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ANTIHYPERTENSIVE PEPTIDES PRODUCED BY INDIGENOUS LACTIC ACID BACTERIA FROM DADIH ORIGIN

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ABSTRACT

In this study the capability of *Lactobacillus plantarum* ssp. *plantarum* DG17 and *Lactococcus lactis* ssp. *lactis* DK12 isolated from dadih origin was considered to generate antihypertensive peptides in reconstituted skim milk (RSM) medium followed bymeasuringangiotensin converting enzyme (ACE) inhibitor activity, protein profile and protein sequence. Theisolates showed high ACE inhibitor abilities *i.e.* 60.79±6.2% and 61.98±7.8%, with IC_{50} values of 439.9 µg/mL and 442.2 µg/mL respectively. Two smaller molecular weight bands ranging in size from 12 to 14 kDa were recorded in peptides profile of these isolates and assumed as antihypertensive peptides. The peptides sequence with molecule weight less than 3.0kDa were identified by LC Nano/MS and then compared withthe peptide database (Milk Bioactive Peptide Databaseand BIOPEP-UWM).One of the peptide sequence (VVVPPF) generated from *Lactobacillus plantarum* ssp. *plantarum*DG17had high ACE inhibitor activity due to the presence of F residue at the C terminal and V residue at the N terminal. The experimental result indicated that *Lactococcus lactis* ssp. *Lactis* DK12 and *Lactobacillus plantarum* ssp. *plantarum* DG17 isolates were potential developed into a starter culture in functional fermented milk.

Key word: *Lactobacillus plantarum*, *Lactococcus lactis*, ACE inhibitor, antihypertensive peptides

INTRODUCTION

High blood pressure or hypertension is one of the most critical causes of premature death worldwide. Hypertension is responsible for at least 45% of deaths due to heart disease and 51% of deaths due to stroke.

In 2025 it is estimated that around 1.56 billion adults will go through hypertension.It is also stated that hypertension kills nearly 8 million people every year, worldwide and nearly 1.5 million people each year in the South-East Asia (SEA) region (WHO, 2013).The regulation of peripheral blood pressure in hypertension is orderby ACE over the renin angiotensin aldosterone system (RAAS).

Traditionally, the RAAS was considered as an endocrine system with angiotensinogen as a substrate, formed in the liver that is cleaved by renin released from renal juxtaglomerular cell to form decapeptide sangiotensin I. Furthermore, angiotensin I is cleaved by ACE activity of the lungs into the active form of Angiotensin II (Paul *et al*., 2006). Angiotensin II then binds to specific receptor in adrenal cortex, resulting in the release of aldosterone. In this classical view, the cardinal function of the RAAS in maintaining of blood pressure by angiotensin II-induced vasoconstriction and aldosterone-mediated sodium retention in the collecting duct (Wolf and Nielson, 1996; FitzGerald and Murray, 2006). Inhibition of ACE activity stimulates the dilation of blood pressure by inhibiting the production of angiotensin II. Synthetic drugs such as captopril, enalapril, lisinopril and another ACE inhibitor group were commonly prescribed to treat hypertension. However, there were some side effects including dry cough, taste disturbances, angioedema and skin rashes associated with these drugs (Murray and FitzGerald, 2007).

Currently, variety of ACE inhibitory (ACEI) peptides derived from food proteins were reported as natural alternative bioactive peptides (Webb *et al.*, 2010; Udenigwe and Mohan, 2014). Fermented milk and cheese had been reported as a potent ACE inhibitor (Pihlanto e*t al.*, 2009; Hernandez-Ledesma *et al.*, 2011). During fermentation process, lactic acid bacteria (LAB) will generate bioactive peptides and amino acids from their proteolytic activity. The released of bioactive peptides were related to several proteolytic systems which consisting of several distinct intracellular peptideses including endo-peptidases, amino-peptidases, di-peptidases, and tri-peptidases (Christensen *et al*., 1999). Previous study had shown that ACE inhibitor was released from *Lactobacillus helveticus* (Chen *et al*., 2015; Beltran-Barrientos *et al*., 2016), *Lactobacillus delbrueckii* ssp. *bulgaricus, Lactococcus lactis* ssp. *cremoris, Lactococcus lactis* ssp. *diacetylactis, Streptococcus salivarus* ssp. *thermophillus, Lactobacillus acido phillus, Lactobacillus casei, Lactobacillus jensenii* (Sett-

ani and Moscetti, 2010) and *Bifidobacterium longum, Bifidobacterium bifidum* genera (Gonzales-Gonzales *et al*., 2013).

Wirawati *et al*., (2019) isolated and identified 12 indigenous LAB from dadih (naturally fermented buffalo milk from West Sumatera), they classified into three genera, namely *Lactococcus, Lactobacillus,* and *Pediococcus,* with high antimicrobial and proteolytic activity properties. Two isolates i.e. *Lactobacillus plantarum* ssp. *plantarum* DG17and *Lactococcus lactis* ssp. *lactis* DK12 were presumed to have ACE inhibitor activity due to their highest proteolytic properties. The objective of this research was to analyzeand identify bioactive peptides with ACE inhibitory activity from indigenous LAB isolated from dadih.

MATERIALS AND METHODS

Cultures: Two isolates from previous study, namely *Lactococcus lactis* ssp. *lactis* DK 12 and *Lactobacillus plantarum* ssp. *plantarum* DG17 were activated in MRS broth medium and incubated at 37^{o}C for 48 hours.

Fermented Milk: Peptides formation were conducted in reconstituted skim milk (RSM) medium, as described by Quiros *et al*., (2007). This milk was prepared by sterilized 10% skim milk (g/mL) at 95^{o}C for 10 minutes and cooled in room temperature. Each isolates was pre-culture (10^{8}cfu/mL) in a sterile RSM medium and incubated overnight at 30^{o}C.The single pre-culture (3.0% v/v) of LAB were inoculated onto a fresh RSM medium and incubated at 30^{o}C for 48 hour. Fermentation process was stopped by pasteurized fermented milk at 75^{o}C for 1 min. To obtain the supernatant, fermented milk was centrifuged at 9000 rpm for 30 min and then store at -20^{o}C for further analysis.

***In-vitro* assay for ACE inhibitory activity:** The inhibitory activity of ACE was determined using the method described by Daliri *et al.,* (2018a) at Pusat Studi Biofarmaka Tropika (Trop BRC) Laboratory, IPB University. For each assay, 20µL of ACE inhibitory solution with 50µL of 5.0mM HHL in 100mM sodium borate buffer (pH 8.3) containing 0.3M NaCl was incubated at 37°C for 5 min. To initiate the reaction, 10µL of 0.1U/mL ACE solution were added and the mixture was incubated at 37°C for 30 minute. The reaction was terminated by adding 100µL of 1M HCl, and then the reaction mixture was mixed with 1.0mL ethyl acetate. The mixture was vortexed for 60 sec and centrifuged at 2000×g for 5 min. An aliquot (0.8 mL) of the ethyl acetate layer was transferred to a clean tube and evaporated in a water bath. Distilled water (0.8mL) was then added to dissolve the hippuric acid (HA) in the tube. The amount of HA formed was measured at 228 nm using spectrophotometer (Hitachi U2800). The amount of HA liberated from Hip-His-Leu under this reaction conditions without an inhibitor was used as a control. The extent of inhibition was calculated as 100%×[B−(A/B)] where A is the optical density (OD) in the presence of ACE and ACE inhibitory component, B is the OD without ACE inhibitory component. For the determination of IC50, series of dilutions containing 550µg/mL, 450µg/mL, 350 µg/mL, 250µg/mL, 150µg/mL and 50µg/mL were prepared. The amount of peptides required to suppress 50% ACE activity was calculated from the regression curves observed for each fraction.

Protein profile in SDS-PAGE gel: Protein profile of fermented milk was visualized by sodium dodecylsulfatepolyacrylamide gel electrophoresis (SDS-PAGE) technique (Khoiriyah and Fatchiyah 2013). We used α-lactalbumin, carbonyl anhydrase, chicken egg albumin, and bovine serum albumin (Sigma) as a marker with molecular weight 14.2kDa, 29kDa, 45kDa, and 66kDa respectively. Ten percent of discontinuous and separating gel were used in this method. Silver staining was used to visualize the peptides bands.

Identification of peptides by LC Nano/MS: Bioactive peptides profile was analyzed using Nano liquid chromatograph Ultimate 3000 series system tandem Q Exactive Plus Orbitrap RH mass spectrometry (Thermo Scientific). This analisys were carried out at advance research laboratory, IPB University. The methods already described by Daliri *et al*., (2018a). The supernatant was subjected to ultrafiltration with Amicon Ultra-4 nominal cut-off 3.0kDa, at 7500g for 30 min at 4^{o}C. Briefly, a 3.0mL sample was mixed with 12mL of acetonitrile. The mixture was filtered through a 0.22 µm filter membrane. An aliquot of 5µL of each sample filtrate was injected into a PepMap RSLC C18 column (75µm×15cm, 3.0µmpartikel size, 100 pore size, and part number ES 800, Thermo Scientific) the flow rate of 0.3µL/min. The optimal mobile phase was a linear gradient system of solution A (H_2O + acetonitrile 98:2 0.1% formic acid) and solution B (H_2O + acetonitrile 2:98 0.1% ormic acid), 30 minute, 2%-35% solution B; 15 minute, increased from 30% to 90% solution B linearly; 15 minute, 90% solution B, then decreased to 5% solution B and kept for 30 minute.

Afterward, the eluent was analyzed by a high definition mass spectrometer (Thermo Scientific) under the following conditions: the mass spectrometry was in positive ionization mode, the source temperature was 40°C. The mass spectrometric data were collected over the range of 200 to 2000 (m/z). The peptide sequence then identified by

Proteomic Discoverer 2.2 software and then compared to peptide database namely Milk Bioactive Peptide Database (http://mbpdb.nws.oregonstate.edu) and BIOPEP-UWM (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep).

Statistical analysis: Data were analyzed using analysis of variance (ANOVA) at 95% level of significance. All experiments were carried out in five replication and the results presented are a mean ± standard deviation (SD).

RESULTS AND DISCUSSIONS

***In-vitro* assay for ACE inhibitory activity:** The ACE inhibitory activity of *Lactobacillus plantarum* ssp. *plantarum* DG17 and *Lactococcus lactis* ssp. *lactis* DK12 in fermented milk crude extract is shown in Fig 1.

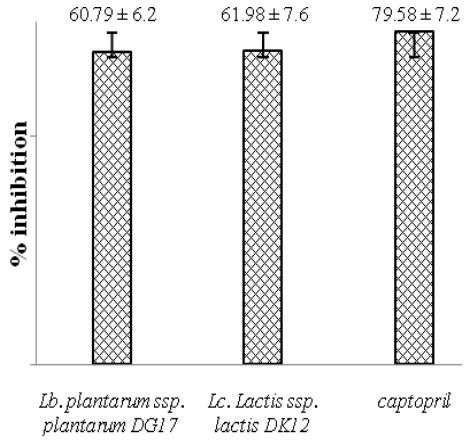

Fig -1: Percent of ACE inhibition by *Lactobacillus plantarum* ssp. *plantarum* DG17 and *Lactococcus lactis* ssp. *lactis*DK12; captopril (control)

There was no significant difference (P>0.05) in inhibitory activity, both isolates showed high activity *i.e.,* 60.79±6.2% and 61.98±7.8% respectively even they were lower than control (captopril). Captopril is a synthetic drug for hypertension treatment.Inhibition concentration (IC_{50}) value was also determined in this study and the result also showed no significant differences between the two isolates, *i.e.*,439.9µg/mL and 442.2 µg/mL respectively. This inhibition activity assumed due to bioactive peptides forming during fermentation process. Previous study demonstrated the various types of milk fermented with *Lactobacillus helveticus, Lactobacillus bulgaricus* ssp. *bulgaricus, Lactobacillus rhamnosus, Lactobacillus acidophilus,* and *Lactococcus lactis* ssp. *cremoris* have shown to contain peptides with variable ACE inhibitory activity (Gobbetti *et al*., 2000; Chen *et al*., 2014; Elkhtab *et al*., 2017). Protein fraction ≤14 kDa in fermented milk with *Lactobacillus casei* HZ1 showed 71.71% ACE inhibitory activity (Han *et al.,* 2012); *Lactobacillus bulgaricus* NCDC and *Lactobacillus fermentum* TDS030603 in fermented camel milk displayed great inhibition on ACE 76.75±1.14% and 73.93±0.74% respectively (Solanki *et al*., 2017). Fermented soy protein with *Pediococcus pentoseceus* SDL1409 generate 65.1 ± 0.78% ACE inhibitory activity (Daliri *et al*., 2018b).

Bioactive peptide structure mechanism during fermentation process was depleted by LAB proteolytic system that hydrolyzed the existed protein therefore peptides and amino acids will released to support their growth. The proteolytic system in *Lactococcus lactis, Lactobacillus helveticus,* and *Lactobacillus delbrueckii* ssp. *bulgaricus* species had been characterized completely. This system including proteases binding in their cell wall and few peptidases (endopeptidase, aminopeptidase, tripeptidase and dipeptidase) was reported by Christensen *et al*., 1999. Savijoki *et al.,* (2006) and Liu *et al*., (2010) reported that LAB proteolytic system consisted of (1) cell envelope protease (CEP) that degrades protein into peptides with 4-30 amino acids residues, (2) peptides transport system, including oligopeptide binding protein, two permeases and ATP-ase enzymes in the role of pores formation and supply the energy, (3) a few of intracellular peptidases that degrades peptides into amino acids.

In this study two isolates from dadih origins showed good ACE inhibitory activity (> 50%) due to their bioactive peptides forming during fermentation process. Even their inhibition activity was lower than a synthetic ACE inhibitory drug (captopril), this result indicated that these isolates have potential to generate antihypertensive peptides for further exploration.

Protein profile in gel SDS-PAGE gel: The result of SDS-PAGE gel showed similar protein profile band in milk fermented by *Lactobacillus plantarum* ssp. *plantarum* DG17 and *Lactococcus lactis* ssp. *lactis* DK12 were shown in Fig 2.

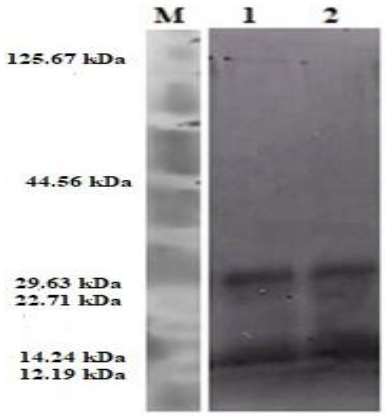

Fig - 2: Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) of fermented milk proteins. Line M: marker; Line 1: milk fermented by *Lactobacillus plantarum* ssp. *plantarum* DG17; Line 2: milk fermented by *Lactococcus lactis* ssp. *lactis* DK12.

Protein content in milk fermented by these isolates was hydrolyzed and denatured as it visualized by the 6 bands forming. The presence of LAB will increased the released of peptides due to their proteolytic properties (Korhonen and Pihlanto, 2006). Two smaller molecular weight bands ranging in size from 12 to 14 kDa were contained in peptides profile. Similar result was reported by Yao *et al*., (2014) which was used *Lactobacillus rhamnosus* GG in cow's milk fermentation. These bands might possess antihypertensive activity in fermented milk. Previous study also exhibit peptides profile on dadih and dangke were ranged between 9.12 kDa-12.38 kDa and physiological activity from these peptides have not considered yet (Soenarno *et al*., 2013).

Identification of peptides sequence by LC Nano/MS: Since there wasa variation in peptides bands of thefermented milk, we determined and identified peptides sequences that possessed antihypertensive (ACE inhibitory) properties. The identification was conducted by LC Nano/MS to the peptides that weighted less than 3.0kDa. The peptides sequence then compared to peptide database (Milk Bioactive Peptide Database and BIOPEP-UWM), only 100% similarity threshold were presented in Table 1. Their sizes varied from 6 to 16 residues.

Table 1: Antihypertensive peptides in fermented milk inoculated by *Lactococcuslactis* ssp. *lactis* DK12 and *Lactobacillus plantarum* ssp. *plantarum* DG17

Parent Protein	Sequence peptides	m/z (da)	MH+ (Da)	charge	ACE-I peptides based on literature	
					Sequence peptides	Reference
Lactococcuslactis ssp. *lactis*DK12						
β-casein	MPFPKYPVEP	602.8035	1204.600	2	MPFPKYPVEP	Saito *et al.,* 2000 Hayes *et al*., 2007
Lactobacillus plantarum ssp. *plantarum* DG17						
β-casein	DELQDKIHPF	621.3148	1241.622	2	DELQDKIHPF	Fan *et al*., 2019
β-casein	EPVLGPVRGPFP	632.8556	1264.704	2	EPVLGPVRGPFP	Villegas *et al*., 2014
β-casein	ELQDKIHPF	563.7984	1126.59	2	ELQDKIHPF	Gobbetti *et al*., 2000
β-casein	MPFPKYPVEP	602.807	1204.607	2	MPFPKYPVEP	Hayes *et al.*, 2007
β-casein	QEPVLGPVRGPFPIIV	859.5015	1717.996	2	QEPVLGPVRGPFPIIV	Quiros *et al*., 2005
β-casein	SLSQSKVLPVPQ	641.8756	1282.744	2	SLSQSKVLPVPQ	Hayes *et al*., 2007
β-casein	VVVPPF	329.2017	657.396	2	VVVPPF	Quiros *et al*., 2009
β-casein	YPFPGPIPN	501.2577	1001.508	2	YPFPGPIPN	Amorim *et al*., 2019
β-casein	YQEPVLGPVRGPFPI	834.9585	1668.91	2	YQEPVLGPVRGPFPI	Torrez-Llanes *et al*., 2011
β-casein	YQEPVLGPVRGPFPIIV	941.0363	1881.065	2	YQEPVLGPVRGPFPIIV	Hafeez *et al*., 2014
β-casein	EPVLGPVRGPFP	632.8556	1264.704	2	EPVLGPVRGPFP	Villegas *et al*., 2014 Hayes *et al*., 2007
β-casein	VLGPVRGPFP	519.8088	1038.61	2	VLGPVRGPFP	Quiros *et al.*, 2005
κ-casein	ARHPHPHLSFM	449.2234	1345.656	3	ARHPHPHLSFM	Ibrahin *et al*., 2017
αS1-casein	FVAPFPEVFG	555.2878	1109.568	2	FVAPFPEVFG	Amorim *et al.*, 2019 Gomez-Ruiz *et al.*,2002
αS1-casein	FVAPFPEVFGK	619.3336	1237.66	2	FVAPFPEVFGK	Torrez-Llianes et al., 2011
αS1-casein	FVAPFPEVFGKEK	498.9373	1494.797	3	FVAPFPEVFGKEK	Contreras *et al*., 2009
αS1-casein	IGSENSEKTTMP	647.3085	1293.61	2	IGSENSEKTTMP	Hayes *et al.* , 2007
αS1-casein	VPNSAEERLH	384.5326	1151.583	3	VPNSAEERLH	Villegas *et al.* , 2014
αS1-casein	VAPFPEVFGK	545.799	1090.591	2	VAPFPEVFGK	Zhao *et al*., 2014

Data in Table 1 indicatesthat only one peptide with 10 residues amino acid was generated from β-casein in fermented milk inoculated with *Lactococcus lactis* ssp. *lactis*. The peptide was in C-terminal part of β-casein at f124-133 (data not shown). This finding was in contrast with Rodriguez-Figueroa *et al*. (2012), who identified 21 new encrypted milk peptides with potent ACE inhibitor activity through a fermentation process

not only from caseins but also from whey proteins.

Meanwhile, fermented milk by *Lactobacillus plan-tarum* ssp. *Plantarum* generated much more peptides (19 peptides) and varied from 6 to 17 residues. These peptides were not only generated from β-casein, κ-casein, and αS1-casein, but also located in C-terminal region. During the fermentation process, a large number of peptides were generated from the C-terminal of β-casein but the N-terminal was resistant to hydrolysis probably due to the presence of several phosphoserine residues at that region (Ha*et al*. 2015). Hydrolysis of the C-terminal region on the other hand could be due to its hydrophobic nature which makes it more accessible for hydrolysis (Chang*et al*., 2014).

This finding indicated that the ability of LAB species to hydrolyze different substrate in RSM medium were very strain dependent. Proteolytic activities and protein hydrolysis patterns differ widely from one strain to another (Jensen*et al*., 2009). The differences possibly due to numerous factors, i.e., CEP gene expression, CEP gene mutation, differences in optimal enzymatic activity condition, although the methodology uses to assess the hydrolysis pattern of CEPs may also somewhat introduce variability in the reported results (Sadat-Mekmene *et al*., 2011; Patel and Hati, 2017).In general, the proteolytic system of LAB in casein exploitation is initiated by a cell-envelope proteinase (CEP) that degrades the protein into oligopeptides that are subsequently taken up by the cells via specific peptide transport systems for further degradation into shorter peptides and amino acids by a concerted action of various intracellular peptidases (Christensen 1999).

Interestingly, in the present study the sequence of one peptide (VVVPPF) had F residue at the C terminal and V residue at the N terminal. We presumed that the peptide has high ACE inhibitor activity. Previous studies reported that peptides with a proline or an aromatic residue (F,W,Y) at their C-terminal positions and a branched aliphatic (V, I or L) amino acid residue at N-terminals showed higher ACE-inhibitory activity (Wu*et al*., 2006). Hydrophobic amino acid residues (P,F,Y) at the C-terminal were found to be very important for substrates or competitive inhibitors to bind to the active site of ACE (Cheung et al. 1980). Our finding was in line withtheir studies, specifically in relationship between amino acid residue at C or N terminal and ACE inhibition activity.

Conclusions

Lactobacillus plantarum ssp. *plantarum* DG 17 and *Lactococcus lactis* ssp. *lactis* DK12 from dadih originshowed great inhibition on ACE activity, therefore,they possibly used as fermented milk starter culture in dairy industry and could be developed as a functional food for antihypertensive management. Antihypertensive peptide produced by these isolates could also be purified and used as nutraceutical or supplement for a patient with high blood pressure symptom.

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