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# PURIFICATION OF POTENT ANGIOTENSIN CONVERTING ENZYME (ACE)-INHIBITORY PEPTIDES DERIVED FROM RED TILAPIA (*Oreochromis* Sp.) BY-PRODUCTS

(Penulenan Peptida Perencat Enzim Penukaran Angiotensin (ACE) daripada Produk Sampingan Ikan Tilapia Merah (*Oreochromis* Sp.))

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**Abstract**

Diet and lifestyle changes are essential alternative treatments for hypertension. Consumers are increasingly more interested in health-promoting ingredients. The use of red tilapia (*Oreochromis* sp.) by-products (RTBP) has been established as a precursor for protein hydrolysate with angiotensin I-converting (ACE)-inhibitory activity. However, the complexity of the protein hydrolysate reduces its potency. Thus, this work aimed to purify and characterise the ACE-inhibitory hydrolysate derived from red tilapia (*Oreochromis* sp.). Thermoase PC10F (EC 3.4.24.27) was used in this study to hydrolyse red tilapia by-products (*Oreochromis* sp.). Meanwhile, ultrafiltration (UF), anion-exchange principle (AEX) and hydrophobic interaction chromatogram (HIC) were applied to purify the hydrolysate. Two molecular weight cut-offs (MWCO) were used for ultrafiltration: 3 kDa and 1 kDa. The 1 kDa hydrolysate showed the highest bioactivity of 85.42% ($IC_{50}$=0.41 mg mL$^-$). Subsequently, the 1 kDa RTBP hydrolysate was purified based on charge using AEX. Positively charged hydrolysate demonstrated significant bioactivity of 72.32%. In the final purification steps, the hydrophobic fractions showed the highest ACE-inhibitory activity obtained through the hydrophobic interaction chromatogram. The chromatogram yielded two fractions of peaks of high ACE-inhibitory activity on the hydrophobic fraction, which were 90.44% and 95.28%, respectively. Thus, ACE-inhibitory hydrolysate of small molecular size, positive charged and hydrophobic contributed significantly to its potency.

**Keywords:** ACE-inhibitory activity, hydrophobicity, red tilapia by-product. protein hydrolysate, purification

**Abstrak**

Penukaran enzim yang dikenali sebagai Angiotensin-1 kepada Angiotensin-2 bertanggungjawab ke atas kenaikan tekanan darah yang menyebabkan hipertensi. Perubahan diet dan gaya hidup perlu diterapkan sebagai pelan rawatan alternatif. Pasaran untuk kompoun penggalak kesihatan didalam makanan semakin dicari-cari pengguna. Sejak sedekad lalu, pelbagai kajian dijalankan ke atas hasil sampingan ikan tilapia (*Oreochromis* sp.) kerana sifat biologinya terutamanya sebagai agen antihipeternsi. Kenaikkan permintaan bekalan terhadap filet ikan turut meningkatkan kadar penangkapan ikan. Maka, terdapat lebihan model pengeluaran yang dapat dieksploitasi dengan cara yang menguntungkan. Di dalam kajian ini,Thermoase PC10F menghasilkan hidrolisat penghambat ACE daripada hasil sampingan ikan tilapia merah (*Oreochromis* sp.). Manakala hidrolisat ini ditulenkan melalui ultrafiltrasi (UF), prinsip pertukaran anion (AEX) dan kromatogram interaksi hidrofobik (HIC). Dua berat molekul 3 kDa dan 1 kDa digunakan didalam proses ultrafiltrasi. Penghambatan aktiviti ACE untuk berat molekul 1 kDa lebih tinggi berbanding 3 kDa dengan jumlah 85.42%. Seterusnya, hidrolisat 1 kDa ditulenkan melalui prinsip pertukaran ion dan hidrolisat bercas positif mempunyai penghambatan aktiviti ACE yang tertinggi (72.32%) berbandingkan dengan hidrolisat bercas negatif. Akhir sekali, proses penulenan dilakukan melalui kromatogram interaksi hidrofobik dan pecahan hidrofobik mempunyai penghambatan aktiviti ACE yang tertinggi. Kromatogram menghasilkan dua bagian puncak hidrofobik yang mempunya peringkat aktiviti penghambatan ACE tertinggi sebanyak 90.44% dan 95.28%. Maka, sifat molekul yang kecil, bercas positif dan hidrofobik merupakan ciri-ciri hidrolisat penghambatan aktiviti ACE yang manjur.

**Kata kunci:** penghambatan aktiviti ACE, kehidrofobikan, produk sampingan ikan tilapia merah, protein hidrosilat, penulenan

## Introduction

Multiple biological activities in fish protein hydrolysate, such as antioxidative, lipid homeostasis regulation, anti-inflammatory, anticancer, neuroprotective and antihypertensive, can be utilised as a functional food ingredient. [1]. ACE inhibitors comprise a novel class of medications used to treat hypertension and heart failure. In addition to their well-known role and ease of availability, synthetic medications include unwanted side effects [2-4]. Thus, the quest for alternative natural ACE-inhibitory peptides has been expanded to various food protein sources, which include aquatic protein sources.

Numerous studies on the freshwater fishes' by-product hydrolysate derived from Grass carp, Tilapia, Snakehead fish, shortfin scad fish and catfish have shown promising antihypertensive properties [4-11]. Tilapia is a freshwater fish that is widely cultured in Asian countries [4] as it multiplies and has low feed requirements [10]. With the rapid increase in fish production to cope with the fish filleting demand, more than 50% of the residuals from the total fish capture become unused as nourishment, which involves nearly 32 million tonnes of the fish by-product that can be processed into a desirable functional food [12, 13]. Tilapia is one of the most important freshwater fish species sold internationally owing to its popularity among people of various cultural, religious and economic backgrounds. Besides, red tilapia scales have between 41% and 84% protein, the highest among residues of fish farms [14, 15].

Protein complexes are among the most essential functional elements for deriving cellular biological functions [16]. Hence, the search begins for antihypertensive bioactive peptides that are inactive within the sequence of the parent protein but can be released during gastrointestinal digestion or food processing like enzymatic hydrolysis [5,13]. However, protein extracted from complex biological systems contains a complex protein combination suspended in a mixture of organic and aqueous solvents [1, 17]. The production of bioactive peptides involves several steps including the screening of enzymes, optimisation of hydrolysis conditions, as well as the purification of protein hydrolysate. Thermoase PC10F was chosen for enzymatic hydrolysis in this study, which is a commercial enzyme for thermolysin. The primary site for thermolysin cleavage specificity accommodates long hydrophobic residues. Thermolysin preferentially cleaves hydrophobic or bulky amino side chains [6,18,19]. Nevertheless, no research on the antihypertensive properties of RTBP hydrolysate using thermolysin or the commercial enzyme, Thermoase PC10F, has been conducted. In addition, purification of the protein hydrolysate-containing peptides is one of the most crucial steps in improving ACE-inhibitory potency. The most common considerations to purify peptides are based on their specific characteristics such as molecular size (ii) charge and (iii) hydrophobicity as peptide activities are heavily influenced by the structure-determined characteristics [4-7, 9-11, 17-23].

The goal of this study was to separate and purified the hydrolysate from a red tilapia (*Oreochromis* sp.) by-products (RTBP) treated with Thermoase PC10F based on molecular size, surface charge and hydrophobicity. The core technique for protein purification was quite straightforward: all impurities were removed while preserving as much of the peptide of interest with high ACE-inhibitory activity as possible.

## Materials and Methods

### Protein hydrolysate preparation

Enzymatic hydrolysis was done using Thermoase PC10F enzyme. The hydrolysis of 2g of sample was mixed with Thermoase PC10F enzyme and hydrolysed with phosphate buffer, pH 7. The ratio of enzyme to sample was 1:160 was incubated at 68 °C for 6 hours in a shaking bath. The sample was immersed in boiling water for 5 minutes to stop the hydrolysis. Then, the hydrolysate (sample) was centrifuged, whereas the supernatant was collected for further analysis.

### Total protein assay (Lowry assay)

Bovine serum albumin (BSA) was used as protein standard at the concentration of 50, 100, 200, 300 and 400 µg mL$^{-1}$. Lowry reagent solution of 1 mL was added to each sample and incubated for 20 minutes at room temperature. Then, 0.5 mL of Follin Ciocalteu's Phenol working reagent was added and mix immediately. Eppendorf tubes were incubated for 30 minutes at room temperature to allow the colour to be developed. After incubation, the values of absorbance were measured by UV-VIS spectrophotometer at 595 ηm followed by the plotting of absorbance.

### Determination of ACE-inhibitory activity: Enzymatic assay with ACE

HHL of 5 mM was prepared using 0.1 M sodium phosphate buffer (pH 8.2) containing 0.3 M NaCl. The same buffer was used for solution preparation of captopril, substrate, hippuric acid, peptides and enzyme dilute. ACE (60 mU mL$^{-1}$) was prepared from the initial unit (2 U mL$^{-1}$) using the same buffer. In each assay, 10 µL of the sample and buffer (control) was pipetted into the sample Eppendorf tube. After that, 30 µL of ACE (60 mU mL$^{-1}$) was added to the sample and gently mixed. Next, the mixture was incubated for 10 minutes at 37 °C. 90 µL of 5 mM HHL was then added to the samples and incubated for another 60 minutes at 37 °C. Finally, 8 µL of 5M HCl was added to stop the reaction and the mixture was filtered through a 0.2 µm PVDF filter into vials.

### HPLC method to determine ACE-inhibitory activity

The samples were analysed with Shimadzu LC-20AP Low Gradient System (SPD-20AV) HPLC by a C18 Thermo Scientific ODS Hypersil HPLC column. The sampler and the column temperature were kept at 4 °C and 30 °C, respectively. The injection volume with the flow rate of 20 µL and 1 mL min$^{-1}$, respectively, for 12 minutes was used with an isocratic solution of 0.1% trifluoroacetic acid in 12.5% acetonitrile. The absorbance was monitored with ultraviolet (UV-VIS) detection at 228 nm. Serial dilutions of HA from 0.02 to 1 mM were applied to determine the calibration curve.

### Calculation of ACE-inhibitory activity

The percentage of ACE-inhibitor activity (ACEi%) was calculated based on the hippuric acid liberated using the hydrolysates sample and control (buffer) as follows (1):

$$ACEi\% = \left[\frac{(HAcontrol) - HAsample)}{HAcontrol}\right] x\ 100 \quad (1)$$

Where (ACEi%) was the percentage of angiotensin-converting enzyme inhibitory activity of hydrolysates; [$HA_{control}$] was the concentration of hippuric acid (HA) liberated using buffer instead of inhibitor in the mixture and; [$HA_{sample}$] was the concentration of hippuric acid liberated in the presence of inhibitors (hydrolysates).

**$IC_{50}$ determination of the hydrolysates**

The $IC_{50}$ value is defined as the concentration of hydrolysates that can inhibit half-maximal of the ACE. Sample or protein hydrolysates that yielded more than 50% ACEi% were then selected and evaluated for ACEi% in different concentrations. The $IC_{50}$ of the hydrolysates with different enzymatic conditions were determined by the regression analysis of plotting the inverse of serially diluted hydrolysates concentration (1/1, 1/2.5, 1/5, 1/10, 1/20) as a function of the inverse of their ACEi% and expressed as mg of protein per mL. The $IC_{50}$ of the peptides were compared with the $IC_{50}$ of captopril as positive standards.

**Determination degree of hydrolysis (DH)**

The degree of hydrolysis (DH) was estimated using the o-phthaldialdehyde (OPA) method as described by Nielsen [24] and Pa'ee [25] with some modifications. The o-phthaldialdehyde (OPA) reagent was freshly prepared, comprising 0.025g of OPA (dissolved in 500 µL of pure ethanol) and 25 µL of β-Mercaptoethanol in 50 mL of 50 mM carbonate buffer pH 10.5. This OPA reagent should be protected from direct light and used within 2 hours, or it can eventually be stored under nitrogen in an ambered glass vial for 1-2 weeks at 4 °C. An aliquot of 400 µL of standard or sample was added into the 3 mL of OPA reagent and mixed for 5 seconds. The mixture was incubated at room temperature before being read at 340 nm at a spectrophotometer by UV-VIS 1800 Shimadzu. Deionised water was used as a blank and leucine dilution as a standard. The DH was calculated using the equation from the method.

**Fractionation and Purification of RTBP hydrolysate: Ultrafiltration (UF)**

Hydrolysates were fractionated using ultrafiltration membranes in a dead-end mode system (Amicon Model 8200 stirred ultrafiltration cell, Amicon Corp., Danvers, MA) utilising the procedures outlined by Roslan [26] with a few minor adjustments.

A stirred ultrafiltration cell has a suspended bar impeller and a stirring hot plate. Flat sheet RC membranes with 1 kDa and 3 kDa MWCO were employed. With a constant pressure at 2.5 bar and continuous stirring speed, an ultrafiltration membrane fractionated the RTBP hydrolysate. Optimal RTBP hydrolysate was separated through a 3 kDa membrane before a 1 kDa membrane. The ACE inhibitory activities and $IC_{50}$ of 1 kDa and 3 kDa permeate were evaluated. Permeate flux was also calculated. The calculation was done as described by Roslan [26] using the following equation (2):

$$Flux = \left(\frac{Total\ quantity\ passed\ throu\quad membrane}{Membrane\ Area}\right)\ L/(m^2.h) \tag{2}$$

Flux is the rate at which a membrane separation process works. It is determined by the properties of the membrane, the transmembrane strain, the system's hydrodynamics, the properties of the solvent and protein, and the amount of protein in the feed.

**Adsorption equilibrium**

The anionic exchanger resin (STREAMLINE DEAE) was used in this work applying a batch adsorption approach. Initially, the resin was washed twice with 10 mM potassium phosphate buffer at pH 7.0 to equilibrate the adsorption conditions. The buffer was decanted once the resin settled at the bottom of the test tube. The RTBP hydrolysate of 5 mL was added and stirred for 10 minutes to adsorb negatively charged peptides to the resin. The resin was then separated from the supernatants by centrifugation. The resin containing the adsorbed protein was eluted with potassium phosphate buffer pH 7.0 containing 1 M NaCl. The resin was regenerated using the same elution buffer. RTBP hydrolysate and supernatant were subjected to total protein assay determination. The difference in concentration between fresh feed RTBP supernatant and concentrated RTBP hydrolysate protein indicates the concentration of trapped RTBP hydrolysate protein. The percentage was calculated using the following formula:

$$Adsorption\ (\%) = \frac{RTBP\ Hydrolysate_{Feed} - Supernatant}{RTBP\ Hydrolysate_{Feed}} \times 100\% \tag{3}$$

**Fractionation of hydrolysate by reverse phase-high performance liquid chromatography (RP-HPLC)**

Ultrafiltered hydrolysate from subsection 2.4.3 was then subjected to RP-HPLC for hydrophobicity fractionation. Shimadzu UFLC system with column (HypersilTM BDS C18 Column, 5 μm, 250×4.6 mm, 130 Å Thermo ScientificTM) was used for the fractionation. An isocratic solution of 100% of 0.1% TFA was used to separate hydrophilic fraction, while 100% of 0.08% of TFA in Acetonitrile was used to separate the hydrophobic fraction. Hydrolysates were collected into 12 fractionations. The absorbance was monitored with ultraviolet (UV-VIS) detection at 323 nm. Each fraction was assayed for ACE-inhibitory activity.

## Results and Discussion

**The production of RTBP hydrolysate with ACE-inhibitory activity**

A high degree of hydrolysis (DH) applying specific proteases can result in a hydrolysate with strong ACE-inhibition activity. RTBP hydrolysate with the highest DH (73.80%) was observed using Thermoase PC10F (E.C. 3.4.24.27). The hydrolysis of peptide bonds containing hydrophobic amino acids was selectively catalysed by Thermoase PC10F, producing hydrophobic peptides with known ACE-inhibitory action [25]. Ghassem [27] used thermolysin to break apart sarcoplasmic and myofibrillar muscle protein derived from *Clarias batrachus* for producing ACE-inhibitory peptide GPPP and IEKPP. GPPP has mixed inhibition mechanisms that are competitive and non-competitive, whereas IEKPP has a competitive inhibition mechanism. Hydrophobic amino acids like glycine and proline (Gly and Pro) may be key factors in ACE-inhibitory activity [25].

A series of purification steps were performed after the hydrolysis, including UF, ion-exchange chromatography and RP-HPLC to separate ACE-inhibitory peptides according to size, charge and hydrophobicity, respectively. According to Pa'ee [28], ACE activity is determined by tracking the amount of hippuric acid (HA) formed as a result of the ACE-catalysed hydrolysis of N-Hippuryl-His-Leu (HHL). As a result, ACE-inhibitory activity increases with increasing concentrations of HA catalysed by the enzyme. The ACE-inhibitory activity value of each purified technique applied to RTBP hydrolysate is shown in Table 1.

Table 1. Overall results for ACE-inhibitory activity of each purification technique

| Procedure | | ACE-Inhibitory Activity (%) |
|---|---|---|
| **Crude RTBP Hydrolysate** | | 70.67 ± 2.40 |
| **Ultrafiltration (1kDa)** | | 85.42 ± 0.98 |
| **Ion exchange Chromatogram (IEX)** | | 72.32 ± 1.01 |
| **Hydrophobic fraction** | Peak 1 | 90.44 ± 4.54 |
| | Peak 2 | 95.28 ± 1.63 |

**The effect of membrane size towards permeates flux and peptide transmission**

Fractionation of RTBP hydrolysate was done by a dead-end UF with two different membrane pore sizes. The cellulose membrane (RC) of MWCO of 3 and 1 kDa were used with a constant pressure of 2.5 bar and a constant stirring speed applied. Permeate flux refers to the rate at which permeate is produced during membrane separation relative to the membrane's total surface area and the amount of time involved [29]. It was shown in Figure 1 that a bigger membrane pore size, 3 kDa, has a higher permeate flux compared to 1 kDa. The line trend in Figure 1 is similar to the previous study done by Roslan [30] at different pH and stirring speeds. The MWCO describes the pore of the membrane, while the membrane represents the molecular weight of a molecule that is rejected at 90% [12, 13]. The bigger the MWCO, the more porous the membrane, whereas smaller MWCO has a smaller membrane pore [31, 32, 33]. The nature of the membrane influences the permeate flow as can be observed that the permeate flow of 3 kDa was faster compared to 1 kDa, which was 4 hours and 5 hours, respectively. The decrease in permeate flow through the membrane was attributable to fouling, which happens due to nonpermeating solutes tending to form a gel on the surface of the membrane, thus affecting the permeate flow [34]. The formation of cake fouling was due to the perpendicular feed flow of the solutions in a dead-end UF and the concentrated suspended solid on the surface of the membrane. Nonetheless, this can be improved by applying a crossflow UF since the feed solution is tangible.

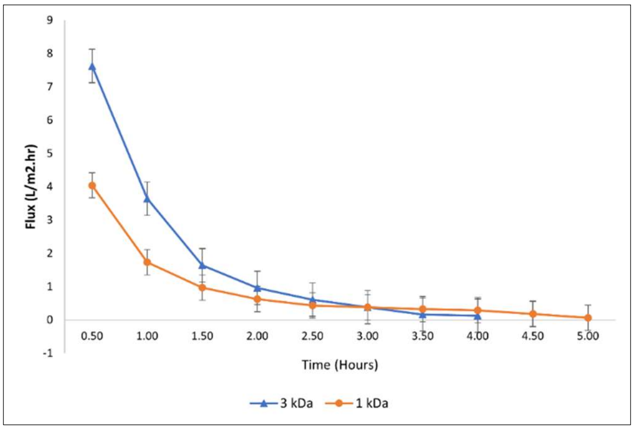


Figure 1. Effects of UF membrane pore size on permeate flux

In addition, the membrane pore size influences peptide transmission. The transmission of peptides through the membrane is highly dependent on the size and direction of membrane pores [26]. In Figure 2, the peptide transmission in 3 kDa was slightly higher compared to 1 kDa, which were 37.40% and 32.62%, respectively. Roslan [26] mentioned that transmissions of peptides through a membrane with a small pore size are lower compared to those of a larger membrane pore size, which may be due to the tendency of a larger peptides size to be retained and accumulated on the membrane surface of the smaller membrane pore size resulting a severe membrane fouling. In his study, the red tilapia by-product hydrolysate was fractionated by a single (10 and 5 kDa) and multilayer (10/5 and 5/5 kDa) membrane pore size. The highest peptide transmission was achieved at a membrane pore size of 10 kDa (87.3%), followed by 10/5 kDa (54.4%), 5 kDa (36.1%) and 5/5 kDa (20.0%). Therefore, it can be concluded that larger MWCO membranes, such as the 3 kDa membrane used in this study, produce greater permeate flux flow and higher peptide transmission.

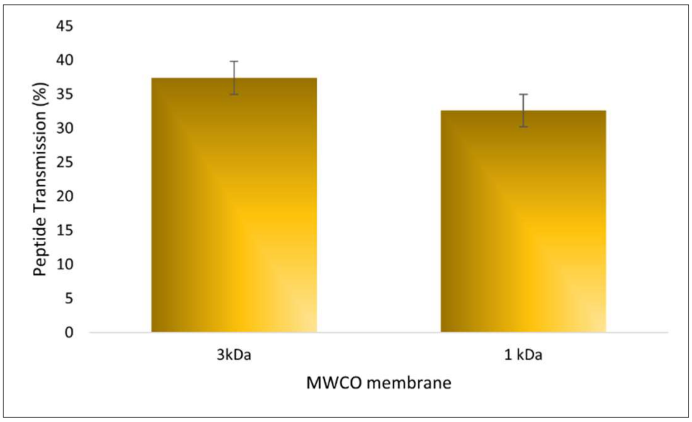


Figure 2. Effects of membrane pore size on peptide transmission

**Effect of size towards potency of ACE-inhibitory activity**

The 1 kDa possessed higher ACE-inhibitory activity compared to 3 kDa, which was 85.42% (Figure 3). Similar trends have been observed by Hua [4] and Ishak [35]. Hua [4] produced an antihypertensive peptide derived from tilapia collagen and the hydrolysate was separated using UF with two molecular sizes of <3 kDa and >3 kDa. The smaller fraction (<3 kDa) exhibited a higher percentage of ACE-inhibitory activity of 74.30% compared to >3 kDa, which was 58.5%. Ishak [35] studied the bioactive peptide derived from shortfin scads waste hydrolysate, (SWH), thus suggesting higher ACE-inhibitory activity of <3 kDa peptides with 81.50%. In this study, the $IC_{50}$ value of 1 kDa RTBP hydrolysate was 0.41 mg $mL^{-1}$, which improved from 0.79 mg $mL^{-1}$ (crude RTBP hydrolysate). The $IC_{50}$ value of purified RTBP hydrolysate was comparable to the pepsin-hydrolysed tilapia frame <1 kDa protein hydrolysate [36]. The $IC_{50}$ value was measured to assess bioactive peptide efficacy. It provides a measure of potency in pharmacological research by indicating the amount or concentration of a compound required to inhibit a biological process by 50% [37]. Therefore, the lower the $IC_{50}$ value, the greater the potency.

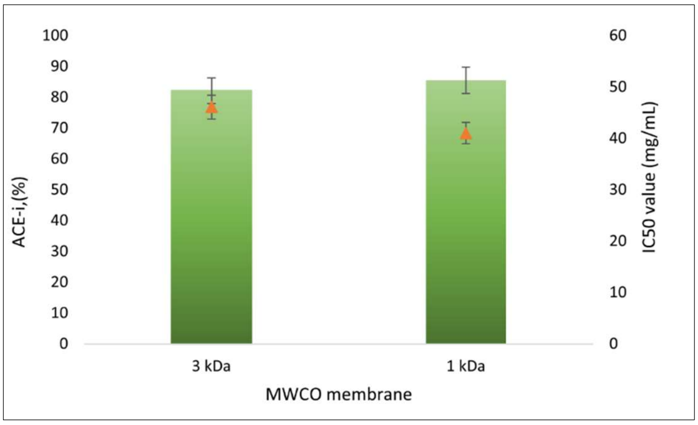


Figure 3. Effects of membrane pore size on ACE-inhibitory activity and $IC_{50}$ value

**The effect of peptides charges**

In most cases, anion-exchange chromatography (AEX) can be used to separate molecules with negative charges from natural and basic molecules [38]. The peptide charged of RTBP hydrolysate was measured using Zeta potential. The electrostatic interactions between proteins and charged surfaces are also pH-dependent, as the charge of proteins depends on the pH of their surroundings [39].

Table 2. Zeta potential measurement of RTBPH

| Sample | Zeta Potential (mV) |
|---|---|
| **1 kDa** | -8.75 ± 0.79 |
| **3 kDa** | -4.82 ± 1.25 |

Both fractions (1 kDa and 3 kDa) were negatively charged (Table 2). Appropriate pH was used to maintain the surface charge of the RTBP hydrolysate and to avoid precipitation due to pH closer to its isoelectric point. The charges on protein molecules can change to accommodate other charged surfaces [39, 40]. The RTBP hydrolysate fraction must be negatively charged to allow the exchange of the peptides to the anionic exchange resin. The anionic exchangers could bind with the negatively charged counterions reversibly. An anion exchanger's counterion is often a weakly binding ion like Cl-, which can be changed out for stronger binding ions including negatively charged amino acids on a protein's surface. Thus, a high salt concentration buffer was used to elute the trapped RTBP hydrolysate.

AEX of 1 kDa RTBP hydrolysate is shown in Figure 4. The absorption percentage of RTBP hydrolysate peptides to the resin was 93.99%. The non-adsorbed peptides consisted of positively charged peptides. The adsorbed peptides (negatively charged) were then eluted several times. ACE-inhibitory peptides can be observed in both the non-bound (non-absorbed) and bound (Elute 1 to 4) fractions. Meanwhile, the non-absorbed fraction (positively charged) has the highest ACE-inhibitory activity of 72.32%, whereas the elutes have the ACE-inhibitory activity from 6.07% to 52.55%. This is similar to the previous study done on Alaska pollack's frame hydrolysate by Je [41] and catfish skin hydrolysate by Sungperm [42], which has higher ACE-inhibitory activity on positively charged fraction. Daskaya-Dikmen [44] and Manoharan [43] suggested that the presence of positively charged on the side chain coupled with other aromatic amino acids would enhance the ACE-inhibitory activity effectiveness.

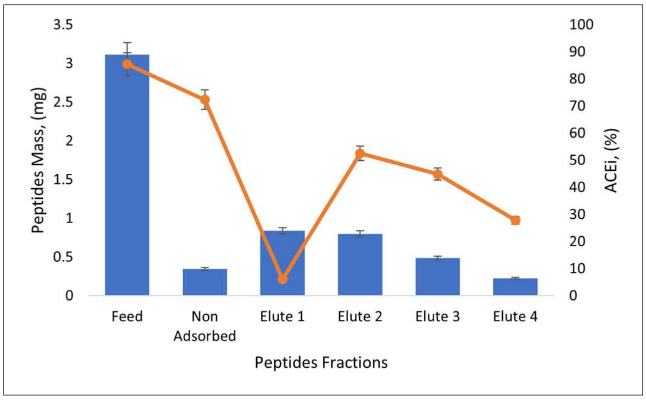


Figure 4. The peptide mass and ACE-inhibitory activity for absorption equilibrium

**The effect of peptides hydrophobicity**

Specific amino acids play a vital role in determining the type of bioactivity (Acquah, et al., 2018). ACE-inhibitory activity is inhibited more effectively by antihypertensive peptides, which typically have aromatic amino acid residues at the C-terminus and hydrophobic amino acid residues at the N-terminus [4], [23], [43–46]. The hydrophobic interaction chromatogram (HIC) was separated using RP-HPLC. The positively charged peptides fraction was further purified, and the fraction of chromatogram's peaks and its ACE-inhibitory activity is shown in Figure 5.

Both hydrophilic and hydrophobic chromatograms produced peaks at 0-5 minutes (Fraction 1) and 5-10 minutes (Fraction 2). The ACE-inhibitory activity of Fraction 1 and Fraction 2 from the hydrophobic fraction showed the highest bioactivity of 90.44% and 95.28%, respectively. However, hydrophilic peptides demonstrated inferior ACE-inhibitory activity in Fraction 1 (47.80%) and Fraction 2 (11.59%) (Figure 5). Ishak [35] observed similar ACE-inhibitory activity (87.99%) from a purified peptide fraction derived from shortfin scad applying RP-HPLC. Pa'ee [25] suggested that hydrophobic peptides tend to possess more ACE-inhibitory activity. In addition, the abundance of the hydrophobic fragment in the RTBP hydrolysate may be due to the enzymatic hydrolysis of Thermoase PC10F. The hydrophobicity of a peptide chain depends on several factors, including the extent to which it is hydrolysed, the processing conditions used, the type of protease employed, the average hydrophobicity value of the protein precursors and the sequence of hydrophobic amino acid residues in the peptide chain [47]. According to several studies, aliphatic amino acids at the N-terminus, positively charged amino acids in the central position, as well as proline, tyrosine, phenylalanine and tryptophan are the main structural components in the C-terminal of ACE inhibitory peptides with high ACE inhibitory action. Additionally, the amino ends of proteins tend to contain hydrophobic amino acids with aliphatic side chains such as glycine, isoleucine, leucine and valine [4, 23, 40, 41, 44]. An ACE-inhibitory peptide that has proline residue at C-terminal tends to have competitive inhibition as mentioned above on *Clarias batrachus* [27], VAP derived from Grass crap [12] and LYPPP and YSMYPP, both of which were derived from snakehead fish [7].

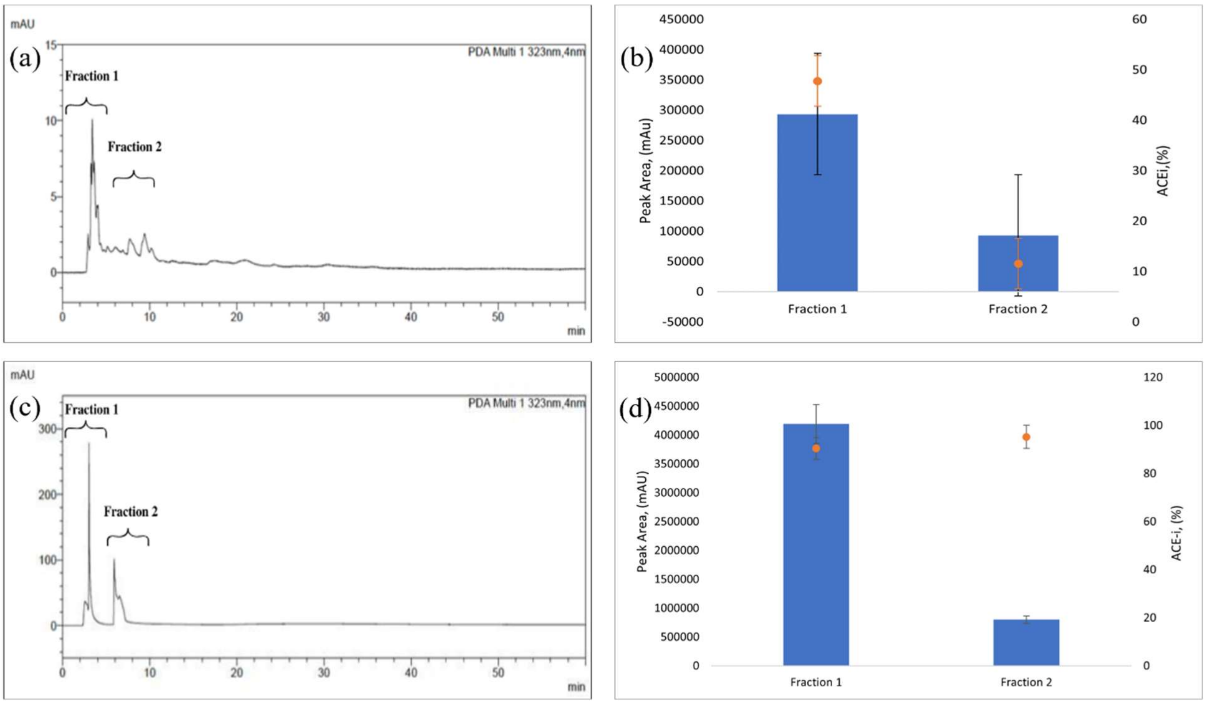


Figure 5. Chromatograms and the ACE-inhibitory activity of purified RTBP hydrolysate (a) Chromatogram fraction of hydrophilic peak (b) Hydrophilic fraction and its ACE-inhibitory activity (c) Chromatogram fraction of hydrophobic peak (d) Hydrophobic fraction and its ACE-inhibitory activity.

## Conclusion

RTBP hydrolysate applying Thermoase PC10F liberated potent ACE-inhibiting peptides. Thermoase PC10F displayed high DH and ACE-inhibitory activity at 73.80% and 70.67%, respectively. 1 kDa RTBP hydrolysate has a stronger ACE-inhibitory activity of 85.42 %. The non-absorbed fraction yielded the highest ACE-inhibitory activity of 72.32%, indicating that it has positively charged characteristics. The purified RTBP hydrolysate yielded two fractions of high ACE-inhibitory activity (more than 90%) on the hydrophobic fraction. The reaction specificity of the Thermoase PC10F allowed the release of hydrophobic peptides. Furthermore, the determination of peptide sequence is crucial to decern its structure-activity relationship, molecular docking and dynamic studies, and other *in silico* studies.

## Acknowledgement

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