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Antibacterial and Antioxidant Activities of Goat Milk Hydrolysate Generated by *Bacillus Sp*. E.13

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Abstract: Hydrolysis of goat milk protein is able to produce peptic hydrolysate that may be biologically active. The study was conducted to evaluate bioactivities of the hydrolysate generated from goat milk. Goat milk proteins were hydrolyzed using protease from *Bacillus sp.* E13 at 55°C, pH 11 for 30 and 60 minutes. Hydrolysates were sequentially fractionated and evaluated for antibacterial and antioxidant activities. Antibacterial assay showed that *Staphylococcus aureus, Listeria monocytogenes, Escherichia coli* and *Salmonella typhimurium* were inhibited by hydrolysate from both hydrolysis time 30 and 60 min. The fraction of <3 KDa from both hydrolysis times showed the strongest bactericidal activity to *E. coli* and *S. typhimurium* with minimum inhibitory concentration (MIC) of 0.058 mg protein/mL. One µg protein/mL of the fraction <3 KDa from 30 min hydrolysis showed scavenging activity which similar to that of 7 µg/mL vitamin C for 2,2'-*azino-bis* (3-*ethylbenzthiazoline*-6-*sulphonic acid* (ABTS) radical and 1.5 µg/mL vitamin C for 2,2 diphenyl 1 picrylhydrazyl (DPPH) radical. The result indicated that the peptide fraction of <3 KDa from 30 min hydrolysis was very potential as antibacterial and antioxidant.

Key words: Bioactivities · Hydrolysate · Goat Milk · *Bacillus Sp*. E.13

INTRODUCTION

Goat milk becomes a particular interest because of its nutritional value and nutraceutical properties such as its bioactive peptides. Active peptides become promising future drug because they can be multifunction and this would reduce the quantity and type of drug which should be administered [1]. Bi-or multi-functional drug may have much improved potencies due to their synergistic effect or may produce fewer side effects than compound acting at a single target. Compared with drug combination, there are several advantages associated with bi- or multi-functional bioactive peptides such as having more predictable pharmacokinetic and pharmacodynamic characteristics because the peptide will be administered as a single medicine [1]. Continuous exploration of bioactive peptides is needed to obtain maximum potential for medical application.

Most of bioactive peptides are not available in the native form but bound within certain proteins. Enzymatic hydrolysis is the commonly method used to release the bioactive peptide from its native proteins. The hydrolysate exhibited advantages such as improvement in water-binding capacity, emulsifying stability, protein solubility and nutritional quality [2] with biological activities beneficial for improving health. Selective hydrolysis can be achieved by application of certain hydrolysis conditions and employing protease from specific sources with desired specificity for peptide bonds adjacent to certain amino acid. Combination of enzyme specificity and hydrolysis condition produce unique peptide sequence that may be physiologically active. Therefore, enzyme and hydrolysis conditions are critical factors to obtain the target peptides and variation on both factors will affect activity of the peptides produced.

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Bioactive peptides present in a complex mixture of hydrolysate need to be fractionated, concentrated and purified to get its maximum effect. There are several techniques for fractionation and isolation of the desired peptide from the non-hydrolyzed protein or other contaminants. Membrane separations are suitable because the technique apply mild working condition. In addition, it is relatively easy to scale up and require lower cost for processing compare to the liquid chromatographic techniques [3]. Ultra-filtration using membrane with specific molecular weight cut off (MWCO) enables us to concentrate the peptide in certain molecular weight by avoiding excessive concentration of salt and to facilitate finding the active peptides [4].

In this study, we used microbial protease to hydrolyze goat milk protein. The protein hydrolysate was then fractionated sequentially using specific MWCO membrane. Then the produced protein hydrolysate was tested for its antibacterial and antioxidant activities.

MATERIALS AND METHODS

Microorganisms: *Bacillus sp.* E13 was used to produce protease. The bacteria was isolated from horse milk and showed highly active protease indicated by clear zone when the bacteria was grown in skim milk agar in preliminary study). *Escherichia coli* (ATCC 25922), *Salmonella Typhimurium* (ATCC 13311) *Listeria monocytogenes* (ATCC 15313) and *Staphylococcus aureus* (ATCC 25923) were used for antibacterial assays.

Milk and Enzyme Preparation: Fresh Etawa crossbreed goat milk was collected from Bogor Agricultural University farm, Indonesia. The milk was defatted by centrifugation at 6000 ×g, 4 °C for 15 min, then hydrolyzed immediately or stored at -20 °C until used. Protease was produced from *Bacillus sp.* isolated previously from local horse milk. The bacterium was cultured in Luria Bertani Broth (Difco, Becton Dickinson and Co, US) containing 0.05 % (w/v) skim milk and incubated at 37 °C for 24 h. The cells were separated by centrifugation 3500 ×g for 20 min. The supernatant was collected, mixed with ammonium sulphate 50 % saturation (w/v) and incubated at 4 °C overnight. Crude enzyme was collected by centrifugation at 10.000 ×g, 4 °C for 20 min. The precipitate was air dried and stored at -20 °C until used. For hydrolysis, crude enzyme was diluted in phosphate buffer saline (Sigma) 10 mM, pH 7.4 (1:2 (w/v)). Protein concentration was analyzed using Quick start ™ Bradford protein assay (Bio-Rad Inc). Standard curve was obtained from reaction between 5 µL of bovine serum albumin at serial dilution 2.5-25 mg/mL and 95 µL Bradford solution. Deionized water was used as blank. Absorbance was measured at λ 600 nm in microtiter plate reader (Labsystems, original Multiscan Ex, Champaign USA).

Enzyme activity assay was conducted according to Bergmeyer *et al*. [5]. Reaction was performed by mixing 250 µL casein 2% (w/v) with 50 µL and 250 µL PBS 0.05 M pH 7, incubated at 37 °C. As much as 500 µL of TCA 0.2 M (Sigma-Aldrich, USA) was added, incubated at 37 °C for 10 min and then centrifuged at 2000 × g for 10 min. As much as 375 µL supernatant was mixed with 1250 µL Na_2CO_3 0,4 M and 250 µL reagent *Folin ciolcateau* (Sigma-Aldrich, USA) (1:2) and then incubated at 37 °C for 20 min. Optical density was measured at λ 578 nm (Spectrophotometer UV-Mini-1240 Shimadzu). Aquadest was used as blank and 5 mM tyrosine (Sigma-Aldrich, USA) was used as standard. One unit (IU) of enzyme was defined as the amount of enzyme needed to produce 1 µmol tyrosine per min.

Enzymatic Hydrolysis: Goat milk was hydrolyzed with protease from *Bacillus sp*. (0.67 IU) with enzyme substrate ratio 1:20 (v/v). Hydrolysis was conducted at 55 °C, pH 11 for 30 and 60 min. The reaction was stopped in boiled water 95 °C for 5 min and centrifuged at 10.000 ×g for 15 min to discard the un-hydrolyzed precipitated protein. The supernatant was adjusted to pH 7 and filtered using 0.45 nm membrane (Acrodisc LC 13 mm, 0, 45 µm, PVDF, Pall Life Sciences, USA). Hydrolysate was sequentially filtered through membrane MWCO 30k, 10k and 3k (AMICON Ultra centrifugal units, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co). Retentate was diluted with the same volume of sterilized deionized water. Protein concentration of each fraction was determined using Quick start ™ Bradford protein assay (Bio-Rad Laboratories Inc). All fractions were evaluated for antibacterial and antioxidant activities.

Antibacterial Assay: MIC (Minimum inhibitory concentration) was performed according to Keeper *et al*. [6] with modification. Briefly, 100 µl of each peptide fraction was prepared in 2-fold dilution series in 96-well round-bottom microplates. As much as 100 µl *E. coli* or *S. typhimurium* suspensions of 10^6 CFU/mL was then

added to the microtiter plate containing peptide fractions. The content of the well (100 µl) was grown onto MacConKey agar plate (BD Difco™, Becton Dickinson and Co, USA) and incubated for 24 h at 37 °C. Additional 24 h incubation was done to ensure no bacterial growth. The MIC was recorded as the lowest concentration of the peptide fraction which produced no visible bacterial growth. Each treatment was done in three replications.

Antioxidant activity: 2,2'-*azino-bis* ([3-*ethylbenzthiazoline*-6-*sulphonic acid*]-diammonium salt) (ABTS) solution (Sigma-Aldrich, USA) was made according to Thaipong *et al*. [7] with some modifications. The working solution was prepared by mixing stock solution of 7.4 mM ABTS radical and 2.6 mM potassium persulphate and allowed to react for 18 h at room temperature in the dark. The solution was diluted with deionized water to obtain an absorbance of 1.1 ± 0.05 at 405 nm. The fresh ABTS was then used for antioxidant assay. As much as 100 µL of each peptide fraction was mixed with 200 µL ABTS in microplate and incubated at room temperature for 15 min to allow the reaction. The absorbance was recorded at λ 405 nm using microplate reader (Labsystems, original Multiscan Ex, Champaign, USA). The control was made by substituting ABTS radical with deionized water, while ABTS control was made by substituting peptide fraction with deionized water. Serial concentrations of Vitamin C p.a (2.5-17.5 µg/mL) was used as standard.

Antioxidant assay using 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) was conducted by as follow. Ethanol 96% was mixed with DPPH to obtain absorbance 1.1 ±0.05 at λ 540 nm. As much as 100 µL of peptide fraction was added to 200 µL DPPH and allowed to react for 30 min at room temperature. Absorbance of the mixture was measured at λ 540 nm. Vitamin C p.a (0.5-2.5 µg/mL) was used to make standard curve [7]. The scavenging activity of peptide fractions to ABTS and DPPH radicals was expressed using equation: Scavenging activity (%) = 100 x (A0-A1)/A0, where A0 was absorbance of ABTS/DPPH and A1 was the final absorbance of sample minus initial absorbance. Result from ABTS and DPPH assays was presented as means of experiments performed in four replications.

Hemolysis Assay: Hemolysis assays were performed using experimental procedure described by Lorenzon *et al*. [8] and Nguyen *et al*. [9]. Freshly prepared chicken red blood cells (RBCs) were washed three times with 0.01 M Tris-HCl (pH 7.4) containing 0.15 M NaCl (Tris-saline). A suspension of 1% (v/v) erythrocytes was made by re suspending cells in Tris-saline. A 100 µl peptide was added with 100 µl RBCs, incubated for 1h, at 37 °C. The samples were centrifuged at 3,000 *x*g for 5 min. A 100 µl of the supernatant was transferred to 96-well microplate and the absorbance was determined at λ 540 nm. The assay was performed in triplicate. A 1% Triton X-100 (Sigma-Aldrich, USA) solution was used as positive control (100% lysis) and Tris-saline as a negative control.

RESULTS AND DISCUSSION

Antibacterial Assay: Hydrolysate of goat milk hydrolyzed by *Bacillus sp*. E.13 protease and its fractions were active to inhibit all test microorganism (*S. aureus, L. monocytogenes, E. coli* and *S. typhimurium*) (Table 1). Minimum inhibitory concentration assay (MIC) showed that hydrolysate and its fractions were more active to Gram negative bacteria. Several reports of protease *Bacillus* showed that protease from that species has been used to hydrolyze protein to produce antimicrobial peptide. Hydrolysis of goat milk casein using *Bacillus* sp. P45 produced antimicrobial peptide against *S. enteritidis*, *E. coli* and *L. monocytogenes* [10].

In this research, we found that Gram positive bacteria (*S. aureus* and *L. monocytogenes*) were relatively more resistant to the most fractions (Table 1) compared to Gram negative bacteria (*E. coli* and *S. typhimurium*). Gram positive bacteria such as *S. aureus* was able to develope an efficient and unique way for controlling resistance mechanisms to antibacterial peptides such as releasing extracellular protease and modifying cell wall. Antibacterial peptide commonly form pore within the bacterial membrane that affect membrane physiology. In Gram positive bacteria, rigidity of the cell wall contributes to the bacterial capability to defend itself against turgor pressure up to 3 to 25 times higher than in the Gram negative bacteria [11]. The ability of Gram positive bacteria to defend itself from antibacterial peptide may also due to their ability to release extracellular protease, such as aureolysin by *S. aureus* which could hydrolyze the C terminal of antibacterial peptide LL-37 and hence the peptide become inactive [12]. Genetic factor such as genes encoding a sensor histidine kinase in *S. aureus* has been also reported to be responsible to develop and maintain antibacterial peptide resistance [13].

Table 1: Minimum inhibitory concentration (MIC) of peptide fractions

Indicator microorganism	*Minimum inhibitory concentration* (MIC) of peptide fractions (mg protein/mL)									
	30 min hydrolysis					60 min hydrolysis				
	Fraction					Fraction				
	Hydro-lysat	>30 KDa	10-30 KDa	3-10 KDa	<3 KDa	Hydro-lysat	>30 KDa	10-30 KDa	3-10 KDa	<3 KDa
S. aureus	>20	>0.42	>0.78	>0.16	>0.22	>17	>0.42	>0.17	>0.16	>0.14
L. monocytogenes	20	>0.42	>0.78	>0.16	>0.22	17	>0.42	>0.17	>0.16	0.14
E. coli	10	0.21	>0.78	>0.16	0.058	8.50	0.42	>0.17	>0.16	0.07
S. Typhimurium	10	0.21	>0.78	>0.16	0.058	8.50	0.42	>0.17	>0.16	0.07

The most active fraction as antibacterial peptide was fraction <3kDa from the 30 min hydrolysis and the Minimum inhibitory Activities (MIC) to *E. coli* and *S. typhimurium* was 0.058 mg protein/mL. The result showed that the fraction <3 kDa was more active against *E. coli* ATCC 25922 than chicken β-defensin with MIC 0.1 mg/mL [14]. In addition, the MIC level of fraction <3kDa from the 30 min hydrolysis was also lower than MIC of fraction A1-45 (0.554 mg/mL) and A1-49 (0.5 mg/mL) from bovine casein hydrolyzed with protease *L. acidophilus* DPC6026 and fraction A1-54 (1.24 mg/mL) [15] indicated the fraction <3 KDa was highly active as antibacterial. Fractions >30 KDa resulting from 30 and 60 min hydrolysis were also active against *E. coli* and *S. typhimurium*. The opposite result was demonstrated by bovine casein peptide of >10 kDa which showed no antibacterial activity [15]. It is possible that fraction >30 KDa in our study originated from shorter peptide which formed oligomer. Some peptides form dimer, trimer or oligomer which increased the molecular weight. As the antibacterial purified peptide from heart of goat (*Capra hircus*) was detected in 6.5, 13 and 19.7 KDa molecule which may arise the possibility of di, tri, or oligomer formation [16].

Antioxidant Activity: Higher antioxidant activity was shown by higher percentage of scavenging activity. In ABTS assay, peptide fraction with molecular weight of 10-30 KDa was the most active to scavenge ABTS radical in comparison to the other fractions at the same concentration (Figure 1). Peptide with <3 KDa could scavenged better than peptides 3-10 KDa. Generally, peptides from the 30 min hydrolysis were better antioxidant towards ABTS compared with that from 60 min hydrolysis except for fraction <3 Kda. In ABTS assay, the highest antioxidant activity was shown by fraction 10-30 KDa resulted from both 30 and 60 min hydrolysis.

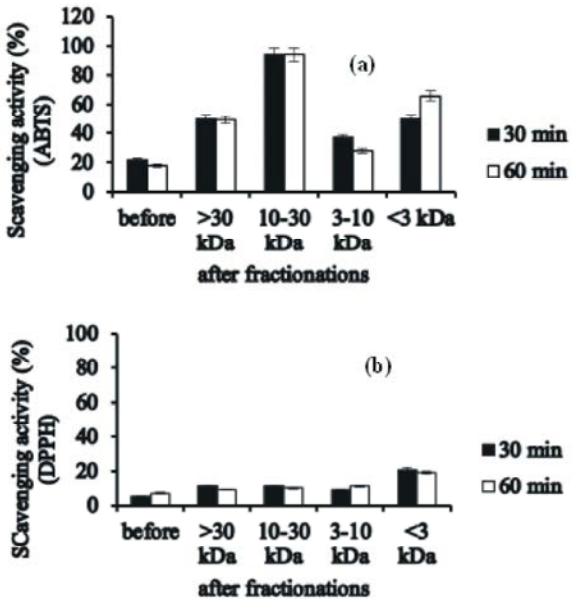

Fig. 1: Scavenging activities of goat milk hydrolyzed at 30 and 60 min, before and after fractions. Scavenging activity was determined based on reduction optical density at λ 405 nm for ABTS assay (a) and λ 540 nm for DPPH assay (b).

The ABTS radical scavenging activity of the fraction 10-30 KDa was 94%. The activity was higher than that of the low molecular weight goat milk protein hydrolyzed with trypsin (± 50%) and subtilisin (80-87%) [17]. Higher molecular weight peptides (10-30 KDa) scavenged ABTS radical are better than lower peptides (<10 KDa). This result is different from other peptide hydrolysates reported previously. Peptide hydrolysate of <10 KDa extracted from velvet antler hydrolyzed by alcalase showed higher ABTS radical scavenging activity compare to peptide >10 KDa fractions [18]. Application of different enzymes and protein may produce peptides with different sizes, sequences and bioactivities.

In DPPH assay, the scavenging activity of the hydrolysate and its fractions were less potent than their activity in ABTS assay (Figure 1b). The most active peptide was fraction <3KDa. Peptide fraction >30KDa, 10-30KDa and 3-10 KDa had similar scavenging activity (Around 10%) for both hydrolysate 30 and 60 min. All fractions showed better result than that of hydrolysate before fractionation. The result showed that fractionation of the hydrolysate enhanced scavenging activity to both ABTS and DPPH radicals. Scavenging activity in DPPH of the hydrolysates and their fractions were lower than that in ABTS assay. Similar result was shown by antioxidant activity of human milk reported earlier [19]. The low value assay is probably due to some limitations of the DPPH method. The reaction of DPPH radical with most antioxidant is slower than in the case of ABTS radical. Moreover, milk contains components whose spectra overlap with DPPH spectra which can distort the spectrophotometric measurement [19]. DPPH also dissolves only in polar matrices and more suitable for lipophilic compounds [20]. ABTS assay is compatible with both aqueous and organic solvent systems and not affected by ionic strength, thus can be used in various media to determine both hydrophilic and lipophilic antioxidant [21]. The result indicated that the goat milk hydrolysate in this research may be more water soluble than lipophilic compound. As in human milk, all these arguments suggest that the DPPH method may not be the best for determination of antioxidant activity for hydrolysate goat milk and its fractions.

Hemolytic assay was used to evaluate possible toxicity of the active hydrolysate fraction. High percentage of hemolytic activity means that the hydrolysates or the fractions are toxic. The most active fraction as antibacterial activity was fraction <3 KDa induced the least hemolysis (3.1 %) (Fig. 2). which imply

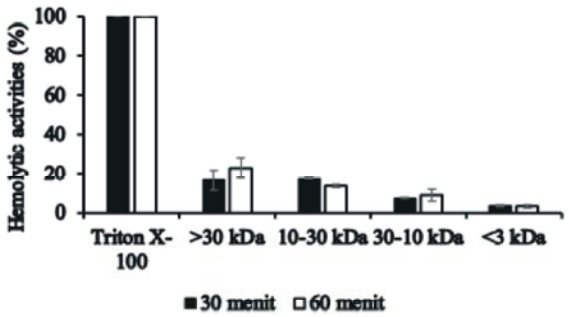

Fig. 2: Percentage of hemolytic activities of peptide fractions from hydrolysate 30 and 60 min hydrolysis, 1% Triton X-100 was a positive control (100% hemolysis).

that fraction <3 KDa is safe for application. The value was also lower than antibacterial peptide Lactocin 160 produced by *Lactobacillus rhamnosus* (8 %). *In vivo* safety evaluation to the rabbit indicated that Lactocin 160 was safe for application [22] which indirectly pointed to safety of goat milk peptide fraction <3 Kda in our study.

Overall results showed that peptides fractions from 30 min hydrolysis, before and after fractionation, had higher antibacterial and antioxidant activities than peptide fractions from 60 min hydrolysis. The highest antibacterial activity was shown by fraction of <3 KDa and the highest antioxidant activity was shown by fraction of 10-30 KDa. Based on the hemolytic assay, peptide fraction of <3 KDa was more safe than fraction of 10-30 KDa. Although antioxidant activity of fraction <3 KDa was not the highest, the fraction was still highly potent as antioxidant as the activity of one µg protein/mL fraction of <3 KDa was similar to 7 µg/mL vitamin C for scavenging to ABTS radical and 1.5 µg /mL vitamin C for scavenging to DPPH radical.

CONCLUSIONS

Peptides from 30 min hydrolysis had antibacterial and antioxidant activities better than peptides from 60 min hydrolysis. Fraction <3 Kda was the most active as antibacterial peptide and fraction 10-30 KDa was the most active as antioxidant peptide. Considering of the safety, peptide from fraction <3 KDa was the most potent as antibacterial and antioxidant peptide.

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