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Fractionation and Identification Antimicrobial Activity of Sumba Mare Milk Protein Against Causative Agent of Subclinical Mastitis

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Abstract: The aims of this study were to fractionate and to identify antimicrobial activity of Sumba mare's milk protein against causative agent of subclinical mastitis. Antimicrobial compounds isolation of whey protein were fractionate using alkaline alumina column. Determination of antimicrobial activity of protein fractions through suseptibility test against bacteria causing subclinical mastitis which have been isolated and confirmed (sugar fermentation, indole, methyl red, voges proskauer, hydrogen sulphide, citrate and catalase) from dairy farms in the area of Bogor. Identification of antimicrobial compounds using HPLC method, there are six main peaks conferring to their different polarities and retention times. Fractionation results of six fractions with different polarity levels were tested for antimicrobial activity against bacteria causing subclinical mastitis (*Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae and Streptococcus pyogenes*). The third whey protein fraction, soluble in acetone exhibited significant inhibition activity on the growth of *S. agalactiae* and *S. pyogenes*.

Key words: Horse Milk · Milk Fraction · HPLC Method

INTRODUCTION

The utilization of mare's milk as a source of food has been known for a long time, especially in Mongolia and Russia because they are similar to human milk and has a therapeutic effect on various diseases [1-3]. Fermented mare's milk products, which are known as Kousmiss, have been used as therapy for patients with digestive disorders and cardiovascular disease [4-6]. Due to its hypoallergenic properties, mare's milk has become an important food source in Europe especially in Italy, France, Hungary and the Netherlands where it has been used as a substitute for cow's milk for children who experience allergic reactions when consuming cow's milk [7, 8]. In addition to having a ratio of whey protein and casein that is similar in composition to human milk proteins, mare's milk is also considered to have high digestibility ability and rich in essential nutrients make it suitable in pediatric dietetics [9]. Mare's milk has a high nutrition value: proteins, fats, lactose and numerous biologically active substances such as minerals and vitamins.

Milk proteins composed of essential amino acids (lactoferrin, immunoglobulin, lysozyme) have a function as an antimicrobial and immunomodulator [10]. Antimicrobial activity is what causes the milk to survive against some spoilage bacteria and to have a longer shelf life [11]. Mare's milk protein contains two primary components, namely casein and whey. The comparison of whey and casein in mare's milk is 1:1.1 [7, 12]. The component of whey milk that plays role as antimicrobial [11] and imunomodulator for children and adults [13] consists of immunoglobulin, lizosim dan lactoferrin [3, 7].

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The study of equine milk in Indonesia has been carried out primarily on Sumbawa horse milk. Research by Hermawati *et al*. [14] showed that Sumbawa horse milk has antimicrobial ability against 9 types of bacterial pathogens in food. Gram-negative bacteria is most sensitive to Sumbawa mare's milk compared to Gram-positive bacteria. The potential therapeutic properties of Sumbawa mare's milk have been studied by Rijatmoko [15] and Pana [16] specifically in investigating antimicrobial activity against *Mycobacterium tuberculosis*. Other studies have shown that colostrum from Sumbawa mare's milk has an antimicrobial ability against *Bacillus anthracis* [17]. The Sumba horse is native to Indonesia on the island of Sumba, East Nusa Tenggara. Sumba's horses have some similarities with the Sumbawa's horse. According to Pickeral [18], the Sumba horse and Sumbawa horse are the same pedigree. The antimicrobial activity in mare's milk has the potential to against diseases as subclinical mastitis in dairy cattle.

The incidence of subclinical mastitis in dairy cattle in Indonesia ranges from 80-97%, [19] and can lead to a decrease of 15-40% in milk production per day, decreased milk quality, rejection of milk by the dairy industry and the high cost of treatment moreover, early culling of dairy cattle. Problems also often arise when mastitis is treated with antibiotics which leads to residues that can affect consumer health problems. The aims of this study was to fractionate and to identify antimicrobial activity of Sumba mare's milk protein against causative agents of subclinical mastitis. Research results are expected to provide information on the antimicrobials fractions of the Sumba mare's milk as an alternative method to prevent subclinical mastitis.

MATERIALS AND METHODS

Milk Sample: A total of 40 Sumba mare's milk samples collected from the third to the fifth month of lactation period. Milk samples were taken in East Sumba and Central Sumba from January to March 2012 and the laboratory experiment was conducted from April to December 2012 in the Laboratory of Veterinary Public Health, Faculty of Veterinary Medicine, Bogor Agricultural Institute and National Veterinary Product Assay Laboratory, Bogor. The first stage of this research was fractionation and isolation of antimicrobial compounds in whey protein of Sumba mare's milk. The second stage was to determinate antimicrobial activity of protein fraction through suseptibility test against causative agents of subclinical mastitis that have been isolated and confirmed from dairy farms in the area of Bogor.

Extraction and Identification of Protein Whey: The whey protein extraction process begins with separation of the mare's milk fat by speed centrifuge 2000 *g* at 4°C, for 30 minutes. Skimmed milk is acidified to a pH of 4.2 [20] by adding 2 N HCl. The solution was then centrifuged at a speed of 10000 *g* at 4°C, for 30 minutes. The sediment (casein) was removed; whey acid was neutralized to pH 6.8 by 2 N NaOH, which then centrifuged at 10000*g* at 4°C for 30 minutes. The whey supernatant obtained was neutral [21-23].

Whey obtained neutral is put through a cartridge seppak cleaning process using column 18 (C-18). The solution containing the protein is collected and evaporated to remove the methanol and other residues, then whey is filtered using a whatman filter 0.2 µm and inserted into the 1 ml tube to be injected on high performance liquid chromatography (HPLC). The mobile phase used was a mixture of distilled water, acetonitrile (ACN) and Trifluoroacetic acid (TFA) with successive comparison of 95:5:0.1% [24]. The UV detector at 220 nm wavelength and a C-18 reversed phase were used. When the whey protein mare's milk was running on HPLC, fractions were observed based on retention times.

Fractionation and Isolation of Antimicrobials in Whey: The isolation process begins with manual fractionation using alkaline alumina column and inserted into the burette. Methanol is slowly poured into the burette and left for one night. The solution is poured into the burette in the order from the lowest polarity to the highest polarity, that is, chloroform, ethyl acetate, acetone, methanol, acetonitril and distilled water. Then include 10 ml of whey protein from the Sumba mare's milk. The compound solution is separated into clear boundaries indicated different components in the Sumba horse milk. The isolation process is completed by opening the burette to accommodate the whey fraction based on the visible boundary wall tubes (Figure 1). Each fraction included into the tube is evaporated completely before distilled water is added and made homogenous with vortex. The tube is then inserted into the sonification machine to remove air bubbles. Before continuing the process of suseptibility, whey fractions are filtered using a whatman filter 0.2 µm [25].

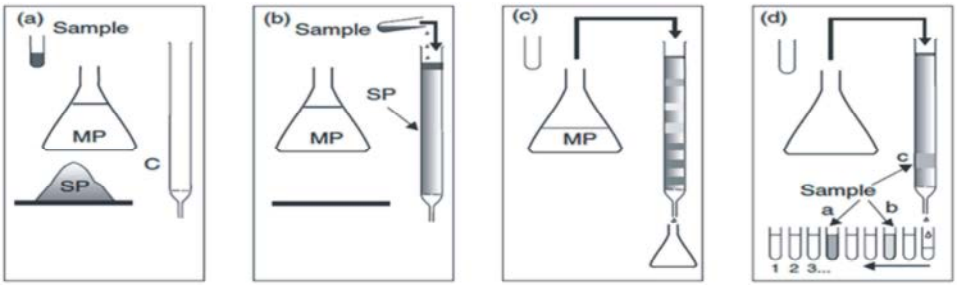

Fig. 1: (a) Components (C: column; SP: stationary phase; MP: movement phase; Sample: mare's milk whey (b) the process of putting the sample into the column (c) the proses of elution (d) isolation sample.

Activity Antimicrobials in Sumba Mare's Milk Against Causative Agents of Subclinical Mastitis: Determination of antimicrobial activity of protein fraction through suseptibility test against causative agent of subclinical mastitis. The principle of suseptibility test is used in the detection of antimicrobials in milk content of Sumba horses and their inhibitory ability. Causative agent of subclinical mastitis (*Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae and Streptococcus pyogenes*) that have been isolated and confirmed from dairy farms in the area of Bogor. The samples were cultured on Nutrient agar, Blood agar and MacConkey agar plates. The isolated bacteria were identified by morphological characteristics and sub culturing on differential and selective media. The bacterial isolates further subjected to biochemical tests for confirmation (Sugar fermentation, Indole, Methyl Red, Voges-Proskauer, Hydrogen Sulphide, Citrate and Catalase) [26]. After the bacterial suspension dries on the surface of the Muller Hinton agar, antimicrobial fractions samples (75 µl), Sumba mare's milk whey (75 µl), whole milk (75 µl) and control (antibiotic and lactoferrin 100 ppm) were included and then incubated at 37°C for 18-24 hours. The isolates were tested for their antimicrobial susceptibility by agar disk diffusion method in accordance with the standard in National Mastitis Council guidelines. The susceptibility of the antimicrobials activity was determined by the inhibition zone diameter [27, 28].

RESULTS

Identification of Whey Proteins, Fractionation and Isolation of Proteins That Exist in the Sumba Mare's Milk Whey: Identification of antimicrobial compounds using HPLC method, there are 6 (six) main peaks with different polarities. The sixth peak appear in different retention time in respectively 4.091, 4.349, 4.516, 5.336, 5.721, 8.779 (Figure 2). Compounds fractionation using alkaline alumina column through elution process. Fractions isolated made the testing of suseptibility easier.

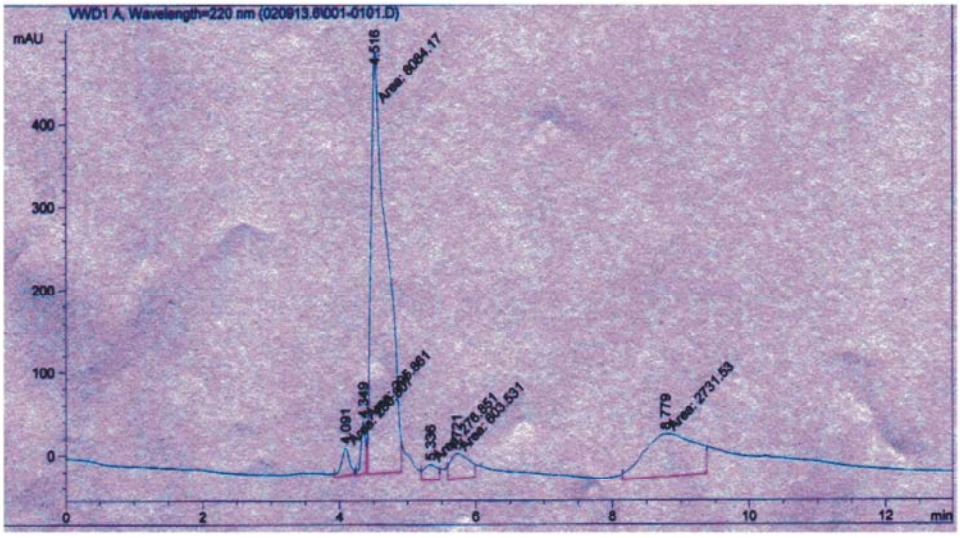

Fig. 2: Six Fraction of whey protein in chromatography.

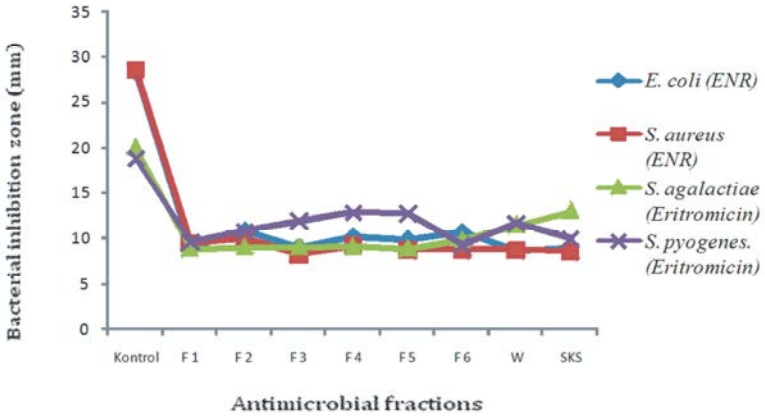

Fig. 3: The antimicrobial activity test in fractions using antibiotics (enrofloxacine, erythromycin) as control (F1: fraction soluble in chloroform, F2: Fraction soluble in ethyl acetate, F3: Fraction soluble in acetone 3, F4: Fraction soluble in methanol, F5: Fraction soluble in acetonitrile, F6: Fraction soluble in soluble distilled water 6, W: whey protein from the Sumba mare' milk, SKS: Sumba mare's milk).

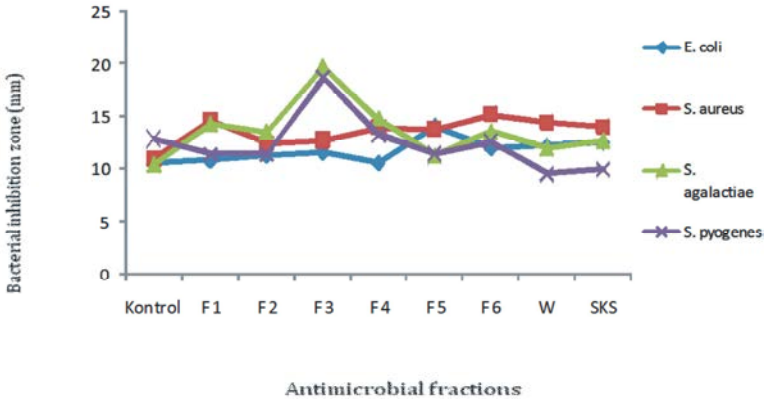

Fig. 4: The testing of antimicrobial ability in fractionation with Lactoferrin as control (F1: fraction soluble in chloroform, F2: Fraction soluble in ethyl acetate, F3: Fraction soluble in acetone 3, F4: Fraction soluble in methanol, F5: Fraction soluble in acetonitril, F6: Fraction soluble in soluble distilled water 6, W: whey protein from the Sumba mare' milk, SKS: Sumba mare's milk)

Alkaline alumina column can bind polar compounds during the fractionation process such that the non polar compounds will appear first, followed polar compounds. The fractions that emerged underwent a coagulation process and was evaporated to remove residual solvent that was mixed during the motion phase. This is to ensure that antimicrobial properties in the fraction was not derived from the solution in the mobile phase.

Activity of Antimicrobials Compounds in Sumba Mare's Milk Against Bacteria Causing Subclinical Mastitis: Determinate of antimicrobial activity of protein fraction through suseptibility. The fractionation resulted in six fractions: chloroform (first), ethyl acetate (second), acetone (third), methanol (fourth), acetonitril (fifth) and distilled water (sixth) fractions that were tested for their antimicrobial activity against (*Escherichia coli, Staphylococcus aureus, Streptococcus agalactiae and Streptococcus pyogenes)*

The testing of antimicrobial ability in the Sumba mare's milk was carried out with two types of control namely antibiotics (enrofloxacine, erythromycin) and lactoferrin (Sigma). Lactoferrin (Sigma) 100 ppm was used because it is thought to have the same funtion as natural antimicrobials contained in milk protein. The testing of inhibition abilities from the use of antibiotics as the control indicated that the Sumbawa mare's milk has antimicrobial activity against the tested bacteria, but its inhibition ability is not as strong as the control (Figure 3). This difference is thought to be due to the lower antimicrobial fraction in the Sumba mare's milk compared with antibiotics.

The inhibition abilities test from the use of lactoferrin as the control indicated that the Sumba mare's milk has antimicrobial activity against causative agen of mastitis subclinical, which can be observed by their inhibitory zone diameter. The suseptibility results illustrated that the Sumbawa mare's milk has antimicrobial activity against causative agents (Figure 4). Fraction 3 was shown to have high inhibition against bacterial growth and was higher than lactoferrin as control. Third fraction whey protein compound which soluble in acetone has inhibition activity on the growth of bacteria *S. agalactiae* and *S. pyogenes* ($P<0.05$).

DISCUSSION

Column C-18 which are non-polar molecules will bind polar molecules; and the polar mobile phase will emerge with polar compounds. This means that the fraction with the earliest exit is the polar fraction while the fraction subsequently is non-polar. The fraction that appears earliest is suspected to consist of larger molecules in compared with the fraction that emerges subsequently.

According to the review Uniacke-Lowe *et al*. [7], it has been stated that whey protein in milk consists of β-lactoglobulin horse (β-Lg), α-laktoalbuminktoalbumin (α-lactalbumin), immunoglobulin (Ig), blood serum albumin (BSA), Lactoferrin and Lysozyme which is similar to cow's milk. The relative amount of whey protein differs significantly between the various species. While whey protein from mare's milk contains lower β-Lg compared with cow's milk, it contains α-lactalbumin and higher Ig. Mare's milk contains a higher amount of Lysozyme and Lactoferrin (which are major contributors to its antimicrobial ability) than cow's milk. Note however that Lactoferrin is also dominant in human milk [12]. Together, IgA, IgG, IgM, Lactoferrin and Lysozyme provide immunity in neonatal and protection against infection [29].

Antimicrobial inhibition ability is affected by characteristics of bacteria such as Gram-negative and Gram-positive bacteria. Bacteria are known as disease causing agents such as *S. aureus* were the leading causes of clinical and subclinical mastitis [30-32]. According to Bekele *et al.* [33] showed that bacteriological examination of mastitis positive quarters revealed *S. aureus* (53.5%), *S. agalactiae* (26.5%), *E. coli* (12.5%). In agreement with Ahmed *et al.* [34], *S. aureus*, *E. coli* and *S, agalactiae* as the bacteria causing subclinical mastitis incidence. Incidence of mastitis caused by *S. aureus* is generally associated with contamination of the human hand and the main pathogen that often causes chronic and subclinical mastitis, while *E.coli* in milk is associated with environmental pollution. *Streptococcus* spp, especially *S. agalactiae*, which is totally dependent on the udder in order to survive in the wild [35, 36]. Based on the results of the study, it can be concluded that there are six fractions in whey protein of the mare's milk. Sumba mare's milk has antimicrobial ability against causative agent subclinical mastitis, with the highest inhibition zone diameter in *S. agalactiae* and *S. pyogenes*.

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