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NON RUMINANT NUTRITION

Soybean meal allergenic protein degradation and gut health of piglets fed protease-supplemented diets

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Abstract

Two experiments were conducted to determine the effects of protease supplementation on degradation of soybean meal (SBM) allergenic proteins (glycinin and β-conglycinin) and gut health of weaned pigs fed soybean meal-based diets. In experiment 1, 2 SBM samples from 2 different sources were subjected to porcine in vitro gastric degradation to determine the effects of protease (at 15,000 U/kg of feedstuff) on degradation of the soybean allergenic proteins. In experiment 2, 48 weaned pigs (body weight = 6.66 kg) were obtained in 2 batches of 24 pigs each. Pigs were individually housed in metabolic crates and fed 4 diets (12 pigs/diet). The diets were corn-based diet with SBM 1 or SBM 2 without or with protease at 15,000 U/kg of diet in 2 × 2 factorial arrangement. Diets were fed for 10 d and pigs were sacrificed on day 10 for measurement of small intestinal histomorphology, permeability of small intestine mounted in Ussing chambers, and serum concentration of pro-inflammatory cytokines. Two SBM sources (SBM 1 and SBM 2) contained 46.9% or 47.7% CP, 14.0% or 14.6% glycinin, and 9.90% or 10.3% β-conglycinin, respectively. Protease and SBM source did not interact on any of the response criteria measured in the current study. Protease supplementation tended to increase ($P = 0.069$) the in vitro gastric degradation of glycinin. Protease supplementation tended to reduce ($P = 0.099$) fluorescein isothiocyanate dextran 4,000 Da (which is a marker probe for intestinal permeability) flow in jejunum, and reduced ($P = 0.037$) serum TNF-α concentration. Protease did not affect small intestinal histomorphology. In conclusion, protease tended to increase gastric degradation of glycinin and reduce gut permeability, and serum concentration of pro-inflammatory cytokines, indicating that the protease used in the current study can be added to SBM-based diets for weanling pigs to improve gut health.

Key words: gut health, protease, soybean meal, weaned pig

Introduction

Although soybean meal (**SBM**) is the most widely used source of amino acids (**AA**) in swine diets, its use in diets for weaned pigs is limited by the presence of allergenic proteins; glycinin, and β-conglycinin (Friesen et al., 1993; Wang et al., 2014). These allergenic SBM proteins are poorly digested by gastric enzymes, and induce immune response in the intestine of young pigs, leading to destruction of tight junction proteins in the intestinal wall, diarrhea, and reduced nutrient utilization (Dreau et al., 1994; Fu et al., 2007; Chen et al., 2011; Zhao et al., 2015). Because

Abbreviations

AA	amino acids
ADF	acid detergent fiber
ADFI	average daily feed intake
ADG	average daily gain
FITC	fluorescein isothiocyanate
G:F	gain-to-feed ratio
IDF	insoluble dietary fiber
IL	interleukin
Isc	short-circuit current
NDF	neutral detergent fiber
PD	potential difference
SBM 1	soybean meal from Volga
SBM 2	soybean meal from Beresford
SBM	soybean meal
SDF	soluble dietary fiber
TDF	total dietary fiber
TEER	trans-epithelial resistance
TNF-α	tumor necrosis factor-α

of the presence of the allergenic proteins in SBM and the relatively high content of fiber in some other feedstuffs of plant origin, more expensive feedstuffs of animal origin are added to diets to meet nutrient requirements of weaned pigs.

Pretreatment of SBM with proteolytic enzymes or microorganisms that degrade allergenic protein can increase the use of SBM in diets for weaned pigs (Cervantes-Pahm and Stein, 2010). Also, supplementation of SBM-based diets with enzymes that can degrade the allergenic proteins in the pig stomach (before the allergenic protein arrive in the small intestine) can potentially alleviate the negative effects of the allergenic proteins. Notably, pretreatment technologies are relatively expensive because of extra costs of pretreatment. However, feed enzymes are typically added to pig diets to improve nutrient availability, and hence their addition in soybean products-based diets for weaned pigs to degrade allergenic proteins and increase nutrient availability may offer a more cost-effective means of mitigating the negative effect of these proteins than some other pretreatment options. The effects of pretreatment of soybean products with proteolytic enzymes or microorganisms on nutritive value of soybean products for weaned pigs have been reported, and various pretreated soybean products are commercially available for livestock feeding (Cervantes-Pahm and Stein, 2010; Kim et al., 2010; Song et al., 2010). However, there is limited information on the effects of supplementing soybean product-based diets with enzymes that can degrade the allergenic proteins in the pig stomach. Protein content of SBM from soybean grown in northern parts of USA is less than that of SBM from soybean grown in southern parts of United States (Grieshop et al., 2003), implying that allergenicity of SBM in pigs can potentially be affected by the source of the SBM. We hypothesized that supplementation of SBM-based diets for weanling pigs with protease results in degradation of allergenic proteins present in SBM in the stomach, leading to reduced allergic reaction in the small intestine, and that effects of supplemental protease on allergenicity of soybean proteins is greater for SBM from the southern than northern parts of United States. The objective of this study was to determine effects of protease on degradation of the soybean allergenic proteins at conditions found in pig stomach; on gut permeability and histomorphology and fecal score of weaned pigs fed diets containing SBM from southern or northern parts of United States.

Materials and Methods

Two experiments were conducted to achieve the objective of this study. The SBM used in the 2 experiments was obtained from 2 sources; SBM 1 (South Dakota Soybean Processors, Volga, SD) and SBM 2 (Southeast Farmers Coop, Beresford, SD). The experimental animal procedures were reviewed and approved by the Institutional Animal Care and Use Committee at South Dakota State University (#17-084A).

Experiment 1

The experiment was conducted to determine effects of protease on in vitro porcine gastric degradation of allergenic proteins present in SBM. Samples of SBM 1 and SBM 2 were ground to pass through 1 mm screen. Each of the ground samples were divided into 8 subsamples to give a total of 16 subsamples. The subsamples were treated with protease (RONOZYME ProAct; DSM Nutritional Products, Kaiseraugst, Switzerland) at 0 or 15,000 U/kg of feedstuff in a 2 × 2 factorial arrangement with SBM and protease as factors and SBM source and protease dosage as levels. The subsamples were then subjected to porcine gastric in vitro digestion as described by Woyengo et al. (2016). Briefly, 4 g of each of the 16 subsamples were weighed into a 500-mL conical flask. A phosphate buffer solution (200 mL, 0.1 M, pH 6.0), HCl solution (80 mL, 0.2 M), and fresh pepsin (8 mL, 20 g/L porcine pepsin, P-0609; Sigma-Aldrich Corp., St. Louis, MO) were added to each flask. Additionally, 2 mL of chloramphenicol (C-0378; Sigma-Aldrich Corp., St. Louis, MO) solution (0.5 g/100 mL) was added in each flask to prevent bacterial growth during the enzymatic hydrolysis. Prior to initiation of pepsin digestion, 66.8 μL of protease was added to the incubation medium. Subsamples were then placed in a water bath at 39 °C for 2 h under a gentle agitation (50 rpm). The hydrolyzed residues were collected by filtration on a nylon cloth, freeze-dried, weighed, and stored at 4 °C for later analysis of glycinin and β-conglycinin proteins. The experiment was done in 3 batches to obtain 12 replicates (4 replicates per treatment for each batch). The experiment was conducted as a randomized complete block design with the flask as experimental unit and batch as block.

The 2 SBM samples were analyzed for DM by oven drying at 135 °C for 2 hr (method 930.15), CP by a combustion procedure (method 990.03), as per AOAC (2012); and for neutral detergent fiber (**NDF**) and acid detergent fiber (**ADF**; Van Soest et al., 1991) on an Ankom 200 Fiber Analyzer (Ankom Technology, Fairport, NY). The 2 SBM samples were also analyzed; soluble dietary fiber (**SDF**) and insoluble dietary fiber (**IDF**) contents of the 2 SBM samples were measured by using the Megazyme Total Dietary Fiber (**TDF**) kit (Megazyme International Ireland Ltd, Wicklow, Ireland) according to AOAC-991.43 and AACC-32-07.01 methods (AOAC, 2012; McCleary et al., 2012). The TDF was calculated as sum of SDF and IDF. The 2 SBM samples were additionally analyzed for isoflavones and saponins concentration using HPLC (Berhow, 2002; Berhow et al., 2006) at the USDA-ARS National Center for Agricultural Utilization Research (Peoria, IL). The SBM and undigested residues collected after porcine in vitro enzymatic hydrolysis were analyzed for glycinin and β-conglycinin proteins by ELISA technique using commercially available kits (Unibiotest Co Ltd, Wahun, China).

Experiment 2

The experiment was conducted to determine effects of protease on gut permeability and histomorphology, serum pro-inflammation cytokines and fecal score of weaned pigs fed

SBM-based diets. Forty-eight pigs (initial body weight = 6.66 ± 0.80 kg; Large White-Landrace female × Duroc male; Pig Improvement Company) weaned at ~21 d of age were obtained from a commercial farm in 2 batches of 24 pigs each. Pigs in each batch were housed individually in 24 pens, and fed 4 diets (6 pens/diet/batch) for 10 d. The 4 diets were an antibiotic-free corn-based basal diet with SBM from the 2 different sources (Table 1) and protease at 2 levels (0 and 15,000 U/kg) in a 2 × 2 factorial arrangement. During the experimental period, the diets and water were offered ad libitum. The occurrence and severity of postweaning diarrhoea were assessed on a pen basis by using a fecal consistency scoring system (Marquardt et al., 1999) on days 1 to 7 of the experimental period. Body weight and feed intake of pigs were determined on day 8 of the experiment for calculation of average daily gain (**ADG**) and average daily feed intake (**ADFI**). Due to limited number of chambers that we had, all the 24 pigs were sacrificed at rate of 8 pigs (balanced for dietary treatment), per day on days 8, 9, and 10 of the experiment for evaluation of small intestinal integrity. Just prior to euthanasia a 5-mL blood sample was collected into a vacutainer tube, stored at 5 °C for 24 hr then centrifuged for separation of serum, which was stored frozen for later analysis of cytokines [interleukin (**IL**)-1β, tumor necrosis factor-α (**TNF-α**), and IL-13]. Upon euthanasia, 10 cm sections of the jejunum (at the middle of small intestine), and ileum (at 70 cm anterior ileo-cecal junction) were immediately collected and placed in ice-cold Ringer's solution (NaCl; 6.72 g/L, K_2HPO_4; 0.42 g/L, KH_2PO_4; 0.05 g/L, $CaCl_2$ dihydrate; 0.18 g/L, $MgCl_2$ hexahydrate; 0.24 g/L, $NaHCO_3$; 2.1 g/L, glucose 1.80 g/L; pH 7.3–7.4) for determination of gut permeability and electrophysiological properties in Ussing chambers technique as described below. Indomethacin (10 μM) was added in the buffer solution at 3 μL/L to help minimize the effects of pro-inflammatory eicosanoids on intestinal tissue electrophysiological properties. Also, 5-cm sections of the jejunum and ileum were obtained (from the same locations where sections for Using Chamber were obtained) and placed into 10% buffered formalin solution for later determination of histology as described below.

Table 1. Ingredient composition of the basal diet

Item	Amount, %
Ingredient, %	
Corn	42.11
Whey powder	20.00
Soybean meal	35.00
Soybean oil	0.49
Limestone	1.05
Monocalcium phosphate	0.11
Salt	0.60
L-Lys.HCl	0.29
L-Thr	0.05
DL-Met	0.10
Pig vitamin premix[1]	0.05
Pig mineral premix[2]	0.15
Calculated composition	
Net energy, Mcal/kg	2.448
Crude protein, %	23.00
Standardized ileal digestible AA content, %	
Lys	1.40
Met	0.40
Met+Cys	0.72
Thr	0.82
Trp	0.27
Ca, %	0.70
Digestible P, %	0.30

[1]Provided the following per kilogram of diet: 11,011 IU vitamin A, 1,652 IU vitamin D3, 55 IU vitamin E, 0.04 mg vitamin B12, 4.4 mg menadione, 9.9 mg riboflavin, 61 mg pantothenic acid, 55 mg niacin, 1.1 mg folic acid, 3.3 mg pyridoxine, 3.3 mg thiamine, and 0.2 mg biotin.
[2]Provided the following per kilogram of diet: 165 mg Zn as $ZnSO_4$, 23 mg Fe as $FeSO_4$; 17 mg Cu as $CuSO_4$, and 44 mg Mn as $MnSO_4$.

Determining intestinal permeability with Ussing chambers

The electrophysiological properties (short-circuit current, **Isc**; potential difference, **PD**; and trans-epithelial electrical resistance, **TEER**) were determined using a Ussing chamber (VCC-MC6; Physiologic Instruments Inc., San Diego, CA) containing pairs of current (Ag wire) and voltage (Ag/AgCl pellet) electrodes housed in 3% agar bridges and filled with 3 M KCl. Intestinal samples for determining gut permeability were transported (while in ice cold Ringer buffer solution) to the laboratory, where they were opened along the mesenteric border. The opened samples were gently stripped of serosal layer. The prepared mucosal tissues (3 tissues per intestinal segment) were placed in tissue holders with an aperture of 1 cm^2, and mounted in the chambers. Ringer buffer solution was added to the serosal and mucosal half chambers (3 mL per each of the half chamber). The chambers were continuously gassed with a mixture of 95% O2 and 5% CO2. The temperature of the chambers was maintained at 37 °C. After mounting tissues in the chambers, the Ussing chambers system was placed in remote mode to allow for Acquire and Analyze software program to obtain data, and tissues were referenced on the Acquire and Analyze Data acquisition software and hardware system. After referencing, 10 mM glucose was added to the serosal bathing solution, which was balanced on the mucosal side with 10 mM mannitol. The Acquire and Analyze software was turned on (to start collecting data) immediately after adding glucose and mannitol. The spontaneous PD was measured using Ringer-agar bridges connected to calomel electrodes, and the PD was short circuited through Ag–AgCl electrodes using voltage clamp that corrected for fluid resistance. The TEER was calculated from the Isc and PD.

Tissues were allowed to equilibrate for 10 to 15 min after the addition of 10 mM glucose to the serosal bathing solution and 10 mM mannitol to mucosal bathing solution. After the equilibration, 30 mM glucose was added to the mucosal side and balanced with 30 mM of mannitol of the serosal side for estimation of active transport of glucose across the mucosa as described below. After equilibration period of 10 to 15 min, fluorescein isothiocyanate (**FITC**)-dextran 4 kDa (#46944, Sigma-Aldrich, St. Louis, MO) was added to mucosal bathing solution for determination mucosal to serosal flux of FITC-dextran 4 kDa. The mucosal to serosal flux of FITC-dextran 4 kDa was measured in darkness by adding 4 kDa FITC-dextran to the mucosal side of the Ussing chambers at a final concentration of 104 nM. Samples (200 μL) were taken from the serosal sides at 0 (just before the addition of FITC-dextran 4 kDa on mucosal side), 15, 30, 45, and 60 min for determination of FITC-dextran 4 kDa concentration (described below), and replaced with 200 μL of buffer solution. After the 60 min of measuring mucosal to serosal flux of FITC-dextran 4 kDa, forskolin (10 μM) was added to both the mucosal and serosal sides of the chambers to determine whether or not the tissues were still alive at the end of Ussing chambers measurements. Significant changes in Isc within 5 min indicated that the tissues were still alive.

Active transport of glucose across the mucosa was calculated as the difference between the Isc values (basal Isc values) that was recorded at the time of adding 30 mM glucose on mucosal side and 30 mM mannitol on the serosal side, and the greatest Isc values that was recorded after the addition of 30 mM glucose on mucosal side and 30 mM mannitol on the serosal side. The concentration of FITC-dextran 4 kDa on serosal side of chambers at 0, 15, and 30, 45, and 60 min was determined by measuring the fluorescence in the collected serosal samples using a fluorescence plate reader (Synergy 2 Multi-detection Microplate Reader, BioTek, Winooski, VT) at 540 nm. The quantity of FITC-dextran 4 kDa that moved from mucosal to serosal side was calculated by multiplying the FITC-dextran 4 kDa concentration on serosal side by the volume of solution on serosal side corrected for the 200 µL samples were taken from the serosal sides.

Gut histomorphological analysis

Samples for histomorphology analysis were sent to the Animal Disease Research and Diagnostic Laboratory at South Dakota State University for staining with haematoxylin and eosin. Villus height (from the tip of the villi to the villus-crypt junction) and crypt depth (from the villus-crypt junction to the base) were measured at 20× magnification using a Nikon microscope (Tokyo, Japan) equipped with a DS2MV Nikon camera (Tokyo, Japan) and NIS Elements software (Tokyo, Japan) in 10 well-oriented villi and crypt columns. The VH-to-CD ratio was calculated.

Determining serum cytokine concentration

Serum concentrations of cytokines were determined using commercially available kits. Serum concentration of TNF-αwas assayed using a Porcine TNF-α Quantikine ELISA Kit (Cat. No. PTA00; R & D Systems, Inc. Minneapolis, MN) according to manufacturer's instructions. Serum concentrations of IL-1β was assayed using a Porcine TNF-α Quantikine ELISA Kit (Cat. No. PLB00B; R & D Systems, Inc. Minneapolis, MN). Serum concentration of IL-13 was assayed using a Porcine IL-13 ELISA Kit (Cat. No. ESIL13, Thermo Scientific, Frederick, MD).

Table 2. Analyzed chemical composition (as is) of SBM samples[1]

Item	SBM 1	SBM 2
Moisture, %	7.39	7.48
Fiber, %		
Neutral detergent fiber	8.53	7.33
Acid detergent fiber	5.38	4.60
Total dietary fiber	22.2	23.9
Insoluble dietary fiber	20.4	21.9
Soluble dietary fiber	1.79	2.02
Crude protein, %	46.9	47.7
Allergenic proteins, g/100 g of crude protein		
Glycinin	29.9	30.6
β-Conglycinin	21.1	21.6
Total isoflavones, mg/g	3.64	3.94
Total saponins, mg/g	3.30	3.51

[1]SBM 1, soybean meal from Volga, SD; SBM 2, soybean meal from Beresford, SD.

Statistical analysis

All data obtained from this study were subjected to analysis of variance using the MIXED procedure of SAS. Main effects of SBM source and protease level and their interactions were determined. Treatment means were separated by the probability of difference when interactions between SBM source and protease level were significant. To test the hypotheses, $P < 0.05$ was considered significant. If pertinent, trends ($0.05 < P \leq 0.10$) are also reported.

Results

The SBM 1 contained similar CP, glycinin, β-conglycinin, isoflavones, and saponins than SBM 2 (Table 2). The SBM samples were similar in content of TDF, IDF, and SDF. The gastric in vitro degradation of β-conglycinin was greater ($P < 0.001$) than that of glycinin (70.3 vs. 50.6%; SEM = 2.15; data not presented). The porcine gastric in vitro degradation of β-conglycinin for SBM 1 was greater ($P = 0.038$) than that for SBM 2 (Table 3). Also, the porcine gastric in vitro degradation of glycinin for SBM 1 tended to be greater ($P = 0.080$) than that for SBM 2. Protease supplementation tended to increase ($P = 0.069$) the porcine gastric in vitro degradation of glycinin. The ADFI was unaffected by SBM source and protease supplementation (Table 4). The ADG and G:F for SBM 1 tended to be greater ($P < 0.10$) than those for SBM 2. However, protease supplementation did not affect ADG and G:F of the pigs.

Jejunal villus height, crypt depth, and villous height to crypt depth ratio were unaffected by SBM source and protease supplementation (Table 5). Source of SBM did not affect the Isc, TEER, and FITC dextran 4 kDa flow in jejunum and ileum (Table 5). Protease supplementation tended to reduce ($P = 0.099$) FITC dextran 4 kDa flow in jejunum. However, protease supplementation did not affect FITC dextran 4 kDa flow in ileum. Also, protease supplementation did not affect the TEER, and basal and glucose-induced Isc in jejunum or ileum. Protease supplementation reduced ($P = 0.037$) serum concentration of TNF-α (Table 6), and numerically reduced serum concentration of IL 1-β. However, protease supplementation did not affect the serum concentration of IL 13. The serum concentration of TNF-α for SBM 1 was greater ($P = 0.031$) than that for SBM 2. However, source of SBM did not affect the serum concentration of IL 1-β and IL 13.

Table 3. Effects of protease and soybean meal source[1] on porcine gastric in vitro degradation of allergenic proteins[2]

	Without protease		With protease[3]			P-value		
Degradation, %	SBM 1	SBM 2	SBM 1	SBM 2	SEM	SBM source	Protease	SBM source × protease
Glycinin	52.21	40.95	59.18	52.58	4.926	0.080	0.069	0.642
β-Conglycinin	72.20	66.95	73.15	69.01	2.186	0.038	0.495	0.803

[1]SBM 1, soybean meal from Volga, SD; SBM 2, soybean meal from Beresford, SD.
[2]Data are means of 12 observations.
[3]Protease (ProAct, DSM Nutritional Products) supplied 15,000 units of protease per kilogram of feedstuff.

Table 4. Effect of protease and soybean meal source[1] on growth performance of nursery pigs during the first 8 d after weaning and fecal score[2]

	Without protease		With protease[4]			*P*-value		
Item[3]	SBM 1	SBM 2	SBM 1	SBM 2	SEM	SBM source	Protease	SBM × protease
ADG, g	54.4	45.0	92.9	29.6	21.3	0.082	0.448	0.379
ADFI, g	96.8	98.1	132.6	97.3	18.1	0.409	0.395	0.648
G:F, g/g	0.503	−0.072	0.519	0.079	0.329	0.096	0.738	0.879
Fecal score[5]	0.90	0.70	0.90	0.80	0.142	0.433	0.793	0.705

[1]SBM 1, soybean meal from Volga, SD; SBM 2, soybean meal from Beresford, SD.
[2]Data are means of 12 observations.
[3]The pig body weights and feed intake were determined on day 8 of the experiment to calculate ADG, ADFI, and G:F.
[4]Protease (ProAct, DSM Nutritional Products) supplied 15,000 units of protease per kilogram of diet.
[5]Fecal score is the mean fecal consistency score: 0, normal; 1, soft feces; 2, mild diarrhea; 3, severe diarrhea. Pigs with a fecal score of ≤1 were considered not to have diarrhea.

Table 5. Effect of protease and soybean meal source[1] on small intestinal histology and permeability to FITC dextran, and electrophysiological properties[2]

	Without protease		With protease[3]			*P*-value		
Item	SBM 1	SBM 2	SBM 1	SBM 2	SEM	SBM source	Protease	SBM source × protease
Jejunal histology								
Villus height, μm	393.0	394.5	407.7	402.8	16.45	0.920	0.499	0.851
Crypt depth, μm	199.1	200.3	206.1	211.6	6.15	0.596	0.155	0.734
VH:CD[4]	1.97	1.99	1.99	1.90	0.074	0.662	0.639	0.484
FITC dextran flow, mg/h								
Jejunum	0.138	0.114	0.068	0.101	0.024	0.841	0.099	0.251
Ileum	0.124	0.089	0.116	0.108	0.031	0.486	0.871	0.669
Resistance, Ω								
Jejunum	72.67	66.81	72.84	84.32	10.82	0.797	0.421	0.430
Ileum	82.38	81.54	96.12	101.90	12.96	0.850	0.199	0.800
Basal Isc, μA/cm^2								
Jejunum	−0.13	0.00	0.01	−0.01	0.030	0.304	0.223	0.297
Ileum	−0.02	−0.06	−0.01	0.02	0.031	0.403	0.352	0.659
SGLT1 dependent Isc, μA/cm^2								
Jejunum	0.11	0.11	0.18	0.12	0.192	0.498	0.238	0.538
Ileum	0.13	0.17	0.11	0.12	0.095	0.828	0.412	0.263

[1]SBM 1, soybean meal from Volga, SD; SBM 2, soybean meal from Beresford, SD.
[2]Data are means of 12 observations.
[3]Protease (ProAct, DSM Nutritional Products) supplied 15,000 units of protease per kilogram of diet.
[4]VH:CD, villus height to crypt depth ratio.

Discussion

The CP content in SBM 1 (46.9%) and SBM 2 (47.7%) samples used in the current study did not differ from the value (47%) that was reported by NRC (2012) for dehulled solvent extracted SBM. Per hundred grams of CP in SBM, the glycinin content (29.9 g for SBM 1 and 30.6 g for SBM 2) and β-conglycinin content (21.1 g for SBM 1 and 21.6 g for SBM 2) in SBM samples used in the current experiment were within the range of values (30 to 39.2 g for glycinin and 18.5 to 31 g for β-conglycinin) that were previously reported for SBM by others (Iwabuchi and Yamauchi, 1987; Delwiche et al., 2007). In North America, the environmental temperature, number of growing days, and day length decreases as one moves from south to north. Protein content in soybean increases with increase in photoperiod and environmental temperature during the growing period (Morandi et al., 1988; Piper and Boote, 1999). The SBM 1 was obtained from Volga, SD, whereas SBM 2 was obtained from Beresford, SD. Most of soybeans that were processed at Volga plant originated from northern parts of South Dakota, whereas most of soybeans that were processed at Beresford plant originated from northern parts of Iowa. Grieshop et al. (2003) reported that CP content of SBM from processing plants in a region that is composed of northern parts of South Dakota was less than that from processing plants in a region that is composed of northern parts of Iowa because of difference between the 2 regions with regard to the number of growing days. In the current study, the CP content in SBM 1 was less than that in SBM 2; however, the difference was marginal.

Sadeghi et al. (2006) reported lower rumen degradability of glycinin than of β-conglycinin, and attributed the lower rumen degradability of glycinin to the presence of disulfide bonds in the glycinin. Glycinin has 2 subunits; acid and basic (Dias et al., 2003; Mo et al., 2006). The β-conglycinin and acidic subunits of glycinin were degraded at faster rate than basic subunits of glycinin during rumen in vitro incubation (van der Aar et al., 1982) or rumen in situ incubation (Romagnolo et al., 1990) of soybean protein, implying that the lower digestibility of glycinin

Table 6. Effect of protease and soybean meal source[1] on serum concentration of cytokines[2]

	Without protease		With protease[4]			*P*-value		
Item	SBM 1	SBM 2	SBM 1	SBM 2	SEM	SBM source	Protease	SBM source × protease
IL-1-β, pl/mL	125.1	138.3	117.9	111.6	10.45	0.742	0.113	0.355
TNF-α, pl/mL	119.8	106.6	107.2	83.9	8.18	0.031	0.037	0.543
IL-13, pl/mL	1,906	4,069	2,673	2,231	936	0.364	0.570	0.172

[1]SBM 1, soybean meal from Volga, SD; SBM 2, soybean meal from Beresford, SD.
[2]Data are means of 12 observations.

[4]Protease (ProAct, DSM Nutritional Products) supplied 15,000 units of protease per kilogram of diet.

is due to the presence of basic subunits in it. The basic subunits of glycinin have greater content of hydrophobic AA than acidic subunits (Mo et al., 2006). The basic subunits of glycinin compared with acidic subunits of the same protein were less soluble at acidic pH (1.5 to 4.0) found in the stomach of pigs (Dias et al., 2003). Thus, lower gastric in vitro degradation of glycinin than of β-conglycinin may be attributed to the presence of more disulfide bonds and hydrophobic polypeptides in the former than in later. Gastric digestibility of protein in feedstuffs such as SBM can potentially be affected by its fiber content, secondary structure of protein, and interaction between protein and other components of SBM. The SBM 1 and SBM 2 were not different in TDF, IDF, and SDF contents. Thus, the tendency for greater in vitro degradation of glycinin; greater in vitro degradation of β-conglycinin for SBM 1 than SBM 2 may have been due to factors other than fiber content. Similarly, Sotak-Peper et al. (2017) reported greater AA digestibility for SBM from northern parts of United States than SBM from southern parts of United States.

Soybean proteins (glycine and β-conglycinin) have been reported to cause allergic reaction in the small intestine of weaned pigs, negatively affecting small intestinal integrity (Li et al., 1990; Dreau et al., 1994; Zhao et al., 2015). Protease supplementation tended to increase porcine gastric in vitro degradation of glycinin, but not of β-conglycinin. The β-conglycinin is glycosylated, whereas glycinin is not (Amigo-Benavent et al., 2009). Non-glycosylated regions of β-conglycinin can be cleaved by proteolytic enzymes such as pepsin, whereas glycosylated regions may not (Amigo-Benavent et al., 2011). Indeed, de-glycosylation of β-conglycinin by PNGase improved its in vitro gastric and small intestinal digestibility (Amigo-Benavent et al., 2011). Thus, the presence of glycans in β-conglycinin may have reduced the accessibility of protease to β-conglycinin, leading to limited effect of the enzyme on digestibility the β-conglycinin.

Piglets were individually housed because the major of objective was determine the effects of dietary protease on gut physiology and immune response, and not growth performance; ADFI for individually housed pigs is less than that for group-housed pigs during the first week after weaning. Thus, the low ADFI of pigs used in the current study could be explained by the high dietary level of SBM, absence of spray dried blood plasma in diets and individual housing of pigs. Protease supplementation did not affect growth performance of the pigs. However, ADG and G:F of pigs fed SBM 1 tended to be greater than that of pigs fed SBM 2. As previously mentioned, the AA in SBM from southern parts of US compared with those in SBM from the northern parts of US are slightly less digestible despite its slightly greater CP and AA in the former than in the latter, implying that the SBM from the southern parts and northern parts are similar in digestible AA content. Thus, the differences between the SBM 1 and SBM 2 with regard to growth performance of pigs may not have been due to differences in supply of digestible AA; it may be explained by greater porcine gastric in vitro degradation of these allergenic proteins for SBM 1 than for SBM 2. It should be noted that the G:F means for diets that are presented in Table 4 are slightly different from those that can simply be calculated from ADG and ADFI means presented in the same table. The G:F value for any given diet in Table 4 was calculated (by SAS program) as summation of G:F values of pigs fed that particular diet divided by number of pigs fed the same diet. The mean generated by this aforementioned method may not be equal to that which is generated by simply diving the mean of ADG by the mean of ADFI, and the difference between the means generated by these 2 methods can increase with increase in data variability.

The source of SBM or protease supplementation did not affect jejunal villous height. Also, protease supplementation did not affect jejunal crypt depth and villous height to crypt depth ratio. Because soybean protein negatively affect integrity of small intestinal in weaned pigs, protease supplementation is expected to result in increased villous height and reduced crypt depth in small intestine by digesting the soybean allergenic proteins. Indeed, Duarte et al. (2019) observed increased villous height of weaned pigs due to protease supplementation. However, in the current study, protease supplementation numerically improved jejunal villous height, but the improvement was not statistically significant. The FITC dextran 4 kDa is marker flow probe that can only pass through larger pores in the gastrointestinal tract wall. Thus, in Ussing chambers, the flow of FITC dextran 4 kDa from mucosal side to serosal side of intestinal mucosa reflects the permeability of the mucosa to the probes hence permeability to toxins and pathogenic microorganisms. Also, in the Ussing chambers, the TEER reflects the size of pores in intestinal mucosa and hence intestinal permeability to toxins and pathogenic microorganisms, whereas Isc reflect net ion movement across the mucosa (Wijtten et al., 2011). In the current study, source of SBM did not affect the TEER and FITC dextran flow in jejunum and ileum, implying that the permeability of jejunum and ileum to toxins was not affected by SBM source. Protease supplementation reduced FITC dextran 4 kDa flow in jejunum, implying that protease supplementation resulted in reduced jejunal permeability to toxins. The reduction in FITC dextran 4 kDa flow in jejunum may have been due to degradation of allergenic proteins by the protease in the upper part of small intestine, leading to reduction in negative effects of the allergenic proteins on permeability of jejunum to toxins. Similarly, expression of tight junction protein (Claudin1) in jejunum of broiler chicken was reduced by replacement of canola meal and DDGS with

38% SBM in diet for the broiler chickens; however, addition of protease to the SBM-containing diet increased the expression of this tight-junction protein to that of SBM-free diet (Cowieson et al., 2017). Also, addition of protease to SBM-based diets for broiler chickens reduced ileal flow of sialic acid (Cowieson et al., 2018), implying that the protease reduced inflammatory injury in small intestinal mucosa. Secretion of sialic acid in the small intestine is increased with an increase in inflammatory injury in the small intestinal mucosa (Pietro et al., 2019). The lack of effect of protease on FITC dextran 4 kDa flow in the ileum may be attributed to the fact that the allergenic proteins had already been digested by dietary protease and pancreatic proteases by the time the digesta arrived in the ileum. Protease supplementation did not affect TEER in the jejunum despite the reduction in FITC dextran 4 kDa by the supplementation. Neirinckx et al. (2011) reported that changes in TEER in jejunum of pigs, horses, dogs, and turkeys did not correspond well to changes in damage to jejunal tissues of these animal species. Moeser et al. (2007) also did not observe correlation between TEER and mannitol flow from mucosal side to serosal side of intestinal mucosa, and suggested that TEER compared with mannitol flow as a measure of barrier is of less clinical significance because it is an indirect measure of barrier function. The Isc in small intestine mucosa mounted in Ussing chambers can increase when there is an increase in net secretion of anions such as Cl and HCO_3 or net absorption of cations such as Na (Wijtten et al., 2011; Moeser et al., 2012). Generally Isc in small intestine of weaned pigs is greater than that of unweaned pigs due to greater secretion of Cl in the former than in the latter; Cl secretion is associated with diarrhoea (Moeser et al., 2012). Protease supplementation or SBM source did not affect the basal Isc. However, it is difficult to explain whether or not protease supplementation or SBM source affected ion secretion or absorption because, for example, anion secretion and cation absorption can change without change in basal Isc if the magnitude of change in anion secretion (amount of negative charges being secreted) is same as the magnitude of change in cation absorption. In the small intestine, most of the glucose is actively absorbed by sodium-dependent glucose transporter 1 protein that absorb glucose together with Na (Moran et al., 2010). Thus, the addition of glucose on the mucosa side of small intestine (balanced with addition of the same amount of mannitol on the serosal side) can result in increased Isc if glucose (and hence Na) is actively absorbed. In the current study, SBM source or protease supplementation did not affect this glucose-induced change in Isc, implying SBM source and protease supplementation did not affect the abundance of sodium-dependent glucose transporter 1 protein in the mucosa of jejunum and ileum of the pigs.

Consumption of food allergens causes degranulation of mast cells in intestinal mucosa (Kraneveld et al., 2012). De-granulatation of mast cells and other immune cells results in increased production of pro-inflammatory cytokines such as TNF-α, IL-1-β, and IL-13 (Theoharides and Kalogeromitros, 2006; Theoharides et al., 2007; Conti et al., 2017). Protease supplementation reduced serum concentration of TNF-α, which may have been due to hydrolysis of allergenic proteins by the protease. However, Duarte et al. (2019) did not observe a reduction in concentration of TNF-α in serum of weaned pigs due to addition of protease to diets. Basal diets fed in the study of Duarte et al. (2019) compared with that fed in the current study contained less amounts of SBM (23% and 27% vs. 35%). Also, basal diets fed in the study of Duarte et al. (2019) contained some animal proteins (blood plasma and poultry meal), whereas the basal diet fed in the current study did not. Thus, the differences between the current study and that of Duarte et al. (2019) with regard to the effect of protease supplementation on serum TNF-α may partly have been due to differences in the composition of basal diets. In the current study, protease supplementation resulted in reduced serum concentration of IL-1-β, but the reduction was not statistically significant. Also, protease supplementation did not affect serum concentration of IL-13. Mast cells differentially release various pro-inflammatory cytokines depending type of activating antigen (Theoharides et al., 2007). Thus, the different effects of protease supplementation on serum concentration of TNF-α, IL-1-β, and IL-13 may be attributed to differential release of these cytokines by activated immune cells in response to the protease supplementation. Serum concentration of TNF-α for SBM 1 was greater than that for SBM 2, but the reason for this is not clear because the gastric digestibility of both glycinin and β-conglycinin were greater for SBM 1 than for SBM 2. It may have been due to the slightly higher content of isoflavones in SBM2 than in SBM 1. Isoflavones have antioxidant and anti-inflammatory activities (Smith and Dilger, 2018), and hence they may reduce production of pro-inflammatory cytokines such as TNF-α.

In conclusion, protease supplementation tended to increase the in vitro gastric degradation of glycinin by 20% and reduce jejunal permeability to FITC dextran 4 kDa by 33%; reduced serum concentration of TNF-α by 16%. Thus, the protease used in the current study can be added in diets for weaned to improve gut health of weaned pigs fed SBM-based diets.

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Conflict of interest statement

Drs. A. J. Cowieson and G. Pappenberger, DSM Nutritional Products, Wurmisweg, Kaiseraugst, Switzerland.

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