Antioxidant activity of soluble protein from natural fermented buffalo milk

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Abstract. Curd from buffalo milk, called dadih, is famous fermented milk from West Sumatra, Indonesia. The milk is fermented naturally by relying on microorganisms which exist nature. Dadih is commonly consumed as breakfast or eaten together with rice. This study aimed to determine the antioxidant activities of dadih from different regency in West Sumatra by using ABTS, DPPH and Fe reducing power methods. The soluble protein of dadih was separated from insoluble protein and fat by centrifugation at 11,000 x g. The supernatant was then assayed for protein content and antioxidant activities. The result showed that among dadih from Agam, Sijunjung and Solok regency, dadih from Agam regency showed lowest protein content but highest antioxidant activities in Fe reducing power with absorbance 1.28 and scavenging activity against radical ABTS (86.46%) or DPPH (82.24%). The result indicated that dadih, especially from Agam regency is very potent as an antioxidant.

Keywords: Antioxidant activity, soluble protein, buffalo milk, dadih

1. Introduction
Dadih is curd made from buffalo milk fermentation from especially ethnic group Minangkabau, West Sumatra, Indonesia. Dadih is very popular in several districts in West Sumatra such as Agam, Bukittinggi, Solok, Limapuluh Kota and Tanah Datar [1] and commonly used as breakfast or eaten together with rice. Due to it nutritious value and flavor, dadih is also used as salad dressing [2]. Dadih is processed traditionally by fermenting buffalo milk using natural microorganism inner a bamboo tube. Fresh cut of bamboo provides unique and enhance flavor. Fermentation occurs spontaneously at room temperature about 30°C for 2-3 days. The milk becomes thick and sour due to lactic acid bacteria in milk and other microorganisms in the bamboo. Commonly dadih was dominated by Lactococcus, Lactobacillus, Bifidobacterium, Streptococcus, and Pediococcus [3]. However, the composition of the bacteria in the final product may vary from time to time, from one to another place, depending on the handling and interaction of the microorganism in the product. Those Lactic acid bacteria were reported able to produce antioxidant [4].

Buffalo milk contains higher protein content compared to cow milk [5] which increase the possibility of more bioactive proteins or peptides. The fermentation process also involves protease enzyme of microorganism which breakdown protein to smaller one, peptides or free amino acid. The smaller protein or peptide may physiologically more active and beneficial for human health than its natural form.
Combination of microorganism in the fermentation process affects the bioactivity of the product. For example, fermented milk obtained from Lactobacillus plantarum Dad 13, Lactococcus lactis and Kluyveromyces marxianus had the low pH and alcohol while the combination of Lactobacillus plantarum Dad13, Lactococcus lactis and Saccharomyces cerevisiae produce high β-carotene content and high antioxidant activity [6].

Isolation of Lactic acid bacteria from dadih obtained from the different region showed different composition. Dadih from Payakumbuh and Sijunjung contain Lactobacillus plantarum while dadih from Padang Panjang contain Lactobacillus sp., Lactococcus sp. and Leuconostoc sp. [5]. It is possible that dadih from the different region showed various bioactivity such as antioxidant or inhibition of pathogenic bacteria. This study aimed to determine the antioxidant activities of dadih from different regency in West Sumatra by using ABTS, DPPH and Fe reducing power methods.

2. Materials and methods

2.1. Dadih and soluble protein preparation

Three days fermented buffalo milk was collected from 3 regencies in West Sumatra: Agam, Sijunjung and Solok, then stored in -20°C until used. Dadih was centrifuged at 11,000 x g, 4°C for 20 minutes. It produced 3 layers. The first layer was a lipid, the second one was soluble protein and the third one was insoluble protein. Lipid and insoluble protein were discarded and the soluble protein was collected using a syringe and put in the new tube. The protein obtained was then filtered using a 0.45 µm membrane. Protein concentration was analyzed using Quickstart™ Bradford protein assay (Bio-Rad Inc). The standard curve was obtained from reaction of 5 µL each serial dilution bovine serum albumin with 95 µL Bradford solution. Deionized water was used as blank. Absorbance was measured at λ 600 nm.

2.2. SDS-PAGE analysis

Protein profile of dadih was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using precast gradient polyacrylamide gel electrophoresis 4-20% (Any kD™ Mini-Protean® TGX gel, Bio-Rad Laboratories Inc.) with coomassie brilliant blue staining (Sigma-Aldrich, USA).

2.3. Antioxidant assay

The antioxidant assay of the protein against to radicals ABTS (2,2’-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) and DPPH (2,2-diphenyl-1-picrylhydrazyl) was conducted as follows [7, 8]: a radical ABTS was prepared by mixing of ABTS 7.4 mM and potassium persulfate (K₂S₂O₈) 2.6 mM (1:1, v/v) and then incubated at room temperature in the dark for 18 hours. The solution was diluted with deionized water until reaching absorbance of 1.1 ± 0.05 at λ = 405 nm. The fresh ABTS was then used for the antioxidant assay. As much 100 µL of soluble protein of dadih was added to 200 µL ABTS mix solution and incubated at room temperature for 15 minutes to allow the protein to react with ABTS. The absorbance of the mixture was measured at λ = 405 nm using microplate reader (Labsystems, original Multiscan Ex, and Champaign, USA).

The procedure of antioxidant assay for DPPH was slightly different from those for ABTS. The DPPH solution was diluted with ethanol until reaching absorbance of 1.1 ± 0.05 at λ = 540 nm. Afterward, 100 µL soluble protein of dadih was added to 200 µL DPPH solution, incubated at room temperature for 30 minutes, and its absorbance measured at λ = 540 nm. Serial concentrations of Vitamin C p.a was used as standard. The antioxidant activities of the soluble protein of dadih to ABTS and DPPH were expressed using the following equation:

\[
\text{Antioxidant activity} = \left[ \frac{A_0 - A_{\text{sample}}}{A_0} \right] \times 100\% 
\]
$A_0$ was absorbance of ABTS/DPPH without protein sample and $A_{\text{sample}}$ was the absorbance of ABTS/DPPH after reaction with protein sample.

Reducing power assay of the soluble protein of dadih was measured as described by Phisut and Jiraporn [9] with some modification. Dadih protein 250 $\mu$L was added to 500 $\mu$L sodium phosphate buffer 0.2 M (pH6.6) and 250 $\mu$L of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 minutes, followed by addition of 250 $\mu$L of 10% trichloroacetic acid (TCA) and centrifuged at 3500 x g at 25°C for 10 minutes. The 1 mL supernatant was added to 1 mL distilled water and 200 $\mu$L 0.1% FeCl$_3$. The mixture was incubated for 10 minutes and the absorbance was measured at $\lambda$ 700 nm. High of absorbance represents high reducing power.

3. Results and discussion

Protein profile of dadih from Agam Sijunjung and Solok was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (figure 1). Generally, the protein profile of dadih from those regions was not different.

![Figure 1. SDS-PAGE dadih from Agam (A), Sijunjung (B) and Solok (C), M: protein marker.](image)

Soluble proteins concentration obtained was different among dadih from Agam, Sijunjung and Solok (table 1). Dadih from Agam showed the lowest concentration compare to dadih from Sijunjung and Solok. Previously, it was reported that total protein of dadih from Agam was 70.6 mg mL$^{-1}$ followed by Solok 69.1 mg mL$^{-1}$ and Sijunjung 50.1 mg mL$^{-1}$ [5], indicated that dadih might be dominated by insoluble protein. Previous research revealed that the different length of fermentation of dadih affected the soluble protein produced. Soluble protein increased from 48-60 hours and decreased after 72-96 hours [10]. This might be associated with the presence of proteolytic enzymes produced by bacteria. Enzymatic hydrolysis of milk affected the consistency of milk which may also change the solubility of protein.

| Region | Protein concentration (mg mL$^{-1}$) |  |
|--------|-----------------------------------|---|
|        | Dadih 1  | Dadih 2  | Dadih 3  | Average |
| Agam   | 1.92     | 2.2      | 1.74     | 1.95    |
| Sijunjung | 2.08    | 1.76     | 2.25     | 2.03    |
| Solok  | 2.41     | 3.02     | 2.76     | 2.73    |
The soluble protein was assayed for antioxidant activities. Two antioxidant assays used in this study were 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH). Antioxidant activities of the proteins were determined by reduction of ABTS or DPPH absorbance after addition of protein. Under the assay condition, 100% of antioxidant activity correspond to complete scavenging of ABTS or DPPH radical. The soluble protein of dadih from Agam showed antioxidant activity against ABTS radical 78-90% (figure 2). This means almost ABTS radicals were neutralized by dadih protein.

Dadih protein from Sijunjung and Solok had lower antioxidant activities than that of Agam. As shown in table 1, the average of soluble protein concentration dadih from Agam was lower than Sijunjung. This indicated that protein concentration did not represent the antioxidant activity. Antioxidant activity of milk product was mainly determined by peptide, enzyme, and sulfur-containing amino acids such as cysteine that were produced during fermentation [11]. Also, the presence of probiotic bacteria such as lactic acid bacteria increased the antioxidant activity of milk product [12]. Bacteria produced a proteolytic enzyme which was hydrolyzed protein yielding peptides. The peptides contributed to enhancing the antioxidant activity of the fermentation milk. In vivo studies was performed to assess the antioxidant activity of the peptides produced during the fermentation of dairy product by the proteolytic strain of Lactobacillus bulgaricus. The result revealed that reactive oxygen species (ROS) decreased in living yeast cell treated by the fermentation product [13].

![Figure 2. Antioxidant activities of the dadih soluble protein from Agam, Sijunjung and Solok region against ABTS radicals.](image)

Another assay was DPPH. Antioxidant activities of the dadih soluble protein against DPPH radical as presented in figure 3. Average of the activity of dadih soluble protein from Agam was also higher than that from Sijunjung and Solok. Generally, dadih protein from Agam, Sijunjung, and Solok showed antioxidant activity was more than 50%, except dadih 2 from Sijunjung. That activity was better than a previous study of a fermentation product from cow milk using single lactic acid bacteria (43.04-48.10%) [6]. This might also due to the combination of many of bacteria species in the natural fermentation process of dadih in bamboo tube.

Study of antioxidant production by Lactic acid bacteria isolated from fermented buffalo milk showed that antioxidant activities were varied depending on the isolate and species. The isolate which found to be identical with Lactococcus lactis subsp lactis and Lactobacillus plantarum showed higher antioxidant activity against DPPH radical compared to other isolates [4]. Lactic acid bacteria of dadih from Agam were composed by Lactobacillus brevis, L. viridescens, L. buchneri. dadih from Sijunjung contained L. plantarum and dadih from Solok composed by L. plantarum, L. fermentum, L. rhamnosus, L. casei, Enterococcus faecium [5].
Figure 3. Antioxidant activities of the dadih soluble protein from Agam, Sijunjung and Solok region against DPPH radicals.

In addition, the difference of antioxidant activities might also due to peptide production by microbial protease enzyme. Different protease enzyme produced by bacteria in dadih resulting peptide or smaller protein with different activities. Enzyme, substrate and time of hydrolysis affect the bioactivity of the protein or peptide [8]. Length of fermentation also influences total lactic acid bacteria and total antioxidant activity. Fermentation of 72 hours produces optimum antioxidant activity, total lactic acid, pH and protein soluble [10].

Antioxidant activity of the dadih protein from Sijunjung was ± 30–60%. Lactobacillus plantarum was isolated from Sijunjung dadih. A similar result was reported in the fermentation of buffalo milk using Lac 13 which was then identified as L. plantarum resulting product with antioxidant activity against DPPH radical 63.79% [4]. This suggested that fermentation milk using similar species of lactic acid bacteria might produce a product with almost similar antioxidant activity.

Another assay was reducing power. Reducing power is associated with the antioxidant activity and this relationship has been established with a bioactive compound of natural product. The Ferric reducing antioxidant power (FRAP) assay measures the presence of reducers such as antioxidant, which indicated by a reduction of Fe3+/ ferricyanide complex to the ferrous form. An increase absorbance of the reaction mixture means an increase in antioxidant activity [14].

Reducing power activity of the soluble protein dadih from Agam was higher than that from Sijunjung and Solok (figure 4) indicated by a high average of absorbance. Although antioxidant activity might be also influenced by vitamin content of dadih but the buffalo milk had similar characteristic. That might contribution of vitamin C in antioxidant activity of all dadih almost similar and the difference could be ignored.

From ABTS, DPPH and Reducing power assay, the antioxidant activity was compared to the antioxidant activity of vitamin C. Standard of vitamin C was made by serial dilution to obtain the standard curve. Vitamin C equivalent of antioxidant activity of the dadih protein was generated based on the curve. The result was shown in table 2.

| Table 2. Average of vitamin C equivalent of dadih from Agam, Sijunjung, and Solok |
|------------------------|-----------------------------|-----------------------------|-----------------------------|
| Origin of dadih        | Vitamin C equivalent (µg mL⁻¹) | ABTS                       | DPPH                       | Reducing power              |
| Dadih1                 | 14.20 ± 0.34                | 26.80 ± 5.31                | 46.73 ± 17.78               |
| Dadih2                 | 9.44 ± 1.65                 | 14.79 ± 6.07                | 20.85 ± 18.36               |
| Dadih3                 | 10.14 ± 0.69                | 24.74 ± 5.67                | 36.28 ± 21.13               |
Generally, averages of vitamin C equivalent of dadih soluble protein from Agam were higher than that from Sijunjung and Solok. As ABTS, DPPH and Reducing power had a different mechanism in action and dadih from Agam showed higher activities, therefore dadih Agam more suitable in various system. This ability makes dadih is very potential to be applied as an antioxidant. Further research is necessary to explore more capability of dadih, especially from Agam region.

4. Conclusion
Antioxidant activities of dadih from Agam were higher than dadih from Sijunjung and Solok in ABTS, DPPH and Reducing power assay. This result suggested that dadih, especially from Agam region, was very potent to be applied as an antioxidant. Fermentation of buffalo milk using a combination of bacteria *Lactobacillus brevis*, *L. viridescens* and *L. buchneri* as contained in dadih from Agam might produce dadih with optimum antioxidant activities.

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