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Research article

Peptide derived *C. striata* albumin as a natural angiotensin-converting enzyme inhibitor

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ABSTRACT

As one of the most popular sources for fish albumin, *Channa striata* has been considered as a promising substitute for human albumin. However, scientific information regarding its genomic and proteomic is very limited, making its identification rather complicated. In this study, we aimed to isolate, characterize, and examine the bioactivity of protein and peptide derivatives of *C. striata* albumin. Fractionation of albumin from *C. striata* extract was conducted using Cohn Process and the yield was evaluated. The peptides were further produced by enzymatic hydrolysis. All these proteins were studied using tricine-SDS PAGE and tested for *in vitro* ACE inhibition. Dry weights of the Fraction-5, where the albumin was more abundant and purer, was 3.8 ± 2.1%. Based on tricine-SDS PAGE analysis, two bands of protein, *e.g.,* approximately 10 and 13 kDa, were detected with highest intensity found in Fraction-5, which might be albumin of *C. striata*. An increasing trend of ACE inhibition by the fractions was observed, ranging from 7.09 to 22.99%. The highest ACEI activity was found in peptides from alcalase hydrolysis with molecular size <3 kDa (56.65 ± 2.32%, IC_{50} 36.93 μg/mL). This value was also statistically significant compared with the non-hydrolyzed Fraction-5 and Parental Fraction, which were 23.48 ± 3.11% ($P < 0.05$) and 13.02 ± 0.68% ($P < 0.01$), respectively. Taken together, these findings suggest a promising potential of peptide-derived *C. striata* albumin for natural antihypertensive agents.

1. Introduction

Albumin is one of the major proteins that represents half of the total protein content in plasma. In healthy adults, albumin concentration is ranging from 3.5 to 5 g/dL. Albumin is synthesized by hepatocytes in the liver, however, stored in a very low concentration in the organ due to its rapid excretion into the bloodstream at the rate of about 10–15 g per day [1]. Its synthesis is positively regulated by hormones, such as insulin and growth hormone, while it is inhibited by pro-inflammatory substances, such as interleukin-6 and tumor necrosis factor-α [2]. It has multiple important physiological functions including modulating colloidal osmotic pressure, binding with a wide variety of endogenous (*i.e.* fatty acids and bilirubin) and exogenous (*i.e.* drugs and toxins) ligands [3].

In clinical practice, most of the clinical use of albumin is based on the capacity to act as a plasma-expander due to its osmotic effect

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[4]. Besides, albumin has also been utilized as a highly sensitive marker for nutritional status, to serve an insight for liver function or the ability to biosynthesize proteins and vital factors to the homeostasis of the body and therapeutic activity based on its non-oncotic properties as well [1,2,4]. However, as an expensive blood product, the use of human albumin arises its own problem. Considering the amount of plasma expanders required in critically ill patients, the use of albumin represents a significant cost [5,6].

Channa striata Bloch (local name: ikan gabus, common name: snakehead fish) is a rich-albumin fish that has been used as a natural remedy in traditional medicine, particularly popular for its used in post operative wound healing and women post-partum [7–9]. The pharmacological properties of this species have been widely reported including its antihypertensive, antimicrobial, anti-inflammatory, antinociceptive, antioxidant and anti-depressant [10–13]. Recently, as one of the most popular sources for fish albumin, *C. striata* has been considered as a substitute for human albumin. However, it is not quite clear that albumin from fish and other species is complementary either in structure or function. Nevertheless, several studies have been reported that the administration of *C. striata* extract has a strong association with the increase of albumin in hypoalbuminemia-related conditions [7,8,14].

Efforts on discovering new bioactive compounds from *C. striata* have been conducted, particularly its endogenous protein and peptide as an anti-hypertension. Several studies have been reported that peptide of *C. striata* showed a potential activity as angiotensin-converting enzyme inhibitor (ACEI) [13,15]. However, those studies did not clearly define which protein of *C. striata* was subjected to generate the potential peptide. Since albumin is known as one of the major proteins in *C. striata*, it was strongly expected that the potential peptide might be obtained from hydrolyzed albumin.

Unfortunately, scientific publications regarding the albumin identification of *C. striata* are very limited. By far, there is no standardized *C. striata* albumin in the market that can be used as standard for the albumin measurement. Even more, information regarding the albumin size is still unclear. Since albumin itself is a protein superfamily with a large number of members with different functions, it became difficult to characterize them based on their function. Report by Li et al. outlined that the only conserved characteristic of the superfamily is the characteristic cysteine motif CCXnC, which are responsible for the formation of the double-disulfide loop of the albumin family, which further contributes to its ligand configuration and binding [16]. However, information regarding genomic and proteomic of *C. striata* itself is also very limited, which made albumin identification based on cysteine motifs is currently unavailable.

In this study, albumin was isolated from *C. striata* extract provided by Mega Medica Pharmaceuticals (MMP), Indonesia, using the Cohn Process method with several modifications. The characteristics of the fractions and the effectivity were evaluated. Furthermore, bioactivity of the protein as an anti-ACE was also evaluated to confirm its identity based on known general function of albumin. In addition, the confirmed albumin fraction of *C. striata* was hydrolyzed by enzymatic process and further fractionated based on their molecular size to generate fractions of albumin peptide with greater ACEI activity.

2. Materials and methods

2.1. Preparation of C. striata extract

C. striata extract was provided by MMP, which complied with all the requirements stated in SNI8074: 2014 *C. striata* albumin extract: quality requirement and processing [17]. *C. striata* extract were passed quality control parameters, such as minimum protein content, microbiology and stability tests have been conducted to ensure that the extract complies with the regulation and is safe to be consumed. The microbiology test comprised of total aerobic microbial count and identification of microbial pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Escherichia coli*), while stability test was evaluated by physical description.

2.2. Cohn Process

Albumin from *C. striata* were fractionated using albumin purification method adapted from Cohn et al. [18] and Raouffina et al. [19] with minor modification especially at temperature for protein precipitation, instead of −3 and -5 °C, we applied 2 – 8 °C range to obtain optimum result [18]. *C. striata* extract was fractionated using different pH and ethanol (EtOH) concentration in a 10 mM acetate buffer. Twenty mL of *C. striata* extract was mixed with 100 mL buffer containing 8% EtOH at pH 7.2. The mixture was incubated at 2 – 8 °C for 15 min and then centrifuged (Julabo, LLC 16) at 10.000 rpm for 30 min to separate the precipitated proteins, which was called Fraction-1. The pellet was removed while the supernatant was replaced into a new container. The supernatant was mixed with 25% EtOH at pH 6.9 and then incubated and centrifuged as the previous steps to obtain Fraction-2. The procedure was continued with 18% EtOH at pH 5.2 and 40% EtOH at pH 5.8 to obtain Fraction-3 and Fraction-4, respectively. Finally, the supernatant from Fraction-4 was mixed with a buffer containing 40% EtOH at pH 4.8 to obtain the pellet from the final system (Fraction-5), which contained suspected albumin fraction. All the steps were conducted in cold conditions. Each pellet was next stored in 2 – 8 °C. The process was conducted in 3 independent replicates.

2.3. Yield analysis

The effectivity of the Cohn Process was evaluated by measuring wet and dry weights of the pellet of each fraction. Wet weight analysis was conducted by directly weighing the pellet after pellet removal step in the Cohn Process. For dry weight analysis, 10 mg pellets of each fraction were dissolved in 1 mL water and incubated overnight at −20 °C to freeze the solution. Frozen solutions were subjected to lyophilization by freeze-drying using Freeze Dryer (Labconco, Freezone 4.5) for 6 h. The dried pellet was weighted using an analytical scale (Mettler Toledo, XS204) to obtain the dry weight. Both wet and dry weights of each fraction were analyzed to obtain

the effectivity of albumin purification by Cohn Process, stated in percentage.

2.4. UV absorbance analysis

The pellets from each fraction were then resolved in distilled water to obtain 10 mg/mL Fraction-1 to Fraction-5. Ten mg of *C. striata* extract was also separately prepared to be used as Parental Fraction. One mL of each fraction was loaded into a cuvette. The UV absorbance of each fraction was scanned using a spectrophotometer (Thermoscientific, Multiskan Go) at wavelength ranging between 200 and 800 nm to determine the UV λ_{max} of the sample [20], which further indicated the presence of protein and other contaminants in each fraction.

2.5. Content analysis

The content of total protein, amino acids, vitamins, and minerals in *C. striata* extract that was used for the Parental Fraction were analyzed by PT. Saraswanti Indo Genetech, Bogor, Indonesia. A Serie assays was conducted to confirm the bioactive compounds presence in the fraction using Ultra-performance liquid chromatography (for amino acids and vitamins) [21], Gas chromatography (for fatty acids) [22], Inductively coupled plasma optical emission spectroscopy (for minerals) [23] and Kjeltec (for total protein) [24].

2.6. Tricine-SDS PAGE

Albumin of *C. striata* is the protein target which has molecular weight <30 kDa, therefore the protein profile of each fraction was analyzed using tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS PAGE) based on procedure described by Schagger [25]. Gradient concentrations of SDS gel, *i.e.,* 4-10-18%, were used to separate proteins based on their molecular sizes. Around 100–200 μg protein from each fraction, 1 μg bovine serum albumin (BSA) and standard obtained from fish albumin Elisa kit were used in the samples. Page Ruler 10–200 kDa unstained protein markers (Thermoscientific, Lithuania) were used as the protein marker. All samples were mixed with SDS loading buffer (4:1, v/v) and incubated in boiling water for 5 min. Twenty μL of each sample was loaded into the gel. Anoda (24.23 g Tris Base in 1 L pure water, pH 8.9) and katoda (12.11 Tris base, 17.92 g tricine, 1 g SDS in 1 L pure water) buffers were used as running buffers. The samples were then electrophorized at 100 V for 80 min. Proteins were visualized by Coomassie brilliant blue R-250.

2.7. Enzymatic hydrolysis of albumin fraction

Lyophilized form of Parental Fraction and Fraction-5 obtained from the Cohn Process was suspended in purified water with a ratio of 1:100 (w/v). Two hundred fifty units of pepsin (Roche, USA) and alcalase (Sigma, MA, USA), were then added to hydrolyze the fraction solutions. The solutions were adjusted to the appropriate temperature and pH of each enzyme, *e.g.,* pepsin (37 °C, pH 2) and alcalase (50 °C, pH 7). After 2 h incubation, each reaction mixture was heated at 98 °C for 10 min to inactivate the enzymes and centrifuged at 3000×*g* for 10 min to remove the Insoluble materials.

2.8. Ultrafiltration of albumin peptide

The peptides from each fraction were further fractionated based on their molecular weight using ultrafiltration membranes (PALL, USA). Six hundreds μL of each fraction were subjected into 10 kDa MWCO tubes by centrifugation at 3000×*g* for 10 min, which sequentially proceeded to the 3 kDa MWCO tube at the same speed and time of centrifugation. This process was used to obtain peptides with different ranges of molecular weight *e.g.,* > 10 kDa, 3–10 kDa and <3 kDa.

2.9. ACEI assay

The effect of each fraction or peptide on inhibiting angiotensin-converting enzyme (ACE) was measured using *in vitro* ACE inhibition (ACEI) assay adapted from Rachmawati et al. [26]. Captopril (250 μg/mL) was used as a positive control. Fifty μL of samples (18.125–250 μg/mL) were mixed with 50 μL ACE (25 mU/mL) and incubated at 37 °C for 10 min. Subsequently, 150 μL of substrate solution (8.3 mM of HHL in 50 mM sodium borate buffer containing 0.5 M NaCl, pH 8.3) was added and the mixture was incubated for 30 min at 37 °C. The reaction was stopped by adding 250 μL of 1.0 M HCl. Five hundred μL of ethyl acetate was then added and centrifuged at 800×*g* for 15 min. Approximately 200 μL of the upper layer was collected into a test tube and evaporated using a heat block (Itabis MHR11) at 95 °C for 15 min. Hippuric acid which was formed was dissolved in 1 mL of distilled water and the absorbance was measured at 228 nm using a spectrophotometer. Percentage of ACEI activity was calculated using Equation (1). The IC_{50} value of the fraction and Captopril (both in range of 18.125–250 μg/mL) was then defined as the concentration of the samples required to inhibit 50% of the ACE activity.

$$\text{Percentage of inhibition} = control \times 100 \quad (1)$$

2.10. Statistical analysis

The statistical differences between the test and control samples were determined by Student's t-test and one-way ANOVA using the StatView software package (Abacus Concepts, Piscataway, NJ, USA). Values were expressed as means ± SD for at least three independent experiments ($p < 0.05$).

3. Result

3.1. Content of C. striata extract

Total content of *C. striata* extract obtained from MMP was analyzed. It contained 80.65% total protein and 21.09% albumin. Fat content of the extract was 2.82%, where palmitic acid, oleic acid, and Omega-9 were the top-three fatty acids with 1.0%, 0.57% and 570.06 mg/100 g, respectively. In addition, 81.71 mg/100 g vitamin B2 and 444.05 mg/100 g calcium were the most dominant vitamin and mineral content found in the extract. Glutamic acid was shown to be the highest amino acid content found in the extract, with another three major amino acids *e.g.,* lysine, aspartic acid and leucine (Table 1).

3.2. Fractionation of C. striata albumin

Twenty mL of *C. striata* extract was subjected to a fractionation method developed by Cohn and colleagues based on albumin solubility difference with other proteins, called Cohn Process. Five systems were applied to separate the albumin from other proteins, wherein different ethanol (8–40%) and pH (4.8–7.2) were used. Products of each system were named as Fraction-1 to Fraction-5. By their appearances, each fraction also showed several visual differences. As shown in Fig. 1A, the color and texture of the precipitated proteins changed from mud-like light brown to bright yellow gel to light yellow gel and finally to white granule. Meanwhile, the different visual appearance of the dry proteins was only found in Fraction-5, where white ribbon-like proteins were found, while the other fractions appeared as yellowish to white granules (Fig. 1B). However, Fraction-4 was excluded from freeze-drying treatment due to its low concentration.

Precipitated proteins obtained from each system were weighted to measure their wet and dry weights. Both wet and dry weights of the obtained proteins were decreased along with the fractionation processes. Interestingly, there was a consistent trend found in all replicates that the precipitated proteins decreased from Fraction-1 to Fraction-4, and then slightly increased in Fraction-5 (Fig. 1). Wet and dry weights of the Fraction-5, where the albumin was theoretically more abundant and purer, were 6.9 ± 3.5% and 3.8 ± 2.1%, respectively. The difference of protein abundance started to be significantly decreased in Fraction-2, which afterwards the decrease was considerably insignificant (Fig. 1C and D). By the assumption that the albumin was lost about 20% from each process stage, hence the dry weight of the albumin from *C. striata* would be approximately 19% compared to that of the crude extract (Parental Fraction).

3.3. UV spectrum profile of Cohn Process fractions

UV spectra profiles of each protein fraction obtained from the Cohn Process were also evaluated and compared to the Parental Fraction. This evaluation was conducted to provide a description regarding purity and the presence of non-protein components in the fractions since the Parental Fraction also contained another component such as thickener agent maltodextrin. As shown in Fig. 2, the UV spectra of Parental Fraction and Fraction-1 were different from that of Fraction-2, -3, -4 and -5. Multipeaks were detected in

Table 1
Total content of *C. striata* extract.

Nutrient	Amount	Nutrient	Amount (mg/kg)
Total protein	80.65%	Amino acid	
Albumin	21.09%	Serine	26905.83
Total fat	2.82%	Glutamic acid	93701.17
Omega-3	80.9 mg/100 g	Phenylalanine	25551.27
Omega-6	253.6 mg/100 g	Isoleucine	29279.34
Omega-9	570.6 mg/100 g	Valin	30973.44
Palmitic acid	1.0%	Alanine	33681.29
Stearic acid	0.3%	Arginine	27422.09
Linoleic acid	0.15%	Glycine	31190.07
Oleic acid	0.57%	Lysine	67173.69
Vitamin		Aspartic acid	56605.67
Vitamin B2	81.71 mg/100 g	Leucine	50050.91
Vitamin E	23.33 mg/100 g	Tyrosine	19175.84
Mineral		Proline	21032.85
Calcium	444.05 mg/100 g	Threonine	31642.64
Magnesium	119.67 mg/100 g	Histidine	12429.50
Zinc	2.42 mg/100 g	Cysteine	11596.38
Fe	5.25 mg/100 g	Methionine	10232.25
Selenium	57.35 μg/100 g	Tryptophan	7181.16

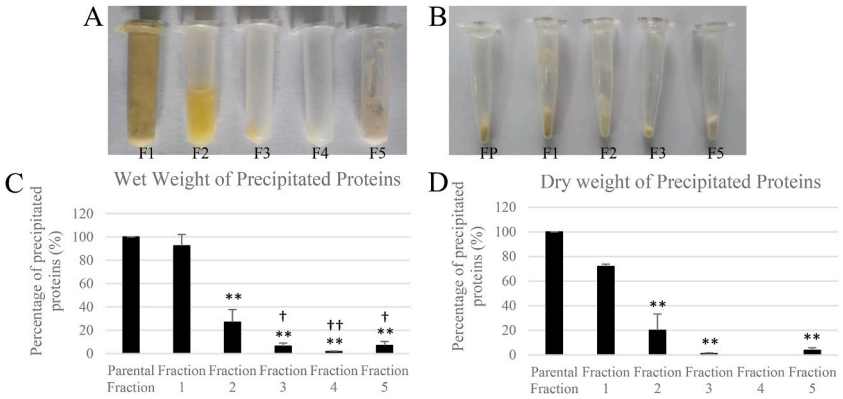

Fig. 1. Precipitated protein generated during the Cohn Process. Five systems that contain different concentrations of ethanol and pH were used to extract albumin from *C. striata* crude extract. (A) Appearances of pellets generated during the fractionation; (B) appearances of proteins after 6 h freeze-drying. Fraction-4 was excluded since its low amount of pellet generated during fractionation. (C) Wet weight of the precipitated protein fractions obtained during the fractionation; (D) dry weight of the precipitated protein fractions obtained during the fractionation; Results are expressed as mean ± SD of three independent replicates. *P < 0.05 compared to Parental Fraction and Fraction-1; **P < 0.01; †P < 0.05 compared to Fraction-2; ††P < 0.01.

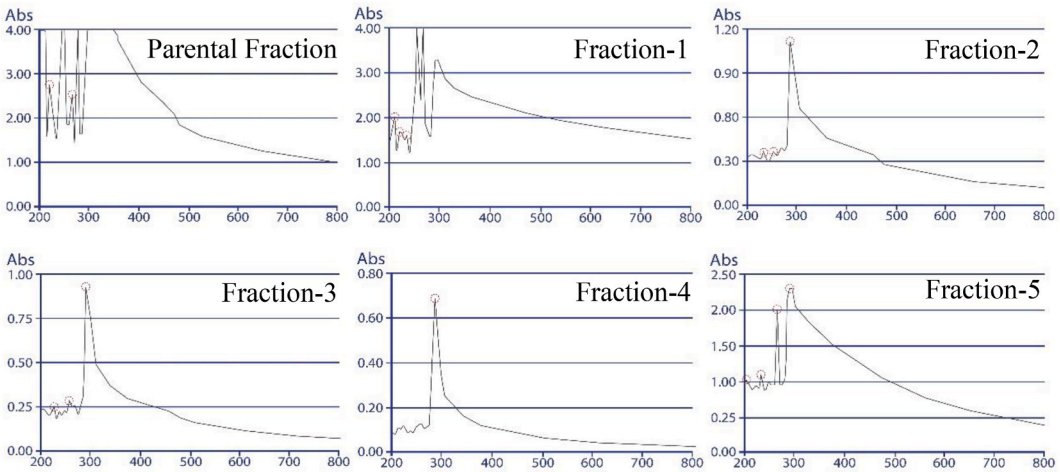

Fig. 2. UV spectra profile of the fractions. The UV spectrum of the protein fractions obtained from the Cohn Process were analyzed using a UV-VIS spectrophotometer in a range of 200–800 nm. This result is representative to three independent replicates.

Parental Fraction and Fraction-1, with maximum absorbances were found at 210–215 nm, which further indicated the present non-protein component in the fractions was major. In another hand, Fraction-2, -3 and -4 showed single peak with maximum absorbance were found at 285 nm, while two peaks detected in Fraction-5 with maximum absorbance at 290 nm (Table 2, Fig. 2). Since protein absorbs maximum wavelength at range 280–290 nm, it was concluded that protein content in Fraction-2, -3, -4 and -5 were considerably pure and present as the major component (Fig. 2).

Table 2
The UV absorbance characteristic of the protein fractions.

Protein Fraction	Peak Detected (nm)	Maximum Absorbance (nm)
Parental Fraction	215, 260	215
Fraction-1	210, 220	210
Fraction-2	285, 255	285
Fraction-3	285,255	285
Fraction-4	285	285
Fraction-5	290, 265	290

3.4. Protein profile analysis

Fig. 3 shows six bands in Parental Fraction, with molecular weight ±40, 25, 20, 15, 13 and 10 kDa. Relatively similar profile was found in Fraction-1, where 4 bands *e.g.,* 40, 15, 13 and 10 kDa were detected. In contrast, only two bands were detected in Fraction-2, -3, -4 and -5, which were 10 and 13 kDa. However, the result clearly showed that two protein bands with molecular weight ±10 and 13 kDa were the major proteins found in all protein fractions, wherein the intensity of the two bands were significantly higher in Fraction-5 (Fig. 3A). As presented in Fig. 3b, increasing concentrations of Fraction-5 were also subjected to tricine-SDS PAGE. Two bands were detected in concentration-dependent manner, where the higher band intensities were detected along with the higher protein concentration.

In this study, BSA and fish albumin were used for comparison purposes. Both albumins showed different protein profiles with that of the fractions. BSA was detected as a single band of 65 kDa, which was in line with its theoretical molecular weight. In other hands, fish albumin showed multi bands ranging from 15 to 60 kDa with major bands detected with approximate molecular weight of 60 kDa (Fig. 3).

3.5. Enzymatic hydrolysis and ultrafiltration

Bioactive peptides of Fraction-5 were produced by enzymatic hydrolysis using two different proteases, *e.g.,* pepsin and alcalase, under optimal conditions. The optimum concentration and incubation time of the enzymes was separately conducted (data not shown). The peptides were then separated based on their molecular sizes using ultrafiltration. The peptides were further subjected to tricine-SDS PAGE to confirm the hydrolysis and ultrafiltration processes were done properly. The non-hydrolyzed Fraction-5 appeared as clear bands with size about 10 and 13 kDa, while the hydrolyzed fractions appeared as smears that indicated the proteins were successfully hydrolyzed into smaller peptide fragments (Fig. 4).

3.6. The effect of protein and peptide fractions on inhibiting ACE

3.6.1. Comparison of ACE inhibition between protein fractions

In this study, ACE inhibitory activity of each fraction was evaluated and compared to Captopril and BSA. As shown in Fig. 5A, positive control showed 56.99 ± 6.48% of inhibition effect, while BSA showed 17.90 ± 1.88%. Remarkably, the protein fractions showed a clear increasing trend on inhibiting ACE, ranging from 7.09 to 22.99%. Compared to the Parental Fraction, Fraction-4 and Fraction-5 showed statistically significant activity, with ACE inhibition effect 19.75 ± 5.02 and 22.99 ± 4.36%, respectively ($p < 0.05$). Although the ACE inhibitory effect of Fraction-1, -2 and -3 were higher than that of Parental Fraction, the differences were statistically insignificant.

3.6.2. Screening of ACE inhibitory activity of peptide fraction derived albumin

As shown in Fig. 5B, the ACEI assay of pepsin- and alcalase-hydrolyzed Fraction-5 was evaluated and compared with the non-hydrolyzed Fraction-5 and Parental Fraction. As seen, the highest inhibition was shown in peptides generated by alcalase hydrolysis with molecular size <3 kDa (56.65 ± 2.32%). This value was also statistically significant compared with the non-hydrolyzed Fraction-5 and Parental Fraction, which were 23.48 ± 3.11% and 13.02 ± 0.68%, respectively ($P < 0.01$). The ACEI activity of the peptide was statistically insignificant compared with that of Captopril (49.09 ± 5.59%). By comparing the peptides generated from the two enzymes, it showed the ACEI activity of the peptides have different trends in regard to their molecular size groups. In pepsin-hydrolyzed peptide, decreasing molecular size showed increasing ACEI activity (up to 47.13 ± 16.80%), while in alcalase, peptides with molecular size >3 kDa showed no significant activity compared with the non-hydrolyzed peptide groups. Peptides with molecular size <3 kDa showed highest ACEI activity in both pepsin- and alcalase-hydrolyzed groups, however, their values were statistically insignificant to each other.

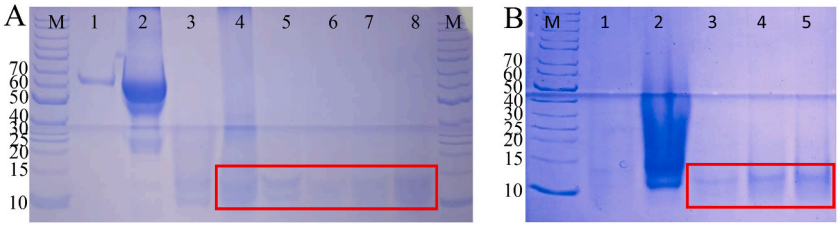

Fig. 3. Protein profile analysis by tricine-SDS PAGE. (A) Protein profile comparison of each fraction. Two proteins major with approximate molecular weight 10–13 kDa were found in all fractions (indicated by red box). M: Protein Marker; 1: BSA 1 μg; 2: Fish albumin from MBS kit; 3: Parental Fraction 50 μg; 4: Fraction-1200 μg; 5: Fraction-2200 μg; 6: Fraction-3200 μg; 7: Fraction-4200 μg; 8: Fraction-5200 μg; (B) increasing concentration of Fraction-5 showed higher band intensity (indicated by red box). M: Protein Marker; 1: Parental Fraction 10 μg; 2: Parental Fraction 100 μg; 3: Fraction-5100 μg; 4: Fraction-5200 μg; 5: Fraction-5300 μg. The figure represents the result of three independent replicates.

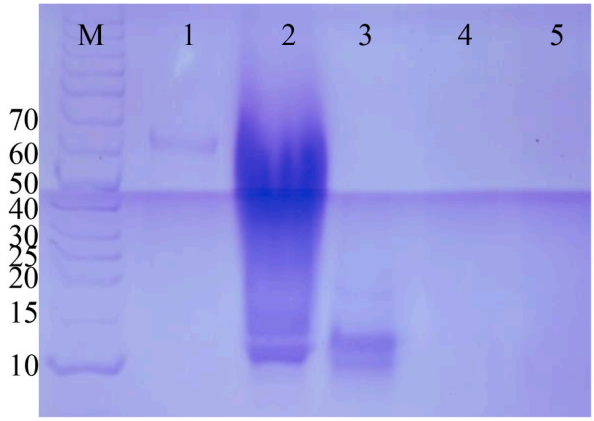

Fig. 4. Tricine-SDS PAGE analysis of with and without hydrolysis of Fraction-5. M: Protein Marker; 1: BSA 1 μg; 2: Parental Fraction 100 μg; 3: Fraction-5200 μg without hydrolysis; 4: Fraction-5200 μg hydrolyzed with pepsin; 5: Fraction-2200 μg hydrolyzed with alcalase. The figure represents the result of three independent replicates.

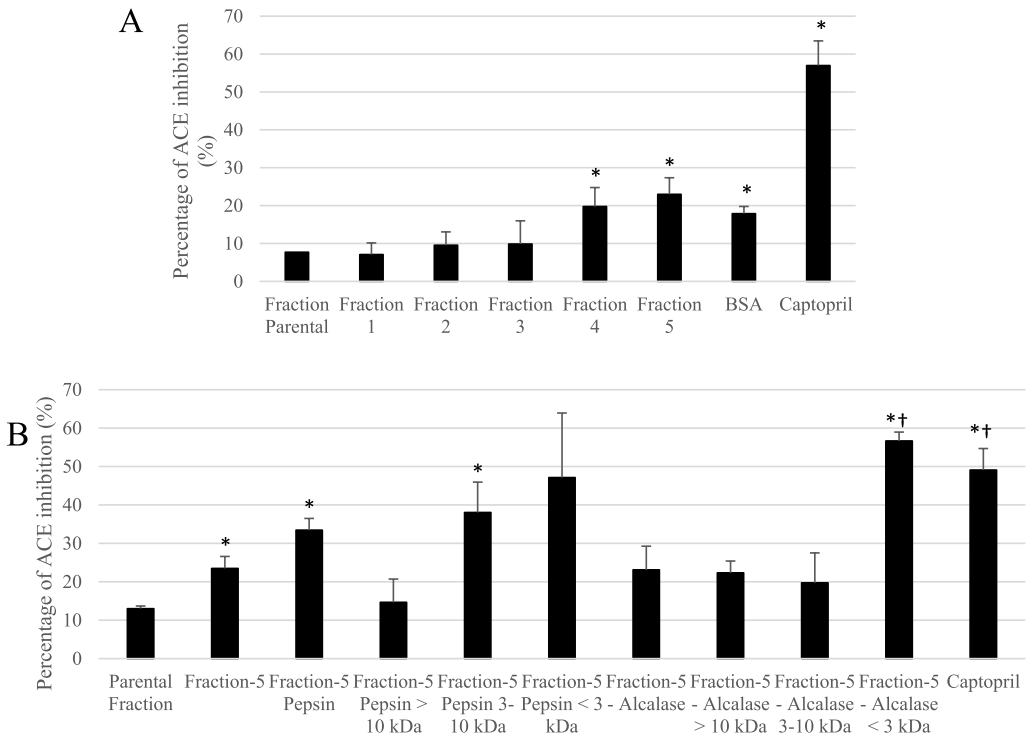

Fig. 5. ACE inhibitory activity of albumin fractions. ACE inhibitory activity of the samples was evaluated by measuring the formation of hippuric acid at 228 nm as the product of HHL conversion by ACE. Captopril (250 μg/mL) was used as the positive control of the experiment. (A) Comparison of ACEI activity between protein fractions. BSA was also compared with albumin fractions obtained from the Cohn Process. (B) Activity of peptides (250 μg/mL) generated from Fraction-5 was evaluated and compared with its non-hydrolyzed fraction. Results are expressed as mean ± standard deviation of three independent replicates. *$P < 0.01$ compared to Parental Fraction. †$P < 0.01$ compared to non-hydrolyzed Fraction-5.

3.6.3. Dose response study

As alcalase-hydrolyzed albumin peptide (<3 kDa) possessed the highest ACEI activity among others, increasing concentrations of the fraction, ranging from 18.125 to 250 μg/mL, were used to conduct the dose response study. The peptide fraction showed ACE inhibitory activity in a dose-dependent manner up to concentration 72.5 μg/mL (Fig. 6). The optimum ACE inhibitory dose of the fraction was shown at 72.5 μg/mL, with IC_{50} value 36.93 μg/mL. These values were statistically insignificant compared to that of the control positive Captopril, with the optimum concentration and IC_{50} were 72.5 and 32.66 μg/mL, respectively) (data not shown).

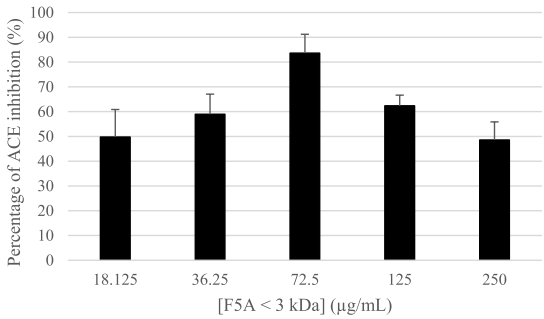

Fig. 6. Dose response study of alcalase-hydrolyzed albumin peptide <3 kDa in inhibiting ACE. Increasing dose of the peptide fractions, ranging from 18.125 to 250 μg/mL, were subjected to ACEI assay procedures. Results are expressed as mean ± standard deviation of three independent replicates.

4. Discussion

It has currently become our major concern to isolate albumin from *C. striata* and to ensure that the albumin was considerably separated from other proteins. Overview of serum albumin purification methods has been summarized by Raoufinia (2016). Several methods, such as affinity precipitation, TCA/acetone precipitation, heat shock method, ammonium sulfate precipitation, ethyl acetate fractionation, and their combination with chromatography, have been reported to successfully purify albumin, however, Cohn Process that developed albumin precipitation using different pH and ethanol conditions emerged as the most suitable approach [19]. The approach was conducted and confirmed to be in line with our recent result, where Cohn Process succeeded in fractionating albumin from *C. striata* into different fractions with good protein purity. It has been reported that Cohn Process resulted in 96–99% of albumin purity with several advantages that were claimed such as suitable for large-scale and industrial production, since ethanol is accessible and inexpensive [27,28]. In addition, several modifications of the Cohn Process were also reported, where chromatography was primarily used as a post-process of the purification method. Liquid chromatography, ion-exchange chromatography and simulated moving bed chromatography were several chromatography methods used to improve the purity of Cohn Process up to 99% for both clinical usage and large scale [19,27].

Protein separation analysis revealed that the approximate molecular weight of *C. striata* albumin was 10 and 13 kDa. In other study, Rahayu et al. (2016) reported that bioactive protein of *C. striata* contained 4 major proteins with approximate molecular weight of 8.3, 10.9, 15.4 and 16.7 kDa, however they never clearly stated which band was *C. striata* albumin [29]. Altogether, these results might indicate that albumin of *C. striata* was much smaller than commonly used albumin such as BSA or HSA whose size were 66.5 kDa. It is quite contradictory with the use of BSA as the protein standard for albumin measurement by far, even in the SNI 8074:2014 as a current national guideline for *C. striata* extract for commercial purposes in Indonesia. It is understandable since there was no available *C. striata* albumin standard in the market currently. However, it brought doubt to several reports on *C. striata* albumin that used BSA as the standard for their albumin measurements.

Immunoblotting using human serum albumin antibodies also did not recognize any proteins in *C. striata* [29], which might also support our finding regarding the difference of albumin in fish and mammals. A study by Andreeva regarding structure of fish albumin reported that fish albumin has structural diversity even within Pisces taxon. Furthermore, the study also suggested that albumin of fish and mammals might be homologous protein. However, the degree of homology between fish and mammalian proteins should be established by comparative analysis of their amino acid sequences or corresponding motifs of albumin genes, which allows combining those genes into one superfamily protein [30].

The enzymatic activity of albumin has been reported in several reports, including ACE inhibitor activities [31–34]. As one of the major pathways involved in hypertension, particularly in its roles in converting Angiotensin I into Angiotensin II that was a major vasoconstriction agent, analysis of ACE inhibition was significantly relevant to antihypertensive activity. In this study, we provide evidence that albumin possesses ACEI properties, however, the activity was considerably low. Low activity on inhibiting ACE by albumin has been discussed by several reports [32,35]. As reported by Fagyas et al. enzymatic activity of ACE appears to be almost completely suppressed by albumin when it is present in its physiological concentration. They also hypothesized that ACE inhibition by albumin is different between circulating ACE and tissue bound ACE [32]. In accordance with that hypothesis, Dzau et al. stated that the plasma ACE represents only a small proportion of the body's total ACE activity, therefore its role is thought to be minimal [35]. Despite its considerably low activity, it is agreed that albumin possesses ACEI activity. It also indicated that there were active sites of the albumin that interact with ACE. In other hands, a number of experiments reported that Captopril, one of the most commonly used ACEI drugs, possesses a strong ACEI activity and commonly used as a positive control both in *vitro* and *in vivo* [26,36,37].

Fish and fishery by-processes extracted peptides and products as ACE inhibitory and anti-hypertensive were reported by Abachi [38], wherein peptides derived from fish muscle crude proteins such as tuna [39], salmon [40], and snakehead fish [13,15], were reported to possess ACE inhibitory and antihypertensive activities *in vitro* and *in vivo*. As one of the major proteins found in *C. striata*, albumin is expected to have a significant contribution to ACEI activity of *C. striata* peptide. In this current report, *C. striata* albumin peptide fractions were generated using enzymatic hydrolysis and ultrafiltration processes. We found that peptide generated from

alcalase hydrolysis with molecular size <3 kDa possessed the highest ACE inhibition in a dose-dependent manner, however the trend was only up to concentration 72.5 μg/mL. With higher concentration, the saturated conditions might be reached resulting reverse activity. In addition, as the fraction was not completely pure substance, the higher the concentration, the higher impurity presence which significantly influenced the inhibitory activity.

This superior activity of the peptides generated using alcalase is also in line with other studies that compared the ACEI activity of fish muscle peptides using several enzymes in *C. striata*, where alcalase has been preferred over other commercial and non-commercial enzymes for the extraction of ACEI peptides [13,15,38]. The use of alcalase in fish protein generally leads to production of short peptides (usually <3 kDa) with hydrophobic amino acids at the C terminal such as proline, tyrosine, tryptophan, phenylalanine or other positively charged amino acid. These characteristics of peptide were critical to make the interaction with the active and non-active sites of ACE possible [38]. Specifically, the biological activity of enzymatic hydrolysates from *C. striata* by alcalase showed the highest activity compared to those by other enzymes, with the presence of proline at their *C*-terminal [15,38]. Proline residue of captopril, a regularly used ACEI drug to treat hypertension, interacts with the ACE active site for inhibitory action, primarily through two histidine residues of ACE [41]. In addition, the presence of proline at the *C*-terminal has been reported to be more stable against gastrointestinal digestion [38]. Those features of biopeptides also fit Lipinski's Rule on predicting the drug-likeness of peptides with the high bio efficacy and bioavailability. These include smaller size and higher hydrophobicity which contribute to their resistance to digestive protease, then reaching the target tissue [38,42].

Alcalase is a common enzyme used in promising peptides production derived from various fish species with therapeutic properties such anti-ACE and anti-hypertension. However, pepsin digestion of certain types of fish resulted in more potent biopeptides than alcalase digestion. Several studies reported that both alcalase and pepsin produce small peptides with molecular size <3 kDa, which are beneficial for anti-ACE peptide screening candidates [38].

Ghassem et al. reported that two ACE inhibitory peptides LYPPP and YSMYPP were generated from *C. striata* crude protein, with IC_{50} values were 1.3 and 2.8 μM, respectively [15]. The main difference between our study with Ghassem et al. (2014) is the sources of the peptides. Ghassem et al. (2014) was using crude protein of *C. striata* [15], while our study specifically used albumin of *C. striata*. Since albumin is also found in crude protein, it might be possible that the biopeptides from Ghassem and ours are not different. However, evaluation using Expasy Protparam software revealed that the peptides generated by Ghassem (LYPPP and YSMYPP) have theoretical pI by 5.52. On the other hand, one of the principles of the Cohn Process is to precipitate protein based on pH. If the pI of the protein is the same as pH in the solution, the protein will precipitate. One of the fractionation steps in the Chon Process were the precipitation at pH 5.2 and 5.8. Theoretically, these two pHs are closer to the theoretical pI of Ghassem's peptides and might likely precipitate the peptides. Meanwhile, our fraction-5 were precipitated at pH 4.8. It might explain the possibility that Ghassem's peptide might be different from the peptides in our study. Another study by Budiari et al. reported 7.85% inhibition of ACE per μg protein resulted from their alcalase-hydrolyzed *C. striata* crude protein [13]. In contrast, the IC_{50} and optimum concentration of our ACEI peptide fraction was 36.93 μg/mL and 72.5 μg/mL, respectively. However, it is not possible to compare the ACEI activity of our peptide with other ACEI peptides produced from *C. striata* crude proteins, since the identification and quantification of the amino acid sequence of our ACEI peptide have not been conducted just yet. Remarkably, our data suggested that the same concentration of *C. striata* albumin peptide and Captopril showed the same strength of ACEI activity. As fish derived biopeptides, whether from the whole, byproducts and/or processed for ACEI/antihypertensive has been reported to have no cytotoxicity effect [38], our current findings might indicate that *C. striata* albumin peptide could be a promising natural source for antihypertensive treatment.

5. Conclusion

Fractionation of *C. striata* extract using Cohn Process has resulted in purer and more concentrated albumin protein fractions. Characterization of *C. striata* albumin of the fractions revealed that the albumin appeared as two bands of protein with molecular size approximately 10 and 13 kDa. Alcalase-hydrolyzed albumin peptide with molecular size <3 kDa showed the highest ACE inhibitory activity (optimum dose 72.5 μg/mL, IC_{50} 36.93 μg/mL), which was statistically significant compared to that of other peptide fractions and the non-hydrolyzed fraction. As in the same concentration the activity of the albumin peptide fraction showed the same strength with that of Captopril, it might also indicate promising basic evidence for the further use of this bioactive peptide as an alternative for natural-based ACEI antihypertensive treatment. For further research, the effect of the *C. striata* albumin peptide on inhibiting ACE in relevant cell lines such as endothelial cells, as well as its mechanism of action as ACEI would need to be explored. In addition, prior to the lack of information regarding genome and amino acids sequences of *C. striata* has intricated the identification and determination of albumin of *C. striata* itself, it is become an urgent matter to conduct whole genome and/or amino acids sequencing of this high commercial value fish species.

Author contribution statement

Guntur Berlian: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Catur Riani: Neng Fisheri Kurniati: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Heni Rachmawati: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Data availability statement

The authors are unable or have chosen not to specify which data has been used.

Conflict of interest

We declare that we have no conflict of interest.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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References

[1] R.N. Moman, N. Gupta, M. Varacallo, Physiology, Albumin, StatPearls, 2021. https://www.ncbi.nlm.nih.gov/books/NBK459198/ (accessed November 30, 2021).
[2] P. Caraceni, M. Tufoni, M.E. Bonavita, Clinical use of albumin, Blood Transfus 11 (2013) s18, https://doi.org/10.2450/2013.005s.
[3] G. De Simone, A. Di Masi, P. Ascenzi, Serum albumin: a multifaced enzyme, Int. J. Mol. Sci. 22 (2021) 1–23, https://doi.org/10.3390/ijms221810086.
[4] T.W. Evans, Review article: albumin as a drug–biological effects of albumin unrelated to oncotic pressure, Aliment. Pharmacol. Ther. 16 (Suppl 5) (2002) 6–11, https://doi.org/10.1046/J.1365-2036.16.S5.2.X.
[5] T.B. Pulimood, G.R. Park, Debate: albumin administration should be avoided in the critically ill, Crit. Care 4 (2000) 151, https://doi.org/10.1186/CC688.
[6] D. Melia, B. Post, Human albumin solutions in intensive care: a review, J. Intensive Care Soc. 22 (2021) 248–254, https://doi.org/10.1177/1751143720961245.
[7] S.Z. Ab Wahab, A. Abdul Kadir, N.H. Nik Hussain, J. Omar, R. Yunus, S. Baie, N. Mohd Noor, I.I. Hassan, W.H. Wan Mahmood, A. Abd Razak, W.Z. Wan Yusoff, The effect of Channa striatus (haruan) extract on pain and wound healing of post-lower segment caesarean section women, evidence-based complement, Alternative Med. (2015), 849647, https://doi.org/10.1155/2015/849647.
[8] S.M.A. Mohd, M.J.A. Manan, Therapeutic potential of the haruan (Channa striatus): from food to medicinal uses, Malays. J. Nutr. 18 (2012) 125–136.
[9] A.M. Mat Jais, Pharmacognosy and pharmacology of Haruan (Channa striatus), a medicinal fish with wound healing properties, Boletín Latinoam. y Del Caribe Plantas Med. y Aromáticas. 6 (2007) 52–60.
[10] M. Rahman, M. Molla, M. Sarker, S. Chowdury, M. Shaikh, Snakehead fish (Channa striata) and its biochemical properties for therapeutics and health benefits, SF J. Biotechnol. Biomed. Eng. 3 (2018) 7–9.
[11] M.A.K. Haniffa, P.A. Jeya Sheela, K. Kavitha, A.M.M. Jais, Salutary value of haruan, the striped snakehead Channa striatus - a review, Asian Pac. J. Trop. Biomed. 4 (2014) S8–S15, https://doi.org/10.12980/APJTB.4.2014C1015.
[12] A.M. Mat Jais, Y.M. Dambisya, T.L. Lee, Antinociceptive activity of Channa striatus (haruan) extracts in mice, J. Ethnopharmacol. 57 (1997) 125–130, https://doi.org/10.1016/S0378-8741(97)00057-3.
[13] S. Budiari, E. Chasanah, M.T. Suhartono, N.S. Palupi, Angiotensin converting enzyme (ACE) inhibitory activity of cude and fractionated snakehead (Channa striata) meat extract, Squalen Bull. Mar. Fish. Postharvest Biotechnol. 13 (2018) 57, https://doi.org/10.15578/squalen.v13i2.345.
[14] A. Mustafa, M.A. Widodo, Y. Kristianto, Albumin and zinc content of snakehead fish (Channa striata) extract and its role in health albumin and zinc content of snakehead fish (Channa striata) extract and its role in health, Int. J. Sci. Technol. 1 (2012) 1–8.
[15] M. Ghassem, A.S. Babji, M. Said, F. Mahmoodani, K. Arihara, Angiotensin I-converting enzyme inhibitory peptides from snakehead fish sarcoplasmic protein hydrolysate, J. Food Biochem. 38 (2014) 140–149, https://doi.org/10.1111/jfbc.12031.
[16] S. Li, Y. Cao, F. Geng, Genome-wide identification and comparative analysis of albumin family in vertebrates, Evol. Bioinf. Online 13 (2017) 1–6, https://doi.org/10.1177/1176934317716089.
[17] Badan Standardisasi Nasional, Ekstrak Albumin Ikan Gabus (Channa Striata) – Syarat Mutu Dan Pengolahan: SNI 8074:2014, 2014.
[18] E. Cohn, L.E. Strong, W.L. Hughes, D.J. Mulford, J.N. Ashworth, M. Melin, Preparat. Propert. Serum 68 (1946) 459–475.
[19] R. Raoufinia, A. Mota, N. Keyhanvar, F. Safari, S. Shamekhi, J. Abdolalizadeh, Overview of albumin and its purification methods, Tabriz Univ. Med. Sci. 6 (2016) 495–507, https://doi.org/10.15171/apb.2016.063.
[20] S. Singh, A. Mishra, A. Verma, A.K. Ghosh, A.K. Mishra, A simple Ultraviolet spectrophotometric method for the determination of etoricoxib in dosage formulations, "J. Adv. Pharm. Technol. Research" "(JAPTR)" 3 (2012) 237, https://doi.org/10.4103/2231-4040.104715.
[21] L. Zhao, X. Zhao, Y. Xu, X. Liu, J. Zhang, Z. He, Simultaneous determination of 49 amino acids, B vitamins, flavonoids, and phenolic acids in commonly consumed vegetables by ultra-performance liquid chromatography-tandem mass spectrometry, Food Chem. 344 (2021), https://doi.org/10.1016/J.FOODCHEM.2020.128712.
[22] J. Ren, E.L. Mozurkewich, A. Sen, A.M. Vahratian, T.G. Ferreri, A.N. Morse, Z. Djuric, Total serum fatty acid analysis by GC-MS: assay validation and serum sample stability, Curr. Pharmaceut. Anal. 9 (2013) 331, https://doi.org/10.2174/1573412911309040002.
[23] M. Welna, A. Szymczycha-Madeja, P. Pohl, Simplified ICP OES-based method for determination of 12 elements in commercial bottled birch saps: validation and bioaccessibility study, Molecules 25 (2020), https://doi.org/10.3390/MOLECULES25051256.
[24] H.K. Mahre, L. Dalheim, G.K. Edvinsen, E.O. Elvevoll, I.J. Jensen, Protein determination—method matters, Foods 7 (2018), https://doi.org/10.3390/FOODS7010005.
[25] H. Schagger, Tricine – SDS-PAGE 1 (2006) 16–23, https://doi.org/10.1038/nprot.2006.4.
[26] H. Rachmawati, I.S. Soraya, N.F. Kurniati, A. Rahma, In vitro study on antihypertensive and antihypercholesterolemic effects of a curcumin nanoemulsion, Sci. Pharm. 84 (2016) 131–140, https://doi.org/10.3797/scipharm.ISP.2015.05.
[27] K. Tanaka, E.M. Shigueoka, E. Sawatani, G.A. Dias, F. Arashiro, T.C.X.B. Campos, H.C. Nakao, Purification of human albumin by the combination of the method of Cohn with liquid chromatography, Brazilian J. Med. Biol. Res. = Rev. Bras. Pesqui. Medicas e Biol. 31 (1998) 1383–1388, https://doi.org/10.1590/S0100-879X1998001100003.
[28] A. Denizli, Plasma fractionation: conventional and chromatographic methods for albumin purification Plazma Fraksiyonlanması: albumin Saflaştırılması için Geleneksel ve Kromatografik Yöntemler Review, J. Biol. Chem. 39 (2011) 315–341.

[29] P. Rahayu, F. Marcelline, E. Sulistyaningrum, M.T. Suhartono, R.R. Tjandrawinata, Potential effect of striatin (DLBS0333), a bioactive protein fraction isolated from Channa striata for wound treatment, Asian Pac. J. Trop. Biomed. 6 (2016) 1001–1007, https://doi.org/10.1016/j.apjtb.2016.10.008.
[30] A.M. Andreeva, Structure of fish serum albumins, J. Evol. Biochem. Physiol. 46 (2010) 135–144, https://doi.org/10.1134/S0022093010020018.
[31] N. V Goncharov, D.A. Belinskaya, A. V Razygraev, A.I. Ukolov, On the Enzymatic Activity of Albumin 41 (2015) 113–124, https://doi.org/10.1134/S1068162015020041.
[32] M. Fagyas, K. Uri, I.M. Siket, G.A. Fulop, V.C. Sato, A. Darago, New perspectives in the renin-angiotensin-aldosterone system (RAAS) II : albumin suppresses angiotensin converting enzyme (ACE) activity in human, 9, https://doi.org/10.1371/journal.pone.0087844, 2014.
[33] D.A. Belinskaia, P.A. Voronina, V.I. Shmurak, M.A. Vovk, A.A. Batalova, R.O. Jenkins, N.V. Goncharov, The universal soldier: enzymatic and non-enzymatic antioxidant functions of serum albumin, Antioxidants 9 (2020) 1–29, https://doi.org/10.3390/ANTIOX9100966.
[34] D.A. Belinskaia, P.A. Voronina, V.I. Shmurak, R.O. Jenkins, N.V. Goncharov, Serum albumin in health and disease: esterase, antioxidant, transporting and signaling properties, Int. J. Mol. Sci. 22 (2021), https://doi.org/10.3390/IJMS221910318.
[35] V.J. Dzau, K. Bernstein, D. Celermajer, J. Cohen, B. Dahlof, J. Deanfield, J. Diez, H. Drexler, R. Ferrari, W. Van Gilst, L. Hansson, B. Hornig, A. Husain, C. Johnston, H. Lazar, E. Lonn, T. Luscher, J. Mancini, A. Mimran, C. Pepine, T. Rabelink, W. Remme, L. Ruilope, M. Ruzicka, H. Schunkert, K. Swedberg, T. Unger, D. Vaughan, M. Weber, The relevance of tissue angiotensin-converting enzyme: manifestations in mechanistic and endpoint data, Am. J. Cardiol. 88 (2001) 1–20, https://doi.org/10.1016/S0002-9149(01)01878-1.
[36] F. Marte, P. Sankar, M. Cassagnol, Captopril, XPharm compr, Pharmacol. Ref. (2021) 1–7, https://doi.org/10.1016/B978-008055232-3.61377-7.
[37] K. Nakamura, K. Naramoto, M. Koyama, Blood-pressure-lowering effect of fermented buckwheat sprouts in spontaneously hypertensive rats, J. Funct.Foods 5 (2012) 406–415, https://doi.org/10.1016/j.jff.2012.11.013.
[38] S. Abachi, L. Bazinet, L. Beaulieu, Antihypertensive and angiotensin-i-converting enzyme (ACE)-inhibitory peptides from fish as potential cardioprotective compounds, Mar. Drugs 17 (2019), https://doi.org/10.3390/md17110613.
[39] Z.-J. Qian, J.-Y. Je, S.-K. Kim, Antihypertensive effect of angiotensin I converting enzyme-inhibitory peptide from hydrolysates of bigeye tuna dark muscle, Thunnus obesus, J. Agric. Food Chem. 55 (2007) 8398–8403.
[40] C.B. Ahn, Y.J. Jeon, Y.T. Kim, J.Y. Je, Angiotensin I converting enzyme (ACE) inhibitory peptides from salmon byproduct protein hydrolysate by Alcalase hydrolysis, Process Biochem. 47 (2012) 2240–2245, https://doi.org/10.1016/J.PROCBIO.2012.08.019.
[41] B.J. Bhuyan, G. Mugesh, Synthesis, characterization and antioxidant activity of angiotensin converting enzyme inhibitors, Org. Biomol. Chem. 9 (2011) 1356–1365, https://doi.org/10.1039/C0OB00823K.
[42] G.B. Santos, A. Ganesan, F.S. Emery, Oral administration of peptide-based drugs: beyond Lipinski's Rule, ChemMedChem 11 (2016) 2245–2251, https://doi.org/10.1002/CMDC.201600288.