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Identification and Molecular Interaction Mechanism Angiotensin Converting Enzyme Inhibitory Peptide from *Bakasang* (Fermented Skipjack Tuna (*Katsuwonus pelamis*))

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Abstract : *Bakasang* is fermented fish product which can inhibit ACE (Angiotensin Converting Enzyme). Therefore, this study aims to purify and identify the molecular interaction mechanism of bioactive peptides from *bakasang* as ACE inhibitors. *Bakasang*'s peptide was isolated. Crude and purified isolates were analyzed its profile by SDS-PAGE. The crude was purified using GFC and identified by LC-MS. Peptides then cleaved by proteinase K using peptide cutter, modeled using PyMol and docked with ACE using HADDOCK Server. Ligplot and Discovery studio 2016 used for analyzing molecular interaction and all visualization was done using Chimera v.1.8. Bioactive peptides in *bakasang* have a molecular weight in between 13 – 43 kDa. From 7 fragmented peptides, there is AQK fragment that has a great potential as ACE inhibitor. That peptide interacted with ACE through hydrophobic and hydrogen interaction on its active site. The conclusion from this study is *Bakasang* has a potential bioactive peptide as ACE inhibitor potential in its bioactive peptide from the fermentation process.
Keywords : ACE inhibitor, *bakasang*, bioactive peptide, molecular docking.

Introduction

Skipjack tuna (*Katsuwonus pelamis*) locally known as "cakalang" is one of the most popular fish in Bitung, North Sulawesi[1]. 'Bakasang' is well known as a typical food of North Sulawesi (Manado). Bakasang' is traditional fermented fish product made from the guts of fish mainly *Katsuwonus pelamis* L as well as other small fish and fish eggs[2]. It Made of Skipjack Tuna meat added by 20% salt fermented by dried under the sun for 7-14 days and can be stored for several months. In Banda, it used as daily diet as a condiment and flavoring agent in various local cuisine[3].

The human ACE (angiotensin-I-converting enzyme) gene is located on chromosome 17q23[4]. It is an enzyme that plays a role in blood pressure regulation. Human ACE has long been regarded as an excellent target for the treatment of hypertension and related cardiovascular diseases[5].Research about ACE inhibitor as antihypertension from some fermented milk products and other foodstuffs had been done[6-8], from fishery products resulting from hydrolysis using certain enzymes[9-11]. Some research showed that the fermentation

process involving lactic acid bacteria that degraded protein to a peptide which has Angiotensin Converting Enzyme (ACE) inhibitor activity.

While there is a few research in ACE inhibitor from fishery fermentation products[3,12]. This research is directed to explore the potential of a local product that has not been studied to produce a new concept that can be used to develop a commercial product with a high economic value for society. All this ime, research in *bakasang* was confined in chemical, microbiological, organoleptic and molecular identification of lactic acid bacteria[13,14], while studies toward biotechnology to find natural ingredients as pharmaceuticals and itsmolecular mechanism is never done. Recently, a search method attribute of the active compound and the most appropriate patterns of interaction involving two molecules called a ligand and receptor through a computational method called molecular docking or in silico method has been developed. This study aims to identify and determine the molecular mechanisms of *bakasang* bioactive peptides.

Material and Methods

Bioactive peptide isolation

Peptide isolation was done by dissolving 100gr *bakasang* into sterile distilled water 1:5 (w/v). It was homogenized and inactivated at 90 °C for 10 mins, continued by centrifugation (Tomy MX–305) 7000g, 4 °C, 20mins. Supernatant was added to ethanol in ratio 1:1 (v/v). The crude bioactive peptide was added by Tris-HCl buffer (pH 6.8) and stored at -20 °C before used.

ACE inhibitor examination

ACE inhibitor examination was done by Cushman and Cheung[15] method with several modifications. The bioactive crude 50 µl was added by ACE solution 50 µl (25 mU/ml) pre-incubated at 32°C 10 mins. That mixture was incubated with substrate 50 µl (Hip-His-Leu 8 mM in HEPES buffer 50 mM contained NaCl 300 mM pH 8.3) for 30 mins at the same temperature. This reaction was ended by adding HCl 1M 200 µl. The solution was extracted with acetyl acetate 1.5 ml and centrifuged 4000 g for 15 mins. Supernatant 1ml was transferred into a new tube and evaporated at room temperature for 2 hours in a vacuum dryer. Then, it was dissolved in 3 ml distilled water and the final concentration was measured by spectrophotometry at 228 nm using UV-vis spectrophotometer. Inhibition activity was measured as percentage inhibition using formula:

$$\%inhibition = \frac{B - A}{B - C} x100$$

A = absorbance by the enzyme and angiotensin-converting enzyme inhibitor compound
B = absorbance of enzyme without inhibitor compound
C = absorbance without enzyme and inhibitor compound

Crude purification and identification

Crude bioactive peptide purification using GFC Sephadex 75 (0.5 g) which was dissolved in distilled water 10 ml and added by Tris-HCl buffer into the column (3 ml volume and 1 ml/5 mins flow rate). The fraction that was alleged containing bioactive peptide subsequently identified using LC-MS. Peptide type prediction was specified by molecular weight that was detected using LC-MS. Chromatography separation using Hypersil Gold column (1.9 µM x 2.2 mM x 100mM). Acetonitrile (eluent A) and distilled water (eluent B) for mobile phase 0.5 ml/minute flow rate, 100% eluent A at 0, 4 and 10 minute.

Molecular docking analysis

The peptide was digested by Proteinase K using peptide cutter program continued by protein modeling using PyMol software[16,17]. The ACE receptor (4C2P) was retrieved from protein data bank PDB database. After both 3D structures were obtained, docking process was done by HADDOCK Server[18]. ACE receptor and ligand interaction were analyzed and used as receptor binding site reference (162, 281, 353, 354, 380, 383, 384, 387, 411, 457, 511, 512, 513, 518, 520, 523). Molecular interaction of the docking results was analyzed using LIGPLOT program and Discovery Studio 2016[19]. All molecular visualization was done using Chimera[20].

Result

ACE inhibitor activity from bioactive peptides crude

ACE inhibitor activity from bioactive peptide was 62.97-77.611% with average 68.80%. This result showed that *bakasang* bioactive peptide had a great potency as ACE inhibitor that might be applied as natural anti-hypertension. Peptide with a lower molecular weight had a strong ACE inhibitory activity. LC-MS identification showed some peptide under 1 kDa in *bakasang* isolate purified GFC.

Bioactive peptide purification using GFC

From 7 fractions which produced from GFC, 3 fractions (4, 5, 6) had been checked for its peptide profile using SDS-PAGE (Table 1 and Figure 1) were chosen for LC-MS identification. It was caused by fraction 1-3 were stuck in the stacking gel while there was not any bioactive peptide in fraction 7 after precipitated by ethanol.

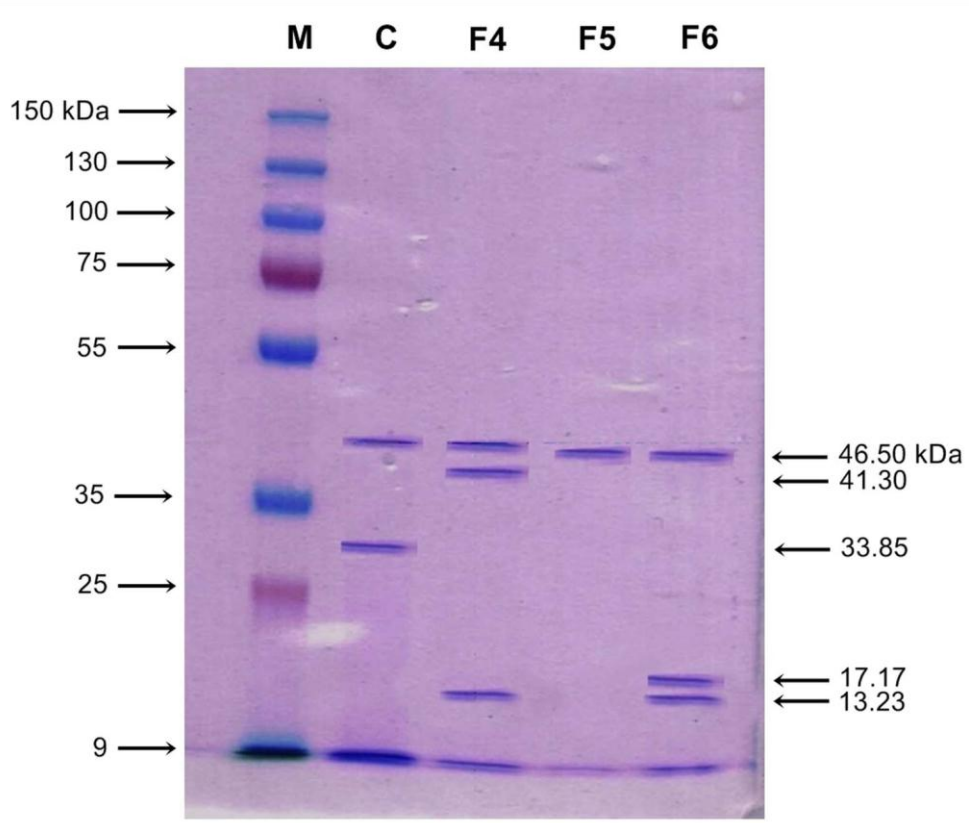


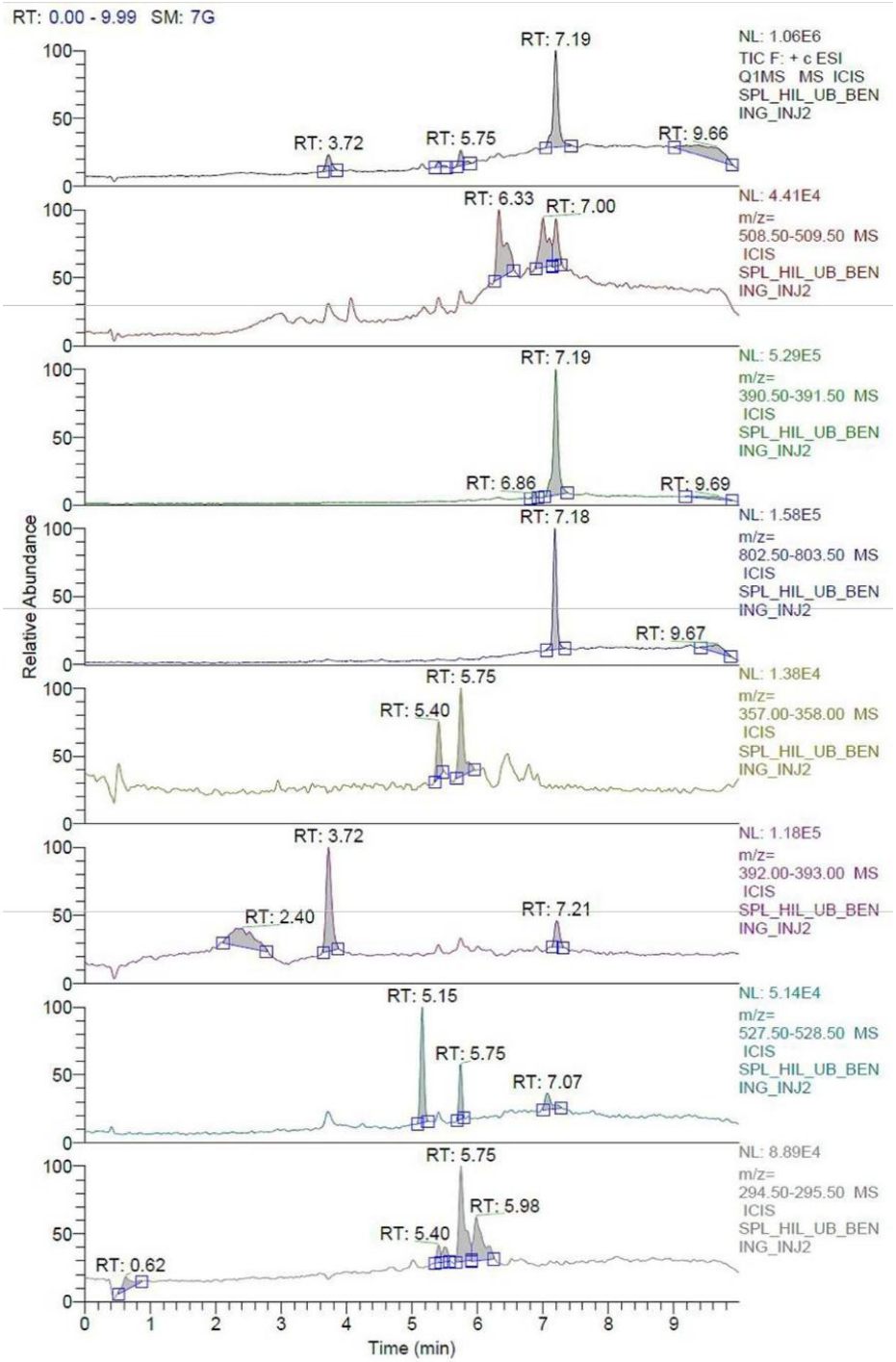
Figure 1: Crude and purified bioactive peptide profile (M: marker, C: crude, F4-6: fraction 4-6)

Table 1. Bioactive peptide molecular weight in crude and purified sample.

Sample	Molecular Weight (kDa)
Crude peptide	46.51; 33.85
Fraction 4	46.51; 41.31; 13.23
Fraction 5	46.51
Fraction 6	46.51; 17.17; 13.23

Isolates identification using GFC with LC-MS

The separation and identification of bioactive peptides isolate purified by GFC, using LC-MS found seven types of peptides can be seen in Table 2 and Figure 2. Bioactive peptide prediction was determined on its molecular weight. From those data, a bioactive peptide in fraction 4, 5, and 6 are short chain peptide such -as



dipeptide Ile-Tyr 294.34 Da, tripeptide Leu-Tyr-Pro 391.47 Da and some similar molecules with more than three amino acids peptide sequences.

Figure 2: Bioactive peptide profile from LC-MS identification

Table 2. LCMS identification result.

Peptide	Molecular Weight (Da)	LCMS
Ala-Leu-Pro-His-Ala (ALPHA)	507.59	508.50-509.50
Phe-Gln-Pro (FQP)	390.44	390.50-391.50
Asp-Met-Ile-Pro-Ala-Gln-Lys (DMIPAQK)	801.96	802.50-803.50
Ile-Lys-Pro (IKP)	356.46	357.00-358.00
Leu-Tyr-Pro (LYP)	391.47	392.00-393.00
Ser-Lys-Val-Pro-Pro (SKVPP)	528	527.50-528.00
Ile-Tyr (IY)	294.35	294.50-295.50

Molecular docking analysis

Fragmented protein by Pro K Peptide fragment was digested into 2-3 amino acid by Proteinase K using peptide cutter software produced 11 fragments as seen in Table 3.

Table 3. Molecular interaction of ACE inhibitor

Receptor	Digested Fragment	Energy
ACE	PHA	-39.1 +/- 2.3
	FQP	-55.8 +/- 0.8
	DMI	-38.3 +/- 6.1
	AQK	***-69.6 +/- 5.2***
	SKV	-50.3 +/- 5.0
	VVP	-28.3 +/- 3.1
	LK	-39.6 +/- 1.5
	PNM	-35.4 +/- 1.3
	IKP	-55.9 +/- 2.8
	LYP	-35.6 +/- 1.9
	IY	-32.3 +/- 2.3

Molecular docking simulation and visualization

AQK fragment is a potential candidate as ACE inhibitor has hydrophobic and hydrophilic interaction. In hydrophobic interaction was found some amino acids that strongly bound with it, i.e.: Phe457, His513, Cys370, Gln369, Ala354, and Ser355. While hydrogen interaction bind to Tyr520, Glu384, Tyr523, His387, His353, Asp377, Glu162, Lys511, and Gln281 (Figure 3). AQK fragment as ACE inhibitor candidate has hydrophobic amino acid (A), polar (Q) and charged polar (K) (Table 4). These results indicate that the C-terminal tripeptide which has activity as ACE inhibitors, not only hydrophobic and charged polar amino acids but also uncharged polar contributed as ACE inhibitor. ACE active side has three sub-parts that have a different character in binding three amino acid C-terminal part of the inhibitor which located on two sides of active homolog i.e.: S1 (antepenultimate), S1' (penultimate) and S2 (ultimate). ACE is preferred substrate or a competitive inhibitor containing hydrophobic amino acid in the third position of the C-terminal. Those subpart and substrate amino acid sequence have to bind appropriately for enzyme and inhibitor interaction. The binding inhibitor or substrate of the enzyme is common at C-terminal of tripeptide.

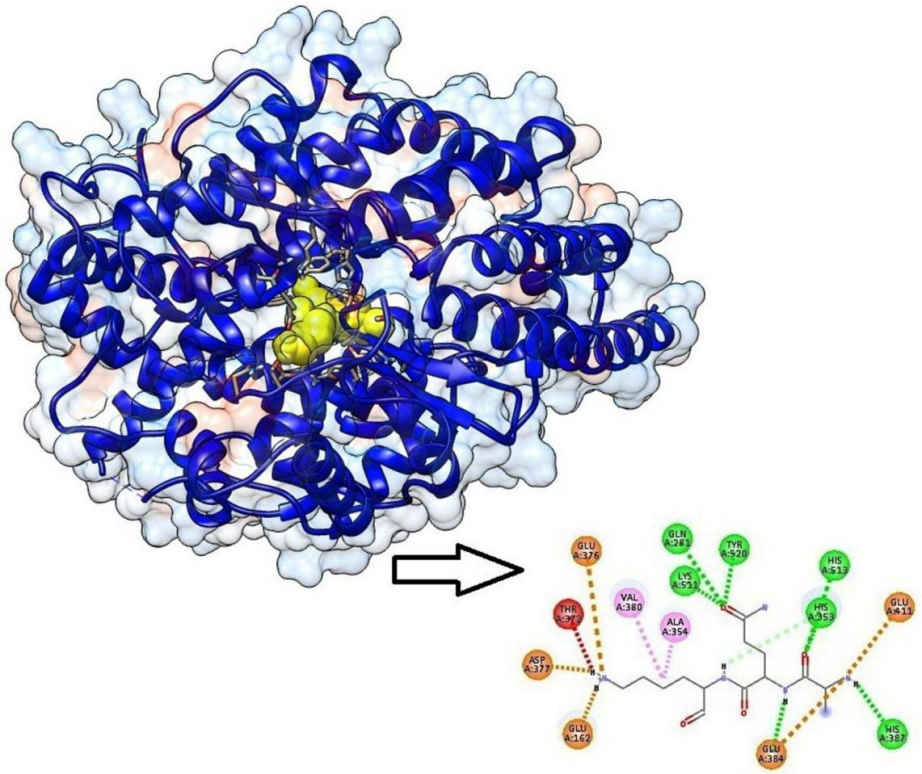


Figure 3: 2D and 3D structure of bioactive peptides binding to ACE

Table 4. Interaction and Residu Peptides from Interaction ACE with its ligans

Receptor	Ligand	Interaction and Residue	
		Hydrophobic	**Hydrogen**
ACE	PHA	Trp279, Ala354, Val380, His383, Glu411, Asp415, Phe457, Tyr523	His353, His513, Glu384, Lys511, Asn277, Gln281
	FQP	Gln281, His513, Val380, His353, Cys352, Leu161, Trp185, Phe512, Trp279	Glu376, Ala354, Glu162, Tyr146, Lys511
	DMI	Phe457, Phe527, Tyr523, His383, Phe512, Val380, Ala354, Trp279	Tyr520, Glu411, His387, Glu384 His513, His353, Lys511, Gln281
	AQK	Phe457, His513, Val380, Cys370, Gln369, Ala354, Ser355	Tyr520, Glu384, Tyr523, His387, His353, Asp377, Glu162, Lys511, Gln281
	SKV	Gln369, Val380, His383, Tyr523, His513, Trp279	His353, Ala354, Asp377, Glu162, Gln384, Glu411, Gln281, Lys511
	VVP	Phe512, His513, His383, Tyr523, Val380, Phe457, Trp279	Tyr520, Lys511, His353, Asn277, Gln281, Glu384, Glu411
	LK	His513, Tyr523, Val380, Ala354, Val518, Ala356, His410, His383	Glu384, Glu411, His353, Arg522, Glu403, His387
	PNM	Tyr523, Asp377, Val380, Phe457, Trp279, Gln281, Ala354, His387, Glu384, Glu411, His383	His513, His353, Tyr520, Lys511, Asn277
	IKP	Ala354, Tyr523, His513, Trp279, Thr282, Val380, Asp453, Phe527, His383	Glu384, His387, Glu411, Asp415, Gln281, His353, Lys511
	LYP	Lys511, Tyr523, His383, Arg522, Ala356, Ser355, Trp279, Val380, Glu162, Gln281, Phe457	Tyr520, Glu384, Glu411, His387, His513, His353, Gln369, Ala354
	IY	His353, Phe457, Tyr523, Phe527, Val518, Ala354, Val380, Val379, His513, His383	Lys511, Glu411, Glu384, Asp415, Gln281, Tyr520

Discussion

Based on the result of bioactive peptide purification, bioactive peptide had a molecular weight in between 13.23 – 46.51 kDa. It can be seen in Figure 1 that there is an accumulation in the lower line of the marker at 9 kDa. Isolated bioactive peptide actually has smaller molecular weight, under 9 kDa, it was proved through LC-MS identification and found some bioactive peptides under 1 kDa. Previous research showed that bioactive peptides as antihypertensive activity has a lower molecular weight[21,22]. In identification of bioactive peptide, a bioactive peptide in fraction 4, 5, and 6 are short chain peptide. Those peptides are potential peptide as ACE inhibitor and anti-hypertension. Some previous research showed that short chain peptide, like di or tripeptide, has potential antihypertensive activity[23]. Moreover, fragmented process has to be done since short chain peptide has stronger ACE inhibitor activity than long chain peptide and single amino acid. Previous research showed that it has higher activity when orally administrated into hypertensive mice compared to a long peptide that can be degraded by digestive enzymes[23]. Furthermore, 2 or 3 peptides arrangement was absorbed faster than the free amino acids[24]. ACE reaction was affected by hydrophobicity character at 3 amino acid residues at C-terminal. The amino acid sequence with hydrophobicity at C-terminal has a potency as ACE inhibitor. Aromatic side chain residue (Phe, Tyr, and Trp), arginine (R) with a positive charge on several peptides and lysine (K) with a positive charge on the C-terminal contributed as ACE inhibitors[25]. Peptides which have high activity ACE inhibitor have Trp, Phe, Tyr or Pro residues at the C-terminus and has branched chain amino acids at the N-terminal[26, 27].

Conclusion

Bakasang has potential ACE inhibitor candidate. LC-MS identification resulted in 7 type of peptides that cleaved with proteinase K and produced 11 peptide fragments. AQK fragment had the highest binding affinity energy than the rest of fragments. Thus, it can be used as an ACE inhibitor candidate. Molecular docking simulation between ACE and AQK fragment suggested two interactions are hydrophobic interactions with some of the amino acids that bind strongly with candidates ACE inhibitors include Phe457, His513, Cys370, Gln369, Ala354 and Ser355, and hydrogen binding to Tyr520, Glu384, Tyr523, His387, His353, Asp377, Glu162, Lys511, and Gln281.

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