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Bioactivities of Peptides from Sumbawa Horse Milk Hydrolyzed by Bromelain

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Abstract

Sumbawa horse milk is used traditionally to cure several diseases such as hypertension, diabetes, asthma and hypercholesterolemia. However, research on enzymatic hydrolysis of Sumbawa horse milk and exploration of their potential bioactive peptide have not yet been reported. The aim of this study was to evaluate the bioactivities of the peptides from Sumbawa horse milk hydrolyzed by bromelain. Sumbawa horse milk was hydrolyzed by bromelain extract at various pH and time of hydrolysis and fractionated by membrane molecular weight cut off (MWCO) 10 KDa. The peptic hydrolysates and fractions obtained were then assayed for antibacterial and antioxidant activities. The results showed that peptide from Sumbawa horse milk hydrolyzed at pH 6 for 60 min has the highest antibacterial and antioxidant activities. The peptide inhibited *Escherichia coli, Salmonella* Typhimurium and *Listeria monocytogenes,* and scavenged 2,2'-*azino-bis* (3-*ethylbenzothiazoline*-6-*sulphonic acid*) (ABTS) radical up to 100 % per 0.1 mg protein/mL. Fractionation using 10 KDa membrane showed that peptide <10 kDa has higher antibacterial and antioxidant activities compared to both before fractionation and fraction >10 kDa. Hemolytic assays using hydrolysate and its fractions did not cause lysis of red blood cells, indicating that they are safe for consumption.

Key words: Sumbawa horse milk, bromelain, hydrolysis, bioactivities

Introduction

Horse milk is minor importance in milk production in comparison to cow and goat milk. Resemblance with human milk in many respect make horse milk have been traditionally important and claimed to have special therapeutic properties. Overall, horse milk is considered to be highly digestible, rich in essential nutrient and possesses an optimum whey: casein protein ratio, making it suitable in pediatric dietetic (Potocnik et al., 2011). Nutritional and therapeutic properties are beneficial for elderly diet. Around 30 million people consume horse milk regularly throughout the world. In Turks, Bashkirs, Kazaks, Mongol, Yakuts and Uzbeks they make lactic-alcoholic beverage called Koumiss (Potocnik et al., 2011). Donmez et al. (2014), reported that triglyceride and cholesterol were decreased but high density lipoprotein (HDL) increase significantly after consumption of Koumiss for 15 days. Administration of koumiss to cancer patients was able to decrease adverse effect of chemotherapy (Uniackle-Lowe et al., 2010).

In Indonesia, Sumbawa horse milk is claimed to cure some diseases such as asthma, hypertension, diabetes and gastrointestinal disorder. The milk and its fraction not only showed some bioactivities but also contain natural microorganism which able to inhibit growth of some pathogenic bacteria. Fractionation of Sumbawa horse milk with different polarity showed that whey protein fraction, soluble in acetone exhibited significant inhibition on the growth of *S. agalactiae* and *S. pyogenes* (Detha et al., 2013). Sujana et al., (2008), reported that Sumbawa horse milk was dominated by *Lactobacilli* and *Weisella/Leuconostoc* and some of these isolate showed antibacterial activity against *Escherichia coli* 25922, *Shigela flexneri, Salmonella* Typhimurium, and *Staphylococcus aureus* 29213. Horse milk not only contain beneficial protein and good microorganism but also contain bioactive peptides that may able to act as antimicrobial, antioxidant, angiotensin converting enzyme (ACE) inhibitor and immunomodulation. These peptides generally remain latent until they are activated by enzymatic hydrolysis. Exploration of potential bioactive peptide derived from Sumbawa horse milk have not been reported. The aim of this study was to evaluate the bioactivities of the peptides from Sumbawa horse milk hydrolyzed by bromelain.

Material and Methods

Material

Fresh Sumbawa horse milk was obtained from Penyaring village, Mojo Utara, Sumbawa Besar.

Bacteria used for antibacterial assay were *Listeria monocytogenes* (ATCC 15313), *Escherichia coli* (ATCC 25922) and *Salmonella* Typhimurium (ATCC 13311).

Methods

Milk and Enzyme preparation

The milk protein was separated from fat by centrifugation 6000 ×g at 4°C for 10 min. The defatted milk was hydrolyzed immediately or stored at -20°C until used. Bromelain was extracted from young pineapple fruit by using acetone extraction (Rowan et al., 1990). Pineapple fruit was peeled and extracted by using food processor. Juice obtained was filtered by Whatman 41 filter, added with acetone in a ratio 1:1 and keep on 4°C overnight. Crude bromelain was collected by centrifugation at 6000 x g for 15 min. Precipitate was air drying and stored at -20°C until used. For hydrolysis, crude enzyme was diluted in phosphate buffer saline 10 mM, pH 7.4 (1:2). Protein concentration was assayed using Bradford reagent (Quick start ™ Bradford protein assay, Bio-Rad Inc) method while enzyme activity assay was conducted according to Bergmeyer and Grassel (1983).

Hydrolysis

Defatted horse milk was hydrolyzed with bromelain (0.1 U/mL) with enzyme substrate ratio 1:4, at 50 °C, pH 5 and 6 for 10, 20, 30 and 60 min. The reaction was stopped in boiled water 95 °C for 5 min. The hydrolysate was centrifuged at 10.000 g for 15 min to discard the precipitated un-hydrolyzed protein, and the supernatant was sterilized using 0.45 nm membrane. Hydrolysate were assayed for antimicrobial and antioxidant activity directly and some was filtered through AMICON Ultra centrifugal units (Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co, MWCO 10 kDa). The retentate and permeate were evaluated for antimicrobial and antioxidant activities.

Peptide analysis and characterization

Peptide profile from pH 6 and 60 min hydrolysis was analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) by using precast gradiend polyacrylamide gel electrophoresis 4-20% (Any kD™ Mini-Protean® TGX gel, BioRad Laboratories Inc) with coomassie blue staining (Sigma-Aldrich, USA).

Hydrolysate from pH 6 and 60 min hydrolysis was run in reverse phase-high performance liquid chromatography (RP-HPLC) according to (Mc Cann et al., 2005). A preparative C-18 column (25 cm x 1.0 cm id); Xterra (Waters Corp.) was used with HP 1050 series HPLC (Waters Corp.) equipped with diode array detector and monitored at 215 nm. The solvents used were: (A) 0.1% (v/v) trifluoroacetic acid (TFA) (Merck Pty. Ltd., Kilsyth, Vic., Australia) in acetonitrile and (B) 0.1% (v/v) TFA in deionized water, at a flow rate 1 mL/min. The HPLC system was equilibrated with 95% solvent A for 5 min, followed by linear gradient of 5-45 (v/v) solvent B over 15 min to elute peptides and a 5 min for re-equilibration.

Antibacterial assay.

Screening of antibacterial activity was conducted by mixing a 100 µL Brain Heart Infusion Broth containing bacteria 10^6 CFU/mL-1 with 100 µL peptic hydrolysate (0.1 mg/mL) (1:1) and incubated at 37 °C for 2 h. As much 10 µL of the mixture was dropped onto Mueller Hinton Agar plate, incubated at 37°C for 24 h. Inhibition was observed based on the growth of bacteria.

Selected peptic hydrolysate was then fractionated and assayed for antibacterial activity. As much as 100 µL Brain Heart Infusion Broth containing bacteria 10^6 CFU/mL was mixed with 100 µL peptic hydrolysate sample (0.1 mg/mL) (1:1) and incubated at 37 °C for 2 h. A serial dilution was made then the mixture was grown on Mueller Hinton agar. The number of colonies was counted after incubation at 37 °C for 24 h. Phosphate buffer saline (10 mM, pH 7.4) was used as control to replace the peptide. All treatments were done in triplicate.

Antioxidant activity.

2,2'-*azino-bis* (3-*ethylbenzthiazoline*-6-*sulphonic acid* (ABTS) solution were made following Thaipong *et al.*, (2006), with some modification. The working solution was prepared by mixing stock solution of 7.4 mM ABTS and 2.6 mM potassium persulphate in equal volume 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 units at 405 nm. The fresh ABTS radical was then used for antioxidant assay. As much as 100 µL peptide fraction (0.1 mg/mL) was mixed with 200 µL ABTS radical in a microplate and incubated at room temperature for 15 min to allow reaction. The absorbance was recorded at λ 405 nm using microplate reader (Labsystems, original Multiscan Ex). Peptic hydrolysate control was made by substituting ABTS radical with deionized water, while ABTS control was made by substituting peptic hydrolysate with deionized water. Serial concentrations of Vitamin C was used as standard (2.5 – 17.5 µg/mL).

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 (0.1 mg/mL) 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 as standard (Thaipong

et al. 2006)[9]. The scavenging activity of peptide fractions to ABTS and DPPH radicals was expressed using equation:

Scavenging activity (%) = 100 x (A0-A1)/A0

A0 was the initial absorbance of ABTS/DPPH and A1 was the final absorbance of sample subtracted by initial absorbance of the peptide fraction. Result from ABTS and DPPH assays was presented as means of experiments performed in three replications.

Hemolysis assay

Hemolysis assays were performed using experimental procedure described by Lorenzon *et al.*, (2012) and Nguyen et al., (2011). Chicken red blood cells (RBCs) were prepared by washing 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 2 h, at 37°C. The samples were centrifuged at 2000 *x* g for 5 min. A 100 µl of the supernatant was transferred to 96-well microplate and the absorbance was determined at 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.

Result and Discussion

Antibacterial and antioxidant activities of peptic hydrolysate

Hydrolysis of Sumbawa horse milk using bromelain at various time and pH, resulted peptic hydrolysate showed antibacterial activity as revealed by the inhibition of bacterial growth (Table 1). Peptic hydrolysate from 60 min hydrolysis and pH 6 was the most potent as it inhibited all bacteria tested, *E. coli*, *S.* Typhimurium and *L. monocytogenes*. Generally, pH 6 yielding better antibacterial activity than that of pH 5, indicated that pH 6 was more suitable to produce bioactive peptide. Further screening for antioxidant activity was only used peptic hydrolysate generating from hydrolysis at pH 6 in order to obtain the most antioxidant peptide from the peptide which had antibacterial activity.

Table 1. Inhibition of bacterial growth after exposed by Sumbawa horse milk hydrolysate for 2 h.

No		Antibacterial activity in hydrolysis condition							
		10 min		20 min		30 min		60 min	
	Bacteria	pH5	pH6	pH5	pH6	pH5	pH6	pH5	pH6
1	*E. coli*	-	+	+	+	-	-	+	+
2	*S.* Thyphimurium	+	+	-	-	-	+	-	++
3	*L. monocytogenes*	+	-	+	+	+	+	-	++

Note: -: no inhibition; +: weak; + +: medium; + + +: strong; + + + +: very strong/no bacteria grew.

Antioxidant activity was defined by measurement of the peptide ability to scavenge ABTS and DPPH radicals. Scavenging activities of peptic hydrolysate from pH 6 was shown at Figure 1.

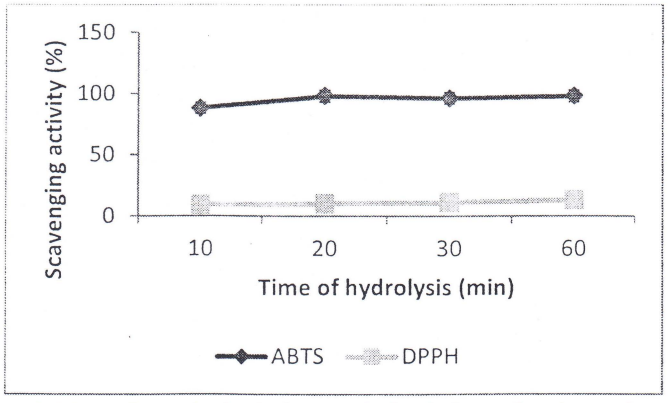


Figure 1. Scavenging activities of peptic hydrolysate derived from Sumbawa horse milk hydrolyzed by bromelain at pH 6.

Scavenging activity to ABTS radicals from peptic hydrolysate of 20 and 60 min was similar (98%) higher than that of 10 min (88%) and 30 min hydrolysis (96%). The activity of peptic hydrolysate to DPPH radicals was very low but the order was similar to ABTS result. The peptic hydrolysate of 60

min hydrolysis showed the highest scavenging activity against DPPH radicals (13%).

SDS-PAGE analysis showed that hydrolysis of Sumbawa horse milk by bromelain for 60 min, which has the highest antibacterial and antioxidant activity, resulting peptide with molecular weight 2-17 kDa (Figure 2). Most of protein with molecular weight >20 kDa such as IgG, Lactoferrin, serum albumin and casein were hydrolyzed generating low molecular weight peptide. Peptic hydrolysate 60 min profiling using HPLC revealed that the peptic hydrolysate was hydrophobic as peak appear in hydrophobic mobile phase (Figure 2).

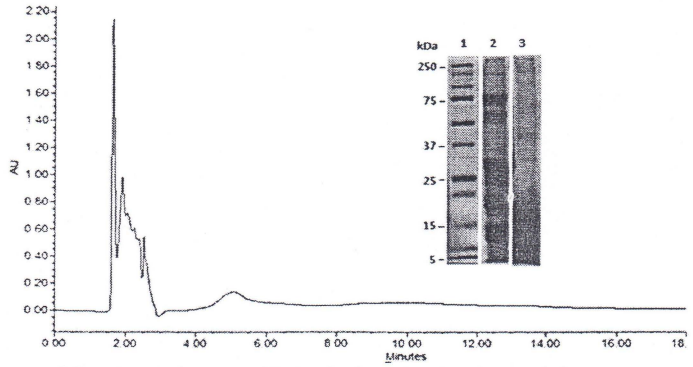
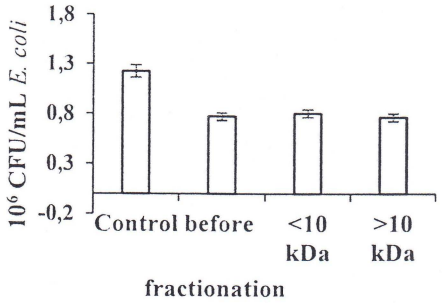


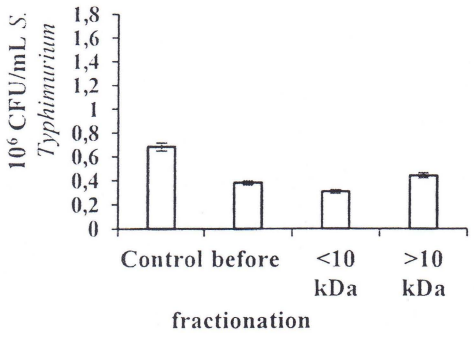
Figure 2. Chromatogram of Sumbawa horse milk hydrolyzed using bromelain extract at pH 6 for 60 min from reverse-phase HPLC obtained via UV/VIS detector wavelength of 215 nm and its SDS-PAGE analysis: 1. Marker, 2.Sumbawa horse milk before hydrolysis, 3. after hydrolysis for 60 min.

Screening bioactivities of peptic hydrolysate showed that peptide from 60 min hydrolysis at pH 6 was the most potent as for antibacterial and antioxidant peptide. The following assay was conducted in order to obtain the active fraction and its safety for further application

Fractionation and bioactivities peptic hydrolysate 60 min, pH 6

Peptide hydrolysate generated from 60 min, pH 6 of hydrolysis was then fractionated using membrane 10 kDa. Antibacterial assay revealed that antibacterial activities against *E. coli* of peptide from hydrolysis 60 min at pH 6, before and after fractionation, was not much different (Figure 3). For *S.* Typhimurium, peptide fraction <10 kDa was more active than before fractionation and fraction >10 kDa. Different from *E. coli* and *S.* Typhimurium, *L monocytogenes* was strongly inhibited by fraction of <10 kDa and >10 kDa, indicated that fractionation of the peptide was very important for antibacterial activity against *L. monocytogenes* (Figure 3). Muro et al., (2013), stated that fractionation using filtration membrane was useful method to semi purified of desired peptide from the non-hydrolyzed protein or other contaminants. Therefore, fractionation was able to enhance bioactivity of peptide.





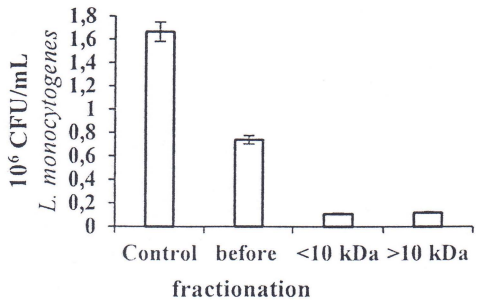


Figure 3. Colony count of antibacterial assay of Sumbawa horse milk was hydrolyzed using bromelain extract at pH 6 for 60 min before and after fractionated by 10 kDa membrane.

Compare to other antibacterial peptides, the activity of fraction of <10 kDa and >10kDa from this research was low. That may due to the peptide need further fractionation using lower MWCO membrane such as 5 or 3 kDa. Hayes et al., (2006), reported that antibacterial activity of the peptide was not observed at peptide >3kDa. Generally, antimicrobial peptide consists of 10-50 residue with low molecular weight such as LL-37, antimicrobial peptide from human defensin (4.5 kDa) (Thennarasu et al., (2010). However some of antimicrobial peptide has higher molecular weight. Antimicrobial peptide isolated from hemolymph of wild *Apis florae* and *Apis carnica*, bees of Saudi Arabia has molecular weight ranges from 14.5 – 15 kDa (Ayaad et al., 2012). In consideration, sometimes antimicrobial peptide were found in di, tri or oligomer form such as antimicrobial peptide purified from heart of goat (*Capra hircus*) was detected in 6.5, 13 and 19.7 kDa (Banerjee et al., 2015), therefore, it is possible that actual molecular weight was lower than which detected on the electrophoresis gel.

As shown in Figure 3, hydrolysate and its fraction showed strong inhibition to *L. monocytogenes*. The bacterium was able to contaminate raw milk (Hunt et al., 2012) or meat (Pradhan et al ., 2010). The ability of peptide derived from Sumbawa horse milk to inhibit *L. monocytogenes* will be able to use as food preservation and control *L. monocytogenes* in food product.

For antioxidant assay, scavenging activity to ABTS radicals at protein content 0.1 mg/mL, before and after fractionation not significantly different (Figure 4). Fraction of <10 kDa was the highest (100%) compare to before fractionation (98%) and fraction of >10kDa (97%). This may due to the concentration of the fraction still high to differentiate the activity. When the concentration was decreased to 0.01 mg/mL, scavenging activity of fraction <10kDa was still high (98%) while the activity of peptide before fraction was 27% and fraction of >10 kDa was 87%. The value of <10 kDa fraction was higher than scavenging activity of rapseed albumin hydrolyzed using alcalase and flavorzyme which resulting value 90% for 0.2 mg/mL (Yu et al., 2013). Scavenging activity to ABTS radical for 10 µg/mL protein content of fraction of <10 kDa was similar to 13 µg/mL vitamin C.

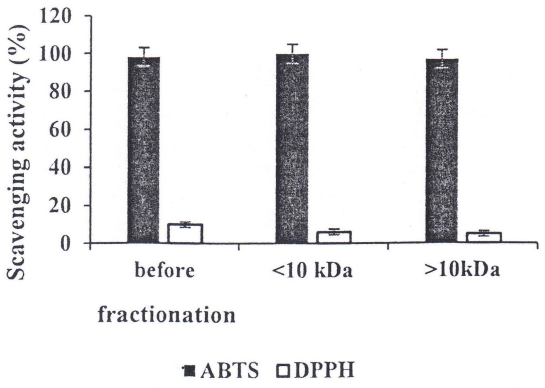


Figure 4. Scavenging activities of Sumbawa horse milk hydrolyzed using bromelain extract at pH 6 for 60 min after fractionated by 10 kDa membrane.

Fractionation using 10 kDa membrane enhance the antioxidant activity indicated by its higher scavenging activity compare to before fractionation. The peptide fraction <10 kDa has the highest activity. The similar result was shown by the peptides from crude protein hydrolysate the aqueous extract of velvet antler (*Cervus elaphus*) prepared by using pancreatin-pepsin, alcalase and neutrase. Peptic hydrolysate <10 kDa showed higher ABTS radical scavenging activity than peptide >10 kDa fractions (Zhao et al., 2011). Filtration using molecular weight cut off membrane was able to increase the antioxidant activity compare to the native protein isolate and hydrolysate (Ajibola et al., 2011).

In DPPH assay, scavenging activity of peptic hydrolysate before fractionation was better than after fractionation using 10 kDa membrane. The decreasing effect of thus separation may be due to from several factors. Interaction between peptide with various sizes maybe needed to optimize their antioxidant activities. The same synergistic interaction between peptide with antioxidant activity was shown by half-fin anchovy (HAHp) peptides. DPPH radical scavenging activities of peptide HAHp1-2III (901.45 Da), HAHp1-2IV (872.37 Da) or HAHp1-2V (1171.60 Da) were enhanced if they were mixed compared with when they were separated[22].

Scavenging activity to DPPH radical for peptic hydrolysate before fractionation was 10% while fraction of <10 kDa was 6% and fraction of >10 kDa was 5%. The value for 100 µg/mL fraction of <10 kDa was similar to vitamin C 1.5 µg/mL. DPPH assay demonstrated very low scavenging activity compare to ABTS assay. This may related to compatibility of assay with the nature of the peptide[23] although both assay were neutralize radical by either direct reduction via electron transfer or radical quenching via H atom transfer[24]. Other possibility was due to a presence of substances whose spectra overlap with DPPH spectra[25].

To evaluate the safety of the peptide was assayed using hemolysis assay. The hemolysis test has been the most commonly used procedure to assess and characterize the toxicities of antimicrobial peptide because it is a sensitive assay to evaluate eukaryotic membrane damage[10]. Peptide derived Sumbawa horse milk showed very low in toxicity indicated by low in hemolysis activity (Figure 5).

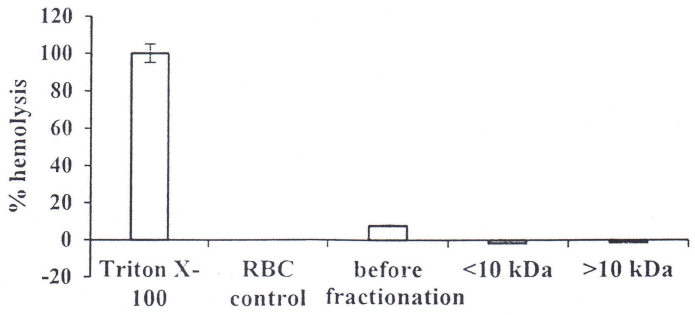


Figure 5. Percentage of hemolytic activity per 0.1 mg protein/mL peptic hydrolysate before fractionation, fractions <10 kDa and >10 kDa. Triton X-100 was a positive control (100% hemolysis) and red blood cell (RBC) was negative control (0% hemolysis).

Hemolysis activity peptic hydrolysate from 60 min hydrolysis at pH 6 of Sumbawa horse milk was 7.67 % while peptides after fractionation were negative. Negative value was obtained from calculation percentage of hemolysis from absorbance of RBC treated with peptide subtracted by absorbance of red blood cell control (0 % hemolysis). Red blood cell alone showed autolysis. The presence of the peptide was able to inhibit autolysis produce absorbance lower than RBC alone. The result indicated that the fraction was safe for further application.

Conclusion

Peptide from Sumbawa horse milk hydrolyzed by bromelain pH 6 from 60 min hydrolysis showed the highest antibacterial and antioxidant activities. The peptide and its fractions inhibited *Escherichia coli*, *Salmonella* Typhimurium and *Listeria monocytogenes*, and scavenged 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS). Peptide fraction of <10 kDa had the highest antibacterial activity and showed strong activity to *Listeria monocytogenes*. The peptide also scavenged ABTS radical up to 100%. The result suggested that peptide fraction of <10 kDa from Sumbawa horse milk potent to be antibacterial and antioxidant

peptide. The peptide is able to be a candidate of food ingredient or food preservation.

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