBM 5%	ESBM 10%	ESBM 15%
Ingredient (%)				
Corn	61.57	59.94	60.05	60.06
Soybean meal	12.07	12.63	6.45	0.38
Wheat short	10.00	10.00	10.00	10.00
Fish meal	5.81	5.81	5.81	5.81
Soy protein isolate	4.00	0	0	0
Enzymolytic soybean meal (ESBM)	0	5.00	10.00	15.00
Limestone	2.32	2.40	2.79	2.49
Sucrose	2.00	2.00	2.00	2.00
Dextrose	1.00	1.00	1.00	1.00
Mineral/vitamin premix[1]	1.00	1.00	1.00	1.00
Lysine	0.16	0.16	0.17	0.18
Threonine	0.02	0.01	0	0
Bone meal	0	0	0.68	1.20
Salt	0	0	0	0.83
Titanium dioxide	0.05	0.05	0.05	0.05
Nutrient level (%)				
Digestible energy (MJ/kg)	13.66	13.66	13.66	13.66
Analysed crude protein	19.01	18.99	19.01	19.04
Analysed Ca	0.79	0.81	0.81	0.80
Analysed P	0.74	0.74	0.73	0.74
Lysine	1.22	1.22	1.22	1.22
Methionine	0.43	0.43	0.43	0.43

[1] Provided the following per kg of complete diet: Vitamin A, 15,000 IU; Vitamin D_3, 3,000 mg; VE (DL-α-tocopherol), 40 mg; Vitamin K_3, 2 mg; nicotinic acid (niacin), 40 mg; D-calcium pantothenate 20 mg; folic acid 2 mg; Vitamin B_{12} 0.2 mg; Choline chloride 1,000 mg; Fe (as ferrous sulfate), 100 mg; Cu (as copper sulfate pentahydrate), 10 mg; Mn (as manganese sulfate) 40 mg; Zn (as zinc oxide), 100 mg; Se (as sodium selenite), 0.3 mg; I (as potassium iodide), 0.5 mg.

replicate) were selected and weighed. Blood was obtained from each pig by cardiac puncture and directly aliquoted into 2-ml sterile vials, one aliquot used liquid sodium heparin (1,000 USP units/ml) as an anticoagulant to obtain the whole blood for lymphocyte tests, another allowed to clot for 4 h for serum preparation. After centrifugation for 10 min at 3,000 g, the serum was aliquoted (100 μl) into 1-ml vials and stored at -20°C for measurements. Pigs were then killed by electric shock, and then spleen, thymus gland, lymph gland in groin, mandible and mesentery were excised (Zhang et al., 1979) and weighed, expressed as the relative weight of body weight (g/kg).

Chemical analyses

The nutrients in the samples were determined according to the standard AOAC methods (1990) for total P (964.06), Ca (935.13), CP (976.05) and crude fat (920.39). Gross energy was measured in an Oxygen Bomb Calorimeter (Model 6300, PARR, Moline, IL). Dry matter was determined by drying a 2 g sample at 65°C to a constant mass. The content of TiO_2 was measured as described by Short et al. (1996). The apparent faecal digestibility of nutrients was calculated as described by Camden et al. (2001).

Trypsin inhibitor in the sample was detected according to the method by Page et al. (2000). One trypsin inhibitor unit (TIU) is defined as an increase of 0.01 absorbance unit per gram dry weight at 405 nm under standard conditions (TIU/g). Urease activity was determined according to the Chinese national standard (GB/T 8622-2006, China) and expressed as milligram ammonia N liberated from 1 g dry weight in 1 min at 30°C, pH 7.0 (mg/g.min). For organic acids estimation, the sample slurry was centrifuged at 10,000 g for 20 min. The supernatant was analysed using HPLC (Model 1100, Agilent Technologies, Inc, Santa Clara, CA, USA) using 10 mM perchloric acid as mobile phase at 40°C as per Andersson and Hedlund (1983). Free amino acids were determined by ninhydrin method (Magne and Larher, 1992). Small peptide (≤500 Da) was quantified using electrophoresis in sodium dodecyl sulfate-polyacrylamide gels (Fling and Gregerson, 1986).

Lymphocyte assay

Peripheral blood T lymphocytes were quantified using the method of E-rosette formation tests described by Brain et al. (1970). Blood samples were diluted with an equal volume of calcium and magnesium-free phosphate buffered saline (CMF-PBS, pH 7.4). The diluted blood was slowly added to the same volume of a lymphocyte separation solution. After centrifugation at 1,000 g for 20 min, a white layer of mononuclear cells at the plasma-Ficoll interface was collected and washed twice with CMF-PBS. The mononuclear cells were diluted with CMF-PBS and counted to 5×10^6/ml. The diluted lymphocytes (100 μl) were incubated with a suspension of 20% bovine serum in CMF-PBS (100 μl) and 2% sheep red blood cell solution (100 μl) at 37°C for 15 min, and then centrifuged for 5 min at 100 g. The cell pellets were harvested and stored at 4°C for 4 h. The cells were differentiated by smear examination (Ranjit, 1984). Erythrocyte rosette-forming cells around which the formation of a cluster (rosette) of cells consisting of more than 3 sheep erythrocytes were counted.

Blood mononuclear cells were isolated as described above and adjusted to a concentration of 1×10^7/ml with CMF-PBS (pH 7.4) for the assay of T lymphocyte subsets. For each sample, 3 tubes containing the following combinations of monoclonal antibodies purchased from Southern Biotechnology Associates (Birmingham, AL, USA) were set up: 10 μl of CD4-phycoerythrin (10^6 cells/μg) and 10 μl of CD3-fluorescein isothiocyanate (10^6 cells/μg); 10 μl of CD8a-phycoerythrin (10^6 cells/μg) and 10 μl of CD3-fluorescein isothiocyanate (10^6 cells/μg); and 10 μl of IgG1-fluorescein isothiocyanate (10^6 cells/μl) and 10 μl of IgG1- phycoerythrin (10^6 cells/μl). Each tube was added 50 μl mononuclear cell suspension, gently mixed and then incubated at 4°C for 20 min in the dark. Then 500 μl of CMF-PBS solution was dispensed into each tube, gently mixed, and left for 10 min at room temperature out of direct light. After 10 min of centrifugation at 800 g, the cells were re-suspended with 500 μl of CMF-PBS. The percentages of CD3+CD4+ and CD3+CD8+ lymphocytes in the mononuclear cell suspension were determined by flow cytometry (Model epicsxl, Beckman Coulter Inc., Miami, FL, USA).

Statistical analysis

One-way ANOVA was employed to determine the effect of ESBM by using the GLM procedure of SAS version 9.0 (SAS Institute, Cary, NC) using pen as the experimental unit. Differences were considered significant using Duncan's multiple-range test at $p<0.05$. Values in the tables were means and pooled SEM.

RESULTS

The hydrolysis of soybean protein with protease is a proprietary process that depresses trypsin inhibitor and urease, and increases the proportions of small peptides, water soluble protein and organic acids (Table 2). In contrast with SBM in this study, the contents of water soluble protein, small peptides and organic acids in ESBM were increased by 631%, 719% and 137%, respectively, but crude fat was decreased by 60%. The activity of trypsin inhibitor and urease were not detected in ESBM compared to SBM.

Table 2. Compositions of soybean meal (SBM) and enzymolytic soybean meal (ESBM)

Item	SBM	ESBM
Dry matter (%)	88.00	88.00
In dry matter (%)		
Crude protein (N×6.25)	46.57	50.38
Amino acid protein	46.24	49.34
Water soluble protein	3.76	27.50
Small peptide (≤500 Da)	3.00	24.57
Crude fat	5.72	2.31
Ash	6.49	6.57
Organic acids	2.38	5.64
Trypsin inhibitor unit (TIU/g)	1,380	Not detected
Urease activity (mg/g min)	0.36	Not detected

The piglets were healthy throughout the experiment with a mortality of less than 2% that was unrelated to dietary treatment. There was no bacterial or viral disease infection. Diets including ESBM at 5 to 15% increased ($p<0.05$) final weight (FW), daily feed intake (DFI) and average daily gain (ADG) of weaned piglets by average 18, 25 and 38%, respectively, compared to the control diet (Table 3). ESBM at 15% increased ($p<0.05$) ADG by 10 and 19%, respectively, compared with ESBM at 5 and 10%, and there was no difference ($p>0.05$) in ADG between ESBM 5 and 10%. Increasing the level of ESBM from 5 to 15% had no effects ($p>0.05$) on DFI for weaned pigs. ESBM added at 10 and 15% improved ($p<0.05$) feed/gain (F/G), but ESBM added at 5% showed no effect ($p>0.05$) on F/G, compared to the control diet.

The apparent faecal digestibility of CP increased ($p<0.05$) by 5 to 16% in the diets containing ESBM at 5 to 15% (Table 4), compared with the control diet. The digestibility of DE, Ca and P in ESBM at 5 or 10% was unaffected compared with the control diet, but apparent faecal digestibility of DE, Ca and P increased ($p<0.05$) by 8, 17 and 10% in 15% ESBM, respectively, compared with the control diet.

The relative weights of spleen of pigs were increased ($p<0.05$) by 15% SEBM, but were unaffected ($p>0.05$) by ESBM 5 and 10%, compared with the control diet (Table 5). ESBM added at 5, 10 and 15% increased ($p<0.05$) the relative weights of thymus by 50.0, 61.1 and 61.9%, respectively. The relative weights in ESBM 10 and 15% was higher ($p<0.05$) than in 5% ESBM. ESBM added at 5 to 15% did not affect ($p>0.05$) the relative weights of lymph glands in groin and mesentery of piglets, but increased ($p<0.05$) the relative weights of lymph glands in mandible by 29.6%, and there were no differences ($p>0.05$) among three levels of dietary ESBM.

Pigs fed diets with SEBM at 5 to 15% had higher ($p<0.05$) percentages of T lymphocytes, CD4+ and CD8+ in peripheral blood of weaned piglets than that with control diet (Table 6). The proportions of T cells and CD4+ were increased ($p<0.05$) by average 9.3 and 36.9% respectively by the inclusion of ESBM, and there were no differences ($p>0.05$) in T cells and CD4+ among the three levels of dietary ESBM. The CD8+ percentages in peripheral blood for ESBM at 10 and 15% were higher ($p<0.05$) than in 5% ESBM, but there was no difference ($p>0.05$) between ESBM at 10 and 15%. For CD4+/CD8+, there was a

Table 3. The effect of enzymolytic soybean meal (ESBM) on the growth performance of weaned pigs[1]

Treatment	IW (kg/pig)	FW (kg/pig)	DFI (g/d)	ADG (g/d)	F/G
Control	8.95^{a}	15.17^{c}	518.98^{b}	225.08^{c}	2.33^{a}
ESBM5	9.04^{a}	17.14^{b}	615.96^{a}	279.37^{b}	2.21^{ab}
ESBM10	8.96^{a}	17.34^{ab}	658.73^{a}	306.13^{ab}	2.16^{b}
ESBM15	9.08^{a}	18.98^{a}	671.16^{a}	348.77^{a}	1.90^{c}
SEM	0.07	0.58	28.55	15.24	0.06

[1] IW = Initial weight; FW = Final weight; DFI = Daily feed intake; ADG = Average daily gain; F/G = Feed/gain.
[a-c] Means without the same super letters within the columns are different ($p<0.05$).

Table 4. The effect of enzymolytic soybean meal (ESBM) on the apparent faecal digestibility of nutrients and digestible energy content in weaned pigs[1]

Treatment	CP (%)	DE (MJ/kg)	Ca (%)	P (%)
Control	71.32^{c}	12.49^{b}	50.76^{b}	50.99^{b}
ESBM 5%	74.91^{b}	12.72^{b}	52.69^{b}	49.15^{b}
ESBM 10%	76.34^{b}	12.78^{b}	52.39^{b}	51.43^{b}
ESBM 15%	82.89^{a}	13.49^{a}	59.19^{a}	56.25^{a}
SEM	1.11	0.16	1.49	1.58

CP = Crude protein; DE = Digestible energy.
[a-c] Means without the same super letters within the columns are different ($p<0.05$).

Table 5. The effect of enzymolytic soybean meal (ESBM) on the relative weights of immune organs of weaned pigs (g/kg body weight)

Treatment	Spleen	Thymus	Lymph gland		
			Groin	Mandible	Mesentery
Control	2.04[b]	1.26[c]	0.69[a]	0.98[b]	1.79[a]
ESBM 5%	2.14[b]	1.89[b]	0.69[a]	1.26[a]	1.75[a]
ESBM 10%	2.09[b]	2.03[a]	0.73[a]	1.28[a]	1.80[a]
ESBM 15%	2.27[a]	2.04[a]	0.73[a]	1.27[a]	1.81[a]
SEM	0.04	0.02	0.02	0.02	0.05

[a-c] Means without the same super letters within the columns are different ($p<0.05$).

difference ($p<0.05$) between 5% ESBM and the control diet.

DISCUSSION

Overcoming growth depression from soybean protein during the first few weeks after pigs are weaned is an important consideration for improving growth and health of pigs. The incubation with microbial protease is one of methods to alleviate the adverse effect of antinutritional factors of soybean meal on the growth performance, nutrient assimilation and potential healthy problem of newly weaned pigs (Caine et al., 1997; 1998; Dierick et al., 2004; Song et al., 2010). Indeed, weaned pigs fed ESBM showed better growth performance and immune function in this study.

Soybean meal high in protein-derived antinutritional factors, such as trypsin inhibitors, urease and allergenic proteins, can cause gastrointestinal disturbances, intestinal damage, increased disease susceptibility and reduced performances of piglets. Bioprocessing of soybean meal by protease is an effective means to relieve the adverse effect of antinutritional factors for animals. In this study, the activity of trypsin inhibitor and urease in ESBM could not be detected. This was supported by a report that the level of trypsin inhibitor decreased as soybean meal treated with protease (Caine et al., 1998).

With the enzymolytic degradation of protein-derived antinutritional factors, the content of soluble protein inevitably increases in soybean meal. Caine et al. (1998) reported that commercial microbial protease pretreatment increased the content of soluble crude protein compared with untreated soybean meal. Importantly, the content of small peptides significantly increased in ESBM in this study, and that small peptides that are able to be absorbed contribute to pigs' nutrition needs further study.

The improvement of ESBM in nutritional values reflected on the better growth performance of weaned pigs in this study. The process of ESBM product includes fermentation and subsequent hydrolyses by microbial protease generated from the former fermentation in this study, which means ESBM sharing the characteristics of high organic acids and soluble proteins from both fermentation and hydrolyses. However, more novel processes are required for better utilization of soybean meal as protein sources.

There are several reports about the effect of commercial protease or fermented soybean meal alone on the growth of monogastric animals. Dierick et al. (2004) reported that there was a non-significant trend for piglets to perform better (growth, F/G ratio) using a microbial exogenous protease in a soybean meal based diet. But, several reports showed that fermented soybean meal increased growth performance and improved F/G ratio in weaned pigs (Feng et al., 2007) and nursery pigs (Jones et al., 2010; Kim et al., 2010).

Similarly, there were several reports on the digestibility of nutrients of weaned pigs fed soybean meal treated with either protease or fermentation. The apparent faecal digestibility of CP and DE increased by ESBM in this study, but Dierick et al. (2004) reported that soybean meal treated with protease did not increase the faecal digestibility of CP and energy of weaned pigs. Caine et al. (1997) found that protease treatment did not improve the apparent digestibility of CP in soybean meal fed to newly weaned

Table 6. The effect of enzymolytic soybean meal (ESBM) on the percentages of peripheral blood T lymphocytes and subsets of weaned pigs

Treatment	T lymphocytes (%)	CD4+ (%)	CD8+ (%)	CD4+/CD8+
Control	59.81[b]	17.83[b]	32.19[c]	0.55[b]
ESBM 5%	65.04[a]	24.21[a]	38.23[b]	0.63[a]
ESBM 10%	65.46[a]	23.84[a]	40.43[a]	0.59[ab]
ESBM 15%	65.54[a]	25.22[a]	41.17[a]	0.61[ab]
SEM	0.82	0.58	0.62	0.02

[a-c] Means without the same super letters within the columns are different ($p<0.05$).

pigs. Feng et al. (2007) reported that fermented soybean meal increased the apparent digestibility of CP and energy for piglets by 17 and 13%, respectively.

Improved nutrition may play a complex and controversial role in animal health. As expected, ESBM increased the relative weights of thymus and mandibular lymph nodes of weaned pigs in this study, indicating that the immune function of weaned pigs may be influenced by feeding ESBM. Transient hypersensitivity to soybean proteins resulting in diarrhea and poor health status is well known in the nutrition of weaned pigs (Li et al., 1990; Dewey, 1993; reviewed by Jezierny et al., 2010). Literature is very limited in using ESBM in weaned pigs. Dierick et al. (2004) reported that there was less residual antigenicity against the soy protein in the stomach and foregut content of piglets on the protease diets and the IgG titers to soy proteins in the sera of protease fed piglets was lowed. Song et al. (2010) found that weaned pigs with 6.58 kg body weights fed diets with fermented soybean meal for 2 weeks were more healthy (less diarrhea) than those fed the diet without fermented soybean meal.

The health status was further investigated by measuring the percentages of T lymphocytes and its subsets in the peripheral blood in this study, and ESBM showed an improvement in the proportions of T cells, CD4+ and CD8+ in this study. The literature in this area is very limited, but there are several indirect reports. Song et al. (2010) reported that plasma from weaned pigs fed fermented soybean meal exhibited reduced immunoreactivity toward α and α' subunits of β-conglycinin and acidic subunits of glycinin, due to a partial hydrolysis of those proteins during fermentation, and this was also proved by the finding that fermented soybean meal showed partial digestion of large proteins with antigenic activity.

In summary, treating soybean meal with *in vitro* fermentation and microbial protease can decrease urease and trypsin inhibitor, and degrade most glycinin and β-conglycinin into small peptides, which improves the nutritional values of soybean protein. Enzymolytic soybean meal partially replacing common soybean meal and soy protein isolate improved feed intake, weight gain and immune function of weaned piglets in this study, showing that ESBM may be a novel soybean protein in the nutrition and health of weaned pigs.

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