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Research Article

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In Vitro Antioxidant Activity of Sesame Milk Fermentation in Human Low Density Lipoprotein (LDL)

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Abstract

Lignan sesaminol triglucoside is a bioactive compound in sesame milk that showed higher antioxidant activity after it is hydrolyzed by β-glucosidase. The objectives of this study were to determine in vitro antioxidant activity of fermented sesame milk (FSM) extract and sesame milk (SM) extract against DPPH and LDL oxidation and to examine phenolic, sesaminol triglucoside content and β-glucosidase activity. Antioxidant activity was examined using DPPH and TBARS assay with LDL as the oxidation substrate. Sesaminol triglucoside was identified with HPLC diode array detector and β-glucosidase activity was determined by measuring hydrolysis rate of ρ-nitrophenyl-β-D-glucopyranoside (ρNPG). After fermentation of sesame milk with L. plantarum Dad 13, the β-glucosidase activity was 70.3±0.023 mU/mL fermented sesame milk, the sesaminol triglucoside content of SM and FSM were 5.65 to 2.56 mg/100 mL of sesame milk, respectively, the phenolic content of SM and FSM were 3.81±0.10 and 7.9±0.08 mg GAE/g dry sesame seed, respectively, radical scavenging activity of SM and FSM 18±0.64, and 45.5±0.37% respectively. Fermented sesame milk inhibited human plasma LDL oxidation by 1.82 compared to unfermented sesame milk. This result related to hydrolysis of sesaminol triglucoside by β-glucosidase which was produced by L. plantarum Dad 13 that resulted in sesaminol. This results suggest that fermented sesame milk extract has in vitro antioxidant activity in human LDL better than sesame milk extract.

Keywords: Fermented sesame milk, Sesaminol triglucoside, DPPH, LDL, TBARS

Introduction

The needs of healthy beverages increased continuously together with awareness of healthy lifestyle. Sesame seed is well known as a food of high antioxidant activity. Sesame milk has opportunity for being processed as vegetable milk like peanut and soybean in regards to its antioxidant compounds and nutrients. Sesame seed contains lignan and tocopherol as primary compounds with lignan has higher antioxidant activity than tocopherol [1,2]. Hydrophobic lignans (sesamin, sesamol and sesamolin) and hydrophilic lignan glucosides (sesaminol, sesamolinol and pinoresinol glucoside) are lignans in sesame seed.

Sesaminol triglucoside in sesame seed is a major bioactive lignan glucoside responsible for several biological activity [1,3]. Sesaminols contained in sesame seeds are very small amount as free form sesaminol but mostly are bound to glucose in the form of lignan mono/di/tri-glucoside, which have no or low antioxidant activity. Sesaminol glucoside can be hydrolyzed by β-glucoside and produced an active aglycone after passing the digestive system in the intestinal track [4]. Free form of sesaminol unbound to glucose is known to have higher antioxidant activity than sesaminol mono/di/tri glucose [5]. Thus, fermentation of sesame milk using β-glucoside-producing lactic acid bacteria which break the glucoside bond and result in more active aglycone compound must increase antioxidant activity of sesame milk.

Ability of lactic acid bacteria to produce β-glucosidase in the vegetable milk fermentation was determined by carbon source availability in the media and the ability of the bacteria to use it. *Lactobacillus plantarum* Dad 13 can produce β-glucosidase during fermentation of sesame milk [6]. The use of β-glucosidase for increasing antioxidant activity properties was done [7]. It was used *L. paracasei* dan *Bifidobacterium longum* as a starter in fermentation of soy milk for 24 hours at 37°C. After fermentation of soymilk the concentration of isoflavon genistein aglicone increased 3-4 folds and daidzein concentration increased 2-3 folds compared to unfermented soymilk. Also fermentation of soymilk with *L. plantarum* and *L. delbruckii* subsp. *lactis* increased aglicone concentration 7 folds compared to unfermented soymilk [8].

Blood is the major transportation systems in human body. The blood contains lipoprotein which classified as Chylomicron, Very Low Density Lipoprotein (VLDL), Intermediate Density Lipoprotein (IDL), Low Density Lipoprotein (LDL), and High Density Lipoprotein (HDL). The highest component of LDL is cholesterol ester, which is highly susceptible to oxidation. Moreover, fatty acids contained in LDL are more than 50% composed of polyunsaturated fatty acid (PUFA). Therefore, oxidation of LDL is one of the cardiovascular disease risk factor. There have been some

reports on the antioxidative activities of sesame seed in inhibition of LDL oxidation. Inhibition of LDL oxidation at 100 ppm level of total phenolic content of black sesame seed (78.4%) was higher than those in white sesame seed (57.3%) in same dose [9]. Rabbits fed on 10% defatted sesame flour containing diets (containing 1% sesaminol glucoside) showed stronger antioxidative activity than rabbits given control diet group, as indicated by the lower 2-thiobarbituric acid reactive substances (TBARS) formed in the liver and serum [10]. The abundant quantities of sesaminol found in serum and liver samples might be responsible for the antioxidative activity. Those facts may point out that although the sesaminol glucosides have no direct role in antioxidative defense system, the intestinal β-glucosidase enzyme may hydrolyze the sesaminol-glucosides complex to free-form lignan and aglycone, which results in the improvement of the antioxidant properties of the sesaminol-glucosides itself.

Fermentation of soybean increased isoflavone bioavaibility for body. Soybean isoflavone aglycone is much easily absorbed by the body than isoflavone glucoside complex. Fermentation of sesame milk produce fermented sesame milk which is rich in sesaminol aglycone to inhibite LDL oxidation. The data regarding the antioxidant activities of fermented sesame milk to scavenge DPPH and inihibite LDL oxidation are still rare. This information is needed to explain the effect of hydrolysis of sesaminol triglucoside to sesaminol aglycone and glucose in sesame milk fermentation for LDL oxidation inihibition. The oxidation of LDL may be inhibited by the consumption of antioxidant from fermented sesame milk which is expected to prevent cardiovascular disease.

Some previous research showed that bioactive compounds which were evaluated with DPPH radical scavenging capacity were not figure information system in the body yet. The other one showed that bioactive compounds which were evaluated with DPPH radical scavenging capacity had low antioxidant activity. In other hand it was evaluated with biological system and showed higher antioxidant activity [11]. Therefore in this research antioxidant activity of lignan glucoside was evaluated with DPPH radical scavenging capacity and TBARS assay with LDL as oxidation substrate for comprehensively information.

The objectives of this study were to determine in vitro antioxidant activity of fermented sesame milk (FSM) extract and sesame milk (SM) extract against DPPH and LDL oxidation and to examine phenolic, sesaminol triglucoside content and β-glucosidase activity.

Materials and Methods

The experiment was conducted in Biotechnology laboratory, Chemistry and Food Biochemistry laboratory of Food Technology Gadjah Mada University. Decorticated sesame seeds of Winas variety were obtained from Brajan Village, Prambanan District, Klaten Regency, Indonesia. Sesame milk was prepared using 12% (w/v) initial concentration of sesame seed.

Lactobacillus plantarum Dad 13 was obtained from FNCC (Food Nutrition Culture Collection) Centre for Food and Nutrition Studies, Gadjah Mada University, Yogyakarta. *Lactobacillus plantarum* Dad 13 which was isolated from *dadih*, a traditional fermentation product of buffalo milk from West Sumatra, Indonesia. The culture stock was kept in 10% glycerol and 10% skim milk with the ratio 1:1 (v/v). One mililiter culture in sterilized 1.5 mL polyethylene screw cap tube was added with 1 mL glycerol-skim milk and stored at -40°C. The strain was activated by adding 1 mL of stock solution with 9 mL of 0.85% NaCl in water, vortexed and then rejuvenated in 10 mL of MRS (De mann Rogosa Sharpe) broth (Oxoid) at 37°C for 18 h.

Sesame milk was made by boiling and steaming of sesame seed process to get the best flavour. It was pasteurized at 75°C for 5 min in autoclave (EYELA MAC 5160), Tiyoda IX Manufacturing Ltd, Japan). The pasteurized milk was inoculated aseptically with an active single culture of *L. plantarum* Dad 13 1% (v/v) and incubated at 37°C for 18 h. Beta-glucosidase activity, sesaminol triglucoside concentration, total phenolic content, and radical scavenging activity were determined at the initial and end of fermentation. Fermented sesame milk was stored at -20°C immediately and freeze dried using a Dynavac FD 300 freeze drier (Rowville, Vic., Australia) until the analysis of sesaminol triglucoside was done. The phenolic content of either sesame milk or fermented sesame milk was determined by adding to human blood LDL. The lipid peroxidation of LDL was determined by measuring the formation of 2-thiobarbituric acid-reactive substances (TBARS). TBARS was calculated as malondialdehyde (MDA) equivalents using as the standard freshly diluted malondialdehy-bis-(dimethyl acetal), i.e., 1,1,3,3-tetraethoxypropane.

Beta-glucosidase activity of fermented sesame milk was determined by measuring hydrolysation rate of ρ-nitrophenyl-β-D-glucopyranoside (ρNPG) [12]. Sesaminol triglucoside analysis used naringenin as internal standar [3]. Determination of DPPH radical scavenging activity [13]. The total phenolic content in the crude extract of sesame milk and fermented sesame milk was determined according to the Folin-Ciocalteu procedures [14].

Inhibition of LDL oxidation using plasma was obtained from healthy human volunteers after 12 h of fasting and dispersed into a tube containing ethylene diamine tetra acetic acid (EDTA) and it was immediately separated by centrifugation (3000g, 10 min). The plasma was then transferred to centrifuges tubes. Solid NaBr was added to adjust the density of plasma to 1.220 g/ml. Then 1.006 g/ml NaBr solution was added and centrifuged at 236.000 g 4°C, for 45 min (Hitachi SCP 85H: 80 T rotor) ultracentrifuge. After separation of the very low density lipoprotein (VLDL) fraction on the top and the next lower fraction, then 1.063 g/ml NaBr solution was added to the remained fraction and centrifuged at 236.000g 4°C for 45 min [15]. The LDL fraction on the top was then collected and stored at -20°C until it was used. The protein content of the fraction was determined with biuret method.

Protein level of LDL was adjusted to 8 mg protein/l with phosphate buffer saline (PBS) pH 7.4. Then 0.4 ml of LDL fraction was incubated with 70 µl 25, 50, 75, 100 ppm phenolic equivalents of sesame milk and fermented sesame milk extract for 5 min. Then 33.3 µl of 50µM $CuSO_4$ was added to induce the LDL fraction and incubated at 37°C for 24 h. After incubation, 33.3 µl EDTA (final concentration 27 mM) were added to prevent any further oxidation.

Determination of thiobarbituric acid reactive substances (TBARS). The extent of LDL oxidation was determined by measuring the TBARS formation. Briefly, 2 ml of 0.67% of thiobarbituric acid (TBA) and 15% of trichloro acetic acid (TCA) in 0.1 M HCL were added to the incubated LDL, and the mixed reagen reacted at 95°C for 1 h and cooled in ice for 5 min. Then the sample were centrifuged at 3000 g for 15 min.

The lipid peroxidation of LDL was determined by measuring the formation of 2-thiobarbituric acid-reactive substances (TBARS), which was measured at 532 nm. The results were in nmol MDA equivalents/g LDL protein. A solution of 1,1,3,3-tetramethoxypropane (TMP) was used as standard and data were reported as means ±SD for four replications.

Statistical analysis. Results are presented as means value ± standard deviation. Data were statistically analysed using one-way ANOVA; pair-comparison of the treatment means was achieved by Duncan's procedure at $P<0.05$ using statistical software SPSS 17 for Windows.

Results and Discussion

Inhibition of LDL Oxidation In Vitro

The antioxidant activity of sesame milk and fermented sesame milk extract were observed individually at four different concentrations of total phenolic content against inhibition of Cu^{2+} induced oxidation of human LDL in vitro by monitoring TBARS formation (Table 1).

Sesame milk and fermented sesame milk extract showed a positive effect of the inhibition of LDL oxidation. TBARS formation of LDL with the addition of fermented or non fermented sesame milk extract was lower than control (LDL+$CuSO_4$). Furthermore, the addition of fermented sesame milk extract in all total phenolic content concentration gave a significantly lower TBARS formation compared to non-fermented sesame milk. The antioxidant activity increased as the concentration increased for each extract as shown by lower formation of TBARS. Hence, a high concentration of total phenolic content in the extract of fermented sesame milk or sesame milk may lead to greater inhibitory effects on the Cu^{2+} induced LDL oxidation. The ability of molecules to donate a hydrogen atom to a radical and the propensity of hydrogen donation which the critical factor that involves free radical scavenging. Among the four levels of total phenolic content of sesame milk used in the experiment, TBARS formation of 75 and 100 ppm total phenolic content of sesame milk was 31.34 and 30.6 nmol MDA equivalents/g LDL protein. It meant that sesame milk ectract inhibited TBARS formation 23.99% and 25.58% which were not significantly different. But, TBARS formation from the addition of 25 ppm total phenolic content of fermented sesame milk was 29.1 nmol MDA equivalents/g LDL protein, which was statistically same with the addition of 75 and 100 ppm total phenolic content of sesame milk. Thus, fermented sesame milk inhibited TBARS formation better than sesame milk. The lower TBARS formation meant the higher antioxidant activity. TBARS formation from the addition of 100 ppm total phenolic content of fermented sesame milk was 21.80 nmol MDA equivalents/g LDL protein and it was same statistically with sesamol standar 75 ppm (20.8 nmol MDA equivalents/g LDL protein). It meant that the addition of 100 ppm total phenolic content of fermented sesame milk inhibited TBARS formation 47,13. Other researchers reported that TBARS formation inhibition of white sesame extract was 40% and black sesame 55%. The male wistar fed on fermented soybean extract with lactic acid bacteria (Biofermentics) at concentrations of 5 or 10 µL/ ml combine with dichromate fed inhibited MDA formation 80% [16].

Table 1: In vitro antioxidant activity of sesame milk and sesame milk fermentation extract in human LDL

Treatments	Concentration of MDA in human LDL (nanomol MDA/mg protein)			Decrease of MDA concentration from control (%)	
Concentrations (ppm)	LDL + Cu^{2+} + sesame milk extract	LDL + Cu^{2+} + sesame milk fermentation extract		LDL + Cu^{2+} + sesame milk extract	LDL + Cu^{2+} + sesame milk fermentation extract
25	36,61 e	29,10 cd		11,21	29,42
50	34,90 e	27,60 c		15,18	33,06
75	31,34 d	25,20 b		23,99	41,80
100	30,60 d	21,80 a		25,58	47,13
LDL + Cu^{2+} +(control)			41,23		
LDL + Cu^{2+} +sesamol 75 ppm			20,8		

- Sesame milk extract equivalen with total phenolic concentration (ppm)
- Mean values within a column followed by the same letters were not significantly different at $p<0.05$ according to Duncan's Multiple Range Test.

During sesame milk fermentation, *L. plantarum* Dad 13 synthetized β-glucosidase enzyme that hydrolyzed the glycosidic bond between sesaminol and glucoside moieties. The hydroxyl group formed after hydrolysis in the C atom no 2 increase the possibility of the formation of a new bond between free aglycone and Cu^{2+} rather than sesaminol in the initial form itself. The hydroxyl group in sesaminol allow the sesaminol compound to serve as a ligand. Ligand is an organic compound or ions able to transfer its electron to the metal core (transition metal ion) for having free electron pair. In this case, the free electron in hydroxyl group was transfered to Cu^{2+}. Cu^{2+} ions, which is bond to the hydroxyl group, were less tend to oxidize fatty acid in LDL. Therefore, the formation of hydroperoxyde were inhibited. Furthermore, the inhibition mechanism of MDA formation in LDL human blood by sesaminol can be hypothesized by regenerating the sesaminol that its consumed by its antioxidant action. The incubation of LDL with copper ion generates hydroxyl radicals, setting of chain reaction that increase the number of the production hydroperoxides. Following this propagation reaction, there is a fragmentation of fatty acids, which leads to the formation of such secondary product as aldehides, ketones, and carbonyls. These secondary products of LDL lipid peroxidation can modify lysine and other residues in apo B protein. Some secondary products of lipid proxidation such as MDA and 4-HNE (4-hydroxynonenal) attach covalently to the apo B component of LDL. The increase in 4-HNE or MDA corresponds largely to the formation of Schiff base products, which in turn can modify apo B. LDL particles that contain

oxidatively modified apo B are recognized in the arterial wall by the scavenger receptors on macrophages, leading to the formation of foam cells. Sesaminol able to inhibit the appearance of Schiff base, high electrophoretic mobility, and the degradation of apo B [15]. Thus, dietary antioxidant such as sesaminol in fermented sesame milk, that inhibite LDL oxidation was needed. Table 2 showed factors that caused increasing of antioxidant activity in fermentation of sesame milk.

Beta Glucosidase Activity, Sesaminol Triglucoside Concentration, Total Phenolic Content and Radical Scavenging Activity in Sesame Milk and Sesame Milk Fermentation

Before fermentation, no β-glucosidase activity was detected in sesame milk. However, during fermentation, *L. plantarum* Dad 13 synthetize β-glucosidase for utilizing glucose in sesaminol triglucoside complex, showed by a higher β-glucosidase activity at the end of fermentation (70.3 ± 0.023 mU/mL fermented sesame milk). The activity of β-glucosidase results in hydrolysis of sesaminol triglucoside and production of the active free aglycone, marked by the decrease of sesaminol triglucoside concentration (5.65 to 2.56 mg/100 mL sesame milk) and the increase of total phenolic content (3.81 ± 0.10 to 7.9 ± 0.08 mg GAE/g dry sesame seed) and Radical scavenging activity of sesame milk with steaming process (20.20 ± 0.64%) was not different statistically with boiling process (18.19 ± 0.64%). Sesame milk with boiling process presented better flavor than steaming process (data was not shown). The best one was fermented resulted 45.5 ± 0.37% of radical scavenging activity at the end of fermentation (Table 2).

Table 2: Beta glucosidase activity, sesaminol triglucoside concentration, total phenolic content and radical scavenging activity of sesame milk and sesame milk fermentation

Parameter	Sesame milk (Initial time)	Fermented sesame milk (end of fermentation)
Beta glucosidase activity (mU/mL fermented sesame milk)	0	70.3
Sesaminol triglucoside concentration (mg/100 ml sesame milk)	5.65	2.56
Total phenolic content (mg GAE/g dry sesame seed)	3.81± 0.10	7.9± 0.08
Radical scavenging activity of sesame milk with boiling process	18.19 ± 0.64a	45.5
Radical scavenging activity of sesame milk with steaming process	20.20± 0.64a	

The decrease of sesaminol triglucoside may be based on the hydrolytic reaction catalyzed by β-glucosidase produced by *L. plantarum* Dad 13. These results pointed out that *L. plantarum* Dad 13 was able to transform sesaminol glucosides to aglycone. The results of aqueous ethanol hydrolysis extracts from sesame oil cake with β-glucosidase indicated those lignans type antioxidant are present both as free phenolic compounds and as aglycone moieties of glycosides in sesame seed [2].

Fermentation of sesame milk with *L. plantarum* Dad 13 gave an enhanced radical scavenging activity by 2.55 times at the end of fermentation compared to sesame milk. Beta-glucosidase enzyme produced by *L. plantarum* Dad 13 appeared to hydrolyze β-1,2-glycoside bond between glucose and lignan molecules in sesame milk fermentation. The free form lignans is more reactive, therefore it showed higher activity in reducing phosphotungstat and phosphomolibdenum in Folin-Ciocalteu reagent, increasing phenolic content in sesame milk fermentation. The increasing radical scavenging activity during fermentation was followed by the increase of total phenolic content increased (Table 2). These results suggested that the hydrolysis of sesaminol triglucoside into sesaminol aglycones contributed to increase antioxidant activity in the fermented sesame milk. The addition of hydroxyl group on the atom C-2 of sesaminol may responsible for the increase of antioxidant activity.

Conclusion and Suggestion

In vitro antioxidant activity of sesame milk fermentation in human LDL better than sesame milk. Fermentation of sesame milk increased antioxidant activity. It was caused by the hydrolysis of sesaminol triglucoside into sesaminol aglycone by β-glucosidase activity released by lactic acid bacteria which contributed to increase total phenolic content, DPPH radical scavenging capacity and inhibition of LDL oxidation [17].

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