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Fractionation of Anticholesterol Bioactive Compounds from Bekasam (Indonesian Fermented Fish Product)

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ABSTRACT

Bekasam functions as an inhibitor of HMG-CoA reductase. Fractionation was required to determine the bioactive peptide which functions as a HMG-CoA reductase inhibitor. Steps taken for this research were the production of bekasam used salt (15%), rice (15%) and *Lactobacillus acidophilus* as a culture starter, extraction and fractionation of *bekasam* to assay its HMG-CoA reductase inhibition. The results showed that six fractions from *bekasam* extract had different inhibition activity. The fraction of *bekasam* extract without evaporation (F1) contained 3 peptides (peptide of 7.69 kD; 10.71 kD and 20.22 kD). Extract free supernatant fraction (F2) contained 4 peptides (peptide of 7.69 kD; 10.71 kD; 20.22 kD and 35.38 kD). Fractions of *bekasam* extract in the F3 contained 2 peptides (7.69 kD and 10.71 kD). Furthermore, fractionation in the F4 can separate only one peptide band with molecular weight 7.69 kD. Peptides were not discovered in the F3 and F4 fraction while F6 and F4 fractions had the higher inhibition fraction to HMG-CoA reductase activity (92.86%). There was peptide 7.69 kD in F4 fraction and lovastatin (148.30 ppm) in F6 fraction.

Keywords: *Bekasam*, peptide 7.69 kD, anticholesterol, HMG-Coa reductase

INTRODUCTION

The 3-Hidroxy-3-Methylglutaryl-Coenzyme A Reductase (HMG-CoA reductase) is an enzyme which has a limiting factor to regulate cholesterol synthesis, especially in the formation of mevalonic acid from Hidroxy Methylglutaryl-Coenzym A (HMG-CoA). The inhibition to HMG-CoA reductase enzyme can reduce cholesterol in

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the hyperlipidaemia (Lyons & Harbinson, 2009; Rinto, 2016). Statins i.e. compactin, pravastatin, lovastatin, simvastatin (Barrios-Gonzales & Miranda 2010) and some peptides i.e. peptide from herbal *Senna obtusifolia* (Chuhua et al., 2008), potato and soy (Liyanage et al., 2008), milk (Kirana et al., 2005) and fermented fish (Kato et al., 2009) are bioactive compounds that reduce HMG-CoA reductase activity and cholesterol.

Some fermented fish product can block activity of HMG-CoA reductase enzyme. *Narezushi* and *Heshiko* extract are Japanese fermented fish products, containing protein fraction (peptides) and non-protein fraction, which have high inhibition for HMG-CoA reductase (Itou & Akahane, 2009; 2010). *Bekasam* extract (Indonesian fermented fish product) also had high inhibition for this enzyme (Rinto et al., 2015a).

Peptide fractions from bekasam which have activity to inhibit HMG-CoA reductase have not been well studied and documented. This study examined the fractionation of bekasam extract and the content of bioactive peptides that had high inhibition activity to HMG-CoA reductase. In addition to bioactive peptides, peptide profiles were identified and amino acid sequencing was done to discover type of peptide from bekasam that functions as an inhibitor of HMG-CoA reductase.

MATERIALS AND METHODS

Materials

Minnows/carps fish (*Rasbora argyrotenia*) was obtained from Indralaya traditional market, South Sumatera, Indonesia. De Man Rogosa Sharpe (MRS) broth medium were purchased from England. Lovastatin, HMG CoA reductase kit assay, were purchased from Sigma Aldrich (USA). A standard molecular weight protein marker (Low Range Protein Ladder) were purchased from Thermo Scientific (Lithuania). *Lactobacillus acidophilus* was screened and isolated from *bekasam*. All other chemicals were of analytical grade and purchased from the local representative of Sigma and Merck.

The Production of Bekasam with *Lactobacillus acidophilus* as a Culture Starter

Minnows/carps fish (*Rasbora argyrotenia*) (1 kg) was used as main raw material for making *bekasam*. *Minnows/carps* fish was gutted, washed and soaked in the starter culture *Lactobacillus acidophilus* (1 L) for 30 minutes. After that, the fish was separated from bacteria culture. Salt (15%) and rice (15%) was added to the fish and then fermented until seven days to produce *bekasam*.

Extraction of *Bekasam*

Extract of *bekasam* was prepared based on Rinto et al. 2015. Briefly, 10 g *bekasam* was homogenised with 40 mL distilled water. The homogenate was centrifuged at 2000 x g, 4°C for 15 minutes. After separating the first supernatant, 50 mL distilled water was added to the precipitate to obtain the second supernatant in the same manner. These two supernatants were mixed and filtered

through membrane 0.45 µm (Biotechlab, Bulgaria). The filtrate was used in enzyme inhibition assay and its lovastatin content analysed.

Fractionation of *Bekasam* Extract

The purpose of fractionation was to separate bioactive peptides from other compounds in the *bekasam* extract. Fractionation was based on the molecule size using filtration membrane (3 kD and 10 kD MWCO, Thermo-Scientific, UK) and membrane filter 0.02 µm (Whatman, Germany). Six fractions were obtained: non-evaporation fraction (F1) was extracted from *bekasam* using aquabides, free supernatant fraction (F2) was result of evaporation of F1; fraction with molecular weight (MW) of > 10 kD (F3), fraction with MW of 3 – 10 kD (F4), fractions with MW of < 3 kD (F5) and fraction with MW< 1 kD (F6). All fractions (F1-F6) were used for assay of their HMG-CoA reductase inhibitory activity.

Lovastatin Assay

Lovastatin content was detected in the free supernatant fraction (F2). Lovastatin is a bioactive compound with molecular weight < 1 kD and since its content in the *bekasam* was low and thus it didn't need to be fractionated. Lovastatin content was measured using spectrophotometer (UV-Mini-1240, Shimadzu). Five mL of the sample was mixed with 20 mL methanol and the mixture shaken for two hours, before it was filtered through membrane 0.45 µm. The filtrate was centrifuged at 120 x g, 4°C for 15 minutes, and the supernatant was separated. Supernatant (0.5 mL) was mixed with 0.5 mL trifluoracetic acid 1% and incubated for 10 minutes. Homogenate (0.5 mL) was placed in 5 mL volumetric flash and filled up with methanol. The absorbance was measured using spectrophotometer at λ = 238 nm. The concentration of lovastatin was calculated in ppm and corresponded to the calibrate curve. Standard lovastatin at concentrations of 6, 8, 10, 20, 30, 40, 50, and 60 ppm were used (Osman et al., 2011).

HMG-CoA Reductase Inhibition Assay

Supernatant from *bekasam* extract (5 mL) was separated and filtered via 0.45 µm membrane and the filtrate was used in the HMG-CoA reductase inhibition assay using HMG-CoA reductase assay kit containing pravastatin as positive control, HMG-CoA as a substrate, HMG-CoA reductase enzyme, NADPH and assay buffer. The procedure followed manufacturer's instructions. The assay was based on the spectrophotometric measurement of decrease in absorbance at λ = 340 nm, which represented oxidation of NADPH by the catalytic subunit of HMG-CoA reductase in the presence of the substrate HMG-CoA. One unit was defined as 1.0 µmole of NADPH converted to NADP+ per 1 minute. Specific activity was defined as µmol/min/mg-protein (units/mg) (Lachenmeier et al., 2012).

Profile Peptides Assay of *Bekasam* Extract Fractions

The peptide profile was analysed by resolving 15% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel in the 1 M Tris-HCl pH 6.8. The protein fraction was loaded into the wells of the gel and electrophoresed using 1x running buffer (24.8 mMTris, 192 mM glycine, 0.1% SDS, in the pH 8.3). A standard molecular weight marker (Low Range Protein Ladder Thermo scientific, Lithuania) was loaded onto the gel to compare the molecular weights of the proteins/peptides in different samples. After electrophoresis, the gels were stained with silver staining mechanism (Giri et al., 2012).

RESULTS

HMG-CoA Reductase Inhibitor (Lovastatin Content in the *Bekasam* Extract)

Lovastatin is a mine bioactive compound which inhibit HMG-CoA reductase enzyme activity. The content of lovastatin in the bekasam from *minnows/carps* fish produced with starter *Lactobacillus acidophilus* was 148.30 ppm (Table 1). This is higher compared with Rinto et al. (2015a) that revealed the statins content in the bekasam was between 20.98 and 106.42 ppm. In addition to statin, bioactive peptide in the bekasam extract also inhibits HMG-CoA reductase enzyme activity.

Table 1
The yield of bekasam fraction, lovastatin content, peptides and inhibition of bekasam extract fraction for HMG-CoA reductase enzyme activity

No	Sample	Yield (%)	Lovastatin (ppm)	Peptides (kD)	Inhibition of HMG-CoA R (%)
1	F1 (Non-Evaporation)	NA*	NA	7.69 10.71 20.22	66,67
2	F2 (extract free supernatant)	15	148.30	7.69 10.71 20.22 35.38	85,71
3	F3 (MW > 10 kD)	3.8	NA	7.69 10.71	69,05
4	F4 (WM 3-10 kD)	1.7	NA	7.69	92,86
5	F5 (MW 1- 3 kD)	0.37	NA	-	85,71
6	F6 (MW < 1 kD)	0.7	NA	-	92,86

Note. NA: Not analysed

HMG-CoA Reductase Inhibitor (Peptides in the *bekasam* Extract)

Peptides in the *bekasam* extract are produced by microorganisms and enzymes in the fermentation process; *Lactobacillus acidophilus* as a culture starter in the fermented process helps to produce peptides. It produces bioactive peptide 6.3 kD as an inhibitor of HMG-CoA reductase (Rinto et al. 2015a). Profile peptides assay of fractions in the *bekasam* extract resulted some peptides (Figure 1). In the fraction of bekasam extract without evaporation (F1) contains 3 peptides, i.e. peptide with molecular weight 7.69 kD; 10.71 kD and 20.22 kD. Extract with free supernatant fraction (F2) contain 4 peptides (7.69 kD; 10.71 kD; 20.22 kD and 35.38 kD). Nevertheless, peptide bands in the F2 was thicker than in the F1. This indicated that the concentration process by evaporation in 70°C could increase the intensiveness of bekasam extract. Fractions of *bekasam* extract in the F3 contain 2 peptides band, i.e. peptide with molecular weight 7.69 kD; 10.71 kD. Furthermore, fractionation in the F4 can separate only one peptide band with molecular weight 7.69 kD. In the F3 and F4 fraction there were no peptides band. This showed that there were no peptides with molecular weight less than 3 kD (Table 1 and Figure 1).

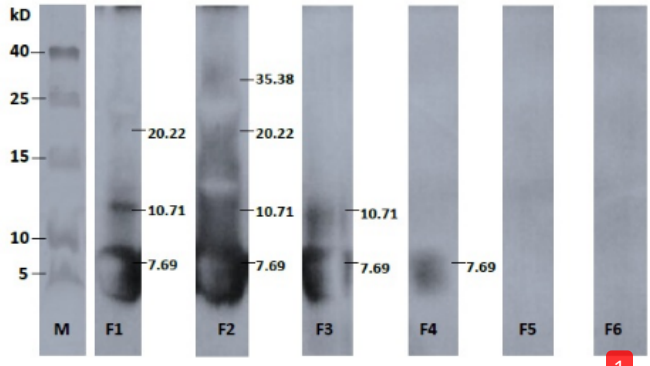

Figure 1. Peptides profile of *bekasam* extract (F1: non-evaporation *bekasam* extract; F2: extract free supernatant; F3: fraction of *bekasam* extract with molecule weight > 10 kD; F4: fraction of *bekasam* extract with molecular weight 3-10 kD; F5: fraction of *bekasam* extract with molecular weight 1-3 kD and F6: fraction of *bekasam* extract with molecular weight < 1 kD)

Inhibition of *Bekasam* Extract

Inhibition assay of each fractions for *bekasam* extract resulted in different inhibition to HMG-CoA reductase enzyme. Overall, inhibition of bekasam extract fractions to HMG-CoA reductase is more than 60%. It indicated that fractions of *bekasam* extract had high level of inhibition to HMG-CoA reductase enzyme. This finding corresponds with a past study

which revealed that the bekasam extract was able to inhibit the activity of HMG-CoA reductase by 64.44% (Rinto et al., 2015a). Crude *bekasam* extract treated without evaporation (F1) had the lowest inhibition. . This caused the concentration of bioactive compounds (peptides) to be lower than the other fractions (Figure 1). The highest inhibition was found inF4 and F5 (92.86%). In F4 fractions contained one band of peptides with a molecular weight of 7.69 kD while there was no band of peptide in F6. This indicated that the F6 fraction of *bekasam* extract involved in inhibition to the enzyme HMG-CoA reductase was lovastatin, while the F4 was a peptide with a molecular weight of 7.69 kD.

DISCUSSION

Fermentation is a chemical process where a substance breaks down into a simpler one. *Bekasam* is one of the fermented fish products. In the fermentation of bekasam, protein is converted to peptides or amino acid by indigenous enzyme and microorganisms. Some microorganisms that are responsible for fermentation is *Lactobacillus plantarum, Lactobacillus mesenteroides, Lactobacillus brevis, Pediococcus*, and *Leuconostoc* (Rhee et al., 2011; Wikandari et al., 2012). Lactobacillus acidophilus are known as lovastatin and peptides producer bacteria which functions as a HMG-CoA reductase inhibitor. Therefore, the use of Lactobacillus acidophilus as a starter culture in the fermentation of bekasam increases the bioactive compounds of HMG-CoA reductase inhibitors (Rinto et al. 2015b).

The extraction and fractionation of *bekasam* produced a peptide fraction (F4) and lovastatin (F6); they displayed high inhibition to HMG-CoA reductase enzyme with a value of 92.86% each . An earlier study showed the inhibitor activity of bekasam extract was 64.44% (Rinto et al., 2015a), it indicates that fractionation process can increase the activity level of inhibition to HMG-CoA reductase enzyme. The evaporation process as are result of *bekasam* extract has an effect on the inhibiting activity causing the peptide to become concentrated. Increasing the concentration and purity of the inhibitor can increase level of inhibition to enzyme activity.

The present of lovastatin in F6 fraction and peptide of 7.69 kD in F4 fraction showed the role of *Lactobacillus acidophilus* as a producer of lovastatin and peptides in the fermentation of *bekasam*. A previous study showed that *Lactobacillus acidophilus* produced lovastatin and peptide as an inhibitor of HMG-CoA reductase. The utilisation of *Lactobacillus acidophilus* as a starter culture in the *bekasam* fermentation improved inhibition of bekasam extract to the activity of HMG-CoA reductase, although the content of lovastatin did not increase. This shows the use of *Lactobacillus acidophilus* as a starter culture in *bekasam* fermentation is important to improve the bioactive peptide as an inhibitor of HMG-CoA reductase enzyme.

CONCLUSION

Fractionation of *Bekasam* extract produces peptide (7.69 kD) and lovastatin (148.30 ppm) which display high inhibition to HMG-CoA reductase enzyme. The use of *Lactobacillus acidophilus* as a starter culture in the fermentation of *bekasam* could increase activity level of peptide (7.69 kD) that functions as HMG-CoA reductase inhibitor.

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