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Research Article
Influences of Palm Sugar, Fermentation Time and Enzyme Hydrolysis on Activities of Angiotensin Converting Enzyme Inhibitory (ACE-I) in Joruk Oci Fish (*Rastrelliger kanagurta*) Hydrolyzate

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Abstract

Background and Objective: Joruk is a traditional fishery product made by salting method followed by a spontaneous fermentation process with lactic acid bacteria. *In vitro* proteins hydrolysis with pepsin and trypsin can produce bioactive peptides. Bioactive peptides produced by joruk hydrolysis can act as antihypertensive. The aims of this study was to determine the influences of palm sugar concentration, fermentation time and enzyme hydrolysis on the activity of Angiotensin Converting Enzyme Inhibitory (ACE-I) of joruk oci fish (*Rastrelliger kanagurta*) hydrolyzates. **Materials and Methods:** The raw material used in this study was Oci fish obtained from Gorontalo, Indonesia. The production of joruk hydrolyzate was added with the concentration of palm sugar 10, 20 and 30% then fermented for 8, 10 and 12 days. Protein hydrolysis was done using pepsin and a combination of pepsin and trypsin. **Results:** The results showed that the dissolved proteins, degree of hydrolysis and ACE-I activities were 6,87-19.40 mg mL^{-1}, 5.53-21.27 and 46.85-67.55%, respectively. **Conclusion:** It can be concluded that the hydrolysate joruk made by hydrolyzed pepsin and trypsin enzymes with the addition of 10% palm sugar and 8 days fermentation time gave the best result and they could improve the activities of ACE-I.

Key words: Joruk, oci fish, fermentation, hydrolysis enzymes, ACE-I

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Angiotensin Converting Enzyme (ACE), also called dipeptidyl carboxypeptidase, is an enzyme that plays an important role in increasing blood pressure through the renin-angiotensin system[1]. ACE catalyze the production of Angiotensin (Ang) II from Angiotensin (Ang) I. The increase of the ACE activity causes an increasing of the formation of Angiotensin (Ang) II, a strong vasoconstrictor for hypertension. Therefore, the inhibition of ACE activity is the main target in the prevention of hypertension.

Some of the synthetic drugs of Angiotensin Converting Enzyme Inhibitory (ACE-I) are captopril, lisinopril, enalapril and ramipril (REF). However, long-term use of synthetic drugs can cause side effects such as dizziness, coughing, skin rashes and impaired kidney function (REF). To prevent or minimize the side effects, peptides from food proteins can be used as ACE-I and considered safer (REF), therefore studies that produce ACE-I isolated from natural ingredients, such as fish, need to be pursued[1].

Oci fish (*Rastrelliger kanagurta*) is a fish that found in Gorontalo, Gorontalo Province, Indonesia and is one of the leading fisheries commodities (REF). Oci fish is the fourth highest captured fishery commodity after snapper, tuna and shrimp (REF). Oci fish is economically high, availability in abundance and has high nutrient content. However, this fish is considered as a highly perishable food, therefore a good handling is needed to maintain freshness of fish and processing to extend its shelf life. Fermentation method is one of traditional method for extending the period of fish storage[1].

Traditional fermentation in recent times has gotten a lot of attention, mainly driven by its health benefits. Foods that produced by fermentation process was claimed to prevent or cure various diseases. Protein degradation during the fermentation of fish and other marine products released amino acids and bioactive peptides which act as antihypertensive[1-4] and antioxidants[1,5,6,7]. One of the traditional fermented products made from fish is bekasam. Commonly, bekasam is produced by adding a cooked rice as a source of carbohydrates to the fish. However, in the Ogan Komering Ulu Timur District, South Sumatra, Indonesia there is a fermented fish product that resemble bekasam, namely joruk. Joruk is produced not only by adding of cooked rice to the fish but also by adding palm sugar. Addition of palm sugar serves as a source of carbohydrates and to cover up the unpleasant aroma that is typically produced by the fermented fish.

Based on Nuraini *et al*.[8] and Hutabarat[9], the addition of cooked rice and palm sugar can reduce pH and increase the number of lactic acid bacteria. Koesoemawardani[10] shown that the addition of 20% of palm sugar produced joruk with a typical aroma of fermented fish, the addition of 25% of palm sugar produced a joruk with a slight aroma of palm sugar and the addition of 50% palm sugar produced a slimy joruk.

Fermentation of joruk was lactic acid fermentation which was natural fermentation with lactic acid bacteria which produced proteolytic enzymes that will degrade fish proteins into bioactive peptides[11-13]. Several methods that can be used to produce bioactive peptides are acid or base hydrolysis, enzymatic hydrolysis and microbial fermentation. Enzymatic protein hydrolysis is an efficient method as it can produce protein hydrolyzates with minimal amino acid damage and expected to increase amino acids with the addition of carbon[12].

Research on the bioactive peptides of ACE-I from hydrolyzates of fermented marine products has been widely carried out. However, the potential of joruk oci fish hydrolyzates as a potential ACE-I agent has not been observed. The aims of this study was to determine the effect of different concentration of palm sugar, the length of fermentation time and hydrolysis of enzymes against the activity of ACE-I joruk oci fish hydrolyzates.

MATERIALS AND METHODS

Materials: Oci fish obtained from fish suppliers in Gorontalo area, Gorontalo Province, Indonesia. Pepsin, trypsin, ACE enzyme, N-Hippuryl-His-Leu hydrate, hepes sodium salt, DPPH 2,2-Diphenyl-1-picrilhidrazyl, sodium chloride, acetic acid, 37% hydrochloric acid, methanol, sodium hydroxide, sodium carbonate, potassium sodium tartrate tetrahydrate, copper (II) sulfate pentahydrate, Folin-Ciocalteu's phenol reagent, bovine serum albumin, trichloroacetic acid, distilled water and sterilized water for injections were obtained from SIGMA Aldrich.

Preparation of joruk oci fish: The process of making joruk oci fish is based on the methods of Wikandariand Yuanita[14] and Nuraini *et al*.[8] with modifications. Fresh oci fish weeded (head, viscera, scales and tails removed) then cut into three pieces and washed with clean water. The fish was weighed 100 g then greased with 25% (w/w) salt and put in a glass jar that was tightly closed and stored in a room prevented from direct sunlight. The salting process lasts for three days. Water produced due to the salting process was removed then the fish was drained. Oci fish added with 3.5% salt, 10% cooked rice and palm sugar with a concentration of 10, 20 and 30%. Then, the mixtures were mixed until evenly distributed and

put into in a glass jar and closed tightly and fermented for 8, 10 and 12 days by storing in a room that was not exposed to sunlight.

Preparation of joruk extract: The process of making joruk extract is based on the method described by Wikandari and Yuanita[14] with modifications. Fifty grams of joruk oci fish was added with 100 mL of distilled water and blended until homogeneous. The solution was then centrifuged for 40 min at 3,000 g. The supernatant was set aside (supernatant 1), while the precipitate formed was added with 50 mL distilled water and then centrifuged for 20 min at 3,000 g (supernatant 2). The supernatant 1 was then added to the supernatant 2 and filtered with a filter paper.

Hydrolysis of joruk oci fish extract: The hydrolysis process of oci joruk extract with pepsin and trypsin refers to Ismanto[15] with modifications. The pH of 20 mL extract with protein content of 5 mg mL^{-1} was adjusted to pH 2 by adding 1 N HCl and then hydrolyzed using pepsin with a ratio of 1:100 (0.01 g of pepsin dissolved in 1 mL water for injections) and incubated in a shaking waterbath at 37°C for 2 h and then the pH was adjusted to pH 7 by adding NaOH 1 M. The trypsin w as added with a ratio of 1:100 (1 mg trypsin enzyme dissolved in 1 mMHCl) and incubated in a shaking waterbath at 37°C for 2 h. The reaction was stopped by heating at 95°C for 10 min and then cooled on ice water.

Analysis of soluble proteins content: The soluble protein content was analysed using the method of Lowry *et al.*[16]. Reagent A made by 10 g of Na_2CO_3 dissolved with 100 mL of 0.5 NaOH. Reagent B made by 1 gram of $CuSO_4.5H_2O$ dissolved with 100 mL of distilled water. Reagent C made by 2 g of K-Na Tartrate dissolved with 100 mL of distilled water. Reagent D prepared by 30 mL of reagent A solution added with 1.5 mL of reagent B and 1.5 mL of reagent C. Reagent E prepared by 20 mL ciocalteu folin diluted to a volume of 200 mL. 1 mL of joruk hydrolyzates was added to the test tube and then added with 1 mL of reagent D. The samples immediately vortexed and incubated at room temperature for 15 min. After incubation, 3 mL of reagent E was added and immediately vortexed and incubated at room temperature for 45 minutes. The absorbance at a wavelength of 750 nm was measured using a spectrophotometer. Soluble protein content was measured by the linear regression of the standard curve of bovine serum albumin (BSA).

Analysis of the degree of hydrolysis: The degree of hydrolysis was determined by the method of Silvestree *et al.*[17] by deposition of 20% TCA (Tri Chloroacetic Acid) to produce 10% dissolved protein fraction and 10% insoluble fraction. 500 µL samples were added with 500 µL of 20% TCA and then homogenized and incubated at 4°C for 30 min. The mixture was then centrifuged at 3,000 g for 20 min. Bovine Serum Albumin (BSA) was used as a standard. The degree of hydrolysis was calculated by the following formula:

$$\mathrm{DH}(\%) = \frac{\text{Soluble protein10\% TCA}}{\text{Total protein content}} \times 100\%$$

Analysis of ACE inhibitory activity: Analysis of ACE inhibitory activity was carried out according to the method of Wenno *et al.*[18] and Cushman and Cheung[19]. 50 µL samples solution was added with 50 mM ACE substrate Hip-His-Leu tripeptide (3.44 mg Hip His-Leu dissolved in a buffer 50 mM at pH 8.3) and then pre-incubated at 37°C for 10 min. The mixture was then added with 50 µL of ACE enzymes (25 m Unit mL^{-1}) and incubated at 37°C for 30 min. The reaction was stopped by adding 200 µL HCl 1 M. For the blank, 50 µL 1 M HCl was added to the mixture before pre-incubation and then added with 1.5 mL of ethyl acetate 0.5 M. The mixture was then vortexed for 10 seconds and then centrifuged at 4,000×g for 15 min. 1 mL of the supernatant was then transferred into a test tube and evaporated at 100°C for 15 min until dried and then cooled at room temperature. Hypuric acid formed was then dissolved in 3 mL of water for injections. The absorbance of 288 nm was then measured using a spectrophotometer. Percent inhibition of ACE inhibitory calculated by the following formula:

$$\text{Percent inhibition of ACE inhibitory}(\%) = \frac{A - B}{A - C} \times 100\%$$

Where:

A : Control (HHL+ACE)
B : Sample (HHL+Sample+ACE)
C : Blank (HHL+Sample)

Statistical analysis: This study carried out by an experimental method. The data were analyzed by using a two way ANOVA with a Randomized Block Design with a 3×3 factorial pattern. If there was a significant difference between treatments, Duncan's multiple range test was used with a confidence level of 95%. To compare the treatment of the use of pepsin and the combination of pepsin and trypsin in the hydrolysis process using the independent samples t test at a confidence level of 95%. Data were analyzed using the IBM SPSS Statistics version 23.

RESULTS AND DISCUSSION

Soluble protein: The soluble protein content of joruk hydrolyzate which hydrolyzed by pepsin was 6.87-8.95 mg mL^{-1}. The soluble protein content of joruk hydrolyzates which hydrolyzed by pepsin and trypsin was 14.78 19.40 mg mL^{-1}. The soluble protein content of joruk hydrolyzates decreases with the increase of concentration of palm sugar. It is expected because palm sugar is not effective as a carbon source for the growth of lactic acid bacteria. Usually, joruk made by adding cooked rice as a source of carbohydrates. The carbohydrate content of the cooked rice was 39.44% and the glucose content was 2.07%[20]. The carbohydrate content of palm sugar was 11.28%[21] and a glucose content 4.46% (DW)[22]. Based on these, it can be seen that the carbohydrate content of cooked rice is higher than palm sugar therefore the addition of rice for joruk production is more effective as a source of carbohydrates than palm sugar. Palm sugar in making joruk is functions as a source of sugar. The addition of high palm sugar (30%) in joruk production provide less nutrients and water needed by lactic acid bacteria to grow and develop therefore the protein degradation process becomes less effective.

Based on Fig. 1, it can be seen that the 8th day of the fermentation was the logarithmic phase of the lactic acid bacteria growth as they optimally utilize water and nutrients found in fish, cooked rice and palm sugar. Hadiwiyoto[23] explained that microbial cells undergo multiplication after adjustment to their growth environment. This phase therefore produce the highest protein content. In the 10th and 12th day of fermentation, the nutrition and water content had begun to decrease so that competition occurred between lactic acid bacteria (entering the stationary phase). In the stationary phase the number of dead and growing lactic acid bacteria was the same. During the death phase, the number of dead lactic acid bacteria was increasing, so that only fewer joruk hydrolyzate proteins degraded. As a result, dissolved protein decreases. Syarief and Halid[24] explained that the amount of nutrients decreases at the stationary phase leading to competition between microbes. The stationary phase will be followed by a phase of death which characterized by a decrease in the number of lactic acid bacteria.

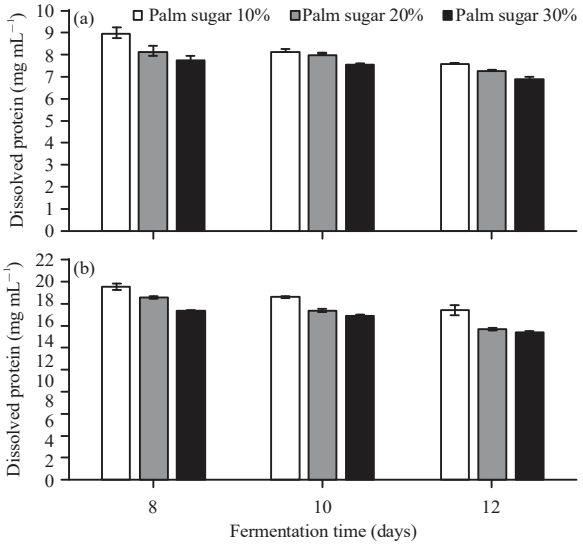

Fig. 1(a-b): The dissolved protein of joruk hydrolyzate which hydrolyzed (a) By pepsin enzyme and (b) By the pepsin and trypsin enzyme

The dissolved protein content of joruk hydrolyzates which hydrolyzed by a combination of pepsin and trypsin enzyme was higher than that hydrolyzed by pepsin alone. This is presumably because combining the enzymes pepsin and trypsin causes more protein to hydrolyze to simple peptides. Increasing production of peptides during the hydrolysis process will increase protein solubility. It was explained that the process of protein hydrolysis, various enzymes in the digestive tract of fish act as protein breakers. In some cases, enzymes such as pepsin, trypsin and chimosin were added to the hydrolysis process to accelerate the rate of proteolysis in fish fermentation[25]. Previous research added that to increase protein solubility and to improve the functional properties of fish proteins, enzymatic hydrolysis can be used because it is the most efficient method[26].

The degree of hydrolysis: Protein degradation by proteolytic enzymes of lactic acid bacteria can be seen from the degree of hydrolysis. The degree of hydrolysis of joruk hydrolyzates which hydrolyzed by pepsin enzyme was 5.53-9.87% (Fig. 2). The degree of hydrolysis of joruk hydrolyzates which hydrolyzed by pepsin and trypsin was 15.60-21.27%. The degree of hydrolysis decreases with increasing concentration of palm sugar and the longer of fermentation time. The degree of hydrolysis by combination of pepsin and trypsin was higher than that which hydrolyzed by pepsin alone. This is occurred because the hydrolysis of proteins using gastrointestinal digestive enzymes can increase the number of peptides and by the combination of pepsin and trypsin can increase the degree of hydrolysis of joruk protein. Pepsin and trypsin have different specificities in hydrolyzing peptide bonds. Pepsin tends to hydrolyze peptide bonds in carboxyl terminals that have aromatic amino acids (phenylalanine, tryptophan, tyrosine and leucine) and trypsin will hydrolyze peptide bonds on the side of carboxyl residues arginine and lysine. The combination of pepsin and trypsin in the production of

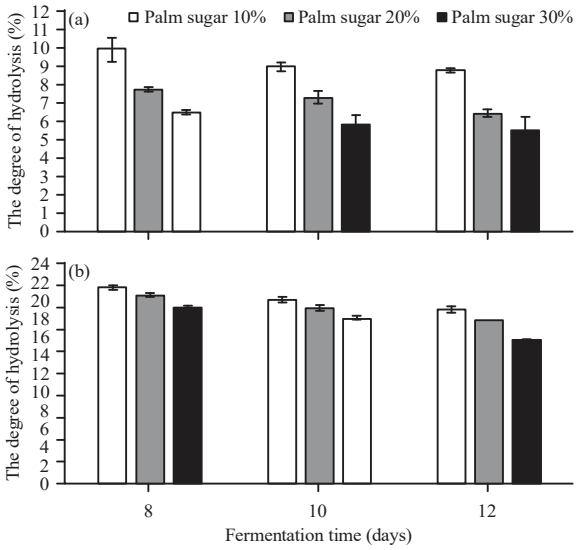

Fig. 2(a-b): The degree of hydrolysis of joruk hydrolyzate which hydrolyzed by (a) Pepsin enzyme and (b) By the pepsin and trypsin enzyme

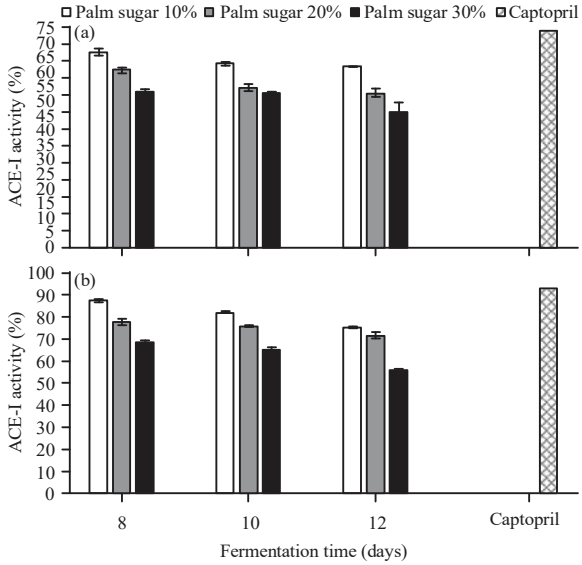

Fig. 3(a-b): ACE-I activity of joruk hydrolyzate which hydrolyzed (a) By pepsin enzyme and (b) By the pepsin and trypsin enzyme

joruk hydrolyzates will increase the degree of hydrolysis. Previous research explained that protein hydrolysis using the pepsin enzyme at pH 2-3 would break down proteins into polypeptides followed by trypsin enzymes at pH 7 to 8 which would break down large polypeptides and peptides[27]. The combination of these enzymes can increase the degree of hydrolysis.

Hydrolysis of fish protein influenced by many factors, including the composition of raw materials, types of enzymes that is used, hydrolysis conditions (temperature, time and pH) and the degree of hydrolysis[28]. The choice of enzymes usually based on the effectiveness and the cost. Protease enzymes from different sources can be used to obtain more selective hydrolysis. This is because each protease enzyme has a different specificity in breaking peptide bonds with certain amino acid residues[29,30].

Activities of angiotensin converting enzyme inhibitory (ACE-I): The activity of ACE-I of joruk hydrolyzates which hydrolyzed by pepsin was 46.85- 67.55% (). ACE-I activity of joruk hydrolyzates which hydrolyzed by pepsin and trypsin enzymes was 56.15-87.84% (Fig. 3). The activity of ACE-I of joruk hydrolyzates which hydrolyzed by pepsin and by the combination of pepsin and trypsin has a tendency to decrease with the addition of higher concentrations of palm sugar. These results can occur allegedly because the concentration of palm sugar affects the dissolved protein content. Whereas dissolved protein was an indicator of the presence of bioactive peptides ACE-I. The higher concentration of palm sugar has an effect on the dissolved protein content, which decreases, so that the degradation of proteins into ACE-I peptides also decreases. This result in the activity of ACE-I of joruk hydrolyzates in the higher concentration of palm sugar (30%) decreased ACE-I hydrolyzate activity.

The degree of hydrolysis is used as a parameter associated with the results of the hydrolysis process, namely as a parameter to measure the level of degradation of proteins by enzymes. The higher the degree of hydrolysis, indicates the greater the number of ACE-I peptides produced. The high degree of hydrolysis also implies that more small molecular weight peptides can be indicated as ACE-I peptides[31].

In this study joruk was hydrolyzed by a combination of pepsin and trypsin with the result that the combination of two enzymes in the hydrolysis process increased ACE-I activity rather than only hydrolyzed with pepsin alone. ACE-I peptides were hydrophobic aromatic peptide chains and positively charged amino acids and the peptides with 2-12 amino acid residues. Peptides with smaller amounts of residue have greater ACE-I activity. Pepsin and trypsin are protease enzymes that widely found in the digestive tract. Pepsin hydrolyzes specific peptide bonds only at carboxyl terminals, namely aromatic amino acids, whereas trypsin hydrolyzes peptide bonds containing carboxyl arginine or lysine groups. Based on the specificity of hydrolysis of pepsin and trypsin, it can be concluded that the hydrolysis of pepsin and trypsin produces bioactive peptides which were sequences of ACE-I peptides.

Inhibition of ACE by ACE-I peptides made possible by the presence of hydrophobic peptide chains (aromatic or side chains branching Tyr, Phe, Trp, Ala, Ile, Val and Met) and positively charged amino acids (Arg and Lys)[32-34]. Tripeptides with Trp, Tyr, Phe and Pro are effective for inhibiting ACE activity[35].

CONCLUSION

The conclusion that can be drawn from this study was joruk hydrolysates that hydrolyzed by pepsin and trypsin enzymes which added palm sugar by 10% with a fermentation time of 8 days gives the highest value of dissolved protein, the degree of hydrolysis and ACE-I activity. This study discovers the potential of joruk hydrolisates as a potential precursor for ACE inhibitor that can contribute to human health. This study will help the researcher to develop further functional food for anti hypertensive activity.

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