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The effect of degradation time in the simulated gastric fluid for bioactive compounds from bekasam

Rinto*, S D Lestari, A Baehaki and Y Sari

Department of Fisheries Product Technology, Faculty of Agricultural, University of Sriwijaya, Indralaya, South Sumatera, Indonesia

*E-mail: rinto@fp.unsri.ac.id

Abstract. Bekasam contains bioactive compounds (lovastatin and peptide) that has function as anticholesterol. This research was aimed to determine the effect of pH and degradation time to lovastatin, peptides, and dissolved protein in bekasam seluang extract (*Rasbora* sp.). This study used a laboratory experimental method with 4 levels of treatment (degradation in simulation gastric fluid pH 2, pH 3 pH 4 and control without simulated gastric fluid). The degradation results were observed every the 2nd hours, the 4th hours, and the 6th hours. Each treatment level repeated 3 times. The stages of the study consisted of making bekasam, extracting of bekasam, calculating the yield of bekasam extract and testing pure bekasam extract in simulated gastric fluid. The analysis was carried out using a descriptive method, which is to describe each parameter obtained. The parameters observed are analysis of lovastatin level, dissolved protein levels, and peptide levels. The results showed that the levels of lovastatin, dissolved proteins and peptides were still detectable after have degraded in simulation gastric fluid an acidity level of pH 2, pH 3 and pH 4 for 2nd to 6th hours.

Keywords: bekasam, lovastatin, peptide, protein, simulated gastric fluid (SGF)

1. Introduction

Processing of fermented fish product has been found in daily life. Some examples of fermented fish products are peda, fish sauce, shrimp paste, wadi, and bekasam. Bekasam is a traditional fermented fish product from Kalimantan and Sumatera. Generally in Sumatera, seluang fish (*Rasbora* sp.) is one of raw material for production bekasam. The processing of production bekasam is done by fermented for 5-7 days, therefore it has distinctive sour aroma and taste. During fermentation process, the protein will be change into some amino acids and bioactive peptides by lactic acid bacteria (BAL). Lactic acid bacteria of *Lactobacillus acidophilus* in bekasam is able to produce secondary metabolites such as lovastatin dan bioactive peptides (Rinto *et al* 2017a).

Bioactive compounds are a chemical compound that can inhibit mechanism formation of cardiovascular diseases such as hypercholesterolemia, hypertension and coronary heart. The survey from sample registration system (SRS) in Indonesia in 2014 showed that coronary heart disease was the highest cause of death in all ages after stroke, which amounted to 12.9%. Rinto and Suhartono

(2016) declared that lovastatin is one of the inhibitors for 3-hidroksi-3-metilglutaril (HMG) Ko-A reductase to decrease the cholesterol levels in blood. Bekasam from bandeng fish (*Chanos chanos*) can produce bioactive peptide which acts as binders for bile salts, it is indirectly can reduce cholesterol levels in the blood (Khoiriyah and Wikandari 2017).

The role of bioactive compounds in the body is obtained when the compound reaches the site of action. Before reacted in the site of action, the bioactive compounds have passed mouth, tongue, pancreas, and stomach. Location of the peptide and lovastatin reaction is in the liver, bekasam extract has degraded first in the stomach before arrived at the site of action. The specific situation in stomach is having acid solution, digestive temperature 37°C, and there is the pepsin which hydrolyzes the protein. The protein (amino acid) will be change when it enter the digestive situation. The protein (amino acid) will be denatured by certain temperature and pH, and will be hydrolyzed by some digestive enzymes such as pepsin enzyme. An acidic solution in the stomach varies between 1-5 pH. In the hungry situation, when the stomach is empty and has pH range 1-2. When we eat, the stomach releases proteases and hydrochloric acid to help the digestion and the increases the pH to 3 and 4. After the food has been digested, the gastric pH been 4 to 5 Therefore the proteins, amino acid and peptides will be changed after entering the digestive system. Currently there is no literature that mentions the relationship between bioactive compound from bekasam extract with pH, temperature and gastric digestive enzymes. The bioactive compounds contained in the bekasam extract are peptides and lovastatins. Peptides on bekasam extract have activity as an inhibitor to cholesterol (Rinto *et al* 2017a). The higher concentration of peptides produce caused the higher effect of decrease in cytosolic blood pressure. Before entered to the body's metabolism, the food had been entered to digestive system. While digestive enzyme and acidity effects the viability of bioactive compounds (Wikandari and Lenny 2016).

The situation in digestive system will make some of bioactive compounds be reactive because effects by pH, temperature and digestive enzyme. Temperature, enzyme, and pH are some factors that can influence the character of bioactive compound after made some reaction. According to (Wikandari and Lenny 2016) contain peptide from bekasam extract are degraded by digestive proteolytic enzyme is 38.42% while the extract was not degraded by digestive enzyme was 35.58%. The stability of index of angiotensin converting enzyme inhibitory peptides derived from soy protein has decreased after incubation for 2 hours at 20°C, 40°C dan 60°C (Wu and Ding 2002). The vitamin C content of pomegranate juice drops to 70% after being digested by the pepsin enzyme, compared to before being digested (Vicente *et al* 2002). Hodgkinson *et al* (2017) revealed that fraction of digestion of whey casein protein is different at pH 3 than pH 5, the more protein digestion at pH 3 than pH 5. The pH treatment affects bioactive compound such as anthocyanin, the higher pH value make the anthocyanin be lower (Fathinatullabibah *et al* 2014). From some of these studies show that pepsin enzyme; temperature and pH affect the viability of bioactive compounds. There is no literature describing the effect of gastric situation on lovastatin and peptide from bekasam extract, therefore this research studied effect of pH and hydrolysis time by pepsin enzyme on simulated gastric fluid on the bioactive compounds from bekasam extract.

2. Materials and methods

2.1. Materials

The main tools used in this study were Whatman 01 filter paper, centrifuge (320 R Universal-USA), waterbath shaker (WNB 14 USA), pH meter (WA-2017), siring filter membrane 0.45, Spectrophotometer UV-Vis (Jenway 6305). The main materials used in this study were seluang fish, salt, rice, aquabides, pepsin SIGMA for analysis, NaCl, $CaCl_2$, HCL, methanol, trifluoroacetic acid (CF_3COOH), standard of lovastatin solution, Lowry reagent, the standard of bovine serum albumin, pp

indicator, NaOH and formaldehyde. All other chemicals were of analytical grade and purchased from the local representative of Sigma and Merck.

2.2. Methods

The research method was conducted by the laboratory experimental method and each stage of the study was repeated 3 times. To obtain data, it consists of several stages, namely making bekasam, calculating the yield of bekasam extracts and degradation of bekasam extracts in simulated gastric fluid. The degraded bekasam extracts consisted of 4 different treatments, i.e:

A1 = Control, used extract without simulated gastric fluid
A2 = Degradation of bekasam extract in pH 2 simulated gastric fluid.
A3 = Degradation of bekasam extract in pH 3 simulated gastric fluid.
A4 = Degradation of bekasam extract in pH 4 simulated gastric fluid.

The degrading treatment in simulated gastric fluid was observed every 2, 4 and 6 hours. The treatment observed at the second hour was given code B, and the sample observed at 6th hour was coded C.

2.2.1. Bekasam. The method for making bekasam seluang according to Rinto *et al* (2017a) with modification, as follows: Seluang fish was weeded (discarded by scales, innards, and other impurities). Fish washed with running water to remove dirt and blood that is still attached. The fish was weighed as much as 250 g and mixed with 15% salt and 15% rice into a closed container and then the fermentation process is carried out at room temperature for 7 days.

2.2.2. Extraction of bekasam. Extraction of bekasam was carried out by single maceration extraction. This method was carried out according to Itou and Akahene (2010), using aquabides solvents: 10 g of the sample was homogenized with 40 mL of aquabides and stirred for 30 minutes at room temperature. The extract was filtered with Whatman 01 filter paper to produce filtrate (1) and residue. Then the residue was macerated again by adding 50 ml of aquabides and stirring for 30 minutes at room temperature. The extract was filtered with Whatman 01 filter paper to produce a filtrate (2). Filters 1 and 2 are mixed, then centrifuged at a speed of 6,000 rpm, at 4°C for 15 minutes to produce supernatants and precipitates. Centrifuged supernatant was then evaporated using a waterbath.

2.2.3. Degradation of bekasam extract. The results of the former extraction applied as in the digestive state in the stomach, the method used refers to Maiti *et al* (2018) with a few modifications as follows: A total of 3.2 mg / mL pepsin was added to 0.03 M NaCl to make an SGF solution (simulated gastric fluid). Added 1 N HCl to Simulated gastric fluid until the solution reaches pH 2, pH 3 and pH 4, 0.5% of bekasam extracts mix with 25 mL of simulates gastric fluid. The sample was homogenized at 37°C and analyzed every 2, 4 and 6 hours.

The parameters observed in this study were the calculation of the yield of bekasam extracts, stability analysis of bioactive compounds including the content of lovastatin, dissolved protein and the content of peptides.

2.2.4. Total extract. Total extract was a comparison between the weight of the extraction and the weight of the total sample before extraction. The calculation of the extracts of the bekasam extract used in the following formula:

$$\text{Percentage of total extract} = \frac{\text{Extraction weigh}}{\text{Weigh of sample before extraction}} \times 100\% \quad (1)$$

2.2.5. Lovastatin assay. The procedure of analysis of lovastatin content was done according to Osman *et al* (2011) using maceration extraction method according to Itou and Akahene (2010): 5 mL

of treated sample mix with 20 mL of methanol (Merck, German) and shake for 2 hours, then filter use a 0.45 μm membrane syringe filter. The filtrate was centrifuged at 3,000 rpm (500×g), 4°C for 15 minutes and then supernatant is separated. 2.5 mL of supernatant is added with 2.5 mL of trifluoroacetic acid 1% (sigma-aldrich, USA), homogenized by vortex and then left for 10 minutes. The final solution was analyzed by a UV-VIS Spectrophotometer use 238 nm of wavelength. Use Lovastatin (Sigma-Aldrich, USA) as standard, make solution of lovastatin with concentrations 6, 8, 10, 20, 30, 40, 50, and 60 ppm.

2.2.6. Dissolved protein assay. Dissolved protein analysis uses the Lowry method. The working principle of the Lowry method was when a protein reacts with phosphomolybdic tungstic reagent in an alkaline which will produce a blue color whose intensity depends on the concentration of protein listed. One (1) mL of the treated sample is mixed with 5 mL Lowry C. Homogenized by vortex and let stand for 10 minutes at room temperature. Added Lowry D 0.5 mL then homogenized by vortex and left for 30 minutes at room temperature. Measured absorbance at ƛ 590 nm. BSA standard curve was made with concentrations of 0, 30, 60, 90, 120, 150, 180, 210, 240, and 300 μg/mL. Regression lines were obtained for the relationship between absorbance and protein concentration

2.2.7. Peptide assay. Determination of peptide compounds was carried out using formol titration (Wikandari and Lenny 2016), as follows: 0.25 mL of the sample extract was dissolved with 4.75 mL of aquabides and put into 250 mL Erlenmeyer. Then the dissolved sample added 10 mL aquabides and + 0.5 mL PP indicator. Then the sample is treated with 0.1 N NaOH until it was colored pink. Samples added 1 mL of 40% formaldehyde solution and titrated with NaOH.

2.2.8. Data analysis. Data analysis was carried out descriptively by describing each test parameter performed. To obtain the research data consists of several stages, namely making bekasam, calculating the yield of bekasam extracts, and degradation of *bekasam* extracts in simulated gastric fluid, analyzing the content of lovastatin, analyzing dissolved protein levels and analyzing peptide compounds.

3. Results and discussion

3.1. Stability of lovastatin compounds

Lovastatin is one of a bioactive compound that has health benefits. Rinto and Suhartono (2016) reported that lovastatin able to reduce cholesterol levels because it is an inhibitor of HMG Ko-A reductase which is a cholesterol-producing enzyme in the body. Stability of lovastatin in simulated gastric fluid must be known to determine how much lovastatin protect itself in digestive situation, so its job as an inhibitor of HMG Ko-A reductase can work. In this research made a standard curve of lovastatin to help calculate linear lovastatin in the sample. Regression line of standard curve of lovastatin is (R^2>0.85) so that the linear equation can be used to calculate the linear number of lovastatin in the sample.

Lovastatin is a product of secondary metabolites from microorganisms, has been studied by Rinto *et al* (2015) that the lactic acid bacteria of *Lactobacilus acidophilus* extract used to produce lovastatin. The presence of the pepsin enzyme in simulating gastric solutions has no effect on lovastatin. This is because the arena of the active side of lovastatin is an ester and miosis lactone, while the pepsin enzyme carries out catalytic or cutting on acidic amino acids and or aromatic of amino acids.

The levels of lovastatin which were degraded in the simulation of gastric solution pH 2 and pH 3 tended to increase during the 2nd hour, 4th and 6th hours. However, the levels of lovastatin degraded in the simulation of pH 4 gastric solution tended to decrease when the 2nd to 6th hour (figure 1). This happens because the main framework of the polyketide in lovastatin in the form of a hydroxyl

hexahydro naptalene ring on the C6 and C8 side chains is damaged due to the release of the group that forms the polyketide framework, also possible double bond damage to the structure or opening of double bonds.

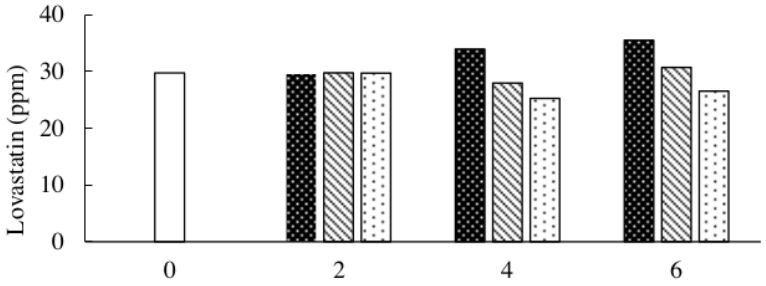

Figure 1. Lovastatin compounds on time and pH of degradation (pH 2: ▓, pH3: ▒, pH 4: ▒).

The lovastatin content in the control which has a neutral pH condition and the absence of pepsin enzyme is the same as the lovastatin content which is degraded by the pepsin enzyme both in the simulation of gastric solution pH 2, pH 3 or pH 4 for 2 hours. This means that the stability of lovastatin which is in the simulation of gastric solution for 2 hours is still the same when the ecstasy has not entered the simulation of gastric solution. At the time of degradation in the simulation of gastric solution for 4 hours or even for 6 hours, the presence of lovastatin is still detected, meaning that the stability of lovastatin is still able to survive the simulation of gastric solution for 2-6 hours.

The duration of degradation shows that the ability of lovastatin to survive is proven by the positive results of the lovastatin content in the test sample. Furthermore, the effect of the acidity level of the gastric solution simulation on the levels of lovastatin was further seen. The effect of the simulated acidity (pH) on the gastric solution seen is the result of the average degradation time of the lovastatin content. The average lovastatin content for each treatment is presented in figure 2.

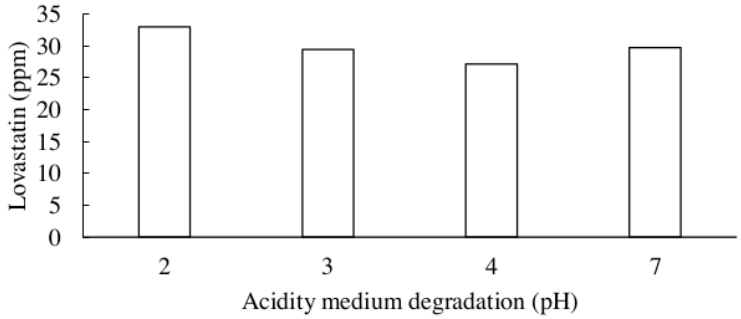

Figure 2. Average of lovastatin content on degradation medium.

Figure 2 shows that the highest level of lovastatin when degraded by simulating pH 2 gastric solution. This is due to two things. The first cause is due to the occurrence of the hydrolysis process on the active structure of lovastatin. According to Mahmoud *et al* (2014) spironolactone compounds have an ester group easily hydrolyzed at pH that is too acidic or basic. Lovastatin has an active group in the form of esters and lactones so that when it is located in a pH 2 the structure is

hydrolyzed and the possibility of a new compound is formed that resembles lovastatin. The second cause is because lovastatin is easier to detect under acidic conditions. Several previous studies conducted acid addition at the lovastatin analysis stage. Osman *et al* (2011) carried out the addition of HCl in the analysis of lovastatin from *Aspergillus terreus* culture. Reddy *et al* (2011) added trifluoroacetic acid in the analysis of lovastatin fermented by *Penicillium funiculosum*.

3.2. Stability of peptides

A peptide is bioactive compounds consisting of 2-50 amino acids. The stability of peptide of bekasam extract cause some acidity levels and pepsin enzyme seen from difference of the number of peptides from sample not degraded and sample degraded. The main sample not degraded is the samples have same treated as a degraded sample. Sample not degraded is bekasam extract homogenized with 25 mL of aquabides, it is neutral pH solution and not add pepsin enzyme. While sample degraded is bekasam extract homogenized with 25 mL of simulated gastric fluid, it has 2-4 acidity levels and added pepsin enzyme then observed every 2nd, 4th, 6th hours.

Determination of the number of peptide is carried out by formol titration method. This method is easiest method and does not cost a lot. Formol titration method principle is neutralized the solution by 0.1 N NaOH to produce dimethylol by adding 40% formaldehyde, where amino group have been bound and did not affect the acid-base reaction. The final of formol reaction is the sample turns pink and not changes for 30 seconds. The amount of peptide in some samples can see in figure 3.

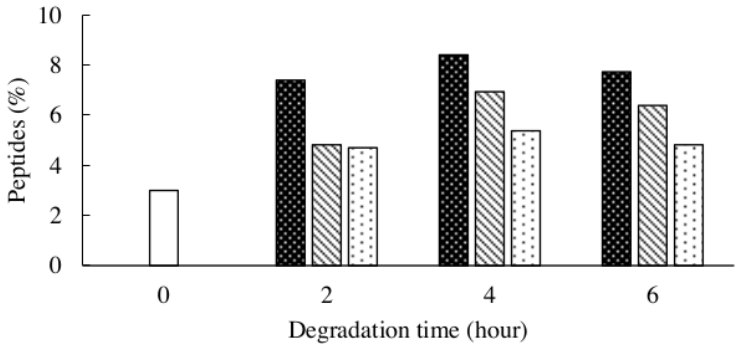

Figure 3. Peptides compounds on degradation time and pH (pH 2: ▓, pH3: ▒, pH 4: ▒).

Pepsin enzyme is one of protease enzymes as an endopeptidase enzyme; it is an enzyme tends to degrade peptide bonds in the inner structure not in the end both of N or C. The main target of the pepsin enzyme is acidly amino acids, namely aspartate amino acid (Asp, D), glutamate acid (Glu, E) and also aromatically amino acids namely phenylalanine (Phe, F), Tyrosine (Tyr, Y) and tryptophan (Trp, W). According to previous research by Rinto *et al* (2017b) the composition of the former fractionated amino acid peptide is KGENYNTGVTPNLRPKAAEVVAFLNKEAIEAIADTMKK. Its formation can be found 7 of 38 amino acid which acidly amino acids and aromatically amino acids. Consider on it statement, pepsin enzyme plays on role almost all of amino acids in *bekasam* extract, the long peptide bond cut is some short peptide bond and automatically can increase number of peptide bond. The 2nd to 4th hours (figure 3) seen that number of peptide is increased on every pH treatment, but the 6th is decreased. It happened because activity of pepsin enzyme is same as general enzyme. The 2^{nh} to 4th hours is increase activity of enzyme, while the 6th hours are decreased activity. The effect of average acidity (pH) on every hour can be seen in figure 4.

The total of peptides in every acidity sample degraded by a simulated gastric fluid is more than sample not degraded (control). It figures how's the higher acidity level of solution treatment, the

more peptides can detected. The acidity level of pH is the most many amounts of peptides detected than acidity level of pH 3 and pH 4. It is assumed that pepsin enzyme that degrades peptides will work optimally at low pH.

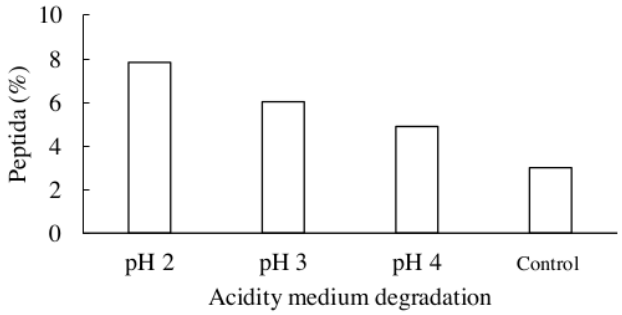

Figure 4. Average of peptides on medium degradation.

3.3. Protein level

Dissolved protein levels are carried out by lowry-folin method. Lowry-folin method has 100 more sensitives than biuret method, so it is effective to measure the sample have little protein content. The Lowry-folin method just only measures the short peptide bond (Alexander and Griffiths 1992). The working principle of the Lowry method is of reduction Cu^{2+} (Lowry B reagent) be Cu^{+} by tyrosine, tryptophan, and cysteine that contained in protein. Cu^{+} ions together with phosphotungstate and phosphomolibdate (Lowry D reagent) be blue solution to absorb the light. The standard protein used is BSA (bovine serum albumin). The result of BSA absorbance is taken in wavelength of 540 nm.

The regression value (R^2) of the standard bovine curve is 0.9737. Its value shows that it significant curve to calculate dissolved protein concentrations. The dissolved protein content can be seen in figure 5.

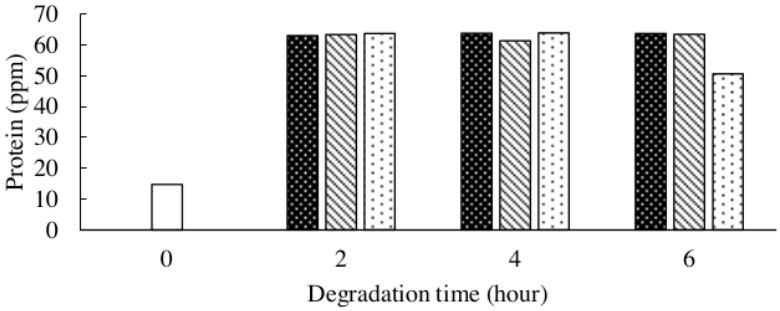

Figure 5. Dissolved protein on time and pH degradation (pH 2: ▓ pH3: ▒, pH 4: ▒).

Bekasam extract is a source of proteins which is derived from seluang fish (*Rasbora* sp.). The containing protein in bekasam extract is a little bite, fermentation processing make the complex proteins broken down by *L. acidophilus*. The dissolved protein of sample not degraded is 14.73 ppm, it the least amount of dissolved protein. The dissolved protein of sample degraded by pepsin enzyme in pH 2, pH 3 and pH 4 on the 2^{nd}, the 4^{th} and the 6^{th} hours is 50.59 ppm-63.73 ppm. Pepsin enzyme is a protein that has 44 N-protein terminals. When detected the absorbance by spectrophotometer, protein in enzyme is detected as dissolved protein. The other condition is protein structure in sample

control more complex than protein structure in sample degraded. Protein in sample degraded be simple protein, as a result simple protein more easy to late and detected than complex protein. The effect of average acidity level (pH) on every degraded hour can see in figure 6.

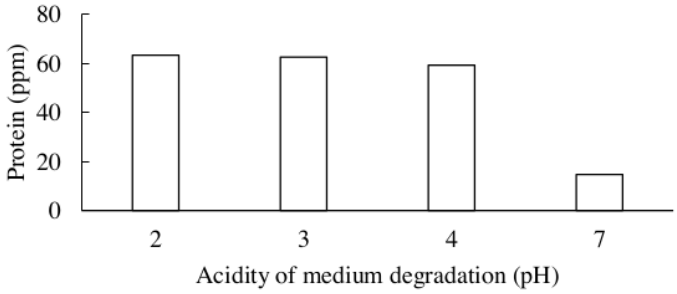

Figure 6. Average of dissolved protein on medium degradation.

Sample degraded on pH 2 simulated gastric fluid is the highest protein content than sample degraded in pH 3 and pH 4. Pepsin enzyme is more active in pH 2 than any pH, so protein in bekasam extracts is destroyed and dissolved. In addition, protein in acid will denature, protein or nucleic acids lose tertiary and secondary structure. The result of the denaturation is making protein easy to late.

4. Conclusion

The conclusions obtained from the research on the effect of pH and degradation time on the simulation of Gastric Solution on seluang bekasam extract were as follows: The yield pasta of bekasam extract was 13.7%. Lovasatin and peptides in bekasam extract still detected after being degraded in pH 2, pH 3 and pH 4 simulation gastric fluid for the 2nd for the 4th hours. The quantity of bioactive peptides after being degraded is increasing due to the enzyme pepsin cutting amino acids and aromatic amino acids in the amino acid chain of the extract so that the pieces of amino acids became more numerous. Examined Lovastatin extracts were more resistant to simulating gastric solutions than bioactive peptides.

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References

Alexander R R and Griffiths J M 1992 *2nd Edition Basic Biochemical Methods* (New York: Wiley-Liss)

Fathinatullabibah, Kawiji and Khasanah L U 2014 Anthocyanin stability of *Tectona grandis* extract in the different of pH and temperature *Applied Journal of Food Science* **3** 60-63

Hodgkinson A J, Wallece O A M, Boggs I, Broadhurst M and Prosser C G 2017 Gastric digestion of cow and goat milk: Impact of infant and young child in vitro digestion condition *Food Chem*. **15** 275-281

Itou K and Akahane Y 2010 Effect of extract from narezushi, a fermented mackerel product, on cholesterol metabolism in wistar rats *J. Fish Sci*. **76** 537-546

Khoiriyah I L and Wikandari P R 2017 Bile salt binding by bekasam that fermented by *Lactobacilus plantarum* B1765 in vitro *Unesa J. Chem*. **6** 41-45

Mahmoud I M, Ibrahim Y M, Shakir M A and El-gied cA A A 2014 Extemporaneous preparations of pediatric oral formulations: Stability studies conducted in *Spironolactone* suspensions, powders and capsules in Saudi Hospital Pharmacies *JGTPS* **5** 1595-1602

Maiti S, Jana S and Laha B 2018 Cationic polyelectrolyte–biopolymer complex hydrogel particles for drug delivery *Design and Development of New Nanocarriers* **6** 223256

Osman M E, Khattab O H, Zaghlol G M and Abd El-Hameed R M 2011 Optimization of some physical and chemical factors for lovastatin productivity by local strain of *Aspergillus terreus* *AJBAS*. **5** 718-732

Reddy D S R, Latha DP and Latha K P J 2011 Production of lovastatin by solid state fermentation by *Penicillium Funiculosum* NCIM 1174 *Drug Invent. Today*. **3** 75-77

Rinto and Suhartono M T 2016 A Review on 3-hidroksi-3-metilglutaril coenzim a reductase and inhibitor: the medies potential of the enzyme inhibitor *Res. J. Pharm. Biol. Chem. Sci*. **7** 1569-1578

Rinto, Dewanti R, Yasni S and Suhartono M T 2015 Isolation and identification of lactic acid bacteria that produced HMG-CoA reductase enzyme Inhibitor from bekasam as a cholesterol reduction agent *Agritech*. **35** 309-314

Rinto, Dewanti R, Yasni S and Suhartono M T 2017b Novel HMG-CoA reductase inhibitor peptide from *Lactobacillus acidophilus i*solated from Indonesian fermented food bekasam *J. Pharm. Chem. Biol. Sci*. **5** 195-204

Rinto, Nopianti R, Herpandi and Oktaviani S 2017a Fractionation of anticholesterol bioactive compounds from bekasam (Indonesian fermented fish product) *J. Trop. Agric. Sci*. **40** 399-406

Vicente A P, Izquierdo, A G and Viguera C G, 2002 In vitro gastrointestinal digestion study of *Pomegranate* juice phenolic compounds, anthocyanins, and vitamin C *J. Agric. Food Chem*. **50** 2308-2312

Wikandari P R and Lenny Y 2016 The effect of degradation for proteolitik enzime to *Angiotensin Converting Enzym* inhibitor from bekasam by *Lactobacillus plantarum B1765 Agritech*. **36** 170-175

Wu J and Ding X 2002 Characterization of inhibition and stability of soy-protein-derived angiotensin I-converting enzyme inhibitory peptides *Food Res. Int*. **35** 367-375

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