



Effects of Coated Compound Proteases on Apparent Total Tract Digestibility of Nutrients and Apparent Ileal Digestibility of Amino Acids for Pigs

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ABSTRACT: Two experiments were conducted to evaluate effects of coated compound proteases (CC protease) on apparent total tract digestibility (ATTD) of nitrogen (N) and energy, and apparent ileal digestibility (AID) of amino acids (AA) and nutrients in diets for pigs. In Exp. 1, 12 crossbred barrows (initial body weight: 20.14±1.71 kg) were housed in individual metabolism crates and allotted into 2 treatments with 6 piglets per treatment according to weight in a randomized complete block design. The 2 diets were corn-soybean meal basal diets with (0.2 g/kg) or without CC protease supplementation. The CC protease supplementation increased ($p<0.05$) the digestible and metabolizable N and energy values and the digestibility and retention rate of N in the diet. The ATTD of energy and nutrients had been improved ($p<0.05$) in the diet supplemented with CC protease. In Exp. 2, 12 crossbred barrows (initial body weight: 20.79±1.94 kg), fitted with T-cannulas at the distal ileum, were blocked by body weight into 2 groups with 6 pigs each. The diets were the same as those in Exp. 1. The CC protease increased ($p<0.05$) the AID of crude protein and some essential AA including arginine, isoleucine and leucine. The AID and ATTD of energy and nutrients had been improved ($p<0.05$) by supplemental CC protease, but the hindgut digestibility of nutrients was unaffected. Overall, the CC protease improved the ATTD of N and energy and AID of some indispensable AA and nutrients in the corn-soybean meal diet for pigs. Therefore, the CC protease supplement could improve the utilization of protein in the corn-soybean meal diet and thus contribute to lower N excretion to the environment. (**Key Words:** Amino Acid, Apparent Ileal Digestibility, Apparent Total Tract Digestibility, Coated Compound Proteases, Nitrogen, Pigs)

INTRODUCTION

Soybean meal (SBM) is one of the most commonly used protein sources for pigs. Most of the proteins in SBM are easily digested, but some proteins including glycinin, protease inhibitor and antigenic proteins are difficult to digest, especially for newly weaned pigs, which may damage intestinal health and impair immune function (Brandon and Friedman, 2002; Sun et al., 2008; Zhao et al., 2008; Wang et al., 2011a;b). Fortunately, with the

developing of feed enzyme technologies, protease treatment of SBM is an alternative means to reduce the antigenic challenge of SBM and improve performance of animals (Rooke et al., 1998; Piao et al., 1999; Yu et al., 2007; Romero et al., 2013). Therefore, the treatment of SBM with appropriate proteases provides a potential method to enhance the utilization of SBM proteins (Adeola and Cowieson, 2011).

Nowadays, increasingly excessive nitrogen emission from animal production has damaged the natural environment and threatened human health both directly and indirectly (He et al., 2006; Shen et al., 2008; Chen et al., 2014). An effective way to improve the nitrogen pollution is enhancing nitrogen digestibility and reducing nitrogen excretion in animal production. Actually, protein sources

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are especially indispensable for pigs (Maxwell and Carter, 2001; Pan et al., 2016). It is, therefore, significant to explore effective proteases to improve the digestibility of protein and thus to decrease nitrogen excretion for successful and sustainable swine production in the future (Brotzge et al., 2014).

Proteases as supplemental feed enzymes have been added to swine diets routinely for many years (Cowieson and Ravindran, 2008; Wang et al., 2011a; b), however, different proteases with different inherent characteristics may elicit divergent responses *in vivo* (Adeola and Cowieson, 2011). In our studies, the enzyme products were compounded with acidic, neutral and alkaline proteases produced by *Aspergillus niger*, *Bacillus subtilis*, and *Bacillus Licheniformis* and coated by corresponding coating layers, which could successively dissolve and work in different microenvironment in the digestive tract. We hypothesized that the coated compound proteases (CC protease) could improve the utilization of nitrogen (N) in different intestinal segments and thus reduce N excretion in corn-SBM diet. Therefore, the objective of these studies was to evaluate effects of the CC protease supplementation on apparent total tract digestibility (ATTD) of N and energy, and apparent ileal digestibility (AID) of amino acids (AA) and nutrients in diets for pigs.

MATERIALS AND METHODS

All procedures used in the experiments were approved by the China Agricultural University Institutional Animal Care and Use Committee (Beijing, China). These studies were conducted in the Metabolism Laboratory of the National Feed Engineering Technology Research Centre (Beijing, China).

Coated compound proteases

The CC proteases with protease activity of 8,000 units/g were in granular form provided by Kemin Industries Co. Ltd. (Zhuhai, China). One unit of enzyme activity is defined as the amount of enzyme liberating 1 μ mol of casein per min at 37°C and pH 5.5. The compound enzymes contain acidic, neutral and alkaline proteases produced by *Aspergillus niger*, *Bacillus subtilis* and *Bacillus Licheniformis*, respectively. Three kinds of proteases were coated in order to reduce the impact of feed pelleting and be released in the related gastrointestinal tract. The acidic protease was coated with heat-resisting membrane, and the neutral and alkaline proteases were both coated by heat-proof and acidity-resistant coating layers, which were not expected to degrade by the gastric acid. Therefore, the CC proteases could successively dissolve and work in acidic, neutral or alkaline microenvironment in the digestive tract.

Animals, diets and experimental designs

Exp. 1 was conducted to evaluate the effects of CC protease on digestible energy (DE) and metabolizable energy (ME) values, N utilization and ATTD of gross energy (GE) and nutrients in corn-SBM diet. Twelve crossbred barrows (Duroc \times [Landrace \times Large White]), with an average body weight of 20.14 \pm 1.71 kg, were housed in individual metabolism crates and allotted into 2 treatments with 6 pigs per treatment according to weight in a randomized complete block design. The base diet was based on corn-SBM (Table 1), and was formulated to meet or exceed the nutrient requirements as recommended by the NRC (1998). The experimental diet was the basal diet supplemented with CC protease (0.2 g/kg). The analyzed composition of the experimental diets is showed in Table 1.

Pigs were housed in an environmentally controlled room (24°C \pm 2°C) in metabolism cages equipped with a feeder and a nipple drinker, fully slatted floors, a screen floor, and urine trays that allowed for the total, but separate, collection of urine and fecal materials from each pig. Pigs were allowed a 10 d period to adapt to the metabolism crates and the environmental conditions of the room. During this period, the pigs were fed a commercial diet and feed intake was gradually increased until the daily feed allowance reached 40 g/kg of the pig's body weight. During the experimental period, feed allowance was equivalent to 40 g/kg of body weight and divided into 2 equal meals (Adeola, 2001).

Exp. 2 was conducted to evaluate the effects of CC protease on the AID of AA and nutrients in corn-SBM diet. Twelve crossbred barrows (Duroc \times [Landrace \times Large White]), with an average body weight of 20.79 \pm 1.94 kg, were surgically fitted with T-cannulas at the distal ileum using procedures adapted from Stein et al. (1998). After 14 days of recovery, pigs were blocked by body weight into 2 groups with 6 pigs each. The diets were the same as those in Exp. 1.

Pigs were housed in individual pens in an environmentally controlled room (26°C, 70% humidity). A feeder and a nipple drinker were installed in each pen. Body weight was recorded at the beginning of the experiment. Feed allowance was equivalent to 40 g/kg of body weight and divided into 2 equal meals fed at 0800 and 1700 hours each day. Water was available at all time throughout the experiment.

Sample collection and analysis

In Exp. 1, individual pig body weight was obtained at the beginning of the experiment. The pigs were adapted to experiment diets for 7 d followed by a 5 d total collection of feces and urine. Feces was placed in plastic bags (one bag per pig) as soon as they appeared in the metabolism crates and were immediately stored at -20°C. A bucket containing

Table 1. Ingredient and chemical composition of the experimental diets (%; as-fed basis)

Item	Control	Coated compound protease ¹
Ingredient composition		
Corn, yellow	67.78	67.76
Soybean meal	21.87	21.87
Wheat bran	5.00	5.00
Soybean oil	2.00	2.00
Limestone	1.10	1.10
Dicalcium phosphate	1.20	1.20
Salt	0.30	0.30
Chromic oxide	0.25	0.25
Vitamin mineral premix ²	0.50	0.50
Chemical composition		
Dry matter	89.7	88.8
Gross energy (MJ/kg)	16.6	16.5
Crude protein	17.4	17.5
Organic matter	84.6	83.8
Essential amino acid		
Arginine	0.90	0.85
Histidine	0.42	0.38
Isoleucine	0.62	0.62
Leucine	1.34	1.39
Lysine	1.02	0.94
Methionine	0.29	0.29
Phenylalanine	0.72	0.71
Threonine	0.65	0.64
Tryptophan	0.18	0.17
Valine	0.78	0.74
Non-essential amino acid		
Alanine	0.87	0.86
Aspartic acid	1.57	1.53
Cystine	0.28	0.27
Glutamic	2.90	2.85
Glycine	0.69	0.64
Proline	1.34	1.35
Serine	0.81	0.78
Tyrosine	0.41	0.40
Total amino acid	15.79	15.41

¹ The diet was supplemented with 0.2 g/kg of coated compound proteases (Kemin Industries Co. Ltd., Zhuhai, China; CC protease). The CC protease, with protease activity of 8,000 units/g measured under the condition of 37°C and pH = 6, were in granular form, and contained acidic, neutral and alkaline proteases produced by *Aspergillus niger*, *Bacillus subtilis*, and *Bacillus Licheniformis*, respectively.

² 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 D₃; vitamin E, 30 IU as dl- α -tocopheryl acetate; 12 μ g of vitamin B₁₂; vitamin K, 3 mg as menadione sodium bisulfate; d-pantothenic acid, 15 mg as calcium pantothenate; 40 mg of nicotinic acid; choline, 400 mg as choline chloride; Mn, 30 mg as manganese oxide; Zn, 80 mg as zinc oxide; Fe, 90 mg as iron sulfate; Cu, 10 mg as copper sulfate; I, 0.35 mg as ethylenediamine dihydroiodide; and Se, 0.3 mg as sodium selenite.

50 mL of 6 N HCl was used to collect urine. Each day, the volume of collected urine was measured and 100 mL/L of

the daily urinary collection was filtered and transferred into a screw-capped bottle and then stored at -20°C until needed for analysis. At the end of the experiment, feces and urine were thawed, pooled by pig, homogenized and sub-sampled. Before analysis, fecal subsamples were dried for 72 h in a 65°C drying oven and ground through a 1-mm screen.

In Exp. 2, the test period lasted 12 d, with a 7 d adaption to the diets followed by a 3 d collection of feces and then a 2 d collection of ileal digesta from 0800 and 1700 hours using the procedures described by Stein et al. (1998). A 200 mL plastic bag was attached to the open cannula using a cable tie. Bags were removed whenever they were filled with digesta, or at least every 30 min and stored at -20°C to prevent bacterial degradation of AA in the digesta. At the end, feces and digesta samples were thawed, homogenized within animal, lyophilized in a vacuum-freeze dryer (Tofflon Freezing Drying Systems, Shanghai, China), and ground through a 1-mm screen for further chemical analysis.

Fecal, urine, digesta and diet samples were analyzed in duplicate for GE using an Automatic Isoperibol Oxygen Bomb Calorimeter (Parr 6300 Calorimeter, Moline, IL, USA). Fecal, digesta and diet samples were also analyzed for dry matter (DM; procedure 4.1.06; AOAC, 2000), ash (procedure 4.1.10; AOAC, 2000) and Kjeldahl N (Thiex et al., 2002). And crude protein (CP) was calculated as N \times 6.25 (Thiex et al., 2002). The chromium content in the diets, digesta and feces was measured using an atomic absorption spectrophotometer (Z-5000; Hitachi, Tokyo, Japan) according to the procedure of Williams et al. (1962). AA, excluding methionine, tryptophan and cystine, were assayed using ion-exchange chromatography with an automatic AA analyzer (L-8900, Automatic Amino Acid Analyzer; Hitachi, Tokyo, Japan) after hydrolyzing with 6 N HCl at 110°C for 24 h. Cystine was determined as cysteic acid and methionine as methionine sulphone after peroxidation with performic acid and pre-column derivation using phenylisothiocyanate (L-8900, Automatic Amino Acid Analyzer; Hitachi, Japan). Tryptophan was determined after hydrolyzing with 4 N LiOH at 110°C for 22 h using high performance liquid chromatography (Agilent 1200 Series; Agilent, Santa Clara, CA, USA).

Calculations and statistical analysis

In Exp. 1, the digestible and metabolizable N and energy, N digestibility and retention rate and the ATTD of GE and nutrients in each diet were calculated (NRC, 1998). In Exp. 2, the AID of AA and CP in digesta was calculated according to Stein et al. (2007). The ATTD and AID of GE and nutrients in each diet were calculated (NRC, 1998), and the hindgut digestibility (HGD) was then calculated as the difference between the concentration of nutrients in the ileal digesta and in the feces.

All data were analyzed with a student's t-test for

Table 2. Daily balance of gross energy (GE) and concentration of digestible and metabolizable energy (DE and ME) in basal diet or diet with 0.2 g/kg proteases for pigs (Exp. 1)

Item	Control	CC protease	SEM	p-value
Daily balance of GE (MJ/d)				
GE intake	14.1	14.0	0.43	0.87
GE in feces	2.1	1.7	0.09	0.02
GE in urine	0.45	0.40	0.06	0.58
DE in diet (MJ/kg)				
As-fed basis	14.2	14.5	0.06	0.01
Dry matter basis	15.8	16.3	0.07	<0.01
ME in diet (MJ/kg)				
As-fed basis	13.7	14.0	0.10	0.05
Dry matter basis	15.3	15.8	0.11	0.01
ME/DE	0.96	0.97	0.01	0.63

CC protease, coated compound proteases; SEM, standard error of the mean.

ME was calculated as GE intake - fecal energy - urinary energy without considering methane loss.

unpaired data with each animal as an experimental unit. Significant differences were declared at $p < 0.05$, and differences at $0.05 \leq p < 0.10$ were considered as a trend toward significance.

RESULTS

Effects of CC protease on energy and N utilization and ATTD of nutrients in Exp. 1

Pigs fed the corn-SBM diet supplemented with or without CC protease had a similar GE intake (Table 2). The CC protease supplement decreased ($p < 0.05$) fecal GE loss, and increased ($p < 0.05$) the DE and ME compared with the basal diet.

Pigs offered CC protease supplementation had a similar

Table 3. Daily balance of nitrogen (N) and concentration of digestible and metabolizable nitrogen (DN and MN) in basal diet or diet with 0.2 g/kg proteases for pigs (Exp. 1)

Item	Control	CC protease	SEM	p-value
Daily balance of N (g/d)				
N intake	23.5	24.3	0.74	0.50
Fecal N excreted	4.7	3.7	0.18	<0.01
Urine N excreted	4.7	2.9	0.58	0.06
Retained N	14.2	17.7	1.00	0.04
N digestibility	0.80	0.85	0.01	<0.01
N retention rate	0.61	0.73	0.03	0.01
DN in diet (g/kg)				
As-fed basis	22.1	24.2	0.17	<0.01
Dry matter basis	24.8	27.2	0.19	<0.01
MN in diet (g/kg)				
As-fed basis	16.7	20.7	0.72	<0.01
Dry matter basis	18.7	23.3	0.89	<0.01

CC protease, coated compound proteases; SEM, standard error of the mean.

Table 4. Apparent total tract digestibility of nutrients in basal diet or diet supplemented with 0.2 g/kg proteases for pigs (Exp. 1)

Item	Control	CC protease	SEM	p-value
Dry matter	0.86	0.88	0.004	<0.01
Gross energy	0.85	0.88	0.004	<0.01
Crude protein	0.80	0.85	0.006	<0.01
Organic matter	0.87	0.89	0.003	<0.01

CC protease, coated compound proteases; SEM, standard error of the mean.

N intake, but a decreased ($p < 0.05$) fecal N loss by 20.6% and tended to a decreased ($p = 0.06$) urinary N loss (Table 3). The CC protease supplement improved ($p < 0.05$) the digestibility and retention rate of N by 5.6% and 19.9%, respectively. The digestible and metabolizable N values were increased ($p < 0.05$) by the CC protease supplementation. The ATTD of DM, GE, CP, and organic matter (OM) in diet supplemented with CC protease was greater ($p < 0.05$) than in the basal diet (Table 4).

Effects of CC protease on AID of AA and nutrients in Exp. 2

The AID of CP was increased ($p < 0.05$) by 8.8% in diet with CC protease compared with that in basal diet (Table 5). The CC protease supplementation improved the AID of arginine, histidine, isoleucine, and leucine by 4.9%, 14.5%, 6.8%, and 9.3%, respectively. The AID of some dispensable

Table 5. Apparent ileal digestibility of crude protein and amino acids (AA) in basal diet or diet supplemented with 0.2 g/kg proteases for pigs (Exp. 2)

Item	Control	CC protease	SEM	p-value
Crude protein	0.68	0.74	0.012	0.01
Indispensable AA				
Arginine	0.82	0.86	0.008	0.01
Histidine	0.69	0.79	0.012	<0.01
Isoleucine	0.74	0.79	0.012	0.01
Leucine	0.75	0.82	0.012	<0.01
Lysine	0.82	0.81	0.013	0.67
Methionine	0.82	0.85	0.013	0.08
Phenylalanine	0.79	0.82	0.014	0.14
Threonine	0.67	0.68	0.013	0.60
Tryptophan	0.72	0.72	0.013	0.96
Valine	0.71	0.72	0.013	0.62
Dispensable AA				
Alanine	0.67	0.74	0.017	0.02
Aspartic acid	0.75	0.76	0.011	0.38
Cystine	0.58	0.61	0.036	0.62
Glutamic	0.76	0.79	0.022	0.28
Glycine	0.52	0.52	0.027	0.82
Proline	0.71	0.68	0.050	0.63
Serine	0.73	0.75	0.012	0.25
Tyrosine	0.71	0.81	0.028	0.03

CC protease, coated compound proteases; SEM, standard error of the mean.

AA including alanine and tyrosine were also increased by the CC protease.

The AID and ATTD of DM, GE, and CP were greater ($p < 0.05$) in corn-SBM diet supplemented with CC protease than those in basal diet, but the HGD of these nutrients was not different between treatments (Table 6). There was no difference in AID and HGD of OM between the diets, but the ATTD of OM increased in diet supplemented with CC protease.

DISCUSSION

The need to reduce the N content in the livestock wastewater is more important than any time previously (Chen et al., 2014). The reductions in N excretion are mostly attributed to changes in both urinary and fecal N excretion in animal production (Dong et al., 2014). The provision of a low CP diet could result in a reduction in N excretion (O'Connell et al., 2006); however, it is indispensable to provide sufficient N sources to meet the AA requirements for pigs. Therefore, the method of maximizing the potential available N in the existing diet is very significant. The use of exogenous protein enzymes in swine diets is a common strategy to ameliorate anti-nutritive effects of SBM and to improve the efficiency of N utilization (Kong et al., 2015).

Supplementation of diets with exogenous enzymes to stimulate nutrient digestion and minimize or eliminate the negative effect of anti-nutritional factors has attracted considerable interest within the modern pig industry (Jo et al., 2012). It has been proved that treatment of SBM with

appropriate proteases may improve piglet performance at weaning, which would be a potential method for increasing the amounts of SBM in starter diets for weaned piglets (Rooke et al., 1998). Protease had a positive effect on the ATTD of DM, GE, and CP in weaned piglets (Wang et al., 2011a). However, there are still some insignificant proteases in diets (Ji et al., 2008; Adeola and Cowieson, 2011; O'Shea et al., 2014). The effectiveness of supplying enzyme in pig diets may vary because of the difference of ingredients, age of pigs or enzyme products (Ji et al., 2008). In our study, the compound enzymes containing acidic, neutral and alkaline proteases could successively dissolve and work in acidic, neutral or alkaline microenvironment in the digestive tract, which is a novel and attractive method that proved effective in the corn-SBM diet for pigs.

There was no difference in the DM and GE intake in our study, which meant the pigs in the two treatments were under almost the same physical condition. The fecal GE loss for pigs fed with CC protease supplementation was lower than those fed with the basal diet. This indicated that there was greater DE and ATTD of GE and nutrients in diet supplemented with CC protease, which were successively confirmed by our results. More importantly, the exogenous enzyme supplementation reduced the fecal N excretion and increased N digestibility and retention rate. This may indicated an improvement of the ATTD of CP. Actually, the increased AID of CP and some AA in Exp. 2 had also supported the results. The ATTD of DM, GE, CP, and OM in Exp. 1 was improved by the protease supplementation, which was the same pattern followed by the results measured using the exogenous indicator method in Exp. 2. The numeric differences of the ATTD of nutrients between the two experiments may be attributed to the different methods for collecting and analyzing fecal samples. Certainly, the different physiological conditions of pigs with or without fistula surgery may also result in differences. Actually, the effects of the CC protease on improving the nutrient digestibility were consistent with each other.

Interestingly, it was not our expectation that the proteases, without any amylase or other side-activities capable of degrading starch, would be capable of improving energy digestibility. A similar unexpected but exciting effects were also detected in broilers fed with sorghum with exogenous protease (Selle et al., 2013). Perhaps the coating proteases degraded a significant portion of the indigestible protein in the soybean-meal and thus resulted into greater digestibility of energy and nutrients.

In the current study, the CC protease supplementation improved the AID of some essential AA including arginine, histidine, isoleucine and leucine, and some non-essential AA including alanine and tyrosine. The other AA, especially lysine, tryptophan and threonine, were least responsive to CC protease supplementation, which means

Table 6. Apparent ileal (AID), total tract digestibility (ATTD) and hindgut digestibility (HGD) of nutrients in basal diet or diet supplemented with 0.2 g/kg proteases (Exp. 2)

Item	Control	CC protease	SEM	p-value
Dry matter				
AID	0.64	0.69	0.011	<0.01
ATTD	0.81	0.85	0.007	<0.01
HGD	0.16	0.15	0.009	0.40
Gross energy				
AID	0.66	0.71	0.011	0.01
ATTD	0.79	0.84	0.007	<0.01
HGD	0.13	0.13	0.008	0.93
Crude protein				
AID	0.68	0.74	0.012	0.01
ATTD	0.76	0.83	0.014	0.01
HGD	0.08	0.09	0.017	0.79
Organic matter				
AID	0.68	0.72	0.014	0.14
ATTD	0.83	0.86	0.008	0.04
HGD	0.15	0.14	0.015	0.84

CC protease, coated compound proteases; SEM, standard error of the mean.

that improvements in digestibility of these AA in practical diets are limited. Therefore, the most limiting essential AA supplementation should be considered independently to avoid overestimating the contribution of the enzymes on the limiting AA when diets supplemented with exogenous enzymes are formulated.

The economic benefit of the use of exogenous proteases is through improvement in the digestibility of dietary AA (Romero et al., 2013). The primary mechanism for this increment appears to be the augmentation of dietary protein hydrolysis and increased protein solubility (Caine et al., 1998). Conclusive evidence is not available supporting a positive or negative change in endogenous secretions caused by exogenous proteases (Romero et al., 2013). We assume that the coating proteases can work in different intestine segments to cover the deficit of the endogenous enzyme and thus improve the nutrients digestibility.

There was greater AID and ATTD of DM, GE and CP in corn-SBM diet supplemented with CC protease, but the HGD of these nutrients was not different between treatments. These findings suggested that the effects of the proteases on hydrolysis of protein were mostly taking place in the small intestine rather than in the hindgut. Maybe the proteases have been inactivated or completely degraded, or disturbed by the microorganisms in the large intestine. As the availability of protein post-caecum is considered to be negligible, an improvement in AID of nutrients, especially CP, can be interpreted as beneficial, as it suggests more energy and protein is available to meet the growth demands of the pig (Romero et al., 2013). Therefore, the greater proportion of nutrients, especially energy or proteins, that are available in the small intestine from diet inclusion of CC protease the more beneficial and significant they will be.

CONCLUSION

The CC protease supplementation improved the ATTD of N and energy and AID of some indispensable AA and nutrients in the corn-SBM diet for pigs. Therefore, the CC protease supplement could improve the utilization of protein in the corn-SBM diet and thus contribute to lower N excretion to the environment.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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