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ANGIOTENSIN CONVERTING ENZYME (ACE) INHIBITORY ACTIVITY OF CRUDE AND FRACTIONATED SNAKEHEAD FISH (*Channa striata*) FILLET EXTRACT

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Abstract

The existence of endogenous bioactive protein or peptide with angiotensin-converting enzyme (ACE) inhibitory activity in snakehead fish fillet is promising to be investigated. The purposes of this research were to extract ACE inhibitory endogenous protein or peptide from snakehead fish fillet and to fractionate the active compounds using ultrafiltration. The extraction employed two solvents, i.e. aquadest and 50% ethanol. Fractionation was conducted using ultrafiltration membranes of 10,000; 5,000 and 3,000 Molecular Weight Cut Off (MWCO) to separate the protein or peptide into the sizes of >10 kDa, 5-10 kDa, 3 -5 kDa and <3 kDa. The parameters observed were protein and peptide content, ACE inhibitory activity (*in vitro*) and also protein and peptide profiles. The result revealed that the snakehead fish fillet contained ACE inhibitory endogenous bioactive protein or peptide. The 50% ethanol was more effective in extracting peptide of <10 kDa than the aquadest. Yet, the aquadest was better in extracting higher molecular weight protein of >10 kDa than the 50% ethanol. The fraction of <3 kDa by aquadest had the highest ACE inhibitor activity per μg protein (7.85% inhibition of ACE per μg protein). Thus, the fraction of <3 kDa aquadest is the most promising option for further research and development of natural anti-hypertension compound. From the result, snakehead fish fillet was potential to be utilized as a functional food as well as functional ingredient to fight hypertension.

Keywords: ACE inhibitor, *Channa striata,* peptide, protein, ultrafiltration

1. Introduction

Snakehead fish (*Channa striata*) is a member of freshwater fish, endogenous to tropical countries and found in many territories in Indonesia (Mustafa, Widodo, & Kristianto, 2012). Snakehead fish fillet is not only well-known for its rich nutrition, but also traditionally well known for its healing benefit. Its protein, amino acids, fatty acids and minerals are responsible for its energy recovery and well-being maintaining benefits (Mustafa, Sujuti, Permatasari, & Widodo, 2013; Rahayu, Marcelline, Sulistyaningrum, Suhartono, & Tjandrawinata, 2016). Albumin, arachidonic acid, glycine, arginine and glutamine as well as Zn and Cu were reported to be contributing in wound healing and pain reliever on post-operation, post-accident or post-natal patients (Mat Jais, McCulloch, & Croft 1994; Mustafa et al., 2012; Mustafa et al., 2013). Snakehead fish fillet is rich in albumin that is sufficient enough to supply the albumin needs for hypoalbuminemia and post-operation patients, or for child's growth (Mustafa et al., 2012). The properties of an antipyretic (Zakaria, Kumar, Mat Jais, Sulaiman, & Somchit, 2008), anti-inflammatory (Zakaria et al., 2008) and anti-osteoarthritic (Michelle, Shanti, & Loqman, 2004) were reported to be found in snakehead fish. Recently, Chasanah, Nurilmala, Purnamasari, and Fithriani, (2015) reported that the aquadest crude extract of snakehead fish fillet (50 mg/ml) was potential as an ACE inhibitor with activity equivalent to 1/10 of captopril, the commercial antihypertensi drug (5 mg/ml). Those reported health benefits and nutrition showed that snakehead fish is a very potential source for discovering new bioactive compounds, particularly

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endogenous protein or peptide with ACE inhibitory activity. Hypertension was responsible for 7.5 million deaths, about 12.8% of the total of all deaths (World Health Organization, 2018). Thus the need for natural ACE inhibitor compounds is very high.

Angiotensin-converting Enzyme (ACE; dipeptidyl carboxypeptidase, EC 3.4.15.1) is one of the main regulators in controlling blood pressure. In renin-angiotensin system, ACE will convert the inactive decapeptide angiotensin I to octapeptide angiotensin II, which is a potent vasoconstrictor, by removing dipeptides in C-terminal. In kinin-kalikrein system, ACE inactivates vasodilator bradykinin by hydrolyzing dipeptide PF in C-terminal (RPPGFSPFR). Vasoconstrictor is an agent that cause constriction of blood vessels while vasodilator is an agent that serves to dilate blood vessels. Angiotensin II causes vasoconstriction which leads to increase in the blood pressure, by the activation of the AT1 receptor (AT1R). Angiotensin II also negatively affects kidney retaining water and salts, causing an increase in extracellular fluid volume and finally causing an increase in the blood pressure (Puchalska, 2014).

The exploration of natural ACE inhibitory compound is encouraging especially in terms of functional food scope. This is because of the treatment of synthetic ACE inhibitor (pharmaceutical drug) such as benazepril, captopril and lisinopril, turned out to result in several side effects namely dry cough, allergy, nausea impairment and red blood cell decline (Jao, Huang, & Hsu, 2012). Endogenous bioactive protein or peptide can be a safer alternative as a natural ACE inhibitor. Muchtadi (2004) reported that ACE inhibitor compounds could be found in foodstuff that is rich in protein. Huang, Davidge, and Wu (2013) supported that bioactive compound like protein and peptide had an influential role to lower the blood pressure. Investigations towards the presence of ACE inhibitory bioactive peptide has been conducted by Ghassem, Babji, Said, Mahmoodani, and Arihara (2014) were hydrolyzing sarcoplasmic protein of snakehead fish fillet using alcalase and by Ghassem, Arihara, Babji, Said, and Ibrahim (2011) were hydrolyzing myofibril protein of snakehead fish fillet using thermolysin. Yet, a study of endogenous bioactive protein or peptide with ACE inhibitory activity from snakehead fish fillet is still sparse.

Endogenous protein or peptide from foodstuff can be extracted using various solvents. Nevertheless, the process in finding the effective solvent to extract endogenous protein and peptide is still challenging. The aquadest and 50% ethanol (aquadest:ethanol = 1:1) were used for extraction in this research. Water is a popular solvent in extraction process with its polarity being advantageous to extract polar compounds such as proteins and peptides (Chemat, Vian, & Cravotto, 2012; Puchalska 2014). Ethanol (CH_3CH_2OH) is a part of alcohol that has the lowest toxicity. It has an OH group which can attract polar and ionic compounds, and also the ethyl group (C_2H_5) that can attract non-polar compounds as well (Saleh, 2016). Moreover, the utilization of organic solvent like ethanol in extraction medium could contribute to the precipitation or denaturation of high molecular weight protein so that it left fractions with lower molecular weight in the solution (Chertov et al., 2005). Furthermore, ultrafiltration is used to fractionate proteins or peptides in this research. Ultrafiltration involves simple process that can reduce the degradation, deactivation, and denaturation of the resulted biological products (Cui, 2005).

This research aims to investigate the presence of endogenous bioactive protein or peptide as the ACE inhibitor from snakehead fish fillet and to determine the most active fraction of the snakehead extracts in inhibiting ACE. This research is valuable for further utilization of foodstuff like snakehead fish as functional foods or functional ingredients, particularly to fight hypertension.

2. Material and Methods

2.1. Material

Wild snakehead fish of 100 – 350 g in weight, 28 – 34 cm in length, was obtained from a pond in surrounding Parung (regency), Bogor, West Java, Indonesia. The fish was immobilized by soaking in ice for 30 minutes. The fillet was filleted in a cold condition, and was stored in -20°C before being extracted. Two solvents used to extract the protein and peptide, i.e aquadest and 50% ethanol (aquadest:ethanol = 1:1). The ethanol used was technical grade while all chemical used for analysis were analytical grade (pa).

2.2. Methods

2.2.1. Extraction of protein and peptide

The extraction of protein and peptide followed Falkenberg, Mikalsen, Joensen, Stagsted, and Nielsen (2014) method with some modifications. Frozen snakehead fish fillet was first being thawed before cut into cubics (0.5 x 0.5 x 0.5 cm) and soaked with liquid nitrogen. The cubics were then minced using hand blender. As much as 20g of minced snakehead fish was then macerated using 60 mL of solvent (ratio of fillet : solvent = 1 : 3) for 10 minutes in ice temperature (<10°C). Next, the suspension was homogenized

(homogenizer ultraturrax T25, IKA Labortechnich, USA) at 13,500 rpm for 5 minutes in ice temperature (<10°C). Homogenate was then centrifuged (Beckman Coulter Avanti) at 15344 x g, 4°C for 10 minutes. Supernatant (crude extract) was obtained, which part of it was used right away for protein and peptide content tests while the rest was freeze-dried for ACE inhibitor bioactivity test. The ethanolic crude extract was first evaporated using a rotary evaporator (Buchi) before being freeze dried. SDS PAGE was performed to supernatant without freeze-drying treatment. The remaining unfreeze-dried crude extract was stored at -20°C before proceeding to ultrafiltration.

2.2.2. Ultrafiltration

The proteins or peptides contained in crude extracts were fractionated based on their molecular weight using ultrafiltration membranes of 10,000; 5,000; and 3,000 MWCO. The process produced fraction of proteins or peptides of >10 kDa, 5 - 10 kDa, 3 - 5 kDa and <3 kDa sizes (Ren et al., 2008). The 5,000 and 10,000 MWCO separations were conducted using tangential ultrafiltration (QuixStand benchtop system, GE Healthcare, USA). The 3,000 MWCO separation was undergone using the centrifugal ultrafiltration (Amicon Pro Purification System, Merk, Germany). As much as 4,700 mL of crude extract (aquadest or 50% ethanolic extract) was ultrafiltrated using the 10,000 MWCO membrane. This treatment produced 1,200 mL retentate (fraction >10 kDa) and 3,500 mL permeate. The 3,500 mL of permeate was then ultrafiltrated using the 5,000 MWCO membrane. This treatment produced the 850 mL retentate (fraction 5-10 kDa) and 2,650 mL permeate. The 2,650 mL permeate of 5,000 MWCO membrane fractionation was further ultrafiltrated using the 3,000 MWCO membrane. This treatment produced an output of the 1,650 mL retentate (fraction 3-5 kDa) and 960 mL permeate (fraction <3 kDa). Each fraction was analyzed for its protein and peptide content, while the rest were freeze-dried for bioactivity analysis. Each of the ethanolic fractions (>10, 5-10, 3-5 and <3 kDa fraction of 50% ethanol extraction) was evaporated using a rotary evaporator before being freeze dried. SDS PAGE protocol was conducted to the un-freeze-dried fractions.

2.2.3. Protein content analysis

This step referred to Lowry protein assay method (Lowry, Rosebrough, Farr, & Randall, 1951). Bovine Serum Albumin (BSA) was used as the standard for measuring protein content.

2.2.4. Peptide content analysis

The peptide content analysis followed the method of Church, Swaisgood, Porter, and Catignani (1983) with modification, employing the spectrophotometry analysis of *O-pthalaldehyde* (OPA). Casein tryptone in phosphate buffer (pH 7.4) was used as the standard for measuring the peptide content.

2.2.5. Angiotensin converting enzyme (ACE) inhibitory activity analysis

This analysis referred to Arihara, Nakashima, Mukai, Ishikawa, and Itoh (2001). As much as 20 µL of samples (10 mg/mL) were added to the 125 µL of substrate buffer (contained 7.6 mM N-*hippuryl-his-leu* hydrate and 608 mM NaCl in 10 mL borate buffer pH 8.3) and then mixed with 15 µL of BSA (10 mg/mL). The mixture was incubated at 37°C for 15 minutes using shaking waterbath. The reaction was initiated by adding 50 µL of ACE enzyme (50 mU/mL) and subsequently was incubated at 37°C for 30 minutes. The reaction was terminated by adding 200 µL of HCl 1 N. The mixture was then vortexed and 1,140 µìL ethyl acetate were added to the mixture to formed hippuric acid. Then it was centrifuged at 10,000 g for 10 minutes. As much as 800 ìL of supernatants were being dried with oven at 95°C for 75 minutes. The formed hippuric acid was then dissolved in 1,000 ìL of aquabidest. The absorbance was measured at 228 nm using UV-Vis spectrophotometer. Concentration of samples used in this study was 10 mg/mL (Yousr & Howell, 2015). The bioactivity was calculated in percentage using the equation:

$$(\%)\ \text{ACE inhibitor (10 mg/ml sample)} = \frac{(A-B)-(C-D)}{(A-B)} \times 100\%$$

where:

A = control absorbance (HHL substrate + ACE enzyme)

B = blank control absorbance (HHL substrate + aquadest)

C = sample absorbance (HHL substrate + ACE enzyme + sample)

D= blank sample absorbance (HHL substrate + sample).

Next, percent inhibition of ACE μg^{-1} protein was obtained by converting percent ACE inhibition of 10 mg/ml sample using protein concentration in 10 mg/ml sample.

2.2.6. SDS PAGE

The SDS PAGE procedure followed the method of Laemmli (1970) with modification. The concentration for the stacking gel was 6% (v/v), and the separating gel concentration was divided based on the sample type : 18% was used for crude extracts and >10 kDa fractions; and 20% was used for fractions of <3, 3- 5, 5 -10 kDa from both of extraction methods (aquadest

and 50% ethanol). Samples were concentrated using TCA 100% and washed by acetone. Then, samples were mixed with 20 μL sample buffer. The mixture was heated using thermoblock at 95°C for 10 minutes. As much as 20 μL of samples were injected into the electrophoresis gel which has been added with the electrophoresis buffer solution. Electrophoresis was run at 100 V, 70 mA for 2.5 hours (Mini-Protean Kit Model, BioRad, USA). After the electrophoresis completed, the gel was stained using coomassie blue. The molecular weight and color intensity of SDS PAGE gel was then determined using the Gel Analyzer version 10.

2.2.7. Statistical analysis

Extraction was conducted in two replications for each type of solvent. Each replication was analyzed in duplicates (Giromini, Fekete, Ian Givens, Baldi, & Lovegrove, 2017), i.e. for protein content, peptide content and ACE inhibitory activitiy analysis. The data were analyzed by variance analysis (ANOVA) and Duncan test at 5% using SPSS version 20. SDS PAGE was conducted only in one repetition for each type of extract (aquadest or 50% ethanol extract).

3. Results and Discussion

3.1. Protein and Peptide Content of Crude Extract and its Fractions

The snakehead fish fillet used in this research (Figure 1) were in accordance to the characteristics as explained in Listyanto and Adriyanto (2009), to be white, thick and without any fine spines or bones. Snakehead fish fillet mainly consisted of white muscle (Gam, Leow, & Baie, 2006)

Table 1 exhibits concentration and total content of protein and peptide of crude extract and its fractions after ultrafiltration. Concentration and total protein content of the aquadest-crude extract and its >10 kDa fraction was higher than the 50% ethanol-crude extract and its >10 kDa fraction ($p<0.05$). These results showed that aquadest was more capable to extract protein with high molecular weight (>10 kDa) than the 50% ethanol solvent. On the other hand, concentration and total protein content of fractions 5-10, 3 -5 and <3 kDa of the 50% ethanol extract was higher than fractions of aquadest extract ($p<0.05$). It means that the 50% ethanol was more capable to extract low molecular weight protein (less than 10 kDa) than the aquadest. These results were also supported by the OPA assay that shown in Table 1. The OPA assay result showed that the concentration and total peptide content of the 50% ethanol-crude extracts and its 5 - 10, 3-5 and <3 kDa fractions were higher than the aquadest-crude extracts and the 5-10, 3-5 and <3 kDa fractions of aquadest ($p<0.05$)

The utilization of 50% ethanol in this research could extract low molecular weight proteins <10 kDa (peptides) better than the aquadest, and vice versa. In biological fluids (i.e. serum and human plasma), the presence of low molecular weight proteins (peptides) are not as much as the presence of high molecular weight proteins. The presence of the low molecular weight protein was also often concealed with the bigger ones. Low molecular weight protein was usually bound to bigger protein carrier such as albumin and immunoglobulin. Albumin acted as the carrier protein that bound the smaller protein. The elimination of high molecular weight protein (i.e. albumin), with a proper protein separation method, can facilitate the detection and identification of low molecular weight protein (Kay et al., 2008). The utilization of organic solvent like ethanol could help in precipitating or denaturizing high molecular weight protein so that it leave the low molecular weight protein in the solution (Chertov et al., 2005). High molecular weight protein could later be separated from low molecular weight protein by centrifugation. Low molecular weight proteins (peptides) are more soluble in an aquaeous solvent so that they can be easily taken in supernatant (Aluko, 2012).

Figure 1. Snakehead fish (*Channa striata*) fillet.

Table 1. Concentration and total content of protein and peptide of snakehead fish (*Channa striata*) fillet crude extract and its fractions

Samples	Volume (mL)	Protein Concentration (mg/mL)		Total Protein Content (mg)	
		Aquadest	50% Ethanol	Aquadest	50% Ethanol
Crude Extract	4,700	12.34 ± 0.01^{g}	4.29 ± 0.01^{e}	57,998.00 ± 66.47^{g}	20,139.50 ± 33.23^{e}
>10 kDa	1,200	20.85 ± 0.06^{h}	10.11 ± 0.02^{f}	25,020.00 ± 67.88^{f}	12,126.00 ± 25.46^{d}
5 -10 kDa	850	0.22 ± 0.00^{b}	0.54 ± 0.00^{d}	187.00 ± 0.00^{a}	459.00 ± 0.00^{c}
3 -5 kDa	1,650	0.17 ± 0.00^{a}	0.27 ± 0.01^{c}	280.50 ± 0.00^{b}	437.25 ± 11.67^{c}
<3 kDa	960	0.17 ± 0.01^{a}	0.29 ± 0.01^{c}	158.40 ± 6.79^{a}	273.60 ± 6.79^{b}
Samples	**Volume (mL)**	**Peptide Concentration (mg/mL)**		**Total Peptide Content (mg)**	
		Aquadest	**50% Ethanol**	**Aquadest**	**50% Ethanol**
Crude Extract	4,700	10.23 ± 0.01^{i}	10.40 ± 0.04^{j}	4,8081.00 ± 66.47^{g}	48,880 ±199.40^{e}
>10 kDa	1,200	9.77 ± 0.11^{h}	9.45 ± 0.05^{g}	1,1718.00 ± 127.28^{f}	11,346.00 ±59.40^{d}
5 -10 kDa	850	4.28 ± 0.03^{c}	6.10 ± 0.03^{f}	3642.25 ± 30.05^{a}	51,85.00 ± 24.04^{c}
3 -5 kDa	1,650	3.27 ± 0.01^{b}	4.89 ± 0.02^{e}	5,403.75 ± 11.67^{b}	8,060.25 ± 35.00^{c}
<3 kDa	960	2.43 ± 0.02^{a}	4.40 ± 0.02^{d}	2,328.00 ± 20.36^{a}	4,219.20 ± 20.36^{b}

Note: different alphabet shows significant difference at $p< 0.05$

The utilization of the 50% ethanol in this research, was expected to be enable the precipitation of high molecular weight protein and hence the recovery of low molecular weight protein was possible. The use of aquadest as single solvent resulted in no precipitation of high molecular weight protein. Therefore, concentration and total protein content of the high molecular weight protein in both crude extracts and in >10 kDa fraction of aquadest extraction were higher compared to those of 50% ethanol extraction. The use of mixed solvent (ethanol:aquadest = 1:1) in this research has proven to be effective in extracting peptides (<10 kDa) compared to the use of aquadest alone. It was also presumed that the mixed solvent could extract both hydrophobic and hydrophilic peptides while a single solvent of aquadest tended to extract limited to hydrophilic peptide. Tamvakopoulos (2006) explained that protein precipitation with organic solvent like ethanol was more effective in extracting non-polar peptides. In addition to its ability to precipitate high molecular weight protein, the introduction of ethanol in extraction medium could dissociate smaller proteins from its carrier (Feig, 2009). The dissociation resulted in low molecular weight protein to pass through the ultrafiltration membrane. It may be the underlying reason why the fraction of >10 kDa extracted by 50% ethanol yielded lower number in total peptide content compared to that by aquadest.

3.2. Profiles of Protein and Peptide from SDS PAGE

SDS PAGE was performed to confirm the protein and peptide profile of the snakehead fish crude extracts and fractions. Figure 2 exhibits the result of SDS PAGE. As there was no visual image of protein or peptide of fraction of 3 – 5 kDa and <3 kDa for both solvents, the SDS PAGE results of those items are not displayed. The low protein concentration in 3 – 5 kDa and <3 kDa fractions made it complicate to be observed in SDS PAGE gel. Figure 3 and 4 displays the color intensity of bands and the molecular weight of protein or peptide from Gel Analyzer ver. 10 computation.

Aquadest-crude extract had 12 protein bands with molecular weight >10 kDa while 50% ethanol- crude extract only possessed 8 bands. Proteins with molecular weight of 104, 90, and 38 kDa that observed in aquadest extract were not present in ethanolic extract. It proves that aquadest extraction yields more high-molecular-weight protein than 50% ethanol extraction. In SDS PAGE, the darker proteins or peptides bands are the richer their protein or peptide

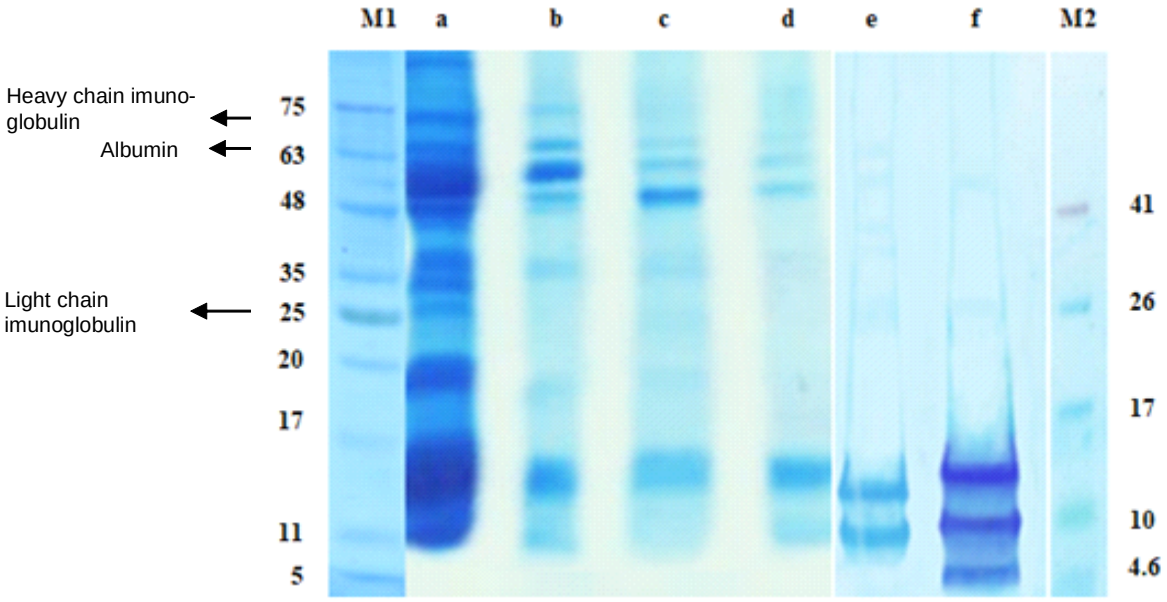

Figure 2 Protein profile based on SDS PAGE result, (M1) broad range molecular weight protein marker, (M2) low molecular weight protein marker (a) crude extract (aquadest), (b) crude extract (50% ethanol), (c) >10 kDa fraction (aquadest), (d) >10 kDa fraction (50% ethanol), (e) 5 – 10 kDa fraction (aquadest), (f) 5 – 10 kDa fraction (50% ethanol).

content. The color intensity of protein band in crude extract of 50% ethanol seemed weaker than that of aquadest, as shown in Figure 2, 3 and 4. It also confirmed that the amount of high molecular weight protein extracted by 50% ethanol was not as much as in aquadest extraction. The results of Lowry and OPA assays supported this idea as well. Furthermore, the >10 kDa fraction of aquadest extraction contained more protein compared to that of 50% ethanol extraction.

The 50% ethanol extraction could precipitate most of albumins and immunoglobulins in crude extract. It has been explained that high molecular weight proteins such as albumin and immunoglobulin conceals the presence of low molecular weight proteins (Kay *et al.,* 2008). Albumins in snakehead fish are about 67.6741 kDa (Syukroni & Trilaksani, 2017) while its immunoglobulins are 72 kDa (heavy chain) and 27 kDa (light chain) (Rauta, Mohanty, Gamayak, & Sahoo, 2013). Both aquadest and 50% ethanol extracts had 64 kDa protein band that was suspected to be albumins. Meanwhile, the protein with molecular weight 73 kDa in crude extract of aquadest and 50% ethanol was presumably heavy chain of immunoglobulins. Albumins and heavy chain immunoglobulins of the 50% ethanol- crude extract had weaker color band intensity than the aquadest-crude extract (figure 2, 3 and 4). This also indicates that 50% ethanol can help to precipitate most of albumins and heavy chain of immunoglobulins in crude extract. The aquadest-crude extract contained protein band of 27 kDa that was suspected to be light chain of immunoglobulins. In case of 50% ethanolic extract, protein band of 27 kDa (considered to be light chain of immunoglobulins) previously did not present in the crude extract, turned out to be only present in the 5-10 kDa fraction of 50% ethanol (figure 2, 3 and 4). The absence of 27 kDa or light chain of immunoglobulins proteins in 50% ethanol crude extract were suspected due to the concealment of these light chain of immunoglobulin by bigger proteins or due to certain interactions between these light chain of immunoglobulins with other proteins in 50% ethanol crude extract. Nevertheless, the color intensity of the immunoglobulins' band light chain in 5-10 kDa fraction of 50% ethanol, was still weaker than the color intensity of the immunoglobulins' band light chain of aquadest's crude extraction. In the aquadest extracts, proteins with molecular weight of 104 and 90 kDa were not discovered in its >10 kDa fractions. One disadvantage of the fractionation using the ultrafiltration is the possibility of losing the protein or peptide in the extraction caused by the protein adsorption within the porous structure of the membrane (Cui, 2005).

The 5 - 10 kDa fraction of the ethanolic extract has three dominant proteins, i.e. proteins of 11, 9 and 7 kDa; while the 5 - 10 kDa fraction of aquadest extract has only two dominant proteins, i.e. proteins of 11

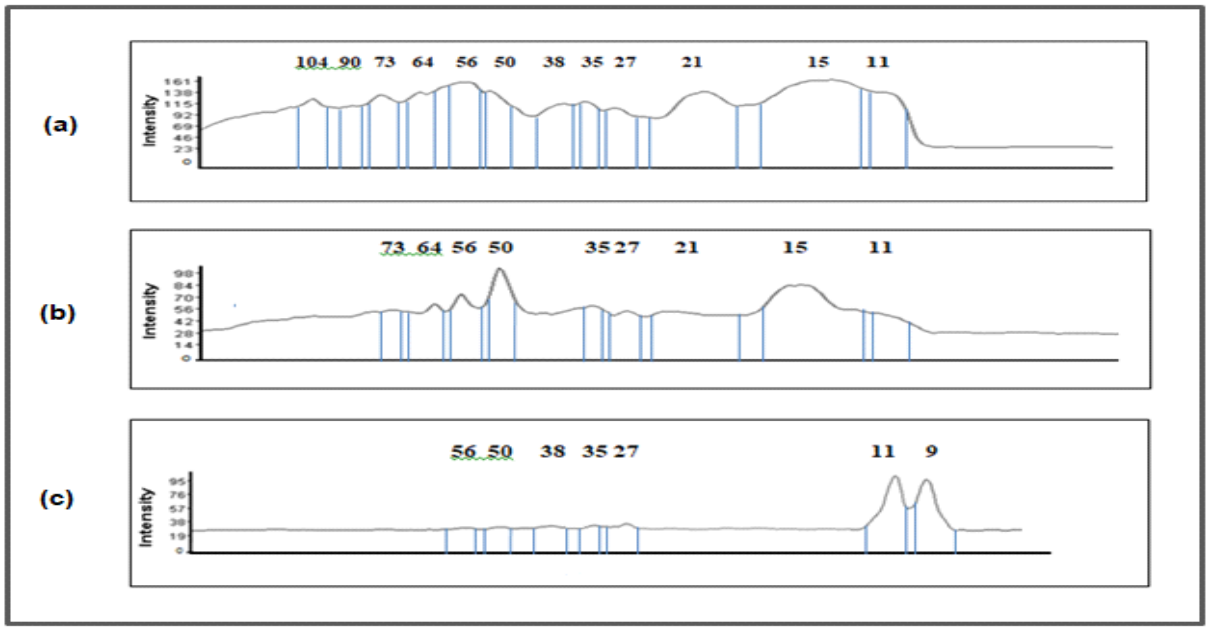

Figure 3 Molecular weight and color intensity of protein and peptide from Gel Analyzer ver. 10 computation (a) crude extract (aquadest), (b) >10 kDa fraction (aquadest), and (c) 5 – 10 kDa fraction (aquadest).

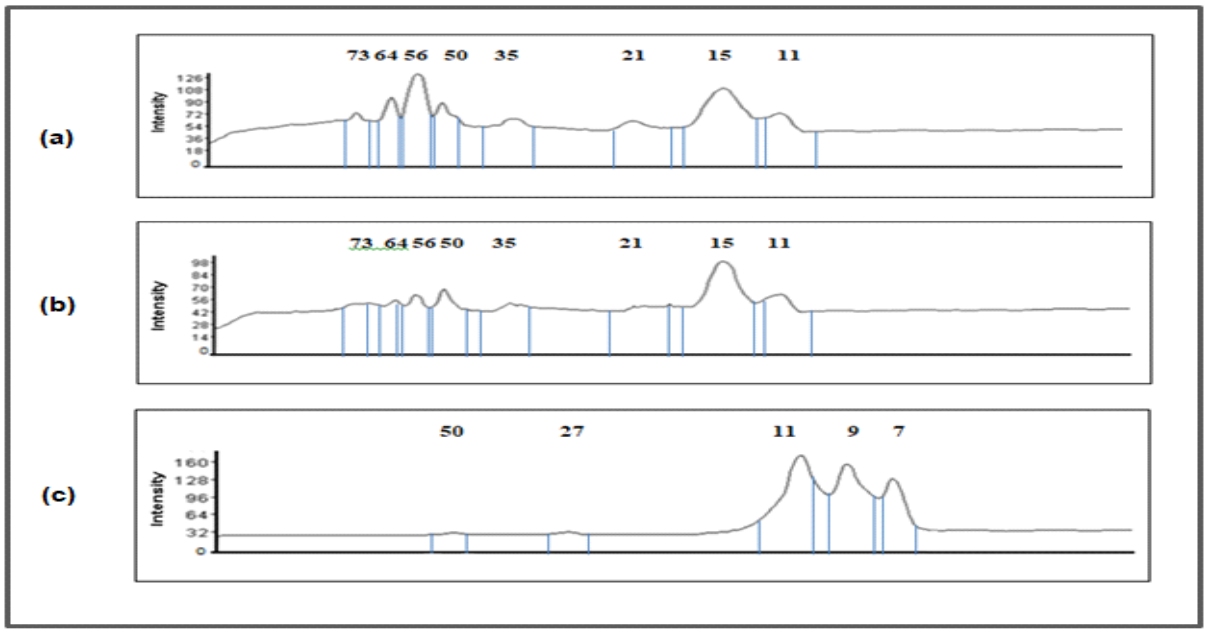

Figure 4 Molecular weight and color intensity of protein and peptide from Gel Analyzer ver. 10 computation (a) crude extract (50% ethanol), (b) >10 kDa fraction (50% ethanol), and (c) 5 – 10 kDa fraction (50% ethanol).

and 9 kDa proteins. It also shows that 50% ethanol was able to extract more low molecular weight proteins than aquadest. Fraction 5-10 kDa of both aquadest and ethanol 50% extracts contained residual protein (>10 kDa fraction) in low intensity. Proteins of 56, 50, 38, 35, 27 kDa were observed in the aquadest-extract, while protein of 50 and 27 kDa were observed in the 50% ethanol extract. Raghavan and Kristinsson (2009) reported similar phenomenon. They fractionated the protein of hydrolyzed (cryotine 25%) Nile tilapia using 30 kDa and 10 kDa membranes and the SDS PAGE results showed that fraction of 10-30 kDa was also including >30 kDa fraction, though with weaker intensity. According to Georgiou, Rice, and Baker

Table 2 Percentage of ACE inhibitory activity of snakehead fish (*Channa striata*) crude extract and fractions

Samples (10 mg/mL)	% ACE inhibitory activity at 10 mg/ml sample	
	Aquadest	50% ethanol
Crude Extract	81.62 ± 0.03^{e}	86.77 ± 0.08^{h}
>10 kDa	77.11 ± 0.16^{c}	83.73 ± 0.11^{f}
5 - 10 kDa	83.42 ± 0.00^{g}	95.14 ± 0.08^{i}
3 - 5 kDa	75.80 ± 0.00^{b}	82.10 ± 0.08^{e}
<3 kDa	71.42 ± 0.01^{a}	80.36 ± 0.00^{d}

Note: Different letters show significant differences at p<0.05 Duncan test.

Table 3. Protein concentration at 10 mg/mL samples and ACE inhibitor percentage per ìg protein

Samples	Protein concentration (mg/mL) in 10 mg/mL samples		% ACE inhibitor per µg protein	
	Aquadest	50% ethanol	Aquadest	50% ethanol
Crude Extract	5.63 ± 0.28^{f}	4.19 ± 0.08^{e}	0.73 ± 0.01^{c}	1.04 ± 0.01 ^{d}
>10 kDa	6.57 ± 0.16^{h}	6.31 ± 0.11^{g}	0.59 ± 0.01^{a}	0.66 ± 0.00^{b}
5 - 10 kDa	0.63 ± 0.00^{b}	1.00 ± 0.01^{d}	6.78 ± 0.03^{g}	4.76 ±0.01^{e}
3 - 5 kDa	0.55 ± 0.00^{ab}	0.87 ± 0.01^{cd}	6.89 ± 0.00^{h}	4.77 ± 0.03^{e}
<3 kDa	0.45 ± 0.00^{a}	0.78 ± 0.00^{c}	7.85 ± 0.14^{i}	5.14 ±0.01^{f}

Note: Different letters show significant differences at p<0.05 Duncan test.

(2001) the numbers of cut off size of each membrane were in average, thus it would result in such normal distribution of higher and lower pores' size in that respective membrane. Moreover, the presence of the >10 kDa fraction in the 5-10 kDa fraction of the 50% ethanol-extract might also be caused of the inadequate concentration of ethanol to precipitate high molecular weight protein and to dissociate low molecular weight protein from the higher ones as well. The ethanol concentration unfolding the high molecular weight protein in variety ways depending on the protein type (Tanaka et al., 2001). Even more, aquadest extraction could not precipitate the high molecular weight protein. Due to that reason, the 5-10 kDa aquadest-extract fraction had more protein impurities band than the 5-10 kDa ethanolic extract fraction. Furthermore, the 9 kDa protein band which previously did not present in the aquadest crude extract turned out to be appearing in the 5-10 kDa fraction. Meanwhile, proteins of 9 and 7 kDa which previously did not appear in the crude extract of the ethanolic crude extract appeared in the 5-10 kDa fraction. Same as the case in the absence of 27 kDa proteins in 50% ethanol crude extract, the absence of those low molecular weight proteins in its crude extract was suspected due to the concealment of those smaller proteins by bigger proteins or due to certain interactions between those proteins with other proteins.

3.3. ACE inhibitory activity in the crude extract and its fractions

The analyses were conducted *in vitro.* The concentration of samples used was 10 mg/mL. The percentage of the samples' ACE inhibitory activity at 10 mg/ml concentration is presented in Table 2. At this concentration, ethanol 50% extracts exhibited better performances in inhibiting ACE than the aquadest extracts (p<0.05). For each fractions of aquadest and ethanol 50% extractions at 10 mg/mL, fraction 5-10 kDa had the highest activity, followed by fractions of >10 kDa, 3-5 kDa and finally <3 kDa. Chasanah et al. (2015) reported that captopril with a concentration of 1 mg/ml had ACE inhibitory activity of 90.32%. The concentration of the snakehead fish fillet extract used for ACE inhibitory activity analysis

in this research (10 mg/ml) was tenfold compared with the concentration of captopril (1 mg/ml) used by Chasanah et al. (2015). Hence, the snakehead fish fillet extract in this study had a lower ACE inhibitory activity than the commercial standard of 1 mg/ml captopril.

Though we found that the 5-10 kDa fraction of ethanolic extract showed the highest ACE inhibitor activity when tested at 10 mg/mL (Table 2). However after those ACE inhibitor percentage data were converted into percent of ACE inhibition per μg protein data (Table 3), we found that the best fraction in inhibiting ACE was the <3 kDa fraction of aquadest extract (inhibiting 7.85 % of ACE per μg protein). This bioactivity was significantly different compared to the other samples' bioactivity ($p<0.05$). The ACE inhibitory activity of the <3 kDa fraction of aquadest is certainly still lower than ACE inhibitory activity of captopril. Arora and Chauhan (2013) reported that the IC_{50} of captopril was 23 nM. ACE inhibitory peptides from food have certain advantage when compared to ACE inhibitory drugs. Peptides from food usually have lower in vitro ACE inhibitory activity compared to ACE inhibitory drugs, so they have no harmful side effects, such as dry cough and angioedema (FitzGerald & Meisel, 2000). As part of everyday food, peptides appear more natural and safe for consumers. As shown in Table 3, fractions of <10 kDa (5-10, 3-5 and <3 kDa) had higher per μg protein ACE inhibitory activity compared to >10 kDa fraction and its crude extract. ACE inhibitor compounds are often low molecular weight proteins or peptides. Erdmann, Cheung, and Schröder (2008) explained that ACE inhibitors are commonly peptides containing 3-13 amino acids and have low molecular weight (<3 kDa). Aluko (2012) also reported that in order to provide an antihypertensive effect in vivo, ACE inhibitory peptides must be absorbed through the intestinal epithelium and reach the cardiovascular system in active form. Low molecular weight proteins or peptides are more resistant to enzymatic hydrolysis in the digestive tract. Small peptides also easier to absorp into the blood circulation where they are transported to various target organs than the bigger ones. Conversely, high molecular weight proteins will be easily degraded by digestive enzymes become inactive or even more active form. Short chain peptides (up to 5 residual amino acids) or hydrophilic peptides containing >25% charged amino acids (like lysine and arginine) and <25% hydrophobic amino acids are soluble peptides that are easily extracted by water/ aquadest (Puchalska, 2014). Endogenous ACE inhibitor peptide generally contains proline in its C-terminal. Peptides with ACE inhibitory activity may as well contains lysine, arginine, tryptophan, phenylalanine, tyrosine at its C-terminal and aliphatic branched-amino acid at its N-terminal. Di-or tripeptides, especially those containing proline residue at its C-terminal, are common examples of peptides resistant to digestive enzymes. Short chain peptides containing 2 to 3 amino acid residues are absorbed faster than free amino acids (Erdmann et al., 2008; Li, Le, Shi, & Shrestha, 2004).) Snakehead fish fillet was reported to contained amino acids phenylalanine, tyrosine, alanine, isoleusine, leusine, proline, valine, methionine, arginine and lysine Zuraini et al., (2006) which is contributing to ACE inhibitory activity.

The results of this study indicate that snakehead fish fillet contained ACE inhibitory endogenous protein or peptide. Snakehead fish fillet can be a source of ACE inhibitor compounds. The <3 kDa fraction of aquadest extraction had the highest μg^{-1} protein ACE inhibitory activity, indicating that it is potential for further research to develop a natural ACE inhibitor.

4. Conclusion

The result of this research revealed that the crude extracts of snakehead fish fillet and all of its fractions, had ACE inhibitor activity with various inhibition activities. This shows that the snakehead fish fillet contains ACE inhibitor protein and peptide. The fraction of <3 kDa extracted by aquadest had the highest ACE inhibitor activity μg^{-1} protein (7.85 % inhibitor ACE μg^{-1} protein). Thus, the fraction of <3 kDa aquadest is the most promising option for further research and development of natural anti-hypertension compound. In this research, we concluded that snakehead fish fillet was potential to be utilized as a functional food or functional ingredient to fight hypertension.

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