Antihypertensive Bioactive Peptides From Hydrolysates of Soy milk Yoghurt (Soygurt)

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Abstract. Soyghurt is a product of fermented soy milk which beneficial nutrients and functional food. This study was conducted to isolate and characterize bioactive peptides from soyghurt hydrolysates which potential as antihypertensive drugs. Soyghurt precipitated by the addition of HCl 1M and centrifuged. Pepsin as a proteolytic enzyme was used to hydrolyzed soyghurt with enzyme/substrate ratio (1/20) at 37 °C and pH 4.5 by interval of hydrolysis time 2, 4, 8 and 16 hours. The quantity of protein hydrolysates determined by Lowry method and the peptide pattern analyzed by SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Antihypertensive activity of soyghurt hydrolysates determined by angiotensin converting enzyme (ACE) inhibitory assay. Protein hydrolysates with the highest inhibition capacity purified by gel filtration column G-15. The results of this study show that soygurt hydrolysates with the highest of ACE inhibition obtained by 16 hours of hydrolysis with the highest degree of hydrolysis (51.6%) and antihypertensive activity 90.2%. This soygurt hydrolysates could potentially be used as natural antihypertensive agent.

Keywords: Antihypertensive, bioactive peptides, soybean yoghurt
1. Introduction

One of diseases that affects many modern societies is high blood pressure. Recently, high blood pressure (hypertension) was a major risk factor in cardiovascular disease. High blood pressure is one of the main factors causing stroke, heart attack, and kidney failure. Many factors triggers hypertension such as the consumption of salt, cholesterol, excessive alcohol and caffeine, obesity and genetic predispositions that can not be controlled [1].

Angiotensin converting enzyme (ACE) is a key enzyme which plays an important role in regulating blood pressure in humans. ACE catalyzes the hydrolysis of inactive angiotensin decapetide to vasoconstrictor angiotensin II, an octapeptide that plays a role in regulating blood pressure. In addition, ACE will also cut a vasodilatator bradykinin into inactive fragments to raised blood pressure [2].

Some of synthetic drugs such as ACE inhibitor agent has been widely used in the treatment of hypertension [3]. The use of synthetic antihypertensive drugs contain some risks and may cause side effects, such as hypotension, decreased renal function, a dry cough, and fetal abnormalities. To solved this limitations many researchers considered to find out the alternative natural ACE inhibitor which not risk and not addictive at all. Some of ACE inhibitors which has extensively studied among others derived from animal protein and vegetable protein [4].

Milk protein is one of bioactive peptides source that can serve as an antihypertensive [5]. These peptides can be produced by enzymatic hydrolysis with proteolytic enzymes such as pepsin or papain or through by lactic acid fermentation of bacteria [6]. Some of milk bioactive peptides capable in inhibiting ACE activity in vitro and in vivo [7], [8], [9]. These peptide fragments have also been found to have antihypertensive properties in animals and humans [10].

Over the past decade, there has been increasing use of food sources producing bioactive peptides in addition to dairy products, for example in cereal products. Fermented cereal products containing peptides that have the effect of lowering blood pressure [11]. Several other studies shown that foods containing animal protein or vegetable protein is an important source of bioactive peptides. The plant sources include cereals (wheat, barley, maize, rice) pseudo cereals (buckwheat and spinach), legumes (soy, lentils, beans), brassica species and others (sunflower) is also sources of bioactive peptides. The presence of bioactive peptides in cereals and legumes that can contribute to an increase in the value of quality protein and add functionality as a functional food that can be consumed daily [12].

Soygurt or soybean yoghurt is a functional food product with highly protein content and soy ingredients processing are continuously improved. For example in fermented soymilk, like the traditional food (soy sauce and tempeh), it has been known a significant role of antihypertensive peptides by acts as an ACE inhibitor [13]. [14]. Another antihypertensive peptides from soybean product are "chunggugjang" from Korean food derived from fermented soybeans with Bacillus subtilis CH1023 [15] and soybean paste [16], soy sauce [17], [18], natto and tempeh [19], and another soy fermentation products [20].

Study on soygurt hydrolysate by proteolytic cutoff as antihypertensive bioactive peptides has not been done. Therefore this study conducted to investigate soygurt bioactive peptides from local soygurt produced on industrial scale as a source of antihypertensive agents. This objective of research is to isolate and characterize an antihypertensive bioactive peptides from soygurt hydrolysate by enzymatic treatment with pepsin. We are expected that the ACE inhibitory activity from this hydrolysates are more better than commercial ACE inhibitor so its could be applied as an alternative antihypertensive drug.

2. Materials and Method

2.1 Sample and reagents

Fresh soygurt purchased from Local Supplier in Depok Regions West Java Indonesia. Other reagents consist of Pepsin (Porcine gastric mucosa, activity 0.8-2.5 units g-1 protein) was purchased from Sigma Chemical Co. (USA), the enzyme ACE (Angiotensin Converting Enzyme, rabbit lung) were
obtained from Sigma Chemical Co. (USA). N-Hippuryl-His-Leu (Hydrate Powder) ≥ 98% (HPLC grade), sodium dodecyl sulfate (SDS) 10% (Sigma Sigma Chemical Co., USA); Lowry Lowry solution I and II, Gel Acrylamide solution (30% T; 2.67C) Bio-Rad; Bis-Acrylamide Sigma, Resolving Buffer (1.5M Tris-HCl pH 8.8 Bio-Rad); Stacking Buffer (0.5M Tris-HCl pH 6.8 Bio-Rad); Ammonium peroxyde disulfate (APS) 10%; N, N,N,N'-Tetramethylethylenediamine (TEMED); Sephadex G-15 (medium), 0.01 M phosphate buffer solution pH 7.2; Running buffer Sigma; Staining solution Coomasie blue R-250 Bio-Rad; 0.25M phosphate buffer pH 7.4. hydrochloric acid (HCl, Merck), sodium acetate, acetic acid (Merck), trochloroacetic acid (TCA, Merck).

2.2 Preparation of soygurt hydrolysates
Soygurt hydrolysate prepared by dissolved in distilled water and the pH set to 2 with the addition of HCl 1M. After coagulation, the fraction separated from the whey protein precipitates by centrifugation (5,000g, 10 min). Protein precipitates were dissolved again in distilled water to obtain a protein concentrate 10% (w/v). To the precipitate was added pepsin with ratio of enzyme: substrate (1:5) and incubation carried out at 2, 4, 8 and 16 hours at 37°C in 0.05 M acetate buffer pH 4.5 [21]. The optimum conditions of pepsin hydrolysis of soygurt analyzed by SDS-PAGE electrophoresis. During the incubation process, approximately 5 mL of hydrolysate mixture is taken at 2, 4, 8 and 16 hours and the degree of hydrolysis measured by Alder-Nissen method [22]. After incubation was completed, each of the hydrolysate mixture was heated at 98°C for 5-10 minutes to inactivate the enzyme. The mixture was centrifuged at 12000 rpm and the supernatant measured an antihypertensive activity by using ACE inhibitory assay. Samples dried in a freeze dry for 24 hours and then stored at -20°C before analysis.

2.3 Determination of protein molecular weight of hydrolysate
Protein degradation and molecular weight of hydrolysates was identified by SDS-PAGE (Sodium Dodecyl Sulphate Acrylamide Gel Electrophoresis) standard methods [23], with a Mini-Protean II Slab Electrophoresis Cell (Bio Rad). Soygurt hydrolysates from enzymatic hydrolysis denatured in sample buffer (Tris-Cl 150 mM pH 6.8 SDS 25%, β-mercaptoethanol, 25% glycerol and 2.5 mM Bromophenol blue) and boiled for 10 min. Electrophoresis SDS-PAGE performed at 16% separating gels by the voltage 200 volts for 60 minutes. Visualization of protein bands staining by Coomasie brilliant blue 0.1% (w/v).

2.4 ACE-inhibitory activity of Soygurt Hydrolysates
The ACE-inhibitory activity was measured by monitoring the release of hippuric acid from hippuryl-Lhistidyl-L-leucine (HHL) using ACE solution [24]. The ACE solution contained 5 mM HHL, 200 mM potassium phosphate buffer (pH 8.3), 25µL ACE from rabbit lung. After 30 min of incubation at 37°C the reaction was stopped by addition of 200 µL of 1N HCl. The hippuric acid formed was extracted with ethyl acetate and removal of ethyl acetate by heat evaporation. The hippuric acid formed was measured by uv-vis spectrophotometer at 228 nm. The percentage of ACE inhibition was calculated based on the absorbance value of the samples :

ACE inhibition (%) = (A-B)/(A-C)

where A = absorbance of solution without sample, B = absorbance of solution with sample, C = Absorbance of blank solution (without ACE solution and sample).
3. Results and Discussions

3.1 Hydrolysis of soygurt

The enzymatic hydrolysis using pepsin enzyme on soygurt hydrolysates was monitored by the SDS-PAGE method and the degree of hydrolysis (DH) was calculated by the Adler-Nissen method. This has been shown that hydrolysis reaction using pepsin enzyme can achieve high DH value with various peptides sizes. SDS-PAGE analysis of soygurt hydrolysates revealed that smaller peptides (<10 kDa) were successfully produced and has a great potential to be used as an antihypertensive. Based on the SDS-PAGE analysis (Figure 1) shows that the optimum hydrolysis of soygurt precipitates by pepsin digested obtained at 16 hours with lost of intensity of the higher bands on molecular weight (MW) of protein hydrolysate (25-37 kDa) and increase in the lower molecular weight of protein bands (<10 kDa).

![Figure 1. SDS-PAGE pattern of soygurt hydrolysates. M : Protein markers. 1 : negative control (not hydrolysed soygurt), 2 : hydrolysis 0 hours, 3 : hydrolysis of 2 hours, 4 : hydrolysis of 4 hours, 5 : hydrolysis 8 hours, 6 : hydrolysis 16 hours.](image)

Loss of higher MW of protein fractions indicated that the molecular digest from enzymatic hydrolysis would be eliminate protein bands to produce biopeptide fractions with a lower molecular weight. No bands were visible for the MW>20 kDa after 16 hours incubation indicated that the hydrolysis of protein had the optimum time at 16 hours. According to the results of the quantitatively analysis of the soluble proteins level of hydrolysate, it was found that soluble protein with lower molecular weight increased after 16 hours hydrolysis as follows in Figure 2.

![Figure 2. The soluble protein of hydrolyzed soygurt 0-16 hours (treatment by TCA).](image)
The results of enzymatic hydrolysis of soygurt indicate that the protease preparation is efficient to form smaller peptides by pepsin hydrolysis. In comparison to the chemical hydrolysis, enzymatic hydrolysis is preferable due to several advantages, such as mild reaction conditions, low undesirable products, and high product quality and yield [25].

3.2 Degree of hydrolysis (DH)

The DH for soygurt protein hydrolysates was determined according to the method of Adler-Nissen [22]. The DH gives an initial indication for the change in the molecular integrity. During protein hydrolysis, the large complex structured protein molecules are broken down into smaller sized peptides and specific amino acids [25]. The DH was continuously monitored during enzymatic treatment of soygurt hydrolysis. As shown in Figure 3, the unhydrolyzed soygurt showed an average DH value of 5.2%. In the case of enzymatic hydrolysis, the DH increased significantly (\( P < 0.05 \)). The highest DH value of 51.6% was achieved after enzymatic treatment of soygurt for 16 h with pepsin enzyme.

![Figure 3. The degree of hydrolysis of soygurt (0-16 hours)](image)

Degree of hydrolysis is affected by the length of incubation and ratio of enzyme used. The enzyme concentration is one of the factors that affect the rate of degradation of proteolytic enzymes. In this process, soygurt hydrolysates will be digested by the proteolytic enzyme in the active site of pepsin to produce an oligopeptides with the small molecular weight. Schematic reaction of proteolytic hydrolysis can be seen at Figure 4.

![Figure 4. Schematic representation of complex enzyme-substrate with 5 binding site. The position of the peptide bond to the substrate is calculated from left to right where the disconnection occurred in bonding P1-P1’ in the pepsin active site’][26].
Pepsin is a proteolytic enzyme that will break the peptide bonds in several amino acid residues such as leucine, phenylalanine, and tyrosine triptopan. The mechanism of peptide bond disconnection occurs at position P1-P1' which is occupied by the amino acid residues polar through the mechanism of acid-base reactions with active sites occupied by the carboxyl group of Asp-32 and Asp-215 [27].

3.3 ACE-Inhibitory Activity
Determination of antihypertensive activity was performed by ACE-inhibitory assay refers to method developed by Cushman and Cheung. Based on investigation it was found that the highest antihypertensive bioactive peptide was obtained at 16 hours of hydrolysis with % ACE inhibition of 90.99% (Figure 5). Increasing of antihypertensive activity in fraction hydrolysates probably caused by a large part of the protein fraction has been degraded into more active oligopeptides. According to Lee et al. [21], the proteolytic hydrolysis of goat milk casein fraction gave the highest inhibitory activity (87.8%) on a casein hydrolysates after pepsin hydrolysis at 48 hours.

![Figure 5. Antihypertensive activity of soygurt hydrolysates (ACE assay).](image)

Soy foods such as soy sauce, miso, and natto show antihypertensive effect by inhibiting ACE, the key enzyme of renin-angiotensin system [18], [19], [20], [28]. Shimakage et al. [29] had identified 5 novel ACE inhibitory peptides containing Ile-Ile and Ile-Asp from protease-treated hikiwari-natto, whose ACE inhibitory activity was about 1.4 times higher than that of protease-untreated hikiwari-natto. In addition, they identified 8 novel ACE inhibitory peptides containing Phe-Phe-Tyr-Tyr and Trp-His-Pro derived from protease-treated soymilk, whose ACE inhibitory activity was about 36 times higher than that of protease-untreated soymilk. The results show that ability in inhibiting ACE of soygurt hydrolysates is greater than untreated soygurt.

4. Conclusions
In this study, we conclude that enzymatic hydrolysis of soygurt by pepsin enzyme has been effective to provide an antihypertensive bioactive peptide with the percent of ACE inhibition up to 90%, so it could be potentially used as an antihypertensive agents for hypertension treatment.

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