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Supplemental protease improves *in vitro* disappearance of dry matter and crude protein in feather meal and copra meal for pigs

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ABSTRACT - The aim of the present study was to determine the effects of supplemental protease on *in vitro* disappearance of dry matter (DM) and crude protein (CP) in feed ingredients for pigs. The test ingredients were three sources of feather meal (FM 1, FM 2, and FM 3), meat and bone meal, soybean meal, corn dried distillers grains with solubles, copra meal (CM), palm kernel meal, wheat, and corn. In experiment 1, *in vitro* ileal disappearance (IVID) of DM and CP were determined to represent the digestion and absorption of the stomach and small intestine of pigs. In experiment 2, *in vitro* total tract disappearance (IVTTD) of DM was determined to represent the digestion and absorption of the stomach, small intestine, and large intestine of pigs. The control group had 99% of a test ingredient and 1% of wheat bran. Protease originating from *Bacillus* spp. was supplemented to the control diet at the expense of wheat bran to make a treatment group (100,000 U/kg of sample). Protease supplementation increased the IVID of DM in FM 1 (39.8 vs. 37.5%) and CM (45.0 vs. 42.2%). In the protease supplementation group, the IVID of CP also increased in FM 1 (48.0 vs. 43.8%) and CM (85.3 vs. 77.5%). The supplemental protease increased the IVTTD of DM in FM 1 (43.9 vs. 42.1%), FM 3 (55.6 vs. 52.7%), and meat and bone meal (85.9 vs. 84.3%). Therefore, nutrient utilization of FM and CM can be improved by supplemental protease originating from *Bacillus* spp. based on *in vitro* assays.

Keywords: digestibility, enzyme, feed additive, pig

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1. Introduction

Proteins are expensive nutritional elements that are essential for maintenance and productive functions of pigs (NRC, 2012). The use of alternative protein sources such as byproducts has increased due to the price fluctuations of conventional protein sources. However, the digestibility of alternative protein sources is generally lower than the conventional protein sources (Son et al., 2019).

In the gastrointestinal tract of pigs, proteins need to be hydrolyzed by endogenous protease into small peptides and amino acids to be utilized by the host. The digestibility of crude protein (CP) varies depending on ingredients for pigs (NRC, 2012; Stein et al., 2016; Son et al., 2019). The capacity of digestive protease may be insufficient when a large quantity of protein exists in the digesta due to a high-protein ingredient used, such as feather meal (FM; approximately 80% CP), a poultry byproduct (Grazziotin et al., 2006; NRC, 2012).

The use of exogenous protease potentially improves the utilization of protein and lowers the fecal excretion of nitrogen (Tactacan et al., 2016; Pan et al., 2017). Pearlzyme® is a serine protease originating from *Bacillus* spp. The supplementation of this protease into poultry diets improves the growth performance (Kim et al., 2015; Ndazigaruye et al., 2019). To our knowledge, the effects of this protease on the nutrient digestibility of swine feed ingredients have not been documented.

In vitro experiments are a time-saving and inexpensive method compared with *in vivo* assays. The *in vitro* procedures have been extensively used to determine the effects of supplementation of enzymes on nutrient digestibility of feed ingredients and diets (Kong et al., 2015; Park et al., 2016a) based on the high correlation between *in vitro* and *in vivo* data (Noblet and Jaguelin-Peyraud, 2007; Park et al., 2012; Son et al., 2017). Therefore, in the present study, we determined the effects of a novel source of protease supplementation on *in vitro* disappearance of dry matter (DM) and CP for swine feed ingredients.

2. Material and Methods

Pearlzyme® (EC 3.4.21.14), a commercial protease product, was provided by Solton Biochem Inc. (Seoul, Republic of Korea). Pearlzyme® with a molecular weight of 28 kDa was purified and characterized from *Periserrula leucophryna*. This protease maintains activity at a high temperature of 50 to 60 ℃ and is stable at pH 4-12 (Joo et al., 2001).

Ten test ingredients were three sources of FM (FM 1, FM 2, and FM 3), meat and bone meal, soybean meal (SBM), corn dried distillers grains with solubles (DDGS), copra meal (CM), palm kernel meal (PKM), wheat, and corn. Before the *in vitro* analysis, the test ingredients were finely ground (<1 mm), and each one was supplemented with either protease mixed evenly with wheat bran or only wheat bran at 1.0% to make up the enzyme group or the control group, respectively. The concentration of supplemental Pearlzyme® in the mixed feed sample was 100,000 unit/kg. All experiments and analyses were conducted in a biosafety level 1 containment facility in Seoul, Republic of Korea (37°32'24.8" N 127°04'27.5" E). The test ingredients were analyzed for DM (method 930.15), ash (method 942.05), CP (method 990.03), neutral detergent fiber (NDF, method 2002.04), and acid detergent fiber (ADF, method 973.18) as described in AOAC International (2016).

In experiment 1, the two-step *in vitro* assay was conducted to determine *in vitro* ileal disappearance (IVID) of DM as described in Boisen and Fernández (1995; Figure 1). Briefly, 1 g of a ground ingredient

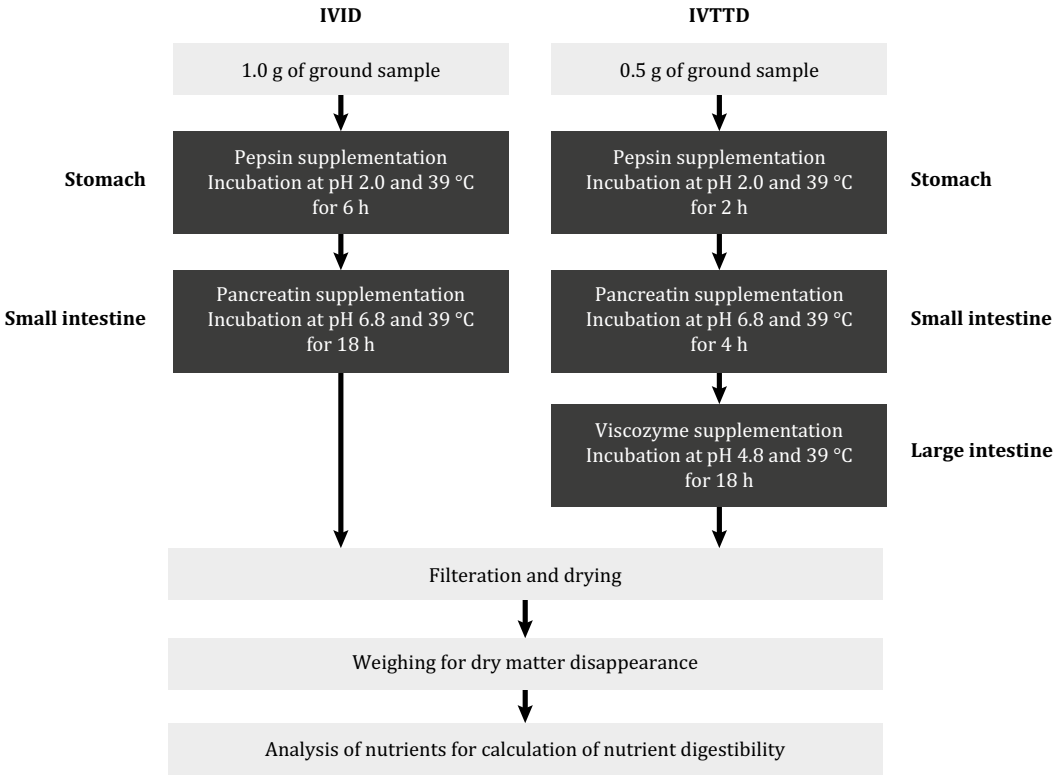

Figure 1 - *In vitro* ileal disappearance (IVID) procedure and *in vitro* total tract disappearance (IVTTD) procedure (Boisen and Fernández, 1995; Boisen and Fernández, 1997; Recharla et al., 2019).

sample was transferred into 100-mL conical flasks. Twenty-five milliliters of sodium phosphate buffer solution (0.1 M, pH 6.0) and 10 mL of HCl (0.2 M, pH 0.7) were added. To simulate digestion conditions in the stomach, the pH was adjusted to 2.0, and 1 mL of freshly prepared pepsin solution (10 mg/mL; ≥250 units/mg solid, P7000, pepsin from porcine gastric mucosa, Sigma-Aldrich, St. Louis, MO, USA) was added to the samples. Test flasks were incubated in a shaking incubator at 39 °C for 6 h as the final procedure of the first step. After the incubation, 10 mL of sodium phosphate buffer solution (0.2 M, pH 6.8) and 5 mL of NaOH (0.6 M, pH 13.8) were added. Then, the pH was adjusted to 6.8. Thereafter, 1 mL of freshly prepared pancreatin solution (50 mg/mL; 4 × USP, P1750, pancreatin from porcine pancreas, Sigma-Aldrich, St. Louis, MO, USA) was added. After incubation in a shaking incubator at 39 °C for 18 h, 5 mL of 20% sulfosalicylic acid solution were added, and samples were left for 30 min at room temperature to precipitate the indigestible protein. The samples were then filtered through pre-dried and pre-weighed glass filter crucibles (Filter Crucibles CFE Por. 2, Robu, Hattert, Germany) containing 400 mg of Celite. Test flasks were rinsed twice with 1% sulfosalicylic acid solution, and 20 mL of 95% ethanol and 99.5% acetone were added twice to the glass filter crucibles. Glass filter crucibles with undigested residues were dried at 80 °C for 24 h. After conducting the two-step procedure, undigested residues on filter crucibles were collected for analysis of CP concentration to determine the IVID of CP.

In experiment 2, the *in vitro* total tract disappearance (IVTTD) of DM was determined using the three-step procedure (Boisen and Fernández, 1997; Figure 1). The first and second steps were similar to the IVID procedure. In the third step of the IVTTD procedure, 10 mL of 0.2 M EDTA solution were added to the samples. The pH was then adjusted to 4.8. Samples were supplemented with 0.5 mL of multi-enzyme (V2010, Viscozyme®, Sigma-Aldrich, St. Louis, MO, USA) as a substitute for microbial enzymes and incubated in a shaking incubator for 18 h at 39 °C. After incubation, the samples were then filtered, and the undigested residues were collected and dried as previously described in the IVID procedure except for the drying condition (at 130 °C for 6 h).

The IVID or IVTTD of DM was calculated using the following equation (Park et al., 2016b):

$$\text{IVID or IVTTD of DM (\%)} = (DM_{TI} - DM_{RS}) \div DM_{TI} \times 100$$

in which DM_{TI} (g) is the amount of test ingredient as DM basis and DM_{RS} (g) is the amount of residue after *in vitro* digestion procedures.

After the two-step *in vitro* assay, the residues of test ingredient and Celite were collected and analyzed for CP. Then, IVID (%) of CP was calculated using the following equation (Akonjuen et al., 2019):

$$\text{IVID of CP (\%)} = [(DM_{TI} \times CP_{TI}) - (DM_{RS} \times CP_{RS})] \div (DM_{TI} \times CP_{TI}) \times 100$$

in which CP_{TI} and CP_{RS} (% DM) are the CP concentrations (%) expressed as DM basis in the test ingredient and the residue, respectively.

Data were analyzed using the GLM procedures of SAS (Statistical Analysis System, version 9.4). The model included the protease supplementation as a fixed variable. Least squares means for IVID and IVTTD of DM and IVID of CP for each ingredient were calculated. Each flask was considered as the experimental unit. The statistical significance of each treatment effect was declared at $P<0.05$ and tendency at $P<0.10$. Variables were analyzed according to the following mathematical model:

$$Y_{ij} = \mu + T_i + \varepsilon_{ij},$$

in which Y_{ij} = *in vitro* disappearance of nutrients from *j*-th experimental unit of the *i*-th treatment, μ = general constant, T_i = fixed effects of protease supplementation, and ε_{ij} = random error associated with the *j*-th experimental unit of the *i*-th treatment.

3. Results

Dry matter, ash, CP, NDF, and ADF concentrations in the test ingredients ranged from 88.1 to 97.0%, 1.5 to 36.9%, 9.1 to 88.0%, 9.8 to 65.5%, and 6.0 to 47.1%, respectively (Table 1). In experiment 1,

supplemental protease increased (P<0.05) the IVID of DM in FM 1 and CM (Table 2). The protease supplementation also increased (P<0.05) the IVID of CP in FM 1 and CM (Table 3). In addition, supplementation of protease tended to increase (P = 0.081) the IVID of CP in FM 2.

In experiment 2, the supplemental protease increased (P<0.05) the IVTTD of DM in FM 1, FM 3, and meat and bone meal (Table 4). Supplemental protease tended to increase the IVTTD of DM in corn DDGS (P = 0.077), PKM (P = 0.058), and corn (P = 0.083).

4. Discussion

The analyzed concentrations of DM, ash, CP, NDF, and ADF in the test ingredients were mostly within the values in the literature (NRC, 2012). The NDF and ADF concentrations in coconut byproducts, palm kernel byproducts, and corn DDGS vary among the studies (Stein et al., 2009; Park et al., 2012; Stein et al., 2016). The deviations of fiber concentrations among the previous studies were likely due to the processing methods for oil extraction in coconut byproducts and palm kernel byproducts (Lee and Kim, 2017) and bioethanol production in corn DDGS (Singh and Cheryan, 1998).

Table 1 - Analyzed chemical composition of feed ingredients (% as-is basis)

Item	Dry matter	Ash	Crude protein	NDF	ADF
Feather meal 1	96.5	2.5	88.0	-	-
Feather meal 2	97.0	1.8	77.3	-	-
Feather meal 3	94.8	2.2	72.1	-	-
Meat and bone meal	95.4	36.9	46.8	-	-
Soybean meal	90.1	6.4	48.5	9.8	6.7
Corn dried distillers grains with solubles	88.8	6.0	27.3	37.7	13.3
Copra meal	89.6	6.9	21.8	54.1	32.1
Palm kernel meal	91.0	4.4	14.9	65.5	47.1
Wheat, hard red	91.9	1.5	11.8	13.6	6.9
Corn, yellow dent	88.1	1.5	9.1	9.8	6.0

NDF - neutral detergent fiber; ADF - acid detergent fiber.

Table 2 - *In vitro* ileal disappearance of dry matter (%) in feed ingredients with or without protease supplementation in experiment 1[1]

Item	Supplemental protease[2]		SEM	P-value
	0%	1%		
Feather meal 1	37.5	39.8	0.30	0.006
Feather meal 2	43.9	45.0	0.67	0.320
Feather meal 3	55.1	55.0	0.58	0.912
Meat and bone meal	69.0	69.7	1.96	0.810
Soybean meal	74.8	75.1	0.33	0.558
Corn dried distillers grains with solubles	50.6	51.7	0.86	0.394
Copra meal	42.2	45.0	0.43	0.020
Palm kernel meal	26.5	26.1	0.13	0.111
Wheat, hard red	82.6	82.6	0.33	0.934
Corn, yellow dent	83.3	83.7	0.37	0.511

SEM - standard error of the means.
[1] Each least squares mean represents three observations except for the control group in copra meal (two observations).
[2] Supplemental protease was supplemented at 1.0% (100,000 U/kg of mixed sample).

The lower IVID of DM in the plant-derived feed ingredients compared with the IVTTD of DM observed in the present work coincides with the previous experiments (Kong et al., 2015; Park et al., 2016b). The greater values in the IVTTD of DM are attributed partially to the presence of fiber-degrading enzymes in the IVTTD procedure and the substrates in the ingredients.

The IVID of CP in feed ingredients more accurately represents protein and amino acid availability of pigs compared with the IVTTD of CP, because the small intestine of pigs is probably the only section where amino acids are absorbed (Holmes et al., 1974; Sauer et al., 1980; Stein et al., 2007). Furthermore, in the total tract digestibility assays of protein and amino acids, microorganisms may influence the metabolism of amino acids such as deamination, bacterial synthesis, or endogenous secretion that can reduce the accuracy of availability in protein and amino acids. Therefore, the IVID of CP was determined in the present work.

The IVID and IVTTD of DM in plant feed ingredients used in the current study were similar to the values in the literature (Kong et al., 2015; Park et al., 2016b) except for corn DDGS. The low DM digestibility of

Table 3 - *In vitro* ileal disappearance of crude protein (%) in feed ingredients with or without protease supplementation in experiment 1[1]

Item	Supplemental protease[2]		SEM	P-value
	0%	1%		
Feather meal 1	43.8	48.0	0.33	<0.001
Feather meal 2	39.9	46.1	1.88	0.081
Feather meal 3	56.7	58.7	1.32	0.330
Meat and bone meal	73.8	75.6	1.15	0.322
Soybean meal	89.4	90.2	0.66	0.446
Corn dried distillers grains with solubles	70.8	70.8	0.90	0.998
Copra meal	77.5	85.3	0.78	0.006
Palm kernel meal	65.3	65.7	0.24	0.370
Wheat, hard red	89.4	88.7	0.25	0.119
Corn, yellow dent	84.0	83.6	1.29	0.824

SEM - standard error of the means.
[1] Each least squares mean represents three observations except for the control group in copra meal (two observations).
[2] Supplemental protease was supplemented at 1.0% (100,000 U/kg of mixed sample).

Table 4 - *In vitro* total tract disappearance of dry matter (%) of feed ingredients with or without protease supplementation in experiment 2[1]

Item	Supplemental protease[2]		SEM	P-value
	0%	1%		
Feather meal 1	42.1	43.9	0.38	0.026
Feather meal 2	45.8	47.8	0.69	0.102
Feather meal 3	52.7	55.6	0.32	0.003
Meat and bone meal	84.3	85.9	0.41	0.045
Soybean meal	93.9	94.2	0.35	0.591
Corn dried distillers grains with solubles	54.0	55.9	0.56	0.077
Copra meal	69.6	70.1	0.24	0.193
Palm kernel meal	36.1	37.6	0.42	0.058
Wheat, hard red	87.9	88.4	0.18	0.119
Corn, yellow dent	87.1	88.2	0.33	0.083

SEM - standard error of the means.
[1] Each least squares mean represents three observations.
[2] Supplemental protease was supplemented at 1.0% (100,000 U/kg of mixed sample).

corn DDGS in the present work appears to be mainly due to the relatively high fiber contents compared with values in the literature (Jones et al., 2010; NRC, 2012; Rojas et al., 2016). The fiber content variability in corn DDGS is largely due to different corn ethanol production processes including corn processing, fermentation method, and heat treatment (Stein and Shurson, 2009; Kim et al., 2012; Stein et al., 2016). High dietary fiber concentrations in swine diets are known to negatively affect nutrient digestibility (Noblet and Jaguelin-Peyraud, 2007; Choi and Kim, 2019; Choi et al., 2020).

In the present study, the IVID and IVTTD of DM and IVID of CP in some FM sources were less than those in other feed ingredients and were increased by the supplemental protease. Although FM contains high CP contents, a large proportion of CP in FM is keratin, which has a great degree of cross-linking by disulfide bonds, resulting in low CP utilization of FM (Grazziotin et al., 2006; Brandelli et al., 2010).

Pearlzyme®, a chymotrypsin-like serine protease originating from *Bacillus* spp., is activated by trypsin in the gastrointestinal tract and enhances protein and amino acid digestibility of non-ruminants mainly by acting on binding complexes with arginine or lysine at the outside of the helical structure in feed ingredients (Rothberg and Axilrod, 1968; Joo et al., 2001; Kim et al., 2015). The amounts of arginine and lysine concentrations in FM are relatively greater than those in other feed ingredients, and additionally, the ileal lysine digestibility in FM is less than those in other feed ingredients (NRC, 2012). Taken together, the large magnitude of protease effects on CP digestibility in FM and CM is likely due to the large quantity of indigestible arginine and lysine in this ingredient (Sundu et al., 2006; Sundu et al., 2009; NRC, 2012). However, in the current study, the responses of supplemental protease were different among the FM sources. A possible explanation is that time and temperature conditions varied by manufacturers can reflect the digestibility and protein composition of FM (Papadopoulos et al., 1986; Zhang et al., 2014). Additionally, protease supplementation increased the IVID of DM and CP in CM, which is also likely due to the large quantity of substrates for protease in CM.

Although SBM contains 49% CP, the digestibility of DM and CP in SBM was not affected by supplemental protease in the present work. This is likely due to the fact that most proteins in SBM are easily digested and absorbed (ileal CP digestibility ranges from 88 to 90%) without additional protease (NRC, 2012; Son et al., 2019). The lack of effects of supplemental protease on CP digestibility in corn DDGS may be partially explained by the fermentation process during the bio-ethanol production procedure that involves diverse microbial enzymes including proteases (Stein and Shurson, 2009; Johnston and McAloon, 2014). The reason for the lack of responses by exogenous protease in PKM is unclear as the indigestible CP content in PKM is similar to CM (Son et al., 2014). In addition, the indigestible arginine and lysine concentrations in these ingredients are also similar (NRC, 2012). Palm kernel meal may have some amino acid linkages or factors that potentially inhibit the action of protease. A possible explanation is that the protein in PKM is bound to the fiber matrix, resulting in poor accessibility to protease. Further research is warranted to identify the reason for the lack of protease effects on PKM. The lack of responses of supplemental protease in wheat and corn was expected as there is very limited amount of substrates (12 and 9% CP, respectively; Table 1) for exogenous protease, and CP digestibility in these ingredients is high (89 and 84%, respectively; Table 3) even without exogenous protease.

In the current study, the effect of supplemental protease alone was tested. Further research on the interaction between supplemental protease and other supplemental enzymes on nutrient digestibility for pigs would also be an interesting topic.

5. Conclusions

Nutrient utilization of feather meal and copra meal can be improved by supplemental protease originating from *Bacillus* spp. based on the present *in vitro* assays.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

Conceptualization: B.G. Kim. Data curation: D.U. Ha. Formal analysis: D.U. Ha. Investigation: D.U. Ha and H. Choi. Methodology: D.U. Ha and H. Choi. Supervision: H. Choi and B.G. Kim. Validation: H. Choi. Writing-original draft: D.U. Ha and H. Choi. Writing-review & editing: B.G. Kim.

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