



# Screening of Novel Angiotensin I Converting Enzyme Inhibitory Peptides Derived From Enzymatic Hydrolysis of Salmon Protamine

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Received 05 Aug. 2016; Accepted 07 Sep. 2016

**ABSTRACT:** Angiotensin I converting enzyme (ACE) inhibitory peptide is widely recognized as useful therapeutic approach in the treatment of hypertension. Bioactive peptides from natural sources, including marine fish, are more considered because it has no harm effects. The objective of this study is screening the presence of potential ACE inhibitory peptide from salmon protamine. ACE inhibitory peptide was purified from salmon protamine after 16 hours of hydrolysis by various enzymes and centrifuged using 3 kDa molecular weight cut off (MWCO) ultrafiltration membrane. The peptide sequences were analyzed by Liquid Chromatography Tandem Mass Spectrometric (LC-MS/MS). ACE inhibitory activity was measured using Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC). The results indicated that trypsin hydrolysate had the highest ACE inhibitory activity compared to the other hydrolysates with IC<sub>50</sub> value 135.96 µg/ml. LC-MS/MS analysis of tryptic protamine identified two major peaks with three peptide sequences, Ser-Ser-Arg-Pro-Ile-Arg (SR-6), Ser-Ser-Ser-Arg-Pro-Ile-Arg (SR-7), Pro-Arg-Arg-Ala-Ser-Arg (PR-6) which sourced from salmine of Chum Salmon (*Oncorhynchus keta*). The ACE inhibitory peptides from Salmon Protamine still has not been reported previously, therefore it can be beneficial for preventing hypertension.

**Author Keywords:** Angiotensin I Converting Enzyme (ACE), Antihypertensive, Bioactive Peptide, Chum Salmon (*Oncorhynchus keta*), Enzymatic hydrolysate, Salmon Protamine

## INTRODUCTION

Nowadays, the inhibition of ACE activity is commonly known as a functional therapeutic agent for preventing and curing hypertension. It has been in use for the last two decades and the tendency to use them will be continuously increased [1]. Currently, several synthetic antihypertensive drugs based on ACE inhibitors have been clinically used such as captopril, enalapril, alacepril, and lisinopril [2]. Even though these synthetic ACE inhibitors could be possibly utilized as antihypertensive drugs, unfortunately they still have some undesirable side effects such as coughing, allergic reactions, taste disturbance, and skin rashes [3]. Thus, developments and investigations to explore a beneficial and economical ACE inhibitors are required to prevent hypertension.

One of the most popular and favorite fish in the world is Salmon. It is considered to be healthy due to its high nutritional value and pharmacological activity. Previous studies have investigated ACE inhibitory peptides from Salmon by-product such as fillet and residuals [4], skin [5, 6], and pectoral fin [7]. However, the ACE inhibitory peptides from Salmon Protamine still has not been reported.

The objectives of this study were to hydrolysis protein using different proteases, and the protein hydrolysate was assessed for bioactivity including angiotensin converting enzyme (ACE) inhibitory activity. Furthermore, the sequences of the bioactive peptides were determined by LC-MS/MS [8].

## MATERIALS AND METHODS

Salmon Protamine were treated with a single protease, with an enzyme to protein ratio of 1:50 (w/w) using different temperatures which were based on the enzymes' activities: trypsin (37 °C),  $\alpha$ -chymotrypsin (37 °C), pepsin (37 °C), and thermolysin (60 °C). The enzymatic digestions of the salmon protamine were kept at pH 8, except for pepsin, which was adjusted to pH 1.3. After incubation for 16 hours, the hydrolysis was stopped by centrifugation at low temperature (14,000 rpm, 10 min, 4 °C) in ultrafiltration membrane (3 kDa MWCO). The filtrate (<3 kDa) was lyophilized and kept at -20 °C for further assay or analysis.

The ACE inhibitory activity was determined according to the method reported by Cushman et al [9] with partial modification. The sample solution containing 30  $\mu$ l of 2.5 mM hippuryl-L-histidyl-L-leucine (HHL) as a substrate and 10  $\mu$ l of inhibitor (at an indicated concentration) in 200 mM borate buffer containing 300 mM NaCl (adjusted to pH 8.3) was pre-incubated at 37 °C for 5 minutes. The control solution was prepared using the same buffer but without inhibitor. Afterwards, 20  $\mu$ l of 2 mU/ml ACE in 200 mM borate buffer was added to the sample solution and control solution, individually. The reaction mixture was incubated statically at 37 °C for 30 minutes and then shaken in a thermostatically controlled shaker incubator (200 rpm) at 37 °C for 30 minutes. The reaction was quenched under acidic conditions by adding 1 M HCl (60  $\mu$ l). Ferulic acid 0.2 mg/ml (10  $\mu$ l) was used as an internal standard for normalizing variation derived from different samples. HHL and its hydrolyzed product, hippuric acid (HA) were analyzed using an HPLC equipped with a C<sub>18</sub> column. The resulting HA was detected using a UV detector fixed at 228 nm. The ACE inhibition (%) was determined based on the following equation:

$$[1 - (\Delta A_{\text{inhibitor}} / \Delta A_{\text{control}})] \times 100$$

where  $\Delta A_{\text{inhibitor}}$  was the peak area of HA in the reaction mixture by the presence of peptide as ACE inhibitor and  $\Delta A_{\text{control}}$  was the peak area of HA in the reaction mixtures without peptide as ACE inhibitor. Definition of ACE activity: One unit (U) of ACE activity was defined as the amount of enzyme required to catalyze formation of 1  $\mu$ mol of HA from HHL per minute at 37 °C.

The peptide sequences in the lyophilized hydrolysate were further identified using LC-MS/MS analysis and database matching. Freeze dried peptides were dissolved in 5% ACN (Acetonitrile) and 0.2% FA (Ferulic acid) in deionized water for LC-MS/MS analysis. LC-MS/MS analysis was performed using a Thermo LCQ DECA XP MAX system with an electrospray ionization (ESI) source (Thermo Scientific Inc., USA). Samples were loaded onto a BioBasic C<sub>18</sub> column with diameter 150  $\times$  2.1 mm, particle size 5  $\mu$ m. The mobile phase consisted of Solution A (100% deionized water and 0.1% FA) and Solution B (100% ACN and 0.1% FA) and was kept at a flow rate of 200  $\mu$ l/min. The MS/MS raw data were acquired using Thermo-XCalibur™ (Thermo-Scientific) then processed into MGF files using Mascot Distiller v2.3.2.0 (Matrix Science, London, UK). The resulting MGF files were searched using the Mascot search engine v2.3 (Matrix Science, UK).

## RESULTS AND DISCUSSIONS

ACE inhibitory activity of each hydrolysates is shown in Figure 1. Captopril is used as positive control. All hydrolysates have potential to inhibit ACE, but compared to other hydrolysates, the highest inhibition was shown in tryptic hydrolysate with 94.82% followed by chymotrypsin, thermolysin and pepsin with the inhibition of 79.11%, 70.20%, 48.66% respectively.

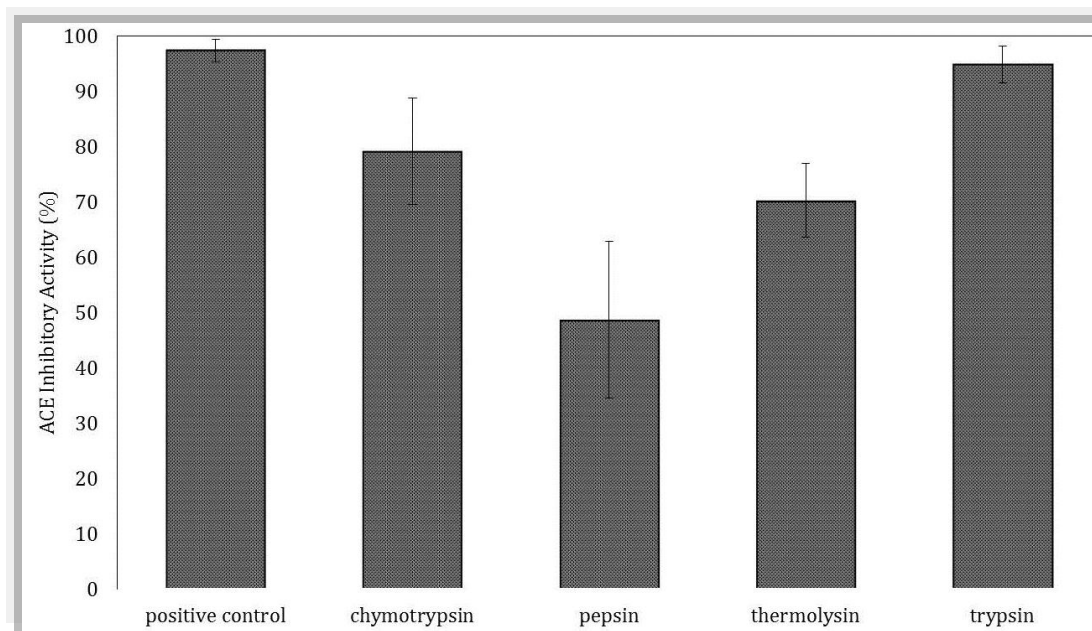
Tryptic hydrolysate, because it has the highest ACE inhibition, further was analyzed for IC<sub>50</sub> of ACE inhibition activity. The IC<sub>50</sub> or the half maximal inhibitory concentration represents the concentration of a peptide that is required for 50% inhibition of its target enzyme. To find out the IC<sub>50</sub> value of crude hydrolysates, the relative ACE inhibition was first determined for various concentrations of peptide; afterwards, the IC<sub>50</sub> was evaluated by plotting the curves of relative ACE inhibition against six different peptide concentrations (Figure 2).

The IC<sub>50</sub> value of tryptic hydrolysate was considered as a low inhibition. The low IC<sub>50</sub> value may be due to cumulative and synergistic effects of various active peptides present in each hydrolysate [10].

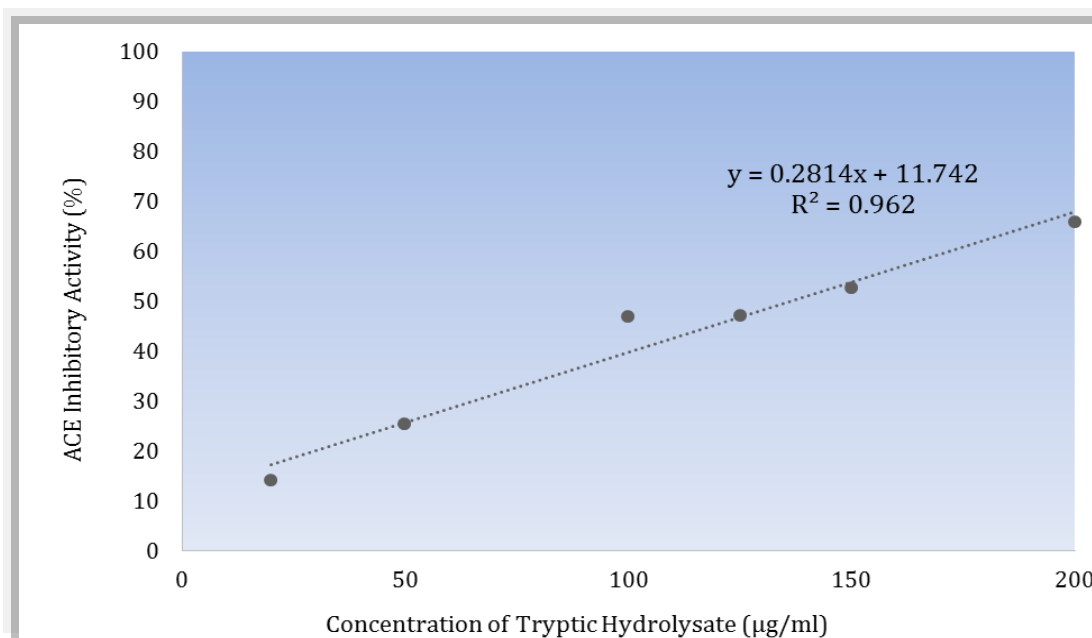
To characterize the peptide identities, the lyophilized tryptic hydrolysate was subjected into LC-MS/MS for analysis of ACE inhibitory peptides. Two major peaks were observed in the LC-MS chromatogram. Through LC-MS/MS analysis and database-assisted identification, peptides derived from Salmon Protamine are compared to the predicted peptides forecasted by peptide sequence application. All the sequences are summarized in Table 1.

LC-MS/MS analysis indicated two major peaks with three peptide sequences. A peptide was located at the first peak with retention time at minute 1.74, whereas two other peptides were located at the second peak with retention time at minute 10.70 and 10.95 respectively. Based on Mascot Distiller database search, for triply

charged the peptide with m/z at 247.91 was identified as Pro-Arg-Arg-Ala-Ser-Arg (PRRASR), for doubly charged the peptide with m/z at 358.60 as Ser-Ser-Arg-Pro-Ile-Arg (SSRPIR) and m/z at 402.03 as Ser-Ser-Ser-Arg-Pro-Ile-Arg (SSSRPIR).



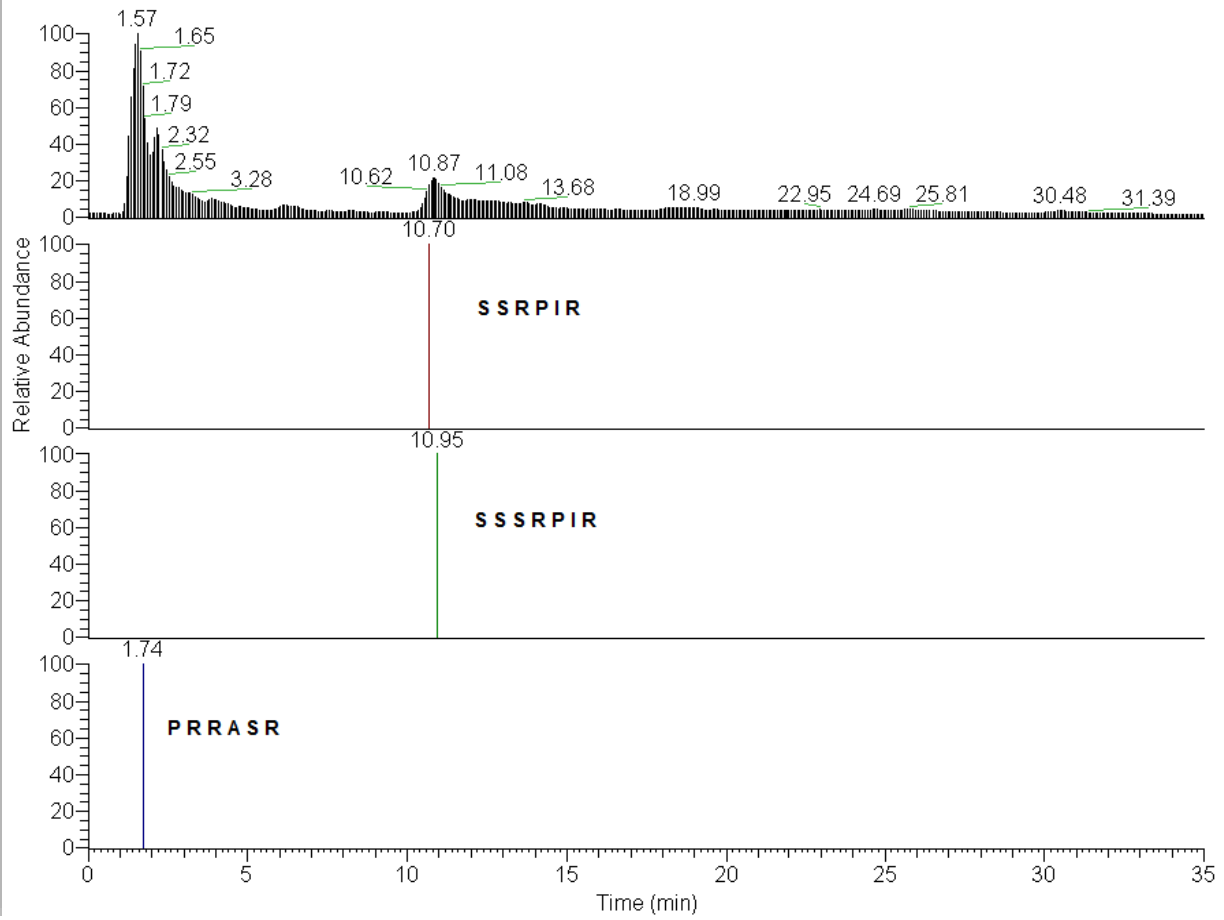
**Figure 1.** ACE inhibitory activities of enzymatic hydrolysates from Salmon Protamine.



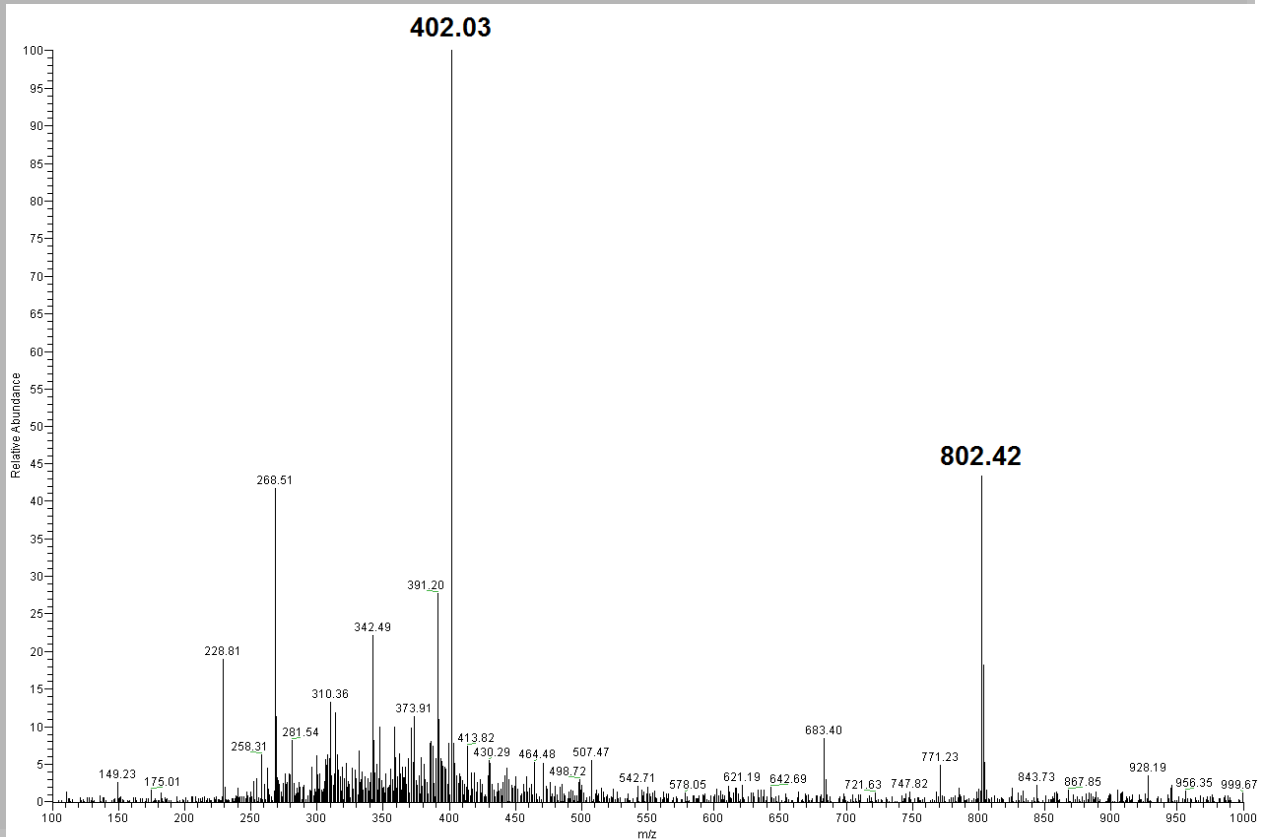
**Figure 2.** IC<sub>50</sub> of tryptic hydrolysate from salmon protamine.

**Table 1.** Comparison of tryptic hydrolysate and predicted tryptic peptide sequences.

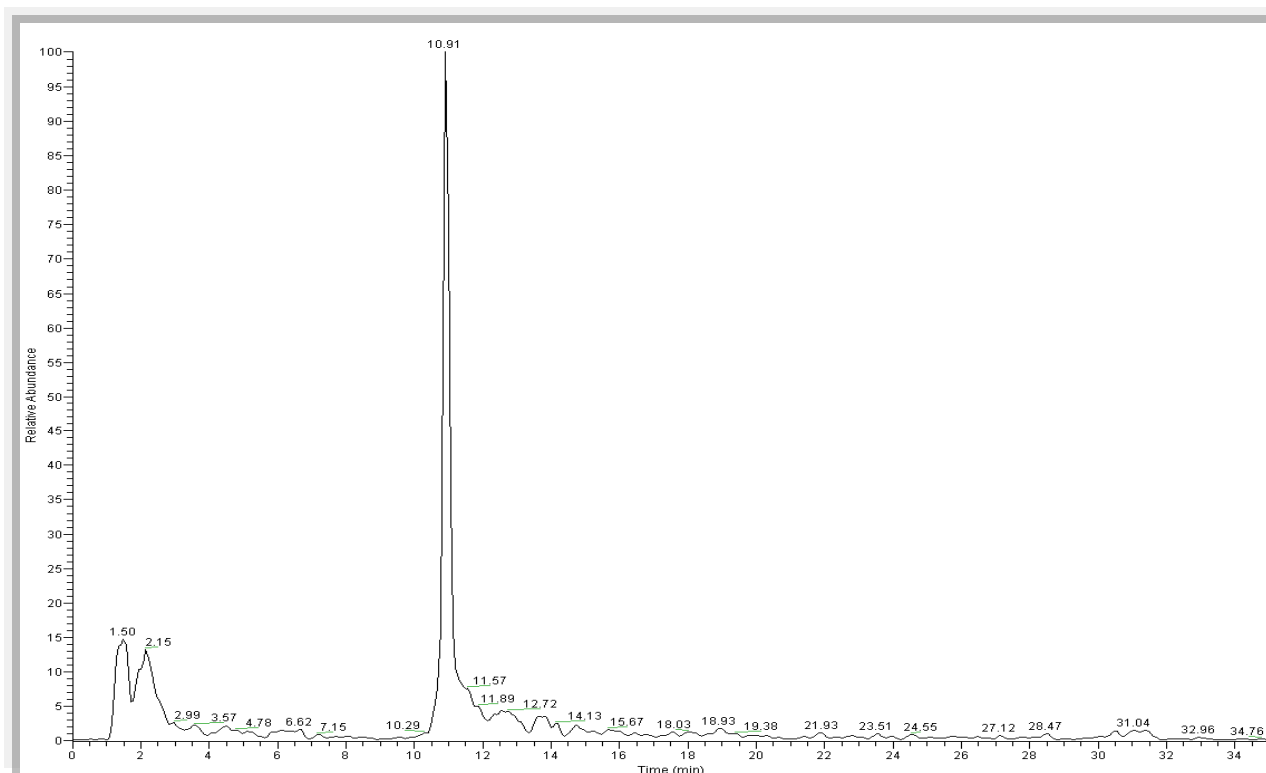
Tryptic Hydrolysate Peptides			Predicted Tryptic Peptides		
m/z	Position	Sequence	m/z	Position	Sequence
802.0454	6-12	SSSRPIR	802.4530	6-12	SSSRPIR
715.1854	7-12	SSRPIR	428.2728	15-17	RPR
740.9328	16-21	PRRASR	333.1881	19-21	ASR



**Figure 3.** LC-MS chromatogram of tryptic hydrolysate



**Figure 4.** LC-MS chromatogram of peptide SSSRPIR



**Figure 5.** Mass spectrum of peptide with  $m/z$  402.03 for doubly charged and  $m/z$  802.42 for singly charged.

Recently, salmon protamine widely used for pharmaceutical excipients. This cationic peptide derived from salmon milt. Protamine itself has a molecular mass of approximately 4000 Da with about 70% of the basic amino acid arginine. Basically, protamines belong to a diverse protein family of arginine rich peptides. Peptides derived from protamine could be useful to observe the differential *in vitro* antimicrobial activity of a 12-residue-long arginine-rich peptide and examined against bacterial and parasite microbes. Protamine C-terminal fragment can be utilized as a potential new antimicrobial peptide [11].

In addition to medical and antimicrobial use, Salmon Protamine is also expected could be used as antihypertensive agent. Higher level of arginine can be investigated for hypertension therapy. Since the arginine-rich peptides also exhibited moderate *in vitro* ACE and renin inhibitory activities, it is also possible that more than one mechanism was involved in producing the antihypertensive effects [12].

Digestion of salmon protamine by various digestive enzymes has resulted the release of bioactive peptides. Chymotrypsin, pepsin, thermolysin and trypsin are some of commonly used and widely distributed commercial enzymes. According to the result of this research, tryptic hydrolysate of salmon protamine has the highest ACE inhibitory activity. It indicated that bioactive peptides hydrolysis strongly affected by protease. Trypsin is widely used to produce ACE inhibitory peptides. However, other proteinases (chymotrypsin, pepsin, thermolysin) as well as enzymes from bacterial and fungal sources have been utilized to generate bioactive peptides [13].

## CONCLUSION

Angiotensin I Converting Enzyme (ACE) Inhibitory peptides were screened from enzymatic hydrolysis of salmon protamine using various enzymes. Tryptic hydrolysate has the highest ACE inhibitory activity. LC-MS/MS analysis of tryptic protamine identified two major peaks with three peptide sequences, Ser-Ser-Arg-Pro-Ile-Arg (SR-6), Ser-Ser-Ser-Arg-Pro-Ile-Arg (SR-7), Pro-Arg-Arg-Ala-Ser-Arg (PR-6) which sourced from salmine All of Chum Salmon (*Oncorhynchus keta*). According to this study, it can be concluded that bioactive peptide derived from salmon protamine has a potential ACE inhibitory peptide. Further study of ACE inhibitory peptide from Salmon protamine and the antihypertensive effect on spontaneous hypertensive rat (SHR) is highly needed in order to discover a new innovation in the treatment of hypertension.

### Competing interests

The authors declare that they have no competing interests.

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