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Article Title	**Hydrolysis Conditions of Porcine Blood Proteins and Antimicrobial Effects of Their Hydrolysates**
Running Title (within 10 words)	Antimicrobial effects of porcine blood protein Hydrolysates
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Special remarks – if authors have additional information to inform the editorial office	
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Conflicts of interest List any present or potential conflict s of interest for all authors. (This field may be published.)	The authors declare no potential conflict of interest.
Acknowledgements State funding sources (grants, funding sources, equipment, and supplies). Include name and number of grant if available. (This field may be published.)	This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA, 116034-03-1-HD030) and by the Regional Animal Industry Center at Gyeongnam National University of Science and Technology (GnTech).
Author contributions (This field may be published.)	Conceptualization: Jin, SG Data curation: Jin, SG Formal analysis: Jin, SG Methodology: Choi JS Software: Jin, SG Validation: Jin, SG. Investigation: Choi JS Writing - original draft: Choi JS, Yim DG Writing - review & editing: Jin, SG Choi JS, Yim DGChoi Y, Kim Y, Kim CS. (This field must list all authors)
Ethics approval (IRB/IACUC) (This field may be published.)	This manuscript does not require IRB/IACUC approval because there are no human and animal participants.

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Hydrolysis Conditions of Porcine Blood Proteins and Antimicrobial Effects of Its Hydrolysates

running title: Antimicrobial effects of porcine blood protein Hydrolysates

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Abstract

In the present study, we determined the degree of hydrolysis of porcine blood plasma proteins, albumin, and globulin hydrolyzed by six proteases (alcalase, neutrase, flavourzyme, protamex, trypsin, and papain) for various reaction times. Moreover, antimicrobial activities of hydrolysates against five pathogenic microorganisms (*Bacillus cereus, Staphylococcus aureus, Salmonella* Typhimurium, *Escherichia coli*, and *Shigella flexneri*) were investigated. Alcalase, trypsin, and papain hydrolysates of the three porcine blood proteins showed higher degree of hydrolysis values than hydrolysates produced by the other three proteases. Degree of hydrolysis of the three porcine blood proteins hydrolyzed by the six proteases failed to increase after 2 h of hydrolysis. In antimicrobial tests, hydrolysates (hydrolysis time of 2 h) showed antibacterial activity only against *B. cereus.* Albumin hydrolysates showed higher antimicrobial activity than globulin and plasma hydrolysates. Albumin hydrolysates obtained with flavourzyme, protamex, and trypsin showed higher antimicrobial activity than those obtained with the other three proteases.

Key words: Porcine blood, hydrolysate, protease, antimicrobial activity, *Bacillus cereus*

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Introduction

Meat consumption is increasing every year worldwide. Livestock husbandry and slaughter are also increasing steadily (Sans and Combris, 2015). In Korea, of all meat consumed over the past decades, pork has been consumed in considerably larger quantities than chicken or beef. In 2014, pork consumption was 20.9 kg per capita; the number of pig heads was 10,090,000; and the number of pigs slaughtered was 15,661,000 (Korea institute for animal product quality evaluation, 2015; Ministry of Agriculture Food and Rural Affairs, 2015). On average, approximately 3.35 L of blood can be obtained during slaughter of a standard pig (Jang et al., 2011; Korea Testing Laboratory, 2015). Based on the number of pigs slaughtered in Korea in 2014, the total volume of blood would be approximately 52 million liters. However, most of the blood from slaughterhouses is discarded, with high disposal costs. Only a small quantity of blood is used as raw material for traditional food such as *Sundae*, soup, and blood sausages in Korea (Hurtado et al., 2012; Korea Testing Laboratory, 2015; Yu et al., 2006). All over the world, only a few countries in Europe use blood as animal feed resource (Han and Park, 2011; Torrallardona, 2010). Porcine blood is composed of 79.14% water, 19.40% organic matter, and 1.46% inorganic matter. It contains 18.22% of protein content, including albumin (2.08%), globulin (1.99%), fibrinogen (0.12%), and hemoglobin (14.02%) (Korea Testing Laboratory, 2015). Until now, porcine blood is known to consumers only as raw material for food.

Protein hydrolysate, consisting of a mixture of free amino acids and peptides, is obtained through protein hydrolysis, and certain protein hydrolysates are known to have specific functional properties (Guo et al., 2009; Lafarga and Hayes, 2014). In enzymatic hydrolysis, these bioactive substances are being developed in various forms depending on the kinds of protein and enzyme used, reaction conditions, and purification method (Jain and Anal, 2016; Nilsang et al., 2005). When proteins are hydrolyzed by enzymatic hydrolysis, their molecular weights decrease, and their secondary and tertiary structures change. Such hydrolysis can also increase the exposure of hydrophobic groups and ionizable groups (Davis et al., 2005). Therefore, peptides generated by enzymatic hydrolysis may show different physical properties than native protein. Generally, bioactive peptides are low-molecular-weight peptides (2-30 amino acids in length) with bioactivities that depend on their amino acid composition and sequence. However, these peptides may be inactive in the native protein form (Korhonen and Pihlanto, 2006; López-Fandiño et al., 2006). In particular, it has been reported that bioactive peptides produced by commercial proteolytic enzymes exhibit various biological activities such as anti-inflammatory, ACE-inhibitory, antioxidant, and antimicrobial activities (Escudero et al., 2013; Hu et al., 2011; Qian et al., 2016; Yu et al.,

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2006). Nowadays, consumers do not want food products containing synthetic preservatives or synthetic antimicrobial agents. Therefore, many researchers have attempted to replace synthetic food additives with natural materials, and bioactive peptides derived from porcine blood proteins are a potential, economical resource.

In previous studies on blood proteins, antihypertensive peptides were isolated by hydrolyzing bovine and porcine hemoglobin proteins (Adje et al., 2011; Yu et al., 2006). In addition, several studies have found antimicrobial activity in bovine hemoglobin hydrolysates (Adje et al., 2011; Hu et al., 2011; Nedjar-Arroume et al., 2006) and antioxidant activity in porcine plasma and hemoglobin proteins (Chang et al., 2007; Wang et al., 2008; Xu et al., 2009). Because the amount of blood collected at slaughter is expected to increase gradually every year, research pertaining to utilization of blood generated by the industrialization of pig slaughter is required. However, no previous study has reported antimicrobial activity of hydrolysates from porcine blood plasma proteins, albumin, or globulin. Therefore, in the present study, degree of hydrolysis (DH) of porcine blood plasma proteins, albumin, and globulin hydrolyzed by six commercial proteases (alcalase, neutrase, flavourzyme, protamex, trypsin, and papain) was measured on the basis of reaction time. In addition, the antimicrobial activity of hydrolysates against five pathogenic microorganisms (*B. cereus, S.aureus, S.* Typhimurium, *E. coli*, and *S. flexneri*) was examined.

Materials and Methods

Collection of porcine blood and separation of blood proteins

Whole porcine blood was freshly obtained immediately after slaughter and immediately used for plasma preparation. Ethylenediaminetetraacetic acid was added as anticoagulation agent to fresh porcine blood at 2 g/L and mixed well. Blood was immediately placed in ice slash and brought back to the laboratory within 30 min. Samples were centrifuged (Supra 25K, Hanil Science Industrial Co., Ltd., Incheon, Korea) at 8,000×*g* for 15 min at 4°C for plasma separation. Globulin and albumin proteins were isolated from the separated plasma using a modified cold ethanol method (Cohn et al., 1946).

For the separation of globulin and albumin proteins from the plasma, the plasma separated by centrifugation was cooled in an ice water bath. Cold ethanol was then added to the plasma at a final concentration of 7.4%. Next, centrifugation was performed at 10,000 ×*g* for 20 min to remove fibrinogen and antihemophilic factor. After that, ethanol was added to the supernatant at a final concentration of 24% and centrifugation was carried out again under the same conditions (10,000 rpm for 20 min). The resulting precipitate (globulin) was stored at 15°C.

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Ethanol was added to the separated supernatant at a final concentration of 60%. The precipitate (albumin) obtained after centrifugation was stored at 15°C.

Preparation of blood protein hydrolysates

The blood proteins obtained in the previous steps were subjected to single protease hydrolysis using the following proteases: alcalase, flavourzyme, neutrase, protamex, papain, and trypsin (Novozymes, Denmark). The characteristics of commercial enzymes used in this study were decribed in Table 1. In brief, 1 g of blood protein was dissolved in 50 mL of 25 mM sodium phosphate buffer (0.2 M monobasic sodium phosphate + 0.2 M dibasic sodium phosphate), which was adjusted to pH 7.0 with 1 N NaOH and 1 N HCl. The mixture was homogenized with a homogenizer (T-25 basic, Ika Works Inc., Wilmington, NC, USA). To determine the optimum hydrolysis time for each protease, blood protein solutions were hydrolyzed with each protease in a 50°C water bath for 30 min, 1 h, 2 h, 3 h, 4 h, or 5 h. The proteases were added at a ratio of 1% for a particular blood protein. After completion of hydrolysis, the solutions were heated in boiling water for 3 min to inactivate the proteases. The mixture was centrifuged at 8,000×*g* for 25 min using Supra 22K (Hanil Science Industrial Co., Ltd., Incheon, Korea). The supernatant was stored at -20°C until use.

Degree of hydrolysis

Degree of hydrolysis was measured using soluble protein as an indicator. In other words, protein concentration of the supernatant of the hydrolyzed solution was measured by the Lowry method (Lowry et al., 1951). Degree of hydrolysis was calculated using the following formula: DH (%) = (Protein content in samples after hydrolysis/Protein content in samples before hydrolysis) × 100.

Antimicrobial tests

Gram-positive bacteria *B. cereus* KFRI 181 and *S. aureus* ATCC12692 and gram-negative bacteria *S.* Typhimurium ATCC 14028, *E. coli* KFRI 836, and *S. flexneri* ATCC 11836 were obtained from Korea Food Research Institute (KFRI, Seongnam, Korea) and Korea National Microbiological Research Resource Center (KNMRRC, Suwon, Korea). Antimicrobial tests were carried out using disc diffusion method (Lee and Lee, 2010). Bacterial suspension (1 mL) containing 10^8 CFU/mL was spread onto nutrient agar plates (Difco Laboratories, Detroit, MI, USA). The hydrolysates of blood proteins(albumin, glubulin, plasma) used in this antimicrobial test were those at 2 hours of hydrolysis. Then 50 (20 mg/mL), 200 (80

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mg/mL), and 400 (160 mg/mL) μL of each hydrolysate concentrated by air drying (dry oven, JS-lin-2500, Seoul, Korea) was added to 8 mm diameter discs placed on inoculated agar. The inoculated plates were then incubated at 37°C for 48 h. Antimicrobial activity was assessed by measuring the zone of inhibition against the tested organisms and indicated in the following manner: -, no antimicrobial activity; ±, slight antimicrobial activity with inhibition zones of 8.1-15 mm; +, moderate antimicrobial activity with inhibition zones of 15.1-20 mm; ++, clear antimicrobial activity with inhibition zones of 20.1-25 mm; +++, strong antimicrobial activity with inhibition zones of more than 25.1 mm.

Statistical analysis

All measurements were repeated three times. Results are expressed as mean values with standard deviations. Data were statistically analyzed with ANOVA and Tukey's multiple range test. Statistical significance was accepted at $p<0.05$ (SAS, 2003).

Results and Discussion

Degree of hydrolysis of porcine blood proteins

Table 1 shows DH values of porcine blood albumin according to proteolytic enzyme used and hydrolysis time. Trypsin hydrolysate of albumin exhibited the highest DH values at all hydrolysis times, followed by alcalase, papain, protamex, flavourzyme and neutrase hydrolysates. Neutrase and flavourzyme hydrolysates of albumin showed DH values of less than 1% after 5 h of hydrolysis. Trypsin hydrolysate of albumin showed the highest DH values at 1 h of hydrolysis. All DH values did not change significantly at 2 h of reaction time (Table 2). In a previous study, DH of porcine hemoglobin was reported to increase with increasing hydrolysis time (Chang et al., 2007).

Table 3 shows DH values of porcine blood globulin according to proteolytic enzyme used and hydrolysis time. As in the case of albumin, alcalase, trypsin, and papain hydrolysates of globulin showed significantly higher DH values than those produced by the other three proteases. According to the tendency of DH with increasing reaction time (Table 2), DH values of alcalase, protamex, and trypsin hydrolysates of globulin did not change significantly after 1 h of hydrolysis. Papain hydrolysate of globulin showed the highest DH value at 4 h of hydrolysis.

Table 4 shows DH values of porcine blood plasma for the six proteases and reaction times used. Degree of hydrolysis values of porcine blood plasma protein hydrolysates produced by any protease did not exceed 2% after 5 h of reaction time. Virtually no hydrolysis reaction

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was observed for plasma proteins when using neutrase, flavourzyme, or protamex. However, alcalase, trypsin, and papain tended to exhibit higher DH values at 2 h of hydrolysis than at other reaction times (Table 3). The DH values of albumin and globulin hydrolysates were higher than those of plasma protein hydrolysates. Alcalase, trypsin, and papain hydrolysates of blood proteins exhibited higher DH values than neutrase, protamex, or flavourzyme hydrolysates of blood proteins. In addition, the blood protein hydrolysates tended to show the highest DH values with the hydrolysis time of 2 h.

Enzymatic hydrolysis is the most common method for producing bioactive peptides from proteins. For hydrolysates to be suitable for further application, hydrolysis conditions such as the enzyme type and concentration, hydrolysis reaction time, temperature, pH, and substrate-to-enzyme ratio are very important (Najafian and Babji, 2012). Among these conditions, enzyme type is crucial to peptide production. According to Hrckova et al. (2002), alcalase is an endopeptidase capable of hydrolyzing proteins, with broad specificity for peptide binding; it prefers large uncharged residues. Verma et al. (2017) reported that when the pig liver was hydrolyzed with alcalase, trypsin and papain for 0, 2, 4 or 6 hours, as the hydrolysis time increased, the degree of hydrolysis increased. Trypsin showed significantly higher degree of hydrolysis than alcalase and papain at more than 2 hours of hydrolysis. As well as, pig liver hydrolysate obtained from trypsin hydrolysis showed highest functional activities(antioxidant and antimicrobial) followed by papain and alcalase pig liver hydrolysates. In addtion, in a result of Hiidenhovi et al. (2005), the degree of hydrolysis of ovomucin hydrolysed with 10 different enzymes, including alcalase, protamex, and trypsin were not different between a hour of hydrolysis and four hour hydrolysis. Flavourzyme possesses both endoprotease and exopeptidase activities. Its ability to release free amino acids is higher than serine endoprotease alcalase (Hrckova et al., 2002). In a previous study by Qian et al. (2007), when the tuna dark muscle hydrolysate was produced by using six hydrolytic enzymes (alcalase, neutrase, pepsin, papain, α-chymotrypsin, and trypsin), the hydrolysate produced by pepsin showed the highest anti-hypertensive activity among all hydrolysates. Ranathunga et al. (2006) reported that the pepsin hydrolysate had the highest antioxidant activity, although it exhibited the lowest DH. Although pepsin was not one of the proteases used in the present study, results of previous studies suggest that DH may vary depending on the protease used. In addition, the bioactivity of the hydrolysate can also change.

Antimicrobial effect of porcine blood protein hydrolysates

The antimicrobial effects of porcine blood protein hydrolysates on five pathogenic microorganisms (*B. cereus*, *S. aureus*, *S.* Typhimurium, *E. coli*, and *S. flexneri*) are tested and antimicrobial effect was shown only in *B. cereus* (Table 5). Hydrolysates produced by all

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proteases exhibited high DH values at 2 h of hydrolysis, hydrolysates at 2 h were used for antimicrobial activity tests in the present study. The hydrolysates obtained by hydrolyzing porcine albumin, globulin, and plasma proteins with the six proteases did not show any antimicrobial activity against the pathogenic bacteria *S. aureus*, *S.* Typhimurium, *E. coli*, *or S. flexneri*. However, porcine blood hydrolysates showed antibacterial effects on *B. cereus*. Of the hydrolysates generated from the three blood proteins, albumin hydrolysates generally showed higher antimicrobial activity than globulin and plasma hydrolysates. When 50 μL of albumin hydrolysates were added to the discs, hydrolysates by all proteases except papain showed slight antimicrobial activity. Clear antimicrobial activity was observed with the addition of 400 μL of albumin hydrolysates by all proteases except papain. Furthermore, albumin and globulin hydrolysates generated by flavourzyme, protamex, and trypsin showed higher antimicrobial activity than those generated by alcalase or neutrase. Of plasma protein hydrolysates generated by different proteases, only those generated by trypsin exhibited moderate antimicrobial activity (with addition of 200 μL to the discs).

Numerous research studies have been carried out on peptides showing antimicrobial activity, using plant and animal proteins. Among animal proteins, antimicrobial hydrolysates have been isolated and characterized from milk proteins, egg proteins, and blood and muscle hemoglobin proteins (Abdou et al., 2007; Hayes et al., 2006; Jang et al., 2008; Xu et al., 2009). In a previous study by Nedjar-Arroume et al. (2006), 1% hemoglobin solution was hydrolyzed by porcine pepsin at 3% DH, and antibacterial activity of the hemoglobin hydrolysate against nine microorganisms was examined—three gram-negative (*E. coli, Shigella sonnei,* and *Salmonella enteritidis*) and six gram-positive (*Micrococcus luteus* A270, *Listeria innocua, Enterococcus faecalis, B. cereus, Staphylococcus saprophyticus,* and *Staphylococcus simulans*). They found that the total hemoglobin hydrolysate exhibited antimicrobial activity against *M. luteus* A270, *L. innocua*, *E. coli,* and *Salmonella enteritidis*. In addition, 9 out of 26 fractions were found to have antibacterial activity (Nedjar-Arroume et al., 2006). The fractions mentioned above can be divided into two groups on the basis of structure. One group consists of b126-145, a107-136, and a107-141 peptides containing less than 50 amino acid residues. They have an overall positive charge owing to the presence of multiple lysine and arginine residues in addition to a substantial stretch of hydrophobic residues and a higher α-helical structure (Powers and Hancock, 2003). The second group includes a133-141 and a137-141 peptides which are small (5 and 9 amino acids) and positively charged. They show little to no presence of hydrophobic residues and a higher random coil structure. These peptides are known to possess antimicrobial activity against

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gram-positive and gram-negative bacteria with membrane-disruptive and non-membrane-disruptive mechanisms (Mohammad et al., 1995; Powers and Hancock, 2003). Similar findings have been reported by Daoud et al. (2005) and Hu et al. (2011). Hu et al. (2011) reported that a newly discovered peptide located in the central part of bovine α-hemoglobin presented antimicrobial activity against *E. coli*, *S. aureus*, and *Candida albicans*. The sequence of this bovine peptide was similar to that of peptides in sheep, deer, pigs, and humans. Although antimicrobial study using hydrolysates of porcine plasma proteins, albumin, and globulin has not yet been reported, Friedrich et al. (2000) reported that bovine albumin peptides have antibacterial activity against gram-positive bacteria including strains of *Staphylococcus*, *Enterococcus faecalis*, *L. monocytogenes*, and *Streptococcus pyogenes*. Therefore, it is expected that porcine blood albumin hydrolysates may have antimicrobial activity against *B. cereus*. However, in a previous study by Salampessy (2010), a hydrolysate (DH 28.2%) of leatherjacket fish (*Meuschenia* sp.) insoluble muscle proteins generated by bromelain—a proteolytic enzyme—at 4.3 mg/mL concentration was found to be active primarily against *B. cereus* and *S. aureus*, and the sequence of the active peptide was identified as Glu-Gln-Ile-Asp-Asn-Leu-Gln (EQIDNLQ)—an anionic peptide rich in glutamic acid and aspartic acid. However, most antimicrobial peptides reported previously are cationic peptides rich in amino acid residues such as proline, arginine, phenylalanine, glycine, and tryptophan (Abdou et al., 2007; Hayes et al., 2006; Jang et al., 2008; Xu et al., 2009). The antimicrobial activity of anionic peptides has also been reported by Lai et al. (2002). Although the hydrolysates were not structurally and chemically characterized in the present study, the study showed, for the first time to our knowledge, that hydrolysates of porcine blood plasma proteins, albumin, and globulin exhibited antibacterial activity against *B. cereus*. This is the first step in analyzing porcine blood plasma proteins for their functionality. Further detailed studies are needed in the future to determine factors such as the optimal hydrolysis conditions, inhibitory activity against various microorganisms, and peptide sequence.

Conclusions

This study determined the hydrolysis conditions (protease type and reaction time) for porcine blood proteins including plasma proteins, albumin, and globulin and found that these hydrolysates possessed antimicrobial activity against *B. cereus*. To the best of our knowledge, this is the first report indicating the potential of porcine blood protein hydrolysis for the production of bioactive peptides. Further studies are needed to identify the hydrolysate peptides and specific hydrolysis conditions to improve their antimicrobial activity and

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functionality. In the future, instead of chemical preservatives, these will not only be another technologies that can help improve food storage naturally, but also serve as the basis for applications in the medical and pharmaceutical industries.

Conflicts of Interest

The authors declare no potential conflict of interest.

Acknowledgments

This work was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through High Value-added Food Technology Development Program, funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA, 116034-03-1-HD030) and by the Regional Animal Industry Center at Gyeongnam National University of Science and Technology (GnTech).

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Table 1. Characteristics of commercial enzymes

Enzyme	pH	Temperature	Activity	Company
Alcalase 2.4L	6.5-8.5	55-70	2.4 AU/g	Novonordisk Bioindystrials, INc., Denmark
Nuetrase 0.8L	5.5-7.5	45-55	0.8 AU/g	
Flavourzyme 500MG	5.0-7.0	45-55	500 LAPU/g	
Protamex 1.5MG	5.5-7.5	35-60	1.5 AU/g	
Trypsin	7.0-8.0	40-50	1250 unit/mg solid	Sigma Chemical Co., USA
Papain	6.0-7.0	55-65	16-40 units/mg solid	Sigma Chemical Co., USA

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Table 2. Degree of hydrolysis (%) of swine albumin protein by the six proteases and reaction times

$^{a\text{-}e}$ Means with different superscription within the same column differ ($p<0.05$).

Time / Protease	30Min	1h	2h	3h	4h	5h	SEM
Alcalase	6.07^{bB}	9.97^{bA}	8.84^{aAB}	8.51^{aAB}	8.84^{aAB}	9.31^{bA}	0.37
Neutrase	0.13^{cB}	0.00^{eB}	0.17^{cB}	0.00^{cB}	0.00^{cB}	0.99^{dA}	0.10
Flavourzyme	0.33^{c}	0.02^{e}	0.30^{c}	0.41^{c}	0.52^{c}	0.85^{d}	0.09
Protamex	2.11^{cC}	3.43^{dBC}	3.89^{bB}	4.49^{bAB}	4.82^{bAB}	5.41^{cA}	0.27
Trypsin	8.91^{aB}	13.14^{aA}	9.44^{aB}	9.37^{aB}	9.77^{aB}	10.83^{aAB}	0.41
Papain	4.42^{b}	6.93^{c}	4.55^{b}	5.28^{b}	5.21^{b}	6.40^{c}	0.28
SEM	0.78	1.20	0.90	0.88	0.91	0.92	

$^{A\text{-}D}$ Means with different superscription within the same row differ ($p<0.05$).

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Table 3. Degree of hydrolysis (%) of swine blood globulin protein by the six proteases and reaction times

$^{a-d}$ Means with different superscription within the same column differ (p<0.05).

Time / Protease	30Min	1h	2h	3h	4h	5h	SEM
Alcalase	2.96^{abB}	5.44^{aAB}	4.70^{aAB}	5.81^{aAB}	5.32^{bAB}	7.17^{aA}	0.41
Neutrase	0.37^{ab}	0.00^{b}	0.65^{b}	0.00^{b}	0.00^{c}	0.86^{b}	0.14
Flavourzyme	0.00^{b}	0.00^{b}	0.45^{b}	0.16^{b}	0.00^{c}	0.00^{b}	0.07
Protamex	0.12^{bB}	1.73^{bA}	0.20^{bB}	0.53^{bAB}	0.37^{cAB}	0.12^{bB}	0.16
Trypsin	3.46^{aB}	5.07^{aAB}	6.80^{aA}	4.57^{aAB}	5.56^{bAB}	6.18^{aAB}	0.33
Papain	1.60^{abC}	4.45^{aBC}	5.44^{aB}	7.17^{aB}	12.99^{aA}	5.56^{aB}	0.87
SEM	0.40	0.57	0.68	0.73	1.13	0.74	

$^{A-D}$ Means with different superscription within the same row differ (p<0.05).

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Table 4. Degree of hydrolysis (%) of swine blood plasma by the six proteases and reaction times

a-d Means with different superscription within the same column differ ($p<0.05$).

Time / Protease	30Min	1h	2h	3h	4h	5h	SEM
Alcalase	0.80	0.67	1.66^{a}	1.03^{a}	1.20^{a}	1.49^{a}	0.11
Neutrase	0.00	0.07	0.13^{bc}	0.00^{c}	0.00^{b}	0.00^{d}	0.02
Flavourzyme	0.00^{B}	0.00^{B}	0.07^{bcA}	0.00^{cB}	0.00^{bB}	0.00^{dB}	0.01
Protamex	0.00	0.00	0.03^{c}	0.03^{bc}	0.00^{b}	0.00^{d}	0.01
Trypsin	0.68	0.38	1.09^{ab}	0.74^{ab}	0.34^{b}	0.74^{c}	0.10
Papain	0.80	0.67	0.74^{abc}	0.68^{abc}	0.63^{ab}	1.09^{b}	0.09
SEM	0.11	0.10	0.16	0.11	0.11	0.14	

A-C Means with different superscription within the same row differ ($p<0.05$).

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Table 5. Antimicrobial effects of swine blood protein hydrolysates hydrolyzed by six proteases (2h) (cm)

[a-b] Means with different superscription within the same column differ ($p<0.05$).

Pathogenic bacteria	Proteins	Hydrolysates	50 μℓ (20mg/ml)	200 μℓ (80mg/ml)	400 μℓ (160mg/ml)
		Enzymes	Diameter	Diameter	Diameter
B. cereus	Albumin	Alcalase	1.45±0.07	2.05±0.05	2.35±0.21
		Neutrase	1.50±0.00	1.95±0.07	2.10±0.14
		Flavourzyme	1.35±0.06	2.30±0.14	2.35±0.07
		Protamex	1.25±0.03	2.15±0.21	2.30±0.14
		Trypsin	1.30±0.14	2.35±0.05	2.55±0.06
		Papain	-	-	-
	Globulin	Alcalase	-	$1.30±0.10^{b}$	$1.53±0.06^{b}$
		Neutrase	-	$1.21±0.08^{b}$	$1.68±0.08^{a}$
		Flavourzyme	$0.09±0.00^{b}$	$1.58±0.08^{a}$	$1.72±0.03^{a}$
		Protamex	$0.85±0.07^{b}$	$1.58±0.08^{a}$	$1.75±0.05^{a}$

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		Trypsin	1.05±0.07[a]	1.58±0.08[a]	1.800±.10[a]
		Papain			
	Plasma	Alcalase	-	1.43±0.06	1.75±0.05
		Neutrase	0.90±0.10	1.47±0.06	1.73±0.06
		Flavourzyme	-	1.47±0.06	1.67±0.06
		Protamex	0.87±0.06	1.53±0.06	1.77±0.06
		Trypsin	-	1.57±0.06	1.73±0.06
		Papain	-	-	-
Control	Antibiotics		5ul	10ul	50ul
B. cereus	Ampicillin (50mg/ml)		-	1.50±0.05	1.84±0.07
S. aureus	Ampicillin (50mg/ml)		2.540.09	2.81±0.04	2.96±0.07
S. typhimurium	Ampicillin (50mg/ml)		1.14±0.05	2.60±0.05	2.84±0.10
E. coli	Ampicillin (50mg/ml)		2.06±0.08	2.21±0.09	2.55±0.06
S. flexneri	Ampicillin (50mg/ml)		1.80±0.07	2.20±0.06	2.72±0.09

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