Antifungal Activity of Peptide Fractions from Goat, Mare and Soybean Milk to *Candida albicans* and *Trichophyton mentagrophytes*

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Abstract. *Candida albicans* and *Trichophyton mentagrophytes* are pathogenic fungi that could induce disease in animal and human. Limited non-toxic antifungal and the presence of resistance are triggering the search for novel alternative. Antifungal peptides gain more interest since their native protein are very abundant and showed broad spectrum activity. This research was conducted to evaluate antifungal activity of goat, mare and soybean milks hydrolysate and their peptide fraction against *Candida albicans* and *Trichophyton mentagrophytes*. Goat, mare and soybean milk proteins were hydrolyzed using *Bacillus thuringiensis* protease enzyme. Hydrolysate products were fractionated using molecular weight cut off membrane 30 kDa, 10 kDa, and 3 kDa. Each peptide fraction was assayed for antifungal activity against to *Candida albicans* and *Trichophyton mentagrophytes*. The result showed hydrolysis of mare milk, goat milk produced bioactive peptides to *Candida albicans* but less effective to *Trichophyton mentagrophytes*. Generally, the peptide fractions derived from whole, casein and whey from mare milk showed higher antifungal activity compared to the peptide fraction derived from goat or soybean milk. Whole mare milk peptides fractions with molecular weight >3 kDa were very effective to inhibit the growth of *Candida albicans* compared to peptide fractions from goat's milk or soybean milk. Peptide fractions >3 kDa from mare casein and whey protein also showed high activity against to *Candida albicans*, while fractions <3 kDa was more active to inhibit *Trichophyton mentagrophytes*. Based on the result, it can be concluded that the hydrolysates and their peptide fraction from whole milk, casein and whey from mare milk are very potent to be developed as antifungal.

Keywords: Antifungal · goat · mare · soybean · peptide fraction

1 Introduction

Candida albicans is an opportunistic yeast that has pathogenic properties. The presence of *Candida albicans* in animal husbandry is closely related to candidiasis. Candidiasis in poultry commonly don't show any clinical sign because the disease usually occurs after

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predisposing condition. Crop mycosis, cutaneous and comb candidiasis can lead feather loss and morbidity [1]. In dairy cow, it causes inflammation in the mammary glands (mastitis). Candida species are the most frequent yeast among the mycotic mastitis isolated from infected mammary gland and are commonly related with post-treatment with antimicrobial agent [2].

Trichophyton mentagrophytes is the second most common causative agent of dermatophytosis after *Trichophyton rubrum. Trichophyton mentagrophytes* is frequently isolated from dog, cat and sometimes cattle. Those dermatophytes fungi are zoonotic and can be transmitted from animal to human. Some strain showed resistance to antifungal drugs.

Treatment using chemical antifungal to eradicate candidiasis and dermatophytosis has been widely practiced in the community. Long term treatment without veterinarian supervision may result in drug residue remaining in the body which harm to animals. So, it is necessary to look for other alternative to eliminate *Candida albicans* or *Trichophyton mentagrophytes*. The new antifungal should be less toxic, not cause allergy and has broad spectrum antifungal activity. One candidate that able to inhibit the fungal growth is a bioactive peptide derived from milk protein and soy extract.

Milk and soy milk contain complete nutrition (carbohydrates, protein, fat, vitamins, and minerals) which are very beneficial for health. The benefits of milk and soy juice are in the proteins and their hydrolysates. Proteins can be hydrolyzed into bioactive peptides that have broad biological functions [3]. Bioactive peptides are specific protein fragments that have a positive impact on body function or condition. Bioactive peptides can be obtained by several hydrolysis methods, namely: hydrolysis by digestive enzymes, and hydrolysis by proteolytic enzymes produced by microorganisms or plants. The success of obtaining bioactive peptide is commonly determined by the protein source and the specificity of the enzyme. Therefore, the selection of enzymes is an important step to obtain bioactive peptides and will determine the bioactivity of the peptides produced [4]. Previous research has shown that peptides from milk protein have been shown to be able to inhibit the growth of *Candida albicans* [5], but in that study did not specify the size of the responsible peptide which able to inhibit the growth of *Candida albicans*.

This research tries to find bioactive peptides from soy milk, goat milk and mare milk by classifying the size of bioactive peptides based on their molecular weight through the fractionation process. The purpose of this study was to evaluate antifungal activity of goat, mare and soy milks hydrolysate and their peptide fraction against *Candida albicans* and *Trichophyton mentagrophytes*.

2 Materials and Methods

2.1 Materials

Yeast *Candida albicans* BCC F059 and *Bacillus thuringiensis* BCC B3013 was obtained from the IRCVS Culture Collection. Colony propagation was carried out by growing *Candida albicans* in Sabouraud Dextrose Agar media (Difco™) and incubated at 37 °C for 24 h. *Bacillus thuringiensis* was grown in water agar (Bacto agar Difco™) containing 0.05% (w/v) skim milk (Difco™). A clear zone around the bacterium indicated protease production.

Soy milk was obtained by soaking 250 g soybean in water overnight. The skin was peeled and then soybean washed and boiled in water about 10 min. The soybean was put in 1500 water and grinded. The suspension obtained was filtered using filter paper or soft cloth that usually used in tofu production. The filtrate was ready to be hydrolyzed.

2.2 Crude Enzymes

The protease enzyme was isolated from *Bacillus thuringiensis* according to Kusumaningtyas et al. (2016) [6]. *Bacillus thuringiensis* was grown in Luria Bertani Broth (Difco™) medium containing 0.05% (w/v) skim milk which was incubated at 37 °C for 24 h. The culture was centrifuged at 3500 g for 15 min and the enzyme in the supernatant was precipitated with 50% ammonium sulfate. After being incubated overnight at 4 °C, the enzyme pellets were taken by centrifugation of 10,000 g for 15 min. The pellets were air-dried and then stored at -20 °C before used or dissolved in PBS pH 7.4 with a ratio of 1:2 for direct use in hydrolyzing milk protein and soy milk.

2.3 Protein Hydrolysis

The protein used was derived from Etawa crossbreed goat's milk from the Faculty of Animal Science, IPB University, Bogor Indonesia, mare milk from Sumbawa and soybean juice. The fat from goat's milk and mare's milk was separated by centrifugation at a speed of 6000 g (hybrid refrigerated centrifuge, CAX-371, Tomy Seiko, Japan) for 15 min and then the fat was discarded. Whey and casein was separated by precipitation using 1 N HCl. After separation, casein was re-dissolved in water (v/w) by adjusting pH. Whole whey and casein were hydrolyzed with proteases from *Bacillus thuringiensis* for 60 min. The soybean juice was centrifuged at a speed of 6000g to separate the soybean juice with larger particles so that it would not clog during filtration with a multilevel membrane filter. The activity of the protease enzyme *Bacillus thuringiensis* was 0.67 units/mL with an enzyme-substrate ratio of 1:20. Hydrolysis was carried out at 55 °C and pH 7 [7, 8] for 30–60 min. Each peptide from hydrolysis was centrifuged at 14,000 *g* for 15 min. After centrifugation, commonly formed 3 layer, supernatant from the middle layer was moved to new tube.

2.4 Peptide Fractionation

Milk and soy milk that have been hydrolyzed with the protease Bacillus thuringiensis were filtered using a molecular weight cut off membrane filter (Millipore) with membrane sizes of 30kDa, 10kDa and 3kDa to obtain peptides with molecular weight sizes $>$30 kDa, 10–30 kDa, 3–10 kDa, $<$3 kDa.

2.5 Antifungal Activity

Antifungal assay was conducted according Kusumaningtyas et al. [6] with some modifications. A total of 100 μl of hydrolysate or peptide fractions was added to 100 μl of *Candida albicans* 10^6 CFU/mL and incubated at 37 °C for 2 h, then 10 μl of the suspension was taken and diluted by adding 990 µl of sterile distilled water to obtain a suspension of 10 ml with a concentration of C. albicans 10^3 CFU/mL. One ml of suspension was taken and poured into a sterile petri dish. As a control, 1 ml of *Candida albicans* 10^6 CFU/mL suspension without peptide was diluted and poured into a petri dish. Amount of 25 ml of liquid Sabouraud dextrosa agar medium (±55 °C) was poured into each petri dish which had already contained the suspension, stired until homogeneous and incubated at 37 °C for 24 h. Observations were made by counting the number of *Candida albicans* colonies compared with the number grown in a control petri disk containing a suspension of *Candida albicans* alone without peptides fractions addition. Each treatment was made in 3 replications. Similar procedure was applied for antifungal assay to *Trichophyton mentagrophytes.*

3 Results

The most effective peptide fractions derived from mare whole milk to inhibit *Candida albicans* was >10 kDa. Whole milk fraction from goat milk had lower anti *Candida* activity than mare milk fraction, but higher than that of soybean milk. Goat milk peptide fraction which the most effective to inhibit Candida albicans was >30 kDa, while soybean milk peptide fraction at 10–30 kDa. The difference antifungal activity might be due to the difference amino acid composition of the peptide fractions. Antifungal activity of the whole milk fractions from whole goat, mare and soybean milk to *Candida albicans* was shown in Table 1, while that activity to *Trichophyton mentagrophytes* was shown in Table 2.

Table 1. Antifungal activity of peptide fraction from whole goat, mare and soybean milk to *C. albicans*

No	Colony count of *C. albicans* (10^6 CFU/mL)			
	Fraction	Goat milk	Mare milk	Soybean milk
1	Kontrol	1,56 ± 0,00	1,56 ± 0,02	1,56 ± 0,24
2	>30 kDa	0,02 ± 0,01	0 ± 0,00	0,57 ± 0,05
3	10–30 kDa	0,28 ± 0,04	0 ± 0,00	0,40 ± 0,01
4	3–10 kDa	0,39 ± 0,03	0 ± 0,00	0,57 ± 0,06
5	<3 kDa	1,21 ± 0,13	1,29 ± 0,01	0,59 ± 0,07

The result of antifungal assay of the whole milk peptide fractions against *Trichophyton mentagrophytes* were lower than that to *Candida albicans*. It may due to *Trichophyton mentagrophytes* has more complex structure as the mold is multicellular while *Candida albicans* was unicellular fungus. The peptide fractions from goat milk 3–10 kDa and that from mare milk 3–10 kDa showed the better activity than others. Peptide fractions from soybean milk showed very low activity against *Trichophyton mentagrophytes.*

Based on the effectiveness of the test results of the three protein sources (mare milk, goat's milk and soy milk) it was shown that mare milk had the highest antifungal activity

Table 2. Antifungal activity of peptide fraction from milk and soybean milk to *Trichophyton* mentagrophytes

No	Colony count of *Trichophyton mentagrophytes* (10^6 CFU/mL)			
	Fraction	Goat milk	Mare milk	Soybean milk
1	Control	1.22 ± 0.02	1.19 ± 0.02	1.22 ± 0.01
2	>30 kDa	1.22 ± 0.47	0.97 ± 0.38	1.22 ± 0.12
3	10–30 kDa	0.88 ± 0.05	0.61 ± 0.08	1.21 ± 0.08
4	3–10 kDa	0.72 ± 0.08	1.14 ± 0	1.21 ± 0.06
5	<3 kDa	1.22 ± 0.08	1.06 ± 0.19	1.22 ± 0.02

Table 3. Antifungal activity of casein and whey fraction from mare milk to Candida albicans

No	Colony count of *C. albicans* (10^6 CFU/mL)		
	Fraction	casein	Whey
1	Kontrol	6,17 ± 0,78	6,17 ± 0,78
2	>30 kDa	0	0
3	10–30 kDa	0	0
4	3–10 kDa	0	0
5	<3 kDa	0,62 ± 0,05	1,56 ± 0,29

against *Candida albicans* and *Trichophyton mentagrophytes*. The research was continued by separating mare milk from whey and casein to determine the effectiveness of these two components in inhibiting *Candida albicans* and *Trichophyton mentagrophytes*. The results of the Candida albicans inhibition test from casein and mare milk whey are shown in Table 3.

As shown in Table 3, all peptide fractions from both casein and whey of mare milk had high activities to inhibit *Candida albicans*. Peptide <3 kDa showed lower inhibition to *Candida albicans* compare to other fractions. From the Table 1 and 3, peptide fractions >3 kDa from whole milk, casein and whey derived from mare milk revealed high activities to *Candida albicans*. As peptide fractions from mare's whole milk, casein and whey showed very low antifungal activity against *Trichophyton mentagrophytes* (Table 4).

4 Discussion

Proteins that are hydrolyzed by the protease enzyme from *Bacillus thuringiensis* produced hydrolysates containing peptides that are bioactive and non-active peptides. To obtain bioactive peptides, filtration or separation is required using a molecular weight cut off (MWCO) membrane. The filtration is able to concentrate the desired peptide based on a certain molecular weight [9]. The selectivity of the membrane used not only

Table 4. Antifungal activity of casein and whey fraction from mare milk to *Trichophyton mentagrophytes*

No	Colony count of *T. mentagrophytes* (10^6 CFU/mL)		
	Fraction	casein	Whey
1	Control TM	119 ± 0.02	119 ± 0.02
2	>30 kDa	121 ± 0.14	113 ± 0.01
3	10–30 kDa	126 ± 0.61	123 ± 0.12
4	3–10 kDa	94 ± 0.18	100 ± 0.12
5	<3 kDa	49 ± 0.1	81 ± 0.1

separates peptides based on size but also based on aromatic groups [10] and charge [11] which affect their bioactivity.

Inhibition test of bioactive peptides generated from protein hydrolysis of goat's milk, mare's milk and soybean extract using the protease *Bacillus thuringiensis* showed the various antifungal activities. In general, the mare milk peptides were more effective than goat's milk and soybean milk in inhibiting the growth of *Candida albicans*. But, all the peptide fractions less effective against *Trichophyton mentagrophytes*. The difference effectivity of the peptide fraction to both fungi may due to the difference of cell wall or membrane cell structure between those fungi. The mechanism of action of these bioactive peptides is to damage the components and structure of the cell wall, to react with the cell membrane and then to inhibit the synthesis of proteins and nucleic acids. The cell wall of *Candida albicans* mainly consists of glucans, chitin, and mannoprotein [12]. The peptide fraction might be easier to react to the cell wall or membrane cell of *Candida albicans*. It depends on the component of the wall. For example, peptide defensin was effective to Candida albicans, but not effective for fungus which does not contain fungus-specific ceramic was not inhibited [13].

Bioactivity of the peptides obtained from the hydrolysis process was determined by the source of protein and enzymes, so the selection of enzymes and proteins is an important step to obtain active peptides [4]. In this study, the protein sources used were mare milk, goat milk and soy milk because of their nutritional content. Milk protein is converted into peptides through enzymatic processes. The resulting peptides such as isracidin, cappacin, lactoferricin, and lactoferampin obtained from bovine milk hydrolysis were known to have potential antimicrobial effects [14]. Enzymes that play a role in protein hydrolysis can be obtained from animal digestion (trypsin, chymotrypsin, and pepsin), from plants (bromelain and papain) or bacteria such as alkalase from *Bacillus licheniformis* [15]. Each enzyme has specific cleavage site which cut protein in a certain amino acid. The length and amino acid of peptide products will determine their bioactivities. Different protein source or enzyme applied for hydrolysis produced peptides with different bioactivities.

Casein consists of the amino acids S1-casein, S2-casein, -casein, while whey consists of the amino acids -lactalbumin, -lactoglobulin, and lactoferrin which later undergoes the process of fermentation, hydrolysis and enzymatic digestion by digestive enzymes and proteolytic enzymes. Added from the outside will produce bioactive peptides [3]. The difference of effectiveness of the peptide fraction to inhibit *Candida albicans* may due to the difference in amino acid sequences which will affect to antifungal activity.

Whole mare milk peptides fractions with molecular weight >3 kDa were very effective to inhibit the growth of *Candida albicans* compared to peptide fractions from goat's milk or soybean milk. Peptide fractions >3 kDa from mare casein and whey protein also showed high activity against to *Candida albicans*, while fractions <3 kDa was more active to inhibit *Trichophyton mentagrophytes.* The different molecular weight, length and amino acid composition resulting different contact region with target that may resulting different mode of action among the peptides. The action to fungal target can be through inhibition of 1.3 beta glucan synthesis leading to cell lysis, inhibition of chitin synthesis in the cell wall and causing leakage of the cell membrane [13]. Based on the result, it can be concluded that the hydrolysates and their peptide fraction from whole milk, casein and whey from mare milk are very potent to be developed as antifungal.

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References

1. Ibrahim ZY, Ali BH, Ali RK, Jarad SS, Farhan W HM. Avian candidiasis: A review. Intl J Pharma Res. 2020; 12(1): 1–4.
2. Sartori LCA, Santos RC MJ. Identification of Candida species isolated from cows suffering mastitis in four Brazilian states. Arq Bras Med Vet Zootc. 2014; 66(5): 1615–7.
3. Szwajkowsa M, Wolanciuk A, Barlowska J, Krol J ZL. Bovine milk proteins as the source of bioactive peptides influencing the consumers' immune system. Anim Sci Pap Rep 2011, 29269–280. 2011; 29: 269–80.
4. Kumar S, Chouhan S, Sanghi A TU. Antioxidative effect of yak milk caseinate hydrolyzed with three different proteases. Vet World. 2013; 6: 799–802.
5. Kusumaningtyas E, Agustio A, Gholib D, Widiastuti R, Kusumaningrum HD SM. Antifungal Activity of Peptide from Goat Milk Hydrolysate to Candida albicans and Trichophyton mentagrophytes. In: Prosiding Seminar Nasional Teknologi Peternakan dan Veteriner. 2015. p. 189–96.
6. Kusumaningtyas E, Widiastuti R, Kusumaningrum HD SM. Bioactivities and analysis of peptides of Sumbawa horse milk generated by Bacillus thuringiensis protease. JITV. 2016; 21(4): 244–54.
7. Josephine FS, Ramya VS, Devy N, Ganapa SB, Siddalingeswara KG, Venugopal N, Vishnawatha T. Isolation, production and characterization of protease from Bacillus sp. isolated from soil sample. Biotech Res. 2012; 2: 163–8.
8. Patel RK, Dodia MS, Joshi RH SS. Purification and characterization of alkaline protease from a newly isolated halo alkaliphilic Bacillus sp. Process Biochem. 2006; 41: 2002–9.
9. Vandanjon L, Johannsson R, Derouiniot M BJP. Concentration and purification of blue whiting peptides hydrolysate by membrane processes. J Food Eng. 2007; 83: 581–9.

10. Chabeaud A, LeGal Y, Alvarez OM, Berge J, Batista I, Pires C, Thorkelsson G, Delannoy C, Jakobsen G, Johansson I BP. Impact of ultrafiltration and nanofiltration of an industrial fish protein hydrolysate on its bioactive properties. J Sci Food Agr. 2010; 90: 1819–26.
11. Cascales JJL, Zenak S, Torre JG, Lezama OG, Garro A ER. Small cationic peptides: influence of charge their antimicrobial activity. ACS Omega 2018,(3) 5390–5398. 2018; 3: 5390–8.
12. Lenardon MD, Sood P, Dorfmueller HC, Brown AJP GN. Scalar nanostructure of the Candida albicans cell wall; a molecular, cellular and ultrastructural analysis and interpretation. Cell Surf. 2020; (100047).
13. Ullivarri MF, Arbulu S, Garcia-Gutierrez E CP. Antifungal peptide as therapeutic agents. Front Cell Infect Microbiol. 2020; (17 March).
14. Tidona F, Cricione A, Guastella AM, Zuccaro A, Bordonaro S MD. Bioactive peptides in dairy products. Ital J Anim Sci. 2009; 8(3): 315–40.
15. Li Y, Yu J, Goktepe I AM. The potential of papain and alcalase enzyme and process optimizations to reduce allergenic gliadins in wheat flour. Food Chem. 2015; 196: 13388–1345.