Studii şi Cercetări Ştiinţifice
Chimie şi Inginerie Chimică, Biotehnologii, Industrie Alimentară
2022, **23** (1), pp. 063 – 071

Scientific Study & Research
Chemistry & Chemical Engineering, Biotechnology, Food Industry
ISSN 1582-540X

ORIGINAL RESEARCH PAPER

DEGRADATION OF CAPRINE CASEIN BY PAPAIN, AND ITS ANTIBACTERIAL EFFECT TOWARDS *ESCHERICHIA COLI*

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Received: October, 06, 2019
Accepted: March, 23, 2022

Abstract: Caprine milk is one of the animal protein sources with high nutrition and relatively easy to digest. Caprine milk has high protein content (3.4 %) which is potential to produce bioactive peptides such as antimicrobial peptides. This study was to analyze the profile of peptides from caprine casein hydrolyzed with papain and analyze their bioactivity as antibacterial toward food pathogens *Escherichia coli* and *Staphylococcus aureus*. The caprine casein was separated by adding HCl to the defatted caprine milk until the *p*H reached isoelectric point. The coagulated protein was recovered by centrifugation. *α*-Casein (MW 32 kDa), *β*-casein (MW 24 kDa) and other caprine protein were hydrolyzed using papain enzyme (4000-6000 units·g^{-1}) for 15 minutes leaving *κ*-casein (MW 21 kDa) and producing one new peptide band (MW 10 kDa) as seen following SDS-PAGE. Both casein and peptide hydrolysates inhibited *Escherichia coli* but not *Staphylococcus aureus*. Further analysis showed that unhydrolyzed casein reduced *Escherichia coli* growth significantly by 1.6 log after 2 hours of exposure and the inhibition was increased by exposure time. However, the hydrolyzed casein only shows little (non-significant) inhibition after 4 hours incubation with the bacteria.

Keywords: *antibacterial, caprine casein, casein hydrolysates, Escherichia coli, papain*

INTRODUCTION

Milk is known as food with complete nutrition content. Aside from the high nutritional value of milk (carbohydrates, proteins, fats, vitamins, and minerals), bioactive components in milk have important physiological and biochemical functions to human health. Some act as antihypertensive, antioxidative, antithrombotic, hypocholesterolemic, immunomodulatory, antimicrobial, etc. [1]. Milk bioactive components can be derived from protein, fat, vitamins and minerals. Casein, as the main protein source, makes up to 80 % of the total milk protein.

Despite of their high demand, the use of conventional antibiotics is related to various resistance problems in bacteria, including pathogenic bacteria in food. The emerging of bacterial resistance to antibiotics becomes one of the issues that encourage scientists to explore new antimicrobial substances with more natural characteristics. Over the past twenty years, multiple reports have pointed to protein act as antimicrobial peptide precursors which can also boost the immune system against pathogenic microorganisms [2].

Caprine milk is one of animal protein source with high nutritional value and relatively easy to digest. Caprine milk protein content (3.5 %) is higher than cow milk (3.3 %) [3]. The high protein content is potential to produce peptides with beneficial activity. Bioactive peptides are peptide with a specific sequence, that have a positive impact on the body functions. Bioactive milk peptides can be obtained by several hydrolysis methods, such as hydrolysis by digestive enzymes, and by proteolytic enzymes produced by microorganisms or plants [4].

Protease is an enzyme that hydrolyzes the protein substrate. Based on the enzyme origin, there are three types of proteases, namely, protease originated from plants, animals, and microorganisms. Papain, bromelain, and ficin are examples of proteases produced from the plant [5]. Proteases from animals included trypsin, chymotrypsin, pepsin, and rennin [5]. Proteases from microorganisms can be intracellular and extracellular protease such as proteases from *Fusobacterium nucleatum* and *Bacillus subtilis* [5, 6]. Different types of protease enzymes have different substrate specificity which results in peptide fragments with different sequences. Protein fragments can be analyzed by SDS-PAGE. It provides information on the peptide and protein profiles at different molecular sizes.

Proteins from various sources have been identified as a precursor for antimicrobial peptides including those from eggs [7], marine products [8], and milk protein and peptides from cow, sheep, goat, and human [9]. An antibacterial peptide from casein-αs2 bovine milk, Casocidin-I (f165-203) was found to inhibit the growth of *Escherichia coli* and *Staphylococcus carnosus* [10]. Purified casein hydrolysates ovine milk (αs2-Ovine f (165-181)) has been reported to inhibit the growth of *Escherichia coli*, *Listeria innocua*, *Staphylococcus epidermidis*, *Enterococcus faecalis*, *Serratia marcescens*, and *Staphylococcus carnosus* [11]. Study on bioactive peptides from caprine milk (especially Indonesian caprine) has not been actively reported as that of bovine.

The objective of this study was to analyze the profile of peptides from caprine casein hydrolyzed with papain and analyze their bioactivity as antibacterial toward food pathogens *Escherichia coli* and *Staphylococcus aureus*.

MATERIALS AND METHODS

Raw material

In this study, the Etawah Breader Caprine (descendant of Jamnapari goat) was used to collect sample milk. Fresh caprine milk was collected from the milk processing unit, university farm, IPB University. The milk was defatted by centrifugation (2,000 g, 4 °C, 30 min) and pasteurized (72 °C, 15 s). The casein was prepared by isoelectric precipitation at *p*H 4.6 with the addition of 2 N HCl. The precipitate was washed with distilled water [12]. Commercial enzyme papain (4,000-6,000 units·g^{-1}) was purchased from Merck, Darmstadt, Germany. Other chemicals such as sodium dodecyl sulfate (SDS), acetic acid, HCl, glycerol, bromophenol blue and *β*-merchaptoetanol were analytical grade.

Preparation of casein hydrolysates

Casein was dispersed in phosphate buffer *p*H 7 0.05 M at concentration 15 % (w/v). Papain was added at an enzyme: substrate ratio of 1 : 1000 (w/w). Enzymatic hydrolysis was carried out in a water bath at 50 °C for 15, 30, and 45 min. The reaction was stopped by heat treatment at 85 °C for 15 minutes [13]. Hydrolysates were then rapidly cooled in an ice bath. The insoluble material was removed by centrifugation (1,600 g, 5 min) using microcentrifuge (Tomy MRX-152). The supernatant was microfiltered (0.45 µm, Minisart, Sartorius, Germany) and stored at −20 °C until use.

Bacterial strains and culture condition

Escherichia coli ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were cultured in tryptic soy broth media (Oxoid, Hampshire, UK), at 37 °C. Active growing cultures were used in the antibacterial studies.

Gel electrophoresis

SDS-PAGE was performed following method by Laemmli [14], with some modification as described by Sinthusamran *et al.* [15]. Samples were dissolved in 5 % sodium dodecyl sulfate (SDS) at ratio of 1 : 5 (v/v) for defatted caprine milk and caprine casein (unhydrolyzed) sample and 1 : 1 (v/v) for hydrolyzed casein sample. The mixtures were incubated at 85 °C for 1 h in a temperature-controlled water bath (Certomat® WR Shaking Waterbath, Sartorius Stedim Biotech, Germany). The mixtures were centrifuged at 1,600 g for 5 min at room temperature to remove undissolved debris. Solubilized samples were mixed at a ratio of 1 : 1 (v/v) with the sample buffer (0.6 % Tris HCl 1 M, *p*H 6.8; 20 % SDS; 50 % glycerol; 1 % bromophenol blue; 0.9 % double-distilled water; 5 % *β*-merchaptoetanol). The mixtures were kept in boiling water for 2 min. Samples were loaded into polyacrylamide gels consisted of 15 % running gel and 4 % stacking gel and subjected to electrophoresis at a constant current of 50 mA for 4 h and 30 min using Mini Protean 3 Cell unit (Bio-Rad Laboratories, Inc., Richmond, CA, USA). After electrophoresis, the gel was stained with 0.05 % (w/v) Coomassie blue R-250 in 40 % (v/v) methanol and 10 % (v/v) acetic acid. The destaining process was run with 40 % (v/v) methanol and 10 % (v/v) acetic acid. Low molecular weight

Multicolor Low Range Protein Ladder (1.7-40 kDa) Thermo Scientific were used as protein standard to estimate the molecular weight of proteins.

Assay of antibacterial activity

The assay of antibacterial activity was conducted using a disk diffusion test (6 mm which accommodated ± 40 µL sample) at approximately 10^7 CFU·mL^{-1} bacterial test concentration with spread method. Amoxylin 1 mg·mL^{-1} was used as a positive control and phosphate buffer as a negative control. After 18 h of incubation at 35 °C, inhibitory zone diameters were analyzed [16, 17].
The contact method was used in further antibacterial test. One milliliter of casein sample which had a positive effect in the disk diffusion test was added to 1 mL tryptic soy broth medium and inoculated with *E. coli* culture at approximately 10^4 CFU·mL^{-1} concentration. The control that we used in this method was sterile aquadest. Bacterial growth was measured by counting the number of viable cell (colony forming units) on Luria Bertani agar plate (HiMedia) at every 2 hours starting from 0 to 6 hours incubation. Two parallels plates of each dilution were incubated for 24 h and then counted. Each experiment was repeated two times [18].

Statistics

The number of viable cells and inhibitory zone were calculated with standard deviation, and the results are presented as mean values with ± SD. ANOVA and Duncan different tests were run to compare the differences in the inhibitory zone and growth-curves of samples from the control. The differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

SDS-PAGE profile

Defatted caprine milk shows ten protein bands in the SDS profile with apparent molecular weight of 68, 60, 55, 34, 25, 21, 19, 18, 15 and 11 kDa (Figure 1). After coagulation into casein, they all appeared on casein SDS-PAGE profile with addition 12 kDa protein band. The three proteins with high molecular weight (68, 60, and 55 kDa) were much thinner while other proteins (21 and 19 kDa) were thicker than those in the defatted milk. The HCl coagulation is expected to dissolve the protein with molecular weight of 68, 60 and 55 kDa into the whey part, but in our study, they still appeared as very thin protein band. This may be caused by imperfect casein rinsing method, which probably should be more than three times. The presence of protein with molecular weight of 21 and 19 kDa were becaused of hydrolysis process by HCl that we used to separate casein and whey from the milk.
The protein was not totally coagulated into casein aggregate but left in the whey part. Casein has three main protein bands α-casein (34 kDa), β-casein (25 kDa), and κ-casein (21 kDa) [19]. When hydrolyzed by papain at *p*H 7, the α-casein and β-casein protein bands disappeared leaving κ-casein (21 kDa) and one new band of about 10 kDa (Figure 1).

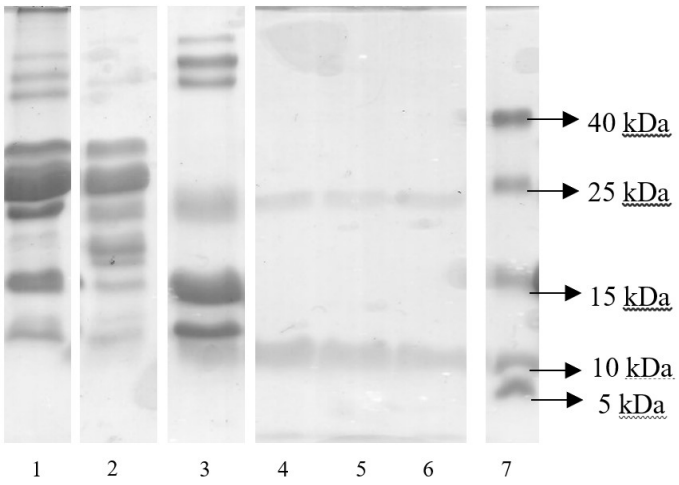

Figure 1. *SDS-PAGE pattern of protein from caprine milk and peptides hydrolysates: 1. Defatted caprine milk, 2. Caprine casein (unhydrolyzed), 3. Caprine whey, 4. Casein hydrolyzed at 15 minutes, 5. Casein hydrolyzed at 30 minutes, 6. Casein hydrolyzed at 45 minutes, 7. Marker*

Hydrolysis casein by papain enzyme in this study was shown by the disappearance of the protein bands in SDS-PAGE analysis. At 30 and 45 minutes hydrolysis time, there was no significant change of the protein bands from the 15 minutes hydrolysis time. This may be due to the strong ability of papain to hydrolyze the casein substrate. In another study, at 37 °C and 10 minutes - 24 hours hydrolysis time, papain was found to be slightly more effective than pancreatin and trypsin in hydrolyzing natrium caseinate with the degree of hydrolysis being 13 - 22 % [19].

Antibacterial activity

Antibacterial activity of caprine casein (unhydrolyzed) and casein hydrolysates were assayed using disk diffusion test toward *Escherichia coli* ATCC 25922. Inhibition was calculated based on the diameter of the clear zone including disk diameter (6 mm) (Figure 2). The concentration of the test bacteria used was approximately 107 CFU·mL^{-1} with volume hydrolysates in each disk being 40 µL.

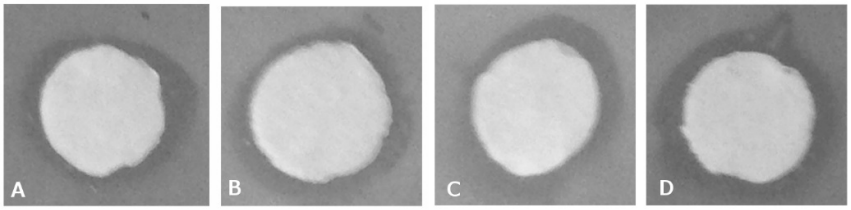

Figure 2. *Caprine casein inhibition zone toward E. coli: (A) caprine casein (unhydrolyzed), (B) casein hydrolyzed at 15 minutes, (C) casein hydrolyzed at 30 minutes, (D) casein hydrolyzed at 45 minutes*

Based on data at Table 1, it can be seen that the casein (unhydrolyzed) and casein hydrolysates have greater inhibition toward *E. coli* compared to *S. aureus* with an average diameter of inhibition zone of more than 7 mm. Inhibition of casein on *E. coli* was shown by both casein (unhydrolyzed) and casein hydrolysates. The inhibitory zone of the casein (unhydrolyzed) and casein hydrolysates toward *S. aureus* were insignificant compared to the negative control. Similar study using larger concentration of goat milk casein (50 % of total solid) showed inhibition both to *E. coli* and *S. aureus,* where inhibition to *E. coli* was greater than to *S. aureus* [20].

Table 1. *Inhibitory zone after treatment with casein and casein hydrolysates*

Casein hydrolysates	**Inhibitory zone**	
	***E. coli* [mm]**	***S. aureus* [mm]**
Caprine casein (unhydrolyzed)	7.70 ± 0.35*	6.00 ± 0.00
Casein hydrolyzed at 15 minutes	7.77 ± 0.17*	6.38 ± 0.33
Casein hydrolyzed at 30 minutes	7.60 ± 0.48*	6.42 ± 0.37
Casein hydrolyzed at 45 minutes	7.69 ± 0.60*	6.32 ± 0.30
Negative control (phosphate buffer)	6.00 ± 0.00	6.00 ± 0.00

*Duncan different test at 95 % confidence interval to the negative control

After 18 h of incubation at 35 °C, inhibitory zone was measured as diameter of clear zone in disk diffusion test (Figure 2). The disk diameter applied was 6 mm. Antibacterial peptide works by interacting with the bacterial membrane component, followed by membrane damage and physiological disorders of the cell wall biosynthesis, cell division, or translocation across the membrane to interact with a cytoplasmic target [21]. It is generally assumed that the positive pole of the peptides interacts with the negative pole of lipids on the outer surface or cytoplasmic membrane. Further, then, the peptides insert and make orientation parallel to the bilayer position, into the cytoplasmic membrane which later result in the release of lipids [21, 22]. Gram-positive bacteria have monolayer lipid and thick peptidoglycan while gram-negative bacteria have lipid bilayer and thin peptidoglycan. The differences between membrane cell characteristics of the gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*) might explain the different inhibitory effects of the casein and its hydrolysates.

Further antibacterial testing was conducted by incubating the bacteria in tryptic soy broth media in the presence of the casein (unhydrolyzed) and casein hydrolysates for various time. This is to observe further antibacterial effect base on contact time with the bacteria. In this method, we only analyzed the inhibitory effect on *E. coli* because it showed more significant inhibition on the agar diffusion method, while *S. aureus* did not show significant inhibition (Figure 2). The results showed that *E. coli* growth in the casein (unhydrolyzed) media was inhibited within the first two hours. The reduced number of *E. coli* was 1.6 log from the initial amount (0 hour) and 2 logs from the control. The number of bacteria was reduced which indicated that the casein (unhydrolyzed) acted as bactericidal. In the presences of casein hydrolysates (15, 30, and 45 min) the growth was not different from the control, even though in the presence of the hydrolyzed casein (especially casein hydrolyzed for 30 minutes) at four hours incubation, the bacterial growth was somehow slower compared to the control (reducing 1.2 log of *E. coli*) (Figure 3).

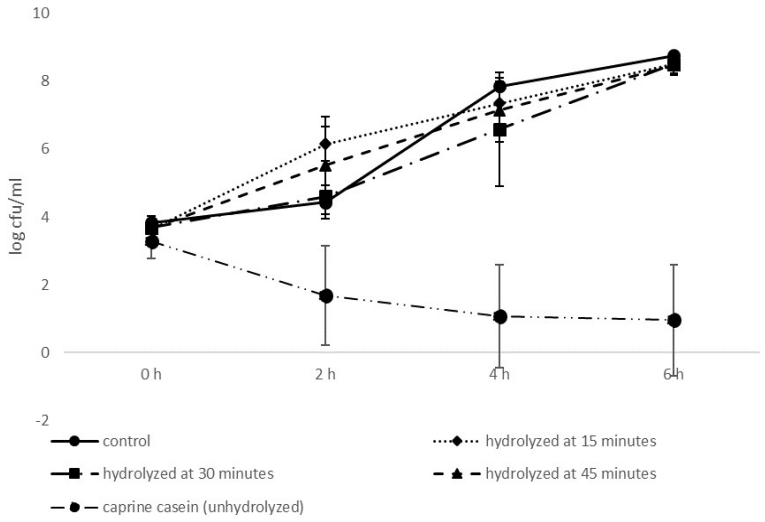

Figure 3. *Inhibition of caprine casein and hydrolyzed casein on the growth of Escherichia coli*

Casecidin αs1 was a peptide derived from bovine casein digested by chymosin, this peptide was able to inhibit the growth of *Staphylococcus, Bacillus subtilis*, *Diplococcus pneumonia*, and *Streptococcus pyogenes* [23]. The antibacterial peptides from casein-αs2 bovine milk, Casocidin-I (f165-203) were effective against *E. coli* and *S. carnosus* [10]. Ovine casein digested by pepsin (s2-Ovine f (165-181)) has been proved to inhibit the growth of *E. coli*, *L. innocua*, *S. epidermidis*, *E. faecalis*, *S. marcescens*, and *S. carnosus* [11]. In this study, the casein (unhydrolyzed) was much more effective as antibacterial compare with its hydrolysates product toward *E. coli* (Figure 3).

Papain enzyme has sulfhydryl functional group and hydrolyzes peptide bond at lysine and glycine amino acids [24]. Differences result from other study may be due to different enzyme used, which has different amino acid specificity. Papain hydrolyzed the peptide bond on lysine and glycine amino acid, while digestive enzymes such as trypsin hydrolyzed at arginine and lysine amino acid; pepsin at alanine, glycine and valine amino acid; and chymotrypsin on tryptophan, tyrosine, and phenylalanine [25]. Usage of different type of enzyme on similar protein substrate can produce different peptides with different amino acid sequences. This might explain the different result obtained in this study.

In this study, the variation of hydrolysis time did not have much difference in the effect of antimicrobial peptides. This may occur because the time variation is not much different, Omara [20] reported that caprine milk casein hydrolyzed by papain for 2 hours with enzyme: substrate ratio of 1 : 100 (w/v) resulted in a degree of hydrolysis of 7.68 ± 0.01 % and inhibition zone on *E. coli* of 10.90 ± 0.06 mm. The degree of hydrolysis indicates the amount of casein that is disgested by papain. The amount of casein digested by papain increased (28.5 %) when the hydrolysis time was prolonged to 5 hours with enzyme: substrate ratio of 1 : 150 (w/v) [26]. Through this observation, it can be stated that a longer hydrolysis time can result in more protein cleaved by the enzymes. In addition, the ratio between the enzyme and the substrate must also be

considered, if there's too little substrate then the peptide produced is not sufficient. This will have an impact on the antimicrobial peptide effect, the less peptide produced, the lesser the effect. However, when hydrolysis time is too long or the ratio of enzyme/substrate is too large can also result in more protein cleaved, resulting in free amino acids that do not have bioactive properties [26].

CONCLUSIONS

Caprine casein hydrolyzed using papain enzyme resulted in the disappearance of α-casein (MW 32 kDa) and β-casein (MW 24 kDa) and leaving κ-casein (MW 21 kDa) and one peptide of about 10 kDa. Both caprine casein (unhydrolyzed) and its hydrolysates produce inhibition zone toward *Escherichia coli* greater than *Staphylococcus aureus*. Further testing with the contact method showed that the caprine casein (unhydrolyzed) can decrease *Escherichia coli* growth significantly which indicates that the caprine casein can behave as bacteriocidal.

ACKNOWLEDGMENTS

This research was supported by the IPB University Research Fund 2013.

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