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FULL PAPER

Influence of palm sugar to the functional properties of bekasam hydrolysate

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Abstract

Hypertension is one of the major global health issues worldwide. One of the methods can be used to treat this disease is by inhibiting the activity of angiotensin converting enzyme, an enzyme that controls the regulation of arterial blood pressure and cardiac output. Bekasam is one of the traditional fermented food made from freshwater fish (oci, *Rastreliger kanaguria*) with the addition of carbohydrate sources (variation concentration of palm sugar i.e. 10%; 20%; and 30%). The objective of this research was to investigate the effect of palm sugar concentration to the ACE inhibitor activity of bekasam after digestive enzyme degradation by trypsin. Hydrolysis process was evaluated by determination of the concentration of soluble protein and the degree of hydrolysis (DH) after treatment and the ACE-inhibitory (ACEI) effect was determined by spectrophotometry method. Compared to the control without trypsin treatment (5.00±0.10 mg/ml), the concentration of soluble protein (mg/ml) of bekasam was increased after trypsin hydrolysis to 16.57±0.55 at 10% of added palm sugar and then decreased to 15.38±0.18 and 10.85±0.28, at 20 and 30% of added palm sugar respectively. The degree of hydrolysis was 21.3±0.3%, 19.7±0.5% and 15.3±0.4%, for 10, 20 and 30% of palm sugar, respectively. The ACE-inhibitory activity was 87.39±0.01, 84.52±0.84, and 77.68±0.63%, for 10, 20 and 30% of added palm sugar, respectively. The results showed that the concentration of palm sugar affects the soluble protein content, degree of hydrolysis and ACEI activity after trypsin hydrolysis with the optimum sugar concentration was 10%.

1. Introduction

Hypertension is one of the major global health issues, owing to its chronic nature, wide prevalence and linkage with increased mortality and morbidity which affects approximately 16 to 37% of the global population (Poulter *et al.*, 2015). Long term hypertension is one of the major risk factors and clinical manifestations of arteriosclerosis, cardiovascular diseases, strokes, heart failures, and chronic renal diseases (Bakris *et al.*, 2014; Lackland and Weber, 2015).

Angiotensin-converting enzyme (ACE, EC 3.4.15.1) is a glycoprotein with carbohydrate moiety composed of mannose, galactose, fructose, N-acetylneuraminic acid, and N-acetyl-glucosamine (Murray and Fitzgerald, 2007). ACE, found mainly in the capillaries of the lungs, is a key enzyme of the renin-angiotensin system (RAS) which is known as a cascade that controls the regulation of arterial blood pressure and cardiac output. This enzyme indirectly elevates the blood pressure by constricting the blood vessels. Therefore, one way to treat cardiovascular diseases is to inhibit the activity of ACE.

Many peptides derived from food proteins have been recommended to be Angiotensin-converting enzyme (ACE) inhibitors. Fish, as a source for food proteins, can be utilized as an ideal starting material for the production of novel ACE-inhibitory peptides. Annually, a large amount of fish is caught like as animal protein source and raw material in food industries. The abundance of the fish requires some ways for preservation, such as by fermentation. In Indonesia, fish fermentation method

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produced a well-known product called bekasam. Several fermented products use lactic acid bacteria (LAB) which has probiotic potential with beneficial effects on human health.

Enzymatic hydrolysis is a widely used method to release ACE-inhibitory peptides from fish proteins. The effectiveness of this method to generate specific peptide fragments with inhibitory activity mainly depends on the proteolytic enzyme used, hydrolysis conditions and the degree of hydrolysis (DH) achieved. A variety of enzymes including commercial proteases and proteases of microbial origin have been reported to produce ACE-inhibitory peptides from various fish proteins.

There are different methods to obtain ACE-inhibitory peptides from fish. The peptides can be extracted from fish proteins by using solvents and/or proteolytic enzymes (Chiang *et al.*, 2006; Hernandez-Ledesma *et al.*, 2011; Thewissen *et al.*, 2011). Several different proteolytic enzymes were reported to hydrolyze proteins such as alcalase, flavourzyme, thermolysin, trypsin, chymotrypsin, pepsin, papain, neutrase, bacterial and fungal proteases (Van der Ven *et al.,* 2002; Chiang *et al.,* 2006; Thewissen *et al.,* 2011; Gu and Wu, 2013). Thewissen *et al.* (2011) extracted peptides with 70% ethanol and extracts were hydrolysed with trypsin, ficin, or thermolysin. Liepke *et al.* (2001) produced ACE-inhibitory peptides with hydrolysis protein via digestive enzymes such as pepsin, trypsin, or chymotrypsin. Pepsin, trypsin and α-chymotrypsin are other important enzymes that have been utilized in the gastrointestinal simulation of peptide digestion (García-Tejedor *et al.,* 2014; Iwaniak *et al.*, 2014; Maestri *et al.,* 2015). Pepsin has broad cleavage specificity with a preference for peptides containing linkages with aromatic or carboxylic l-amino acids. It preferentially cleaves C-terminal to Phe and Leu, which are important amino acids for the ACE-inhibitory capacity of peptides (Murray and Fitzgerald, 2007; Sornwatana *et al.,* 2015). Trypsin, on the other hand, cleaves the peptide after Lys or Arg. (Kim *et al.,* 1999).

In some studies, the combination of enzymes was used to increase the level of potential ACE-inhibitory peptides due to enzyme specificity (Majumder and Wu, 2010; Ambigaipalan *et al.,* 2015). Indeed, pepsin, α-chymotrypsin and trypsin were combined to simulate the gastrointestinal digestion of food proteins in humans (Guang and Phillips, 2009; Jimsheena and Gowda, 2011; Gu and Wu, 2013). Furthermore, a combination of two or more enzymes may show higher ACE-inhibitory activity than single enzymatic treatment.

ACE-inhibitory peptides can be obtained through fermentation using cultures with proteolytic activities (Hayes *et al.,* 2007). Lactic acid bacteria (LAB) are generally used as a starter culture for ACE-inhibitory peptide fermentation (Hayes *et al.,* 2007; Rizzello *et al.,* 2008; Jakubczyk, 2013; Nejati *et al.,* 2013; Shu *et al.,* 2015). However, studies showed that microorganisms other than LAB such as *Bacillus* spp. (Lee *et al.,* 2015; Moayedi *et al.,* 2016), *Staphylococcus vitulus* (Fernández, *et al.,* 2016), *Saccharomyces cerevisiae* (Vermeirssen *et al.,* 2003; Murray and Fitzgerald, 2007; Jakubczyk *et al.,* 2013), *Debaryomyces hansenii* (García-Tejedor, 2014), *Mucor* spp. (Hang and Zhao, 2012) and *Aspergillus* spp. (Wang *et al.,* 2008) also can be used as Inoculum. Type of inoculum, fermentation conditions and inoculum levels play important roles in ACE-inhibitory peptide production (Jakubczyk *et al.,* 2013; Shu *et al.,* 2015; Fernández *et al.,* 2016).

Some synthetic inhibitors of ACE, such as captopril andenalapril, have proved to be useful as anti-hypertensive drugs (Ondetti *et al.,* 1977; Astawan *et al.,* 1995). However, synthetic inhibitors of ACEI have side effects, such as cough and angioneurotic oedema. Recently, food scientists are developing new angiotensin-I converting enzyme (ACE) inhibitors from natural foods (Murray and Fitzgerald, 2007), with the purpose of reducing the side effects of using ACE inhibitors for the treatment of hypertension. ACE-inhibitory peptides have been found in several fermented products such as miso, nato, yogurt, cheese, etc. (Murray and Fitzgerald, 2007). This research was conducted to investigate the effect of the addition of palm sugar to functional properties such as the soluble protein, the degree of hydrolysis and ACE inhibitor activity of bekasam.

2. Materials and methods

2.1 Materials

The materials used in the study were oci fishes, palm sugar, rice and pepsin. The oci fishes were obtained from Gorontalo, Sulawesi Island while the palm sugar and rice were obtained from central Java area. Trypsin (porcine stomach mucosa) was purchased from Wako Pure Chemical Industry Ltd., Japan.

2.2 Sample preparation

The proximate analysis of the fresh oci fish was conducted. Bekasam of oci fish (50 g) was added with 100 mL aquadest and blended with a food processor (Panasonic) for 5 mins. The bekasam extract was then homogenized with a homogenizer for 10 mins. The bekasam extract was incubated into shaking water bath Taitec Personal-11 for 30 mins at 70°C, and then the extract was cooled at room temperature.

2.3 Hydrolysis protein by trypsin

Bekasam extract was adjusted to pH 2.0 with 1 M of HCl. Trypsin (porcine stomach mucosa) was added into the bekasam extract at an amount of 0.01 g. After 2 hrs of incubation at 37°C the hydrolysate pH was adjusted to 7.0 with 1 M of NaOH and the hydrolysis by trypsin was terminated by heating at 95°C for 10 mins followed by cooling on ice (Salwanee *et al.,* 2013).

2.4 Degree of hydrolysis (DH) analysis

The degree of hydrolysis analysis is the percentage of solubilized protein in 20% trichloroacetic acid (TCA), in relation to the total protein content of the sample. Aliquots of 500 µL of the hydrolysed protein were mixed with 500 µL of 20% TCA solution to obtain the soluble and insoluble fractions in 10% TCA. After 30 mins of incubation at room temperature, the mixture was centrifuged at 3000 x *g*, and the soluble protein content of the supernatant was analysed spectrophotometrically using Lowry *et al.* (1951) method. Protein soluble concentration was obtained by comparing the absorbance of sample and the absorbance of bovine serum albumin (BSA). The degree of hydrolysis was counted following the equation:

$$DH\ (\%) = \frac{Soluble\ protein}{Total\ protein} \times 100\%$$

2.5 ACE-inhibitory activity

ACE-inhibitory activity was determined following the method of Arihara *et al.* (2001). A sample solution of the synthetic peptide at the amount of 6 mL was mixed with 50 mL of 7.6 mM HHL (hippuryl-His-Leu) containing 100 mM borate buffer (pH 8.3) and 608 mM NaCl. Before reacting with ACE, the sample was pre-incubated for 5 mins at 37°C in a water bath. The reaction was initiated by the addition of 20 mL of 60 mU/mL ACE dissolved in borate buffer (pH 8.3) containing 200 mM boric acid and 50 mM sodium tetraborate. The mixture was incubated for 30 mins at 37° C. The reaction was stopped by adding 554 mL of 0.1 M HCl, except for the blank which had already added by 554 mL of 0.1 M HCl before the incubation. The product (hippuric acid) of the reaction was extracted by the addition of 1.5 mL of ethyl acetate and vigorous mixing. Then, the mixture was centrifuged at 2500 rpm (1170 x *g*) for 15 mins. A total of 1 mL of supernatant was collected into another tube and dried at 100°C for 10 mins. The tube was cooled at room temperature for 10 mins and then 1mL of 1M NaCl was added into it. It was stirred with a vortex mixer for 30 s. The hippuric acid liberated by ACE was photometrically determined at 228 nm. The ACE-inhibitory activity was calculated with the following formula:

$$Inhibition\ (\%) = \frac{(Ec - Es)}{(Ec - Eb)} x100\%$$

Where Ec is the control absorbance; Eb is the blank absorbance; and Es is the sample absorbance.

3. Results and discussion

The results of the proximate composition of oci fish are shown in Table 1. The average values for protein, fat, ash and carbohydrate were 65.00±1.20 g/100g, 25.63±0.57 g/100g, 5.69±0.98 g/100g and 3.68±0.34 g/100 g (in % dry weight basis), respectively. Protein was the major component and this indicated that oci fish is a rich source of protein. The protein content of oci fish was similar to the protein content of tuna fish (65.04±1.40 mg/mL), but oci fish had a higher fat and ash content (11.77±1.41 (%db) and 3.12±0.11(%db)) (Salwanee *et al.,* 2013).

Table 1. Proximate composition of oci fish (fresh)

Component	Percentage (%) db)
Ash	5.69±0.98
Protein	65.00±1.20
Lipids	25.63±0.57
Carbohydrate (by difference)	3.68±0.34

3.1 Effects of different palm sugar concentration on the soluble protein content of oci bekasam protein extract

Effects of concentration of palm sugar on the soluble protein content of oci bekasam protein extract, that was fermented for 8 days after hydrolysed by trypsin are tabulated in Table 2. The content of soluble protein in the oci bekasam protein extract before hydrolysis was 5 mg/ml. When the oci bekasam protein extract was hydrolysed by trypsin, the content of soluble protein increased and was significantly different ($p<0.05$). The highest soluble protein of oci bekasam protein extract was obtained with the addition of palm sugar approximately at 10% (16.57%). On the other hand, the lowest soluble protein of oci bekasam protein extract was obtained with 30% addition of palm sugar (10.85%). Higher soluble protein content obtained upon tryptic hydrolysis is probably due to trypsin's specificity, as it is known that trypsin preferentially catalyses polypeptides on the carboxyl side of basic amino acids (arginine or lysine). In this study, amino acid components analysed with LC-MS indicated that there was arginine dan lysine in the oci fish (data not shown). Food protein-derived bioactive peptides are naturally physiologically active peptide fragments encrypted within the sequence of food proteins and can be released through enzymatic hydrolysis and microbial fermentation. Enzymatic hydrolysis is a widely used method to release ACE-inhibitory peptides from marine fish proteins. The

effectiveness of using this method to generate specific peptide fragments with inhibitory activity mainly depends on the proteolytic enzyme used, hydrolysis conditions and the degree of hydrolysis (DH) achieved.

Table 2. Soluble protein content of extract of oci bekasam protein that was added with various concentration of palm sugar, fermentation by trypsin

Palm sugar (%)	Soluble Protein content (mg/mL)
10	16.57±0.55
20	15.38±0.18
30	10.85±0.28

3.3 Degree of hydrolysis and ACE-inhibitory activity of hydrolysates of oci bekasam protein extracts added with different palm sugar concentration

Enzymatic hydrolysis was performed using trypsin. Hydrolysis efficiency was evaluated by measuring the degree of hydrolysis (DH) in the hydrolysates that had been generated using three different palm sugar concentration (Table 3). Overall, the hydrolysis of the oci bekasam protein extracts was the highest rate of hydrolysis during the 8 hours fermentation. The rapid increase in DH indicated that a large amount of peptides were cleaved from oci bekasam protein extracts and released into hydrolysates at different palm sugar added concentration. These results inferred that the higher concentration palm sugar added, the less amount of oci bekasam protein hydrolyzed. The rate of enzymatic cleavage of peptide bonds is an important factor in determining the rate of DH (Benjakul and Morrissey, 1997). These peptides also act as effective substrate competitors to undigested or partially digested compact proteins in the substrate (Nguyen *et al.,* 2011). Decreased hydrolysis reaction rate in oci bekasam protein extract can also be attributed to the limited availability of the substrate, as it is known that the substrate different in the palm sugar concentration.

Table 3. Degree of Hydrolysis of the extract of oci bekasam protein that was added with various concentration of palm sugar and fermented for 8 days

Palm sugar (%)	Degree of Hydrolysis (% db)
10	21.33±0.31
20	19.73±0.50
30	15.27±0.42

Among the concentration of the added palm sugar, the hydrolysis activity on the oci bekasam protein extract with 10% added palm sugar showed the highest rate (21.33%), followed by 20% added palm sugar (19.73%). Whereas, the lower DH values was observed with the 30% palm sugar added (15.27%). The efficiency of trypsin in catalysing the hydrolysis depends on the nature of the oci bekasam proteins extract. Lower DH value at 30% added palm sugar could probably due to the substrate specificity, as it was known that increasing the palm sugar concentration causes the protein component to decrease.

ACE-inhibitory activity of hydrolysate protein of oci bekasam is shown at Table 4. The ACE-inhibitory activity of protein oci bekasam that was added with 10%, 20% and 30% palm sugar and fermented for 8 days were 87.39%, 84.52%, and 77.68%, respectively. Wikandari and Yuanita (2016) indicated that the ACE-inhibitory activity of bandeng bekasam protein extract that was hydrolysed by trypsin enzyme was 69.87%. This indicated that hydrolysate of protein of oci bekasam hydrolyzated by trypsin has higher ACE-inhibitory activity than the extract of bandeng bekasam protein. So, hydrolysates of oci bekasam that was hydrolysed by trypsin had high potential as an ACE inhibitor. The ACE -inhibitory activity causes the peptides to be released by the protease digestion (trypsin). Korhomen and Pihlanto (2006) indicated that such peptides are inactive within the sequence of the parent protein and can be released in three ways: (1) through hydrolysis by digestive enzymes, (2) through the action of proteolytic enzymes derived from microorganisms or plants and (3) through hydrolysis by proteolytic microorganism. Jamhari *et al.* (2013) found that meat extract of Baliu cattle, Kacang goat, native chicken, and local duck after proteolytic reaction used protease can show ACE-inhibitory activity.

Table 4. ACE-inhibitory activity (%) of hydrolysate protein of oci bekasam protein added with various concentration of palm sugar.

Palm sugar (%)	ACE-inhibitory activity (%)
10	87.39±0.01
20	84.52±0.07
30	77.68±0.63

To investigate the effects of palm sugar concentration on ACE-inhibitor activity, samples taken from the hydrolysates at different palm sugar concentrations were subjected to ACE-inhibitor activity assay at a concentration of 2 mg peptide/mL (Table 4). Among all hydrolysates, the ACE-inhibitor activity decreased with increasing palm sugar concentration. The ACE-inhibitor activity of hydrolysate of oci bekasam protein extract added with various concentration of palm sugar resulted in forming simpler peptides inhibiting ACE in the process of liberating hippuric acid from hyppuryl-L-hystidyl-L-leucine (HHL). The hydrolysis of oci bekasam protein extract by trypsin produced many peptides which played the role in inhibiting the ACE activity. ACE inhibitor activity of hydrolysate of oci bekasam was relatively high and thus, has a high potential as an antihypertensive agent. The highest ACE inhibition at a level of 87.39% was observed for the hydrolysates of oci bekasam protein extract that was

added with 10% palm sugar. In particular, ACE-inhibitor activity significantly decreased if the concentration of palm sugar increased which is depicted as a fast decrease in DH. DH is defined as the ratio of the fraction of peptide bonds cleaved to the total number of peptide bonds (Adler-Nissen, 1982), and it has been widely used to evaluate hydrolytic progress.

The positive correlation between DH value and ACE -inhibitory activity has been reported in studies on the proteolysis of canola meal (Wu *et al.,* 2009), cuttlefish muscle (Balti *et al.,* 2010), palm kernel cake (Zarei *et al.,* 2015) and bovine collagen (Zhang *et al.,* 2013) proteins. It has been suggested that reaching a certain level of DH may contribute to the release of more active peptides from protein precursors (Wu, 2009).

4. Conclusion

The result showed that the initial concentration of soluble protein of oci bekasam protein extract was 5.00±0.10 mg/mL. After hydrolysis by trypsin, the soluble protein content increased to 16.57±0.55 mg/ mL, 15.38±0.18 mg/mL and 10.85±0.28 mg/mL for 10%, 20% and 30% added palm sugar respectively. The degree of hydrolysis was 21.33±0.31%, 19.73±0.50% and 15.27±0.42%, respectively and ACE-inhibitory activity were 87.39±0.01%, 84.52±0.84%, and 77.68±0.63%, respectively. Based on the results, the best palm sugar concentration is 10%, due to the higher soluble protein degree of hydrolysis and ACE-inhibitory activity.

Conflict of Interest

The authors declare no conflict of interest in the manuscript.

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