

Antimicrobial and Anti-inflammation Activities of Fraction and Single Peptides Derived from Mare Milk Protein

Kusumaningtyas E¹, Subekti DT¹, Fitaningtyas DFL²

¹Indonesian Research Centre for Veterinary Science, Jl RE Martadinata 30 Bogor, Indonesia

²Faculty of Pharmacy, Pancasila University, Srengseng Sawah Jagakarsa Jakarta, Indonesia
Email: enikusuma@yahoo.com

(received: 24-05-2019 ; revised: 01-07-2019 ; accepted: 23-08-2019)

ABSTRAK

Kusumaningtyas E, Subekti DT, Fitaningtyas DFL. 2019. Aktivitas antimikroba dan anti-inflamasi fraksi dan peptida tunggal derivat dari protein susu kuda. JITV 24(3): 112-121. DOI: <http://dx.doi.org/10.14334/jitv.v24.3.1976>

Susu kuda mengandung peptida bioaktif yang berguna bagi kesehatan manusia dan hewan. Peptida yang terkandung dalam fraksi atau peptida tunggal kemungkinan menunjukkan aktivitas yang berbeda. Tujuan dari penelitian ini adalah untuk mengevaluasi aktivitas antimikroba dan antiinflamasi peptida dalam bentuk fraksi dan tunggal derivat protein susu kuda. Uji antimikroba, fraksi <3 kDa dan peptida tunggal LVNELTEFAK (peptida 1), HPYFYAPELLYYANK (peptida 2), dan LANSLTEFAK (peptida 3) dilakukan terhadap *Escherichia coli* dan *Candida albicans*. Uji antioksidan dilakukan menggunakan metode 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) dan 2,2-diphenyl-1-picrylhydrazyl (DPPH). Pengaruh anti-inflamasi dideteksi dengan cara mengukur interleukin 1 β (IL- 1 β) dan Tumor Necrosis Factor - α (TNF- α) pada mencit setelah pemberian lipopolisakarida (LPS) *Escherichia coli* bersama-sama dengan fraksi atau peptida tunggal. Hasil penelitian menunjukkan bahwa peptida dalam bentuk fraksi memiliki aktivitas antibakteri, antifungi, dan antioksidan terhadap radikal ABTS lebih tinggi daripada semua peptida tunggal (P<0.05). Aktivitas antiinflamasi ditunjukkan oleh peptida 1 dan 2 ditandai dengan penurunan IL-1 β yang significant sesudah perlakuan (P<0.05). Berdasarkan hasil tersebut, disimpulkan bahwa aktivitas antimikroba dan antioksidan fraksi <3 kDa lebih baik daripada aktivitas peptida tunggal. Untuk aktivitas antiinflamasi, peptida tunggal menunjukkan aktivitas yang lebih baik dibandingkan fraksi

Kata Kunci: Antimikroba, antioksidan, anti-inflamasi, peptida, susu kuda

ABSTRACT

Kusumaningtyas E, Subekti DT, Fitaningtyas DFL. 2019. Antimicrobial and anti-inflammation activities of peptide fraction and purified peptides derived from mare milk. JITV 24(3): 112-121. DOI: [HTTP://DX.DOI.ORG/10.14334/JITV.V24.3.1976](http://dx.doi.org/10.14334/jitv.v24.3.1976)

Mare milk protein contains bioactive peptide which is beneficial for human and animal health. Peptides in the fraction and single may show different activities. The objectives of the study were to evaluate antimicrobial and anti-inflammation activities of the fraction and single peptide derived from mare milk protein. Antimicrobial assay was conducted by testing antibacterial and antifungal activities of fraction <3 kDa, single peptide LVNELTEFAK (peptide 1), HPYFYAPELLYYANK (peptide 2), and LANSLTEFAK (peptide 3) against *Escherichia coli* and *Candida albicans*. Antioxidant assay was conducted using 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) methods. Anti-inflammation effect was detected by interleukin 1- β (IL- 1 β) and Tumor Necrosis Factor - α (TNF- α) production in mice after administration of *Escherichia coli*'s lipopolysaccharide (LPS) and combined with fraction or single peptide. Result showed that peptide in fraction form has higher antibacterial, antifungal activities and antioxidant activities against radical ABTS than all of single peptide (P<0.05). Anti-inflammation activity was showed by peptide 1 and peptide 2 which was indicated by significantly decreasing of IL-1 β after treatment (P<0.05). Based on the results, it was concluded that antimicrobial and anti-antioxidant activities fraction <3 kDa was better than single peptide. For anti-inflammation activity, a single peptide showed better activity than fraction.

Key Words: Antimicrobial, antioxidant, anti-inflammation, peptide, mare milk

INTRODUCTION

Mare milk have been consumed by around 30 million people in the world (Potočnik and Gantner 2011),

although it is less popular than cow or goat milk. Administration of fermented mare milk, Koumiss, to rat treated with toxic compound showed improvement in kidney and brain (Abdel-Salam et al. 2010). Mare milk

was also reported to have positive effect on patient with cardiovascular disease and promote wound healing (Jastrzebska et al. 2017). In Indonesia, Sumbawa mare milk is commonly used as therapy for cardiovascular, hypertension and gastrointestinal disorder. However, research about beneficial of Sumbawa mare milk on whole milk and fraction were still limited. Milk protein derivative such as peptides and their bioactivity also has not been widely explored and utilized.

Bioactive peptides usually bound in the native protein and will be active after hydrolysis process. The peptides present as a mix peptide in hydrolysate or single after purified. Peptides in single form may show higher or lower activities compared to hydrolysate. Single peptide can be produced by purification from hydrolysate or synthesize the peptide. Both peptide from purification or synthesis have potential to be active. A single synthetic peptide derived from a modified peptide was reported active to various bacteria such as *Escherichia coli*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Bormann et al. 2017). In addition, peptide KVISMI derived and purified from whey protein showed antibacterial activity against *Staphylococcus aureus* and *Escherichia coli* (Pei et al. 2017).

Previous research reveals that hydrolysate and peptide fraction especially fraction <3 kDa of Sumbawa mare milk showed antibacterial and antioxidant activities (Kusumaningtyas et al. 2016). The fraction contains 13 peptides with various physiochemical characteristic such as charge, structure and isoelectric point. Potential of antibacterial activities of these peptides was predicted based on their charge, structure and hydrophobicity. Peptide with high therapeutic index (TI) was predicted to be better as antibacterial peptide compared to the lower one. Peptide LVNELTEFAK and HPYFAPELLLYANK which was predicted to have high antibacterial activities was synthesized and assayed further in laboratory to obtain their real activities.

In addition, peptides are also able to be modified to obtain new peptides with higher activities. In this research, peptide LVNELTEFAK (TI: 28.69) was modified and synthesized to be LANSLTEFAK (TI: 94.91) to increase their therapeutic index which probably increase its bioactivities. Comparison of bioactivities of peptide fraction <3 kDa with single peptide LVNELTEFAK, HPYFAPELLLYANK and LANSLTEFAK were also evaluated.

MATERIALS AND METHODS

Materials

Bacillus thuringiensis was isolated from mare milk from Bogor, Indonesia. *Escherichia coli* (ATCC 25922), *Candida albicans* (BCC F059) were used for antibacterial and antifungal assays. Mare milk was collected from Sumbawa Island, Indonesia. Sumbawa mares are local horses of Indonesia which have a geographic original distribution on Sumbawa Island, West Nusa Tenggara Province that had been stated through the Decree of Minister of Agriculture No 2917/Kpts/OT.140/6/2011, June 17, 2011

Peptide fraction <3 kDa was obtain from hydrolysis of Sumbawa mare milk using *B. thuringiensis* protease and showed high activity against Gram negative bacteria, *Escherichia coli* and *Salmonella Typhimurium*. Purified peptide The sequence of peptide 1 (LVNELTEFAK) and peptide 2 (HPYFAPELLLYANK) was obtained from selected peptide of fraction <3 kDa which had high therapeutic index prediction (Kusumaningtyas et al. 2016), while peptide 3 (LANSTEFK) was modification of peptide P1 by replacing amino acid valine (V) with alanine (A) and glutamic acid (E) with Serine (S). Those peptides were synthesized by First Base Laboratories, Selangor Malaysia. Molecular weight and purity of the peptides assayed as shown in Table 1.

Fraction preparation

Defatted mare milk was hydrolyzed at 55 °C pH 11 for 30 minutes using crude protease of *Bacillus thuringiensis* (0.67 IU) isolated using ammonium sulfate precipitation according to Kusumaningtyas et al (2016). Hydrolysis was performed at enzyme substrate ratio 1:20 (v/v). The reaction was stopped by freezing immediately at -20°C for 15 min. Lipid and insoluble protein was discarded by centrifugation 11.000 ×g for 15 min. The supernatant was filtered using 0.45 µm membrane (Acrodisc LC 13 mm, 0,45 µm, PVDF, Pall Life Sciences, USA) and then filtered through centrifugal filter MWCO 3k (AMICON Ultra centrifugal units, Merck Millipore Ltd., Tullagreen, Carrigtwohill, Co).

Peptide analysis

Calculation of theoretical physico-chemical properties were conducted using primary structure analysis

Table 1. Molecular weight and purity of the peptides

| Peptide | Sequence | Mass (Da) | Purity |
|------------------------------|------------------|-----------|--------|
| Peptida 1 | LVNELTEFAK | 1162.62 | 96.72% |
| Peptida 2 | HPYFYAPPELLYYANK | 1887.92 | 92.86% |
| Peptida 3 | LANSLTEFAK | 1092.58 | 99.17% |
| Cecropin A (control peptide) | | 4003.4 | 98.77% |

Source: data sheet of peptide 1, 2, 3 and Cecropin A

www.expasy.org (Kusumaningtyas et al. 2016) and therapeutic index was analyzed using <http://split4.pmfst.hr/split/dserv1/?akcija=run>. (Juretić et al. 2009).

Antibacterial assay

Antibacterial assay was conducted according to Keepers et al. 2014 with modification. As much as 100 μ L, *Escherichia coli* suspensions of 10⁶ CFU/ mL was mixed to 100 μ L peptide fraction <3 kDa, peptide 1 or peptide 2 in eppendorf. The 100 μ L of mixed suspension was grown onto Mueller Hinton agar plate (BD Difco™, Becton Dickinson and Co, USA) and incubated for 24 h at 37°C. The viable colonies were counted. Each treatment was done in three replications. Cecropin A was used as control peptide.

Antifungal assay

Briefly, 100 μ L of *Candida albicans* suspensions of 10⁶ CFU mL⁻¹ was added to the eppendorf containing 100 μ L peptide fraction <3 kDa, peptide 1 or peptide 2. The 100 μ L of mixed suspension was grown onto Sabouraud dextrose agar plate (BD Difco™, Becton Dickinson and Co, USA) and incubated for 24 h at 37 °C. The viable colonies were counted. Each treatment was done in three replications. Cecropin A was used as control peptide (modification from Keepers et al. 2014)

Antioxidant assay

Antioxidant assay was conducted using 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) (Sigma-Aldrich, USA) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Sigma-Aldrich, USA) according to modification of methods by (Thaipong et al. 2006). Stock solution of ABTS 7.4 mM was mixed with 2.6 mM potassium persulphate and incubated at room temperature in the dark for 18 hours. The solution was adjusted for absorbance 1.1 \pm 0.05 at λ = 405 nm by diluted in

deionized water. The fresh ABTS was then used for antioxidant assay. Fraction <3 kDa or single peptide 100 μ L (1 μ g /mL) was added to 200 μ L ABTS or DPPH, incubated for 15 minutes for ABTS or 30 minutes for DPPH at room temperature to allow the reaction. Absorbance of the resulting mixture was recorded at λ 405 nm for ABTS and at λ =540 nm using microplate reader (Labsystems, original Multiscan Ex, and Champaign, USA). The scavenging activity of fraction or peptide to ABTS and DPPH radicals was expressed using equation:

$$\text{Scavenging activity (\%)} = 100 \times (A_0 - A_1) / A_0$$

Where A₀ was absorbance of ABTS/DPPH and A₁ was the final absorbance of sample minus initial absorbance. The assays were performed in three replications and the results were presented as means.

Anti-inflammation Assay

Anti-inflammation assay was done according to (Mohamed & Saleem 2014). Mice were treated using: (1) LPS *E. coli* (1 mg/mL, 0.4 mL) as positive control, (2) LPS *E. coli* (1 mg/mL, 0.4 mL) and peptide P1 LVNELTEFAK (1 mg/mL, 0.3 mL), (3) LPS *E. coli* (1 mg/mL, 0.4 mL) and peptide LANSLTEFAK (1 mg/mL, 0.3 mL), (4) LPS *E. coli* (1 mg/mL, 0.4 mL) and fraction <3 kDa (0.1 mg protein/mL, 0.3 mL) (5) NaCl physiologis (0.4 mL). LPS was injected to mice and after 30 minutes, treated mice were injected with fraction, peptide or NaCl physiologis. After 2 hours infection, blood samples were collected for IL-1 β and TNF- α cytokines measurement using mouse IL-1 β ELISA kit and mouse TNF- α ELISA kit (Sigma Aldrich).

Statistical analysis

Experiment data were statistically analysis using Minitab version 18. Significant differences between samples were analyzed using analysis of variance (ANOVA) and continued with Fisher test.

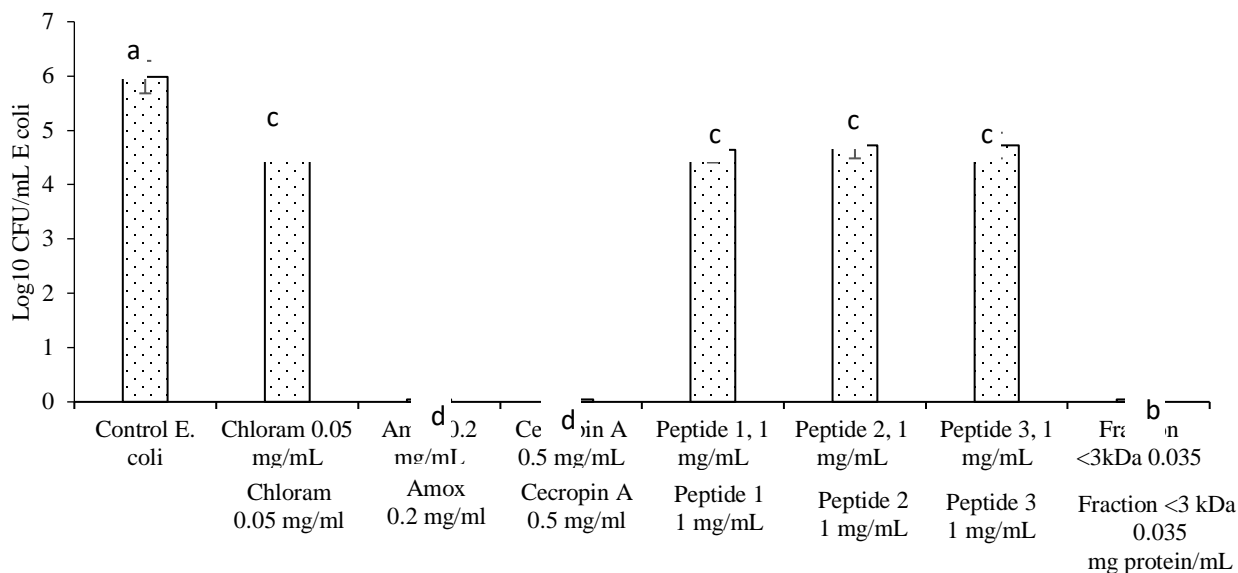


Figure 1. Antibacterial activities of the peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPPELLYYANK), peptide 3 (LANSTEFK) and fraction <3 kDa against *Escherichia coli* ATCC 25922. Chloramphenicol 0.05 mg/mL and Amoxicillin 0.2 mg/mL as positive control antibiotic, peptide Cecropin A as a positive control peptide and *E. coli* without any treatment as negative control. Different letters indicated a significant different (P<0.05).

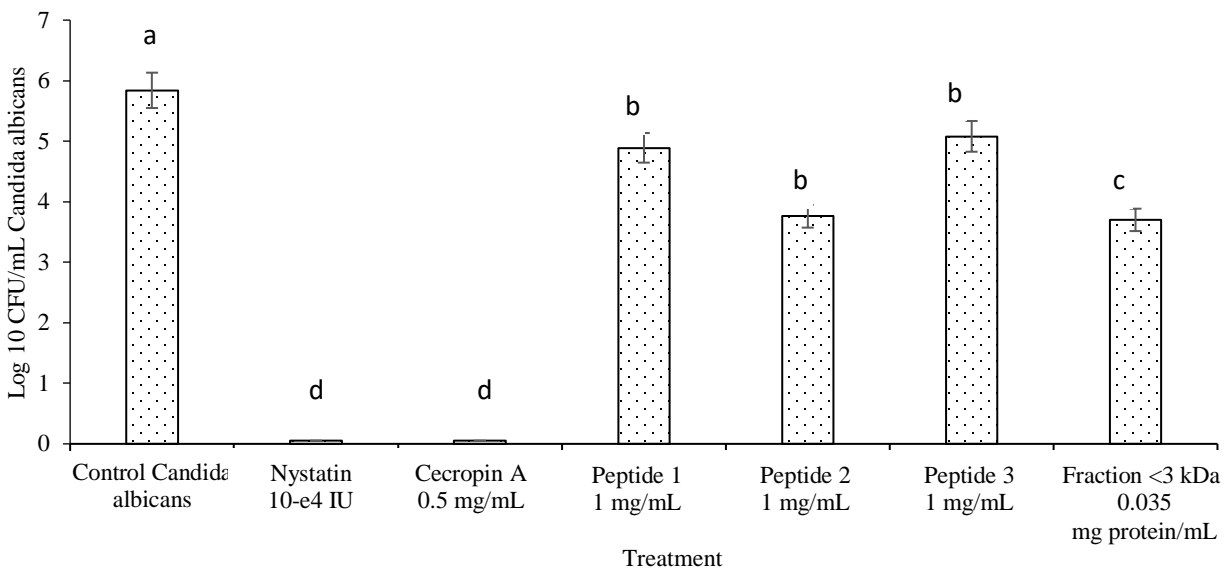


Figure 2. Antifungal activities of the peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPPELLYYANK), peptide 3 (LANSTEFK) and fraction <3 kDa against *Candida albicans* BCC BF059. Nystatin 10,000 IU as positive control of antifungal, peptide Cecropin A as a positive control of peptide and *Candida albicans* without any treatment as negative control. Different letters indicated a significant different (P<0.05).

RESULTS AND DISCUSSION

Antibacterial and Antifungal Activities

Fraction <3 kDa generated from mare milk protein hydrolyzed by *Bacillus thuringiensis* and three of selected peptide derived from these fraction was assayed for antibacterial activities against *Escherichia coli* and antifungal activities against *Candida albicans*. As shown at Figure 1. Fraction <3 kDa was able to inhibit growth of *E. coli* up to 6 log cycles similar with Amoxicillin 0.2 mg/mL and positive control from commercial peptide, Cecropin A 0.5 mg/mL. The synthetic peptide, peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPELLYYANK), peptide 3 (LANSTEFK) showed lower antibacterial activities. Similar to result of antibacterial assay, fraction <3 kDa showed higher activity compared to single peptide in antifungal assay. Previously, fractionation of the hydrolysate was able to increase activity. Antibacterial activity of the hydrolysate increased when it fractionated. Minimum bactericidal activity of hydrolysate to *Escherichia coli* and *Salmonella Typhimurium* was >10 mg protein/mL and increased to be 0.035 mg protein/mL in fraction <3 kDa (Kusumaningtyas et al. 2016). Jovanović et al. (2015) was also reported that fractionation of the peptides was able to increase their antimicrobial activity. Fractionation based on the molecular weight of the peptide may concentrate the peptides which have similar activities. However, the antimicrobial activity was decreased when the peptide was purified into single peptide. Almaas et al. (2011) also reported that pure peptide derived from caprine whey showed less antibacterial effect against *Escherichia coli* K12, *Bacillus cereus* RT INF01 and *Listeria monocytogenes* compared to hydrolysates. This suggested that the fraction contains peptides may work synergistically.

Amoxicillin was included in beta-lactam antibiotic group which worked by binding irreversibly to transpeptidase enzyme (known as penicillin binding protein= PBPs) (Dowling et al. 2017). The bound caused interference in crosslinking peptidoglycan in bacterial cell wall resulting in weakness and lysis in it. Meanwhile, nystatin bound to ergosterol lipid as a part of membrane of fungal cell induced pore formation in cell membrane (Serhan et al. 2014). This pore caused damage and death of the fungi due to disruption in membrane permeability. Mechanism of action of Cecropin A is by binding to negatively charge of bacterial membrane lipid, therefore form a dense layer leading pore formation in membrane (Silvestro et al. 2000). Complex structure (long peptide sequence) and positively charge of Cecropin A made it

able to ion channel into membrane which increased permeability of bacterial or fungal membrane. Consequently, the permeability of bacterial and fungal cell membranes would increase to be very permeable which caused intracellular damage.

Cecropin A works based on the interaction of peptide with negatively charge membrane lipids, therefore electrical charge is a very important factor in the action as antifungal or antibacterial. Positively charge and ability of peptide to form complex with cell membrane might be a key of the effectivity of peptide as an antimicrobial. Positively charge from the peptide will easily bind to negatively charge from bacterial or fungal cell membrane. This is supported by other study which demonstrated that cationic peptides were able to increase cell wall permeability and leading cell membrane damage (Guarna et al. 2006; Manna et al. 2018). Dong et al. (2018) also reported that peptide LK6 or its analogs positively charged +6 or +7 and were able to form alpha helix structures showed high antibacterial activities against *Escherichia coli* and *Staphylococcus aureus*.

Meanwhile, the three peptides tested (peptide 1, 2, 3) have neutral and negative charge therefore their antimicrobial activities were very low. Lee et al (2015) stated that cationic peptides with good antimicrobial activity should be +2 to +7 and consisted of 12 to 100 amino acids. Peptide fraction <3 kDa was a mixture of several peptides with charge -4 to +1, therefore antimicrobial mechanism was not certain. The fraction was effective as antibacterial but less as antifungal. Interaction among peptides in the fraction may contribute to its activity. It may not be only based on positive charge but also other factor such as hydrophobicity, amino acid composition or charge distribution.

Based on the Figure 1, Figure 2 and Table 1, antimicrobial activity of single peptide was determined primary by charge and hydrophobicity. In this research, Cecropin A showed highest antimicrobial activity against *Escherichia coli* and *Candida albicans*, compared to peptide 1, 2, and 3. Cecropin A had therapeutic index prediction only 31.83, lower than peptide 2 and peptide 3, but it showed high charge (+6) and high hydrophobicity +34.74. Yin et al. (2012) reported that balancing of charge distribution and hydrophobicity of the peptide determined antimicrobial activity and peptide toxicity to the mammalian cell. Moreover, antimicrobial activity of the peptide was also determined by amino acid composition in the sequence. Antibacterial peptide commonly was dominated by amino acid L while residue C was dominant in the hydrophobic group of antimicrobial peptides with antifungal activity (Mishra & Wang 2012).

Table 2. Physicochemical analysis and index therapy prediction of assayed peptide

| Sequence | Length (aa) | Mass (Da) | Isoelectric Point | Charge | Hidrophobicity (kcal/ml) | Therapeutic index prediction |
|---|-------------|-----------|-------------------|--------|--------------------------|------------------------------|
| LVNELTEFAK (Pep 1) | 10 | 1162.62 | 6.53 | -1 | +14.89 | 28.68 |
| HPYFYAPELLYYANK (Pep 2) | 15 | 1887.92 | 7.47 | 0 | 11.74 | 64.75 |
| LANSLTEFAK (Pep 3) | 10 | 1092.58 | 6.53 | 0 | +12.68 | 94.91 |
| KWKLFKKIEKVGQNIRDGIIKA GPAVAVVGQATQIAK (Cecropin A; a commercial peptide) | 37 | 4003.8 | 10.94 | +6 | +34.74 | 31.83 |

Source: Kusumaningtyas et al. 2016; Cecropin A data sheet

Fraction <3 kDa was composed of 13 peptides which varied in length, charge and hydrophobicity (Kusumaningtyas et al. 2016). High therapeutic index value might increase therapeutic effect and decrease toxicity of the peptide (Tamargo et al. 2015). The peptide with high antimicrobial activity and low toxicity allowed it to penetrate and disrupt bacterial membrane cell without damage host cell membrane. Commonly, peptide was safe for therapy if it had therapeutic index value higher than 10 (Tamargo et al. 2015). Peptide 1 and peptide 2 which were used in this research were selected based on the therapeutic index prediction. The peptides were synthesized and evaluated for their antimicrobial activities against *Escherichia coli* and *Candida albicans*. Peptide 1 was also modified to increase therapeutic index prediction to obtain peptide with higher antimicrobial activity, namely peptide 3. The physicochemical analysis and index therapy prediction of the peptide 1, 2, 3 and Cecropin A are shown in Table 2.

The result also confirmed that high therapeutic index prediction not always represented the antimicrobial activities. Peptide HPYFYAPELLYYANK and LANSLTEFAK which had therapeutic index prediction higher than Cecropin A, had lower activities in laboratory experiment. Therapeutic index in this study was only prediction using statistics calculation based on amino acid sequence, while therapeutic index experimental was determined based on laboratory trials in biological system such as in animal or human cells. Therefore, it was possible that therapeutic index prediction and therapeutic index experimental produced different result.

Antioxidant activity

ABTS (2,2-azinobis 3-ethylbenzothiazoline-6-sulfonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) were used to measure scavenging activity of the fraction and peptide 1, 2, and 3. Scavenging activities were

determined by reduction of ABTS or DPPH absorbance after addition of peptide fraction or single peptide. Complete scavenging of ABTS or DPPH radical indicated 100 % scavenging activity. Scavenging activity fraction <3 kDa and peptide 1, 2, 3 is shown at Figure 3.

Fraction <3 kDa showed the highest scavenging activity against ABTS and DPPH compare to Peptide 1, 2, 3, although DPPH assay results were not significant different. These indicated that antioxidant activity of peptide in fraction had better activity than in single peptide form. Similar to antimicrobial peptide, it might be due to the peptides in the fraction worked synergistically resulting higher activity. Yousr & Howell (2015) reported that the scavenging activity to DPPH of the selected peptide, YPSPV, 5% antioxidant activity, was lower than their original fraction, EYGF 33 (egg yolk gel filtration fraction 33 which obtained from fraction 33th of egg yolk protein hydrolysate 2 kDa fractionated using sephadex 25) which had scavenging activity of 59.8 %. These data supported that single peptide were not always better than mixed peptides in the fraction.

Anti-inflammation

Inflammation is a component of the innate immune response as a part of host defense against infection and to restore homeostasis in damaged tissues (Manna et al. 2018). Inflammation change to be dangerous if it develops to be excessive and cause organs failure or malfunction. External intervention is needed to maintain the balance of the inflammatory response by organism. LPS is the main activator of the host defense mechanism (Dong et al. 2018). Aggregated LPS which initially binds with liposaccharide-binding protein (LBP) and the primary LPS receptor CD14; then, LPS-LBP-CD14 complexes are transduced by another membrane protein, Toll-like receptor 4 (TLR4), after which they triggered proinflammatory signaling pathways and induce cytokine

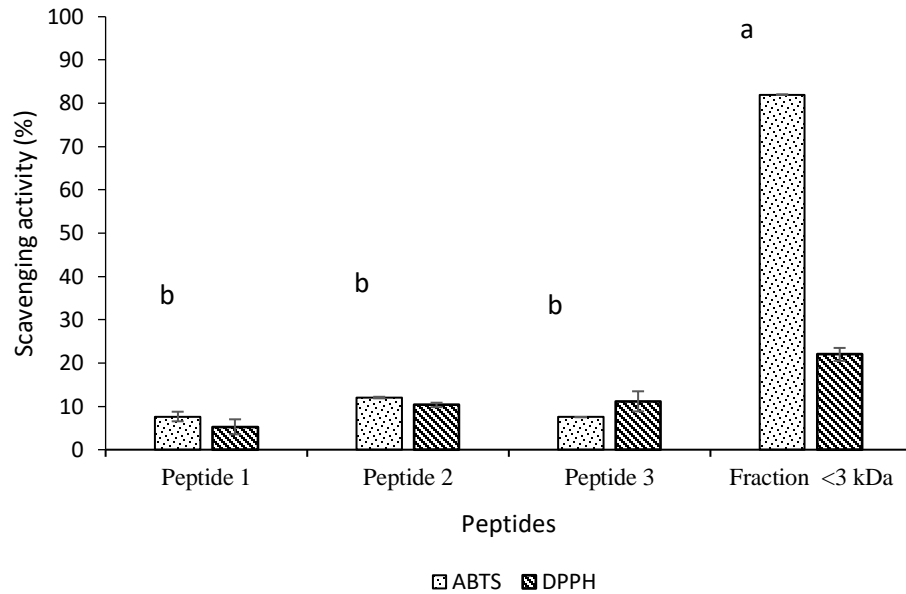


Figure 3. Antioxidant activities of fraction of the peptide 1 (LVNELTEFAK), peptide 2 (HPYFAPELLLYANK), peptide 3 (LANSTEFK) and fraction <3 kDa to [2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)] (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). Different letters indicated a significant different ($P < 0.05$). Assay using DPPH did not show any significant different ($P > 0.05$)

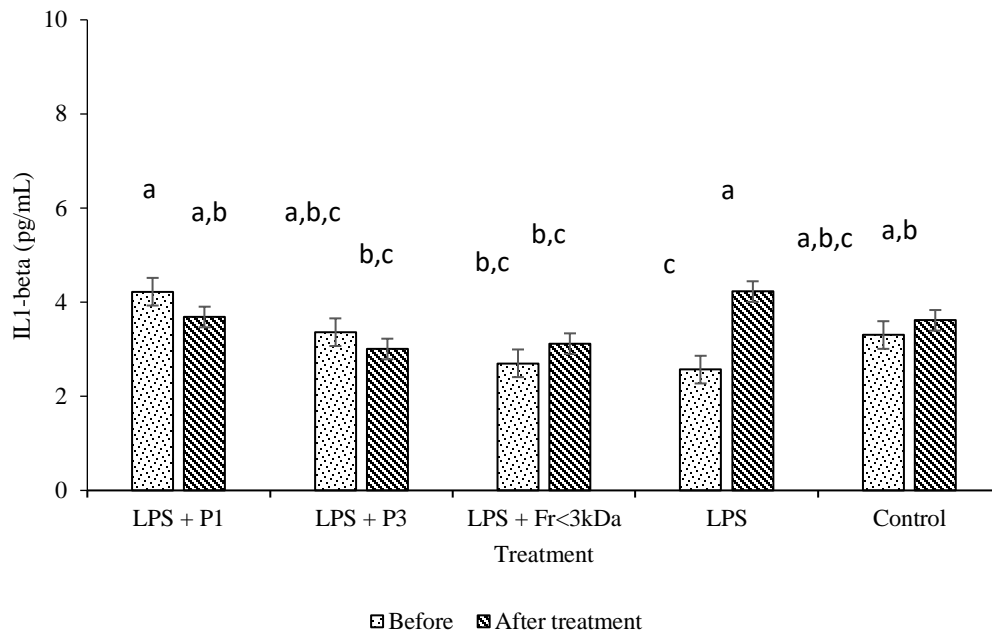


Figure 4. IL 1- β in mice serum before and after treated by lipopolysaccharide *Escherichia coli*, fraction <3 kDa mare milk protein hydrolysate, peptide 1 (LVNELTEFAK) and P3 (LANSLTEFAK). Different letters indicated a significant different ($P < 0.05$).

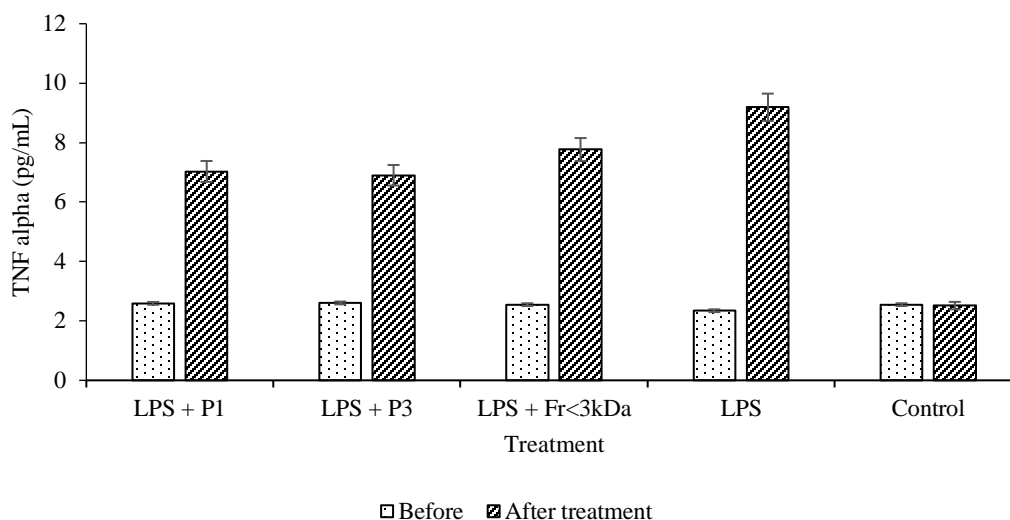


Figure 5. TNF- α in mice serum before and after treated by lipopolysaccharide *Escherichia coli*, fraction <3 kDa mare milk protein hydrolysate, peptide 1 (LVNELTEFAK) and P3 (LANSLTEFAK). TNF alpha, all treatment before or after, did not show any significant different

secretions such as tumor necrosis factor alpha (TNF- α) and interleukins 1 β , interleukins 8 and reactive oxygen species (Dong et al. 2018).

Interleukin-1 (IL-1) α/β were proinflammatory cytokines which were considered as key orchestrators of innate immune response (Garlanda et al. 2013). Production of IL-1 was triggered by injury or infection, including microbial ligand and damage associated with molecular pattern (Rock et al. 2011). IL-1 induces cytokines, chemokines, growth factors and vascular adhesion molecules IL-1 induces cytokines, chemokines, growth factors and vascular adhesion molecules (Garlanda et al. 2013).

Interleukin-1 β is pro-inflammatory cytokines that exerts pleiotropic on a variety of cells and plays key roles in acute and chronic inflammatory which is implicated in pain, inflammation and autoimmune condition (Ren and Torres 2009). Interleukin-1 (IL-1) is not only a potent regulator of innate immune system important for host defense but is also associated with injury and disease in the brain (Giles et al. 2015). Large production of cytokines proinflammation such as IL-1 beta and TNF-alpha harms to organ and cause mortality.

Antiinflammation assay was conducted by measured proinflammation cytokine concentration IL-1 β and TNF- α . The agent which was able to decrease IL-1 β indicated that this agent as anti-inflammatory (Dinarello CA 2011). Lipopolysaccharide (LPS) *E. coli* enhance cytokine IL-1 β production. Treatment with peptide 1(P1: LVNELTEFAK) dan peptida 3 (P3: LANSLTEFAK)

indicated decreasing concentration of IL-1 β compared to mice treated LPS. IL-1 β was also increased in mice serum treated with fraction <3 kDa mare milk. TNF- α increase in mice treated with LPS only or peptide P1 and P3, but TNF- α concentration in mice serum treated with peptides P1 and P3 still lower than mice treated with LPS. Slightly decreasing concentration of TNF- α was also observed in mice treated with fraction 3 kDa. Those results showed that peptide P1, P3 or fraction <3 kDa were able to slightly decrease cytokines inflammation.

TNF- α increased in treatment with LPS or peptide, but TNF- α in serum treated with peptide was lower than by LPS alone. In treatment with fraction <3 kDa, TNF- α also decreased although it was still higher than that of single peptide. The result indicated that both fraction or single peptide was able to decrease proinflammation cytokine TNF- α and act as anti-inflammation agent although the results were not significantly different.

According to Sun and Shang (2015), antimicrobial peptide was possible to act as anti-inflammation by neutralization of lipopolysaccharide, killed bacteria and inhibit production of proinflammation cytokines. Decreasing of proinflammation cytokines was also decreasing of inflammation response. Fotschki et al. (2016) reported that mare milk contained immunomodulating properties decreased Ig E and decreased expression of IL-4 had proinflammatory properties in mice sensitized with allergen. It is possible that bioactive peptides in mare milk are able to act as antimicrobial as well as anti-inflammation agents. Cationic peptide was

reported to be able to act as anti-inflammation by neutralizing and bounding with LPS (Guarna et al. 2006; Lee E, Shin A 2015; Dong et al. 2018). But in this research, decreasing some inflammatory mediators such as TNF α and IL 1 β was able to be triggered by short, negative and positive charge. This indicated that mechanism of bioactive peptide as anti-inflammation did not related to its positive charge. The mechanism has yet not clear.

CONCLUSION

Peptide fraction and purified peptide derived from mare milk protein hydrolyzed using *Bacillus thuringiensis* protease had antibacterial, antifungal, antioxidant and anti-inflammation activities. Generally, fraction <3 kDa had higher antibacterial, antifungal and antioxidant activities compared to single peptide. The result indicated that purification of the peptide in the fraction <3 kDa was not necessary. For anti-inflammation, peptide 1 and peptide 3 were able to reduce IL1- β better than fraction. Therapeutic index based on amino acid sequence should be confirmed by laboratory experiment to determine real antimicrobial activity of the peptide. It is suggested that fraction <3 kDa is a promising agent for future application as antimicrobial and antioxidant

ACKNOWLEDGEMENT

This works was supported by grant from Ministry of Agriculture of the Republic of Indonesia, APBN No 1806.105.002.052A, 2016 and Indonesian Research Centre for Veterinary Science

REFERENCES

- Abdel-Salam AM, Dekheil A, Babkr A, Farahna M Mousa HM. 2010. High fiber probiotic fermented mare's milk reduces the toxic effects of mercury in rats. *N Am J Med Sci.* 2:569–575.
- Bormann N, Kolisza A, Kasper S, Schoen L, Hilpert K, Volkmer R, Kikhney J, Wildermann B. 2017. A short artificial antimicrobial peptide shows potential to prevent or treat bone infections. *Sci Rep* :7 1506.
- Dinarello CA. 2011. Interleukin-1 in the pathogenesis and treatment of inflammatory diseases. *Blood.* 117:3720–3732.
- Dong W, Zhu X, Zhou X, Yang Y, Yan X, Sun L. 2018. Potential role of a series of lysine- / leucine-rich antimicrobial peptide in inhibiting lipopolysaccharide-induced inflammation. *Biochem J.* 475:3687–3706.
- Dowling AM, Dwyer JO, Adley C. 2017. Antibiotics : Mode of action and mechanisms of resistance . *Antibiotics : Mode of action and mechanisms of resistance.* In: (Méndez-Vilas A (Ed), editor. *Antimicrob Res Nov bioknowledge Educ programs.* [place unknown]: Publisher: Formatex Research Center.; p. 536–545.
- Kusumaningtyas E, Widiastuti R, Kusumaningtyas HD, Suhartono MT. 2016. Bioactivities and Analysis of Peptides of Sumbawa Horse Milk Generated by *Bacillus thuringiensis* Protease. *JITV.* 21:244–254.
- Fotschki J, Szyk AM, Laparra JM, Markiewicz LH, Wrowblewska B. 2016. Immune-modulating properties of horse milk administered to mice sensitized to cow milk. *J Dairy Sci.* 99:9395–9404.
- Garlanda C, Dinarello CA, Mantovani A. 2013. The interleukin-1 family: back to the future. *Immunity.* 39:1003–1018.
- Giles JA, Greenhalgh AD, Davies CL, Denes A, Shaw T, Coutts G, Rothwell N, McColl BW Allan S. 2015. Requirement for interleukin-1 to drive brain inflammation reveals tissue-specific mechanisms of innate immunity. *Eur J Immunol.* 45:525-530.
- Guarna MM, Coulson R, Rubinchik E. 2006. Anti-inflammatory activity of cationic peptides : application to the treatment of acne vulgaris. *FEMS Microbiol Lett.* 257:1–6.
- Jastrzebska E, Wadas E, Daszkiewicz T, Pietrzak-Fiecko R. 2017. Nutritional Value and Health-Promoting Properties of Mare ' s Milk – a Review. 62:511–518.
- Jovanović J, Stefanović A, Grbavčić S, Šekuljica N, Elmalimadi M, Knežević-Jugović BBZ. 2015. Peptides with improved antimicrobial activity screened by membrane ultrafiltration from egg white protein hydrolysates Slovak Society of Chemical Engineering Institute of Chemical and Environmental Engineering. In: 42nd Int Conf SSCHE May 25–29, 2015, Tatranské Matliare, Slovakia. [place unknown]; p. 732–739.
- Juretić D, Vukicević D, Ilić N, Antcheva N, Tossi A. 2009. Computational design of highly selective antimicrobial peptides. *J Chem Inf Model.* 49:2873–82.
- Ren K, Torres K. 2009. Role of interleukin-1 β during pain and inflammation. *Brain Res Rev.* 602:57–64.
- Keepers TR, Gomez M, Celeri C, Nichols W and Krause K. 2014. Bactericidal activity, absence of serum effect and time-kill kinetics of Ceftriaxime-Avibactam against β -lactamase-producing Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 58:5297–5305.
- Lee E, Shin A, Kim Y. 2015. Anti-inflammatory activities of cecropin A and mechanism of action. *Arch Insect*

- Biochem Physiol. 88:31–44.
- Manna S La, Natale C Di, Florio D, Marasco D. 2018. Peptides as Therapeutic Agents for Inflammatory-Related Diseases. *Int J Mol Sci.* 19:1–18.
- Mishra B, Wang G. 2012. The importance of amino acid composition in natural AMPs : an evolutionary , structural , and functional perspective. *Front Immunol.* 3:1–4.
- Mohamed MF, Seleem M. 2014. Efficacy of short novel antimicrobial and anti-inflammatory peptides in a mouse model (MRSA) skin infection. *Drug Des Devel Ther.* 8:1979–1983.
- Pei J, Jiang H, Li X, Jin W, Tao Y. 2017. Antimicrobial peptides sourced from post - butter processing waste yak milk protein hydrolysates. *AMB Express.*
- Potočník K, Gantner V. 2011. Mare ' s milk : composition and protein fraction in comparison with different milk species. 61:107–113.
- Rock KL, Lai JJ, Kono H. 2011. Innate and adaptive immune responses to cell death. . *Immunol Rev.* 243:191–205.
- Serhan G, Stack CM, Perrone GG, Morton CO. 2014. The polyene antifungals , amphotericin B and nystatin , cause cell death in *Saccharomyces cerevisiae* by a distinct mechanism to amphibian-derived antimicrobial peptides. *Ann Clin Microbiol Antimicrob.* 13:1–4.
- Silvestro L, Weiser JN. 2000. Antibacterial and Antimembrane Activities of Cecropin A in *Escherichia coli*. *Antimicrob Agents Chemother.* 44:602–607.
- Sun Y, Shang D. 2015. No TInhibitory Effects of Antimicrobial Peptides on Lipopolysaccharide-Induced Inflammation. *Mediat Inflamm.*:1–8.
- Tamargo J, Heuzey J L, Mabo P. 2015. Narrow therapeutic index drugs : a clinical pharmacological consideration to flecainide. *Eur J Clin Pharmacol.* 71:549–567.
- Thaipong K, Boonprakob U, Crosby K, Cisneros-Zevallos L, Byrne D, H. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimation antioxidant activity from guava fruit extracts. *J Food Comp Anal.* 19:669–675.
- Yin LM, Edwards MA, Li J, Yip CM, Deber CM. 2012. Roles of Hydrophobicity and Charge Distribution of Cationic Antimicrobial Peptides in Peptide-Membrane Interactions. . *J Biol Chem.* 287:7738–7745.
- Yousr M, Howell N. 2015. Antioxidant and ACE Inhibitory Bioactive Peptides Purified from Egg Yolk Proteins. *Int J Mol Sci.* 16:29161–29178.