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Extraction of Collagen from the Skin of Kacang Goat and Production of Its Hydrolysate as an Inhibitor of Angiotensin Converting Enzyme

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ABSTRACT

The study was designed to determine the potential of collagen hydrolysate produced from the skin of Kacang goat through chymotrypsin hydrolysis to be used as an inhibitor of *angiotensin converting enzyme* **(ACE). This research was conducted in three replications, with the measured parameters include ACE inhibitory potential and collagen hydrolysate fractionation. The results showed that collagen extraction of Kacang goat skin by chymotrypsin hydrolysis yielded 9.74% (dry matter, v/v) collagen, with pH at 6.6. The extracted collagen contained α1, α2, and β collagen chains with molecular weights of 151 kDa, 141 kDa, and 240 kDa, respectively. Furthermore, the collagen hydrolysis produced protein peptides confirmed at molecular weights of 43 to 107 kDa. The hydrolysate fractionation at molecular weights of <3 kDa, 3-5 kDa, and >5 kDa showed proteins concentrations of 2.33 mg/mL, 3.81 mg/mL, and 3.93 mg/mL, respectively. The hydrolysate fractionation with molecular weight <3 kDa showed to have ACE inhibition activity with the IC_{50} value of 0.47 mg/mL. The study concluded that collagen hydrolysate extracted from the skin of Kacang goat had a promising potential as a source of antihypertensive agent.**

Keywords: antihypertensive; chymotrypsin; collagen of goat skin; hydrolysate; IC_{50}

INTRODUCTION

The research on goats in Indonesia has been focused on improving meat and milk production. The study to explore and evaluate the potential use of animal skin for the production of beneficial derivative compounds such as gelatin, collagen, or bioactive peptide is limited. Goat skin is one of the by-products from slaughterhouses that can be consumed with high protein content. The physical structure of goat skin is composed of connective tissue, which is a micro component of fibrils and fibroblasts that form collagen and elastin tissue. Moreover, raw goat skin contained 60%-70% water, 25%-32% protein, 2.2%-3.2% fiber protein, and 7%-7.3% crude fat (Ajayi & Akomolafe, 2016) (Naffa *et al*., 2019). However, the collagen protein of goat skin has low digestibility due to the strong protein-binding bonds in the form of the triple helix. The collagen cleavage into smaller compounds can be done through enzymatic hydrolysis. The exploration of alternative protein sources in regards to their functional properties has been growing continuously and getting more attention as the trend to use natural food as an additive (Sadeghi *et al*., 2019).

Enzymatic hydrolysis is preferred over chemical hydrolysis since the process can be controlled, and the final product can be further developed (Iltchenco *et al*., 2017). The enzymes used for hydrolysis include pepsin, trypsin, and chymotrypsin (Sujarwanto *et al*., 2018; Winarti *et al*., 2019). The hydrolysis using various digestive enzymes have shown to be able to produce bioactive peptide compounds as antihypertensive agents from the animal products such as meat of buffalo (Sujarwanto *et al*., 2018), foot of chicken (Bravo *et al*., 2019), milk of goat (Widodo *et al*., 2019), the meat of rabbit (Permadi *et al*., 2019), and meat of duck (Winarti *et al*., 2019). The bioactive peptide is able to inhibit the activity of angiotensin converting enzyme in the RAAS system (Renin-Angiotensin-Aldosterone System), which causes hypertension due to the constriction of blood vessels (Hsueh and Wyne, 2011). The ability of enzymes to hydrolyze proteins varies greatly, depends on the specific nature of the enzyme. The chymotrypsin enzyme tends to cut the peptide bonds formed by

large molecular and hydrophobic amino acid residues. Chymotrypsin cleaves peptide bonds at leucine-leucine position, and previous research resulted from the leu-thr-glu-ala-pro-leu-asn-prolys-ala-arg-asn-glu-lys sequence with potential as an ACE inhibitor (Jamhari *et al.*, 2013). Therefore, the use of the chymotrypsin enzyme to hydrolyze the collagen contained in the skin of the goat for the production of oligo-peptide and their potential as an ACE inhibitor has never been studied. This study aims to determine the potential production of collagen-protein hydrolysate extracted from the skin of local Kacang goat using the chymotrypsin enzyme. The collagen hydrolysate can be used as an inhibitor of angiotensin converting enzyme (ACE).

MATERIALS AND METHODS

Materials

The skin was obtained from 1.5 years of male Kacang goat from Gunung Kidul Regency, Yogyakarta, Indonesia. Pepsin (porcine stomach mucosa) and chymotrypsin were purchased from the Sigma-Aldrich Chemical Industry Ltd., Germany. Angiotensin-converting enzyme (ACE) from rabbit lung was obtained from the Sigma Chemical Co., St. Louis, USA, and hippuryl-L-histidyl-leucine (HHL) free base was purchased from Nacalai Tesque, Kyoto, Japan. No animals experimented in the present study. Thus, there was no requirement for ethical approval.

Skin Preparation

Goat skin was prepared from the remaining meat, fat, and dirt. The hairs in the skin were removed by scraped using a knife, then rinsed with running water and drained for 30 minutes until the water does not drip. The skin was cut into small pieces and weighed as much as 100 grams. Before extraction, goat skin was tested for proximate assay (AOAC, 1990).

The Production of Collagen from the Skin of Kacang Goat

Collagen was extracted by the enzymatic method according to Wahyuningsih *et al.* (2018). Small pieces of goat skin, each with a weight of 100 grams, were immersed in 0.1 M NaOH solution at a ratio of 1:10 (w/v) for 10 minutes then the solution was discarded. Extraction was done by immersing the skin in a solution of 0.5 M acetic acid containing 0.1% (w/v) pepsin at a ratio of 1:10 (w/v) for 24 hours at 4°C. The mixture was filtered using Whatman filter paper No. 1. Subsequently, the hydrolysate was precipitated with 2.6 M NaCl overnight at 4°C. The extract was centrifuged at a speed of 4500 g for 30 minutes at 4°C. The pellet was dissolved with 0.5 M acetic acid (1:5 ratio) and dialyzed with 0.1 M phosphate buffer for 24 hours with periodic solution replacement and finally washed with distilled water for 2 hours. The extracted collagen was freeze-drying. The yield of collagen was obtained by the formula below:

Collagen yields (%)= (Dry weight of collagen / Initial weight) x 100%

Characterization of Collagen Extracted from the Skin of Kacang Goat

The extracted collagen was characterized for pH and molecular weight characteristics, following the methodology of Alfaro *et al*. (2013) and Vidal *et al.* (2019b). The pH of collagen was measured by weighing one gram of sample then dissolved into 100 mL of distilled water. The pH of the collagen solution was measured using the pH meter (pH meter, Hanna Instrument). The molecular weight of collagen was determined using SDS-PAGE. The electrophoresis gel used in this procedure contains 15% resolving gel and 4% stacking gel. A collagen sample at the weight of 0.01 gram was dissolved in 20 mM NaOH with 0.1 M buffer phosphate (pH 8.3) in a ratio of 1:5. Collagen was then homogenized using a vortex mixer (Velp ZX4, Velp Scientifica, Italy). A total of 10 μL of collagen solution was mixed with 4 μL of loading SDS buffer, then heated with a water bath at 85ºC for five minutes. The collagen solution was cooled and centrifuged at 3000 rpm for 30 seconds. Collagen samples were loaded into the electrophoresis gel with a voltage of 110V for 150 minutes. The gel was stained using 0.25% Coomassie blue, soaked for 6 hours, then de-stained for 15 minutes. The gel was fixed in a 10% solution of acetic acid, observed, and measured the band formed.

Hydrolysate Production Derivate from the Collagen of Goat Skin

The collagen was hydrolyzed and fractionated to obtain potential bioactive peptide hydrolysate according to Jamhari *et al.* (2013) with a slight modification. Collagen was hydrolyzed with the chymotrypsin enzyme. Collagen was dissolved in a phosphate buffer mixture (pH 8.3) with 20 mM NaOH. The chymotrypsin enzyme at a concentration of 2U/mg/mL was added to the collagen solution, then incubated at 37 °C for 1 hour. The hydrolysis reaction was stopped by heating at 95°C for 10 minutes, then cooled on ice before fractionation. The fractionation of collagen hydrolysate was conducted through two stages of filtering. In the first stage, the hydrolysate was fractionated using a 5 kDa Vivaspin concentrator. Two milliliters of hydrolysate was put in a column/filtrate tube, then put in a centrifuge. Centrifugation was carried out at a speed of 4500 g (4ºC temperature) for 45 minutes. The filtrate of 5 kDa was collected for smaller fractions. The filtrate from the filtration results was taken for the second stage of fractionation using a 3 kDa centrifugal filter. Centrifugation was carried out at a speed of 4500 g (4ºC temperature) for 45 minutes. The filtrate of 3 kDa was collected as a result of <3 kDa filtration. Homogenate was collected as a result of 3-5 kDa dissolved protein. The levels of dissolved protein were tested to determine the concentration of potential bioactive peptides in each fraction.

Hydrolysate of collagen was measured using the waddle method (Wolf, 1983). The samples of fractionated protein were taken at a volume of 5 µL then put in 1 mL of 20 mM NaOH solution and buffer phosphate (pH 8.3) (ratio of 1: 5). The absorbance of the sample was measured at 215 nm and 225 nm wavelengths using a spectrophotometer. The hydrolysate of collagen was calculated by the formula:

Dissolved protein (mg/mL) = (A215 - A225) x28.8

where A215 was absorbance at λ 215 and A225 was absorbance at λ 225.

Determination of ACE Inhibiting Potential

The determination of ACE inhibiting potential was measured following Liu *et al.* (2013). Hydrolysate of collagen solution (207 µL) containing ACE inhibitor peptides with protein concentrations of 0.6, 0.8, 1.0, 1.2, 1.4, and 1.6 mg/mL was mixed with 25 µL of 5.0 mM HHL substrate dissolved in 100 mM borate buffer (pH 8,3) and 300 mM NaCl, and then the samples were pre-incubated for 5 minutes in a water bath at 37°C. The reaction started with the addition of 18 µl 0.018 U ACE dissolved in borate buffer (pH 8.3). Incubation was carried out in a water bath for 30 minutes at 37°C. The reaction was stopped by the addition of 250 µL 0.1 N HCl, except for blanks that were added 250 µL 0.1 N HCl before incubation. The product of the reaction (hippuric acid) was extracted with the addition of 1.5 ml of ethyl acetate and shaken for 3 minutes. The mixture was then centrifuged at a speed of 3,600 rpm for 15 minutes. One milliliter of the supernatant was taken and transferred to another test tube, then dried at 100°C for 60 minutes. The test tube was then cooled to room temperature for 30 minutes. The hippuric acid released by ACE was determined spectrophotometrically, with a wavelength of 228 nm. The percentage of ACE inhibitory activity was calculated using the formula:

Inhibitory activity (%) = [(Ec-Es) / (Ec-Eb)] x 100%

where Ec was absorbance of the control, Es was absorbance of sample, Eb was absorbance of blank.

The concentration of ACE inhibitors to inhibit 50% of the ACE activity is called the IC value (IC_{50}). IC_{50} was obtained by making a regression equation of the inhibitory activity of several serial dilution samples. All measurements were carried out for triplicate. The regression curve of the IC_{50} calculation was described as a sigmoid curve by plotting ACE activity (%) versus the inhibitor concentration and performing a model fit using Bayesian data analysis (von der Linden *et al.*, 2014).

Experimental Designs and Data Analysis

This experiment was designed as an exploratory study using homogenized collagen of goat skin. The obtained data on the characters of collagen in the skin of Kacang goat was shown as descriptive analysis. The molecular weight of collagen was visualized as SDS-PAGE pattern and calculated by regression analysis. The analysis of variance with one way pattern using SPSS was applied to determine the differences among the protein content after various sizes of ultrafiltration application. The ACE inhibition data were collected and calculated statistically for the IC_{50} through the sigmoid regression analysis.

RESULTS

The Characterization of Collagen Produced from the Skin of Kacang Goat

The chemical composition of the skin of Kacang goat and the yields of collagen produced are presented in Table 1 and Table 2. Table 1 shows the chemical composition of the skin of Kacang goat, which protein content more than 30%. It means that goat skin is a good source of collagen protein and has the potential as a source of many derivative-protein products, including bioactive peptides. The yield of collagen extraction derived from the skin of Kacang goat using pepsin in 24

Table 1. Chemical composition of skin of Kacang goat

Compositions	Percentage (%)
Water content	70.19
Ash content	0.36
Fat content	0.49
Protein content	31.05

Table 2. The comparison of collagen characteristics hydrolyzed from the skins of Kacang goat to the other animal's skin

Substrate	Enzyme	Incubation time	Collagen yield (dry weight, %)	pH	Reference
Skin of Kacang goat	Pepsin	24 hours	13.56±0.02	6.6	In this Study
Lamb slaughter by-product	Pepsin	72 hours	12.3	-	Vidal *et al*., 2020
Sheep slaughter by-product			8.88		
Skin of rabbit	Pepsin	48 hours	71	6.3	Martínez-Ortiz *et al*., 2015
Bull hide	Pepsin	24 hours	30.20±0.87	-	(Noorzai *et al*., 2020).
Calf hide,			19.50±0.78		
Cow hide,			26.90±0.32		
Bovine face-pieces			15.40±1.16		
Ox hide			30.10±0.26		

hours of incubation time was obtained at 9.73 g per 100 g sample or 9.73% (Table 2).

Figure 1 shows the two main collagen chains, i.e., α and β band forms. The molecular weights of α1, α2, and β chains of collagen from the skin of Kacang goat were 151 kDa, 141 kDa, and 240 kDa, respectively.

Collagen Hydrolysate of Goat Skin Produced by Chymotrypsin Hydrolysis

Figure 2 shows the results of SDS-PAGE testing of collagen obtained from the skin of Kacang goat hydrolyzed using the chymotrypsin enzyme. The results of the molecular weight pattern of collagen hydrolysate in various hydrolysis times of 15, 30, 45, and 60 minutes were relatively showed the same molecular weight. The molecular weights of collagen hydrolysate after hydrolysis with chymotrypsin were observed between 43 to 107 kDa. The α1, α2, β chains, and the hydrolyzed band (hydrolysis for 15 to 60 minutes) look fading.

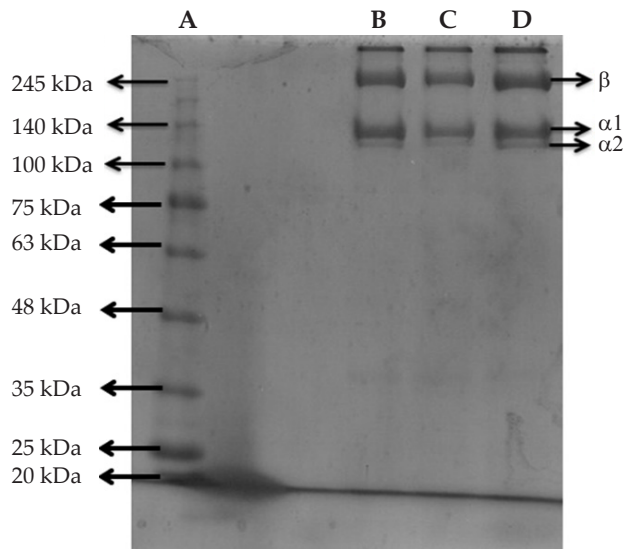

Figure 1. SDS-PAGE patterns of collagen extracted from the skin of Kacang goat; (A) Marker 20 to 245 kDa; (B-D) Collagens isolate batch 1 to 3.

Table 3. Angiotensin-converting enzyme (ACE) inhibitory activity (%) of fractionate protein of collagen hydrolysate with molecular weight < 3 kDa

Protein level (mg/mL)	Protein in assay (mg/mL)	Inhibitory percentage (%)
1.8	22.06	93.07 ± 0.74
1.6	19.61	88.27 ± 0.71
1.4	17.16	80.97 ± 2.75
1.2	14.71	76.20 ± 3.10
1	12.25	69.74 ± 2.79
0.8	9.8	66.02 ± 2.23
0.6	7.35	58.87 ± 3.00

The Potential of Protein Hydrolysate as an ACE Inhibitor Agent

Fractionation is one of the methods of isolating peptides of a certain size by filtration (Liu *et al*., 2013). The results of fractionation of collagen hydrolysate were determined for dissolved protein levels, and their activity tests as ACE inhibitors are presented in Table 3. Ultrafiltration separated the protein fragments based on their molecular weights, and the data showed no significant differences ($p>0.05$) among the filtrates with various filter sizes on the protein concentration (Table 4).

The measurement of ACE was performed to observe the potential of collagen hydrolysate proteins as ACE inhibitors. The IC_{50} was calculated by regression equations between hydrolysate protein content and the inhibition percentage, as presented in Figure 4. The data showed that hydrolysate protein produced by treating the collagen with chymotrypsin followed by ultrafiltration resulted from IC_{50} was 0.47 mg/mL.

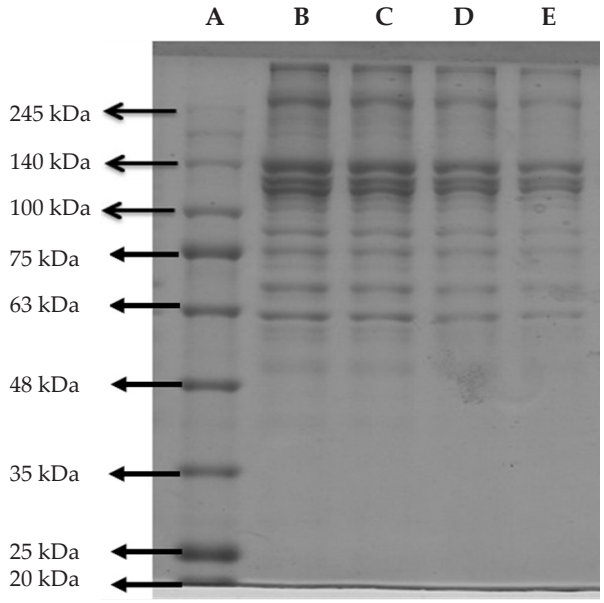

Figure 2. SDS-PAGE patterns of collagen in the skin of Kacang goat hydrolyzed with chymotrypsin at different incubation times; (A) Marker 20 to 245 kDa; (B) 15 minutes; (C) 30 minutes; (D) 45 minutes, and (E) 60 minutes.

Table 4. Protein concentration of fractionation samples with various filtrate sizes

Molecular weight of filtrate (kDa)	Protein content (mg/mL)^{ns}
<3	2.04 ± 0.52
3-5	2.97 ± 0.65
>5	3.69 ± 1.12

Note: ns= Non significant

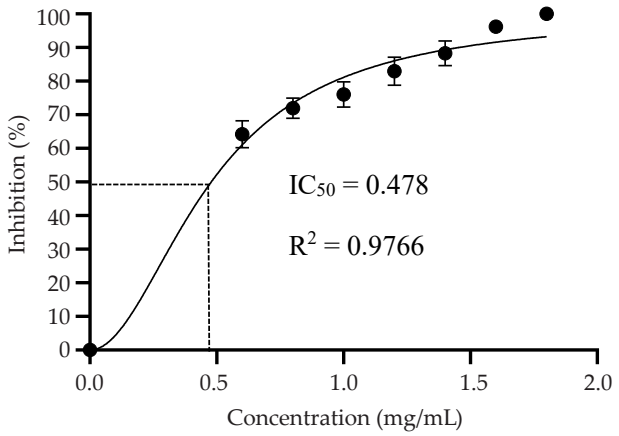

Figure 3. Sigmoid curve between angiotensin-converting enzyme (ACE) inhibition and protein concentration of collagen hydrolysates; (C) 30 minutes; (D) 45 minutes, and (E) 60 minutes.

DISCUSSION

The protein analysis of the skin of Kacang goat was obtained at 30.54%, which is lower than that of Ettawa goat (46.89%) (Kasim *et al.,* 2013) and dog (32.17%), but higher than that of the Nigerian goat (27.36%) and leather of pigs (25.36%) (Ajayi & Akomolafe, 2016). Generally, the collagen content of hiding is around 30% of the total body protein (Chuck-Hernández & Ozuna, 2019). It has the potential for collagen with a high denaturation temperature compared to the skins of other animals. The presence of protein in the skins of livestock can also indicate the potential of the skin to produce collagen structure and product yield. Collagen molecules are triple-helix of three different alpha chains of repeating amino acid units (Gly-X-Y) N, where X and Y are any amino acids. However, X is often a proline, and Y is often hydroxyproline (Noorzai *et al.,* 2020).

The yield of isolated collagen is affected by several factors, such as the condition of raw material, extraction method, incubation time, and temperature. The condition of raw materials allows the condition of different tissue-protein compatibilities so that it affects the enzyme activity and the length of time required to hydrolyze the substrate by the enzyme. Incubation time has a higher influence on the yield of collagen. The longer incubation time will produce the maximal hydrolysis process that eventually will produce higher yields (Noorzai *et al.,* 2020). The skin of sheep has a collagen yield of 8.88% (Vidal *et al.,* 2019b) and the skin of the rabbit calf has a collagen yield of 19.5% (Martinez-ortiz *et al.,* 2015). The rapid hydrolysis rate of collagen tends to increase the number of collagen molecules produced that will be converted and will eventually increase the yield value (Muyonga *et al.,* 2003). The protein content of collagens is affected by the extraction method since the more drastic extraction processes can result in lower protein contents, as they severely affect the protein structure, breaking it into smaller fractions (Vidal *et al.,* 2019a).

The pH of isolated collagen in this study was 6.6. Previous research by Tabarestani *et al.* (2012) showed that collagen solubility was affected by pH values. The pH value of 6.6 is in accordance with the standards of BSN (2014) that the standard pH values range from 6.5 to 8. The yields of collagen extracted from the skin of Kacang goat were tested for molecular weight by SDS-PAGE. The bands formed from SDS-PAGE extract of collagen from the skin of Kacang goat are presented in Figure 1.

The SDS-PAGE of collagen were observed from previous works with different species in the skin of rabbit showing α1, α2, and β chain conformations (Martínez-Ortiz *et al.,* 2015). Collagen containing these two chains can be grouped into type I collagen (Khiari *et al.,* 2014; Luderman *et al.,* 2017), which is a characteristic of collagen originating from the skin. The β chain (dimer) observed in the electrophoresis results show that collagen has intramolecular and intermolecular crosslink components (Lodhi *et al.,* 2018).

One of the characteristics of hydrolyzed proteins is the decreasing of molecular weight (León-López *et al.,* 2019) due to the hydrolysis of the proteins into smaller peptides with smaller molecular weights (Jamhari *et al.,* 2013). The protein concentrations of collagen hydrolysate are 2.33 ± 0.38 mg/mL with the lowest molecular weight fractionation of <3 kDa) (Table 3) with ACE inhibiting potential can be seen in Figure 3. Based on the measurement of IC_{50} of angiotensin converting enzyme described in Figure 3, the inhibition of ACE activity by collagen hydrolysate of the skin of Kacang goat as was measured with IC_{50} value of 0.47 mg/mL. Previous research in the meat hydrolysate of Kacang goat showed the IC_{50} value of 0.31 mg/mL (Jamhari *et al.,* 2013). In addition, IC_{50} of a milk-protein hydrolysate of the goat was 0.005-0.056 mg/mL (Espejo-Carpio *et al.,* 2013). IC_{50} of protein from the leg of native chicken was reported in the level of 0.33 ±0.02 mg/mL (Yuliatmo *et al.,* 2017).

CONCLUSION

Hydrolysis of collagen from the skin of Kacang goat by using chymotrypsin was more susceptible to the decrease in molecular weight with the increase in the duration of incubation as shown by the SDS-PAGE bands of the α1, α2, and β collagen chains. The ultrafiltration could separate the different sizes of oligopeptide and oligopeptide with the lowest molecular weight showed the highest ACE inhibition activity. The potential candidate to be developed for antihypertensive treatment is the small-fraction peptide having a molecular weight less than 3 kDa with the best ACE inhibition with the IC_{50} value of 0.47 mg/mL.

CONFLICT OF INTEREST

We declare that there is no conflict of interest regarding any financial, personal, or organizations related to the material discussed in the manuscript.

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