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Peptides Hydrolysate Derived from Collagen of Snakehead Murrel (*Channa striata*) Skin Demonstrate Anti-cholesterol and Anti-oxidant activities

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ABSTRACT

Anti-cholesterol and anti-oxidant play a crucial role to combat cardiovascular disease (CVD), due to formation of arterial plagues from oxidation of cholesterol. In the past decades, bioactive peptides demonstrating anti-cholesterol and anti-oxidant activities have emerged as the alternative drugs. In this study, acid soluble collagen was extracted from the skin of snakehead murrel and employed to induce secretion of collagenase by *Bacillus licheniformis* F11.4. The collagenases secreted were in turn used to produce peptides hydrolysate and were grouped in two distinct collagenase fractions, designated as fraction D and F. Peptides hydrolysate produced by the fraction D was found to demonstrate HMG-CoA inhibitor activity comparable to pravastatin and limited anti-oxidant activity. Meanwhile, peptides hydrolysate generated using the fraction F demonstrated anti-oxidant activity comparable to BHT (2mM), vitamin C (2mM), and vitamin E (2mM), but limited HMG-CoA activity. Combination of the fraction D and F resulted in substantial HMG-CoA inhibition and anti-oxidant activities.

1. Introduction

Bioactive peptide (BP) is derived from proteins that are hydrolyzed by proteolytic enzymes (Korhonen and Pihlanto-Leppala 2006) or acid. BP has been developed into anti-hypertension, anti-oxidant, anti-thrombotic, and hypo-cholesterolemic drugs, which are powerful to prevent degenerative diseases, such as cardiovascular disease (CVD). Protein for the source of BP can be of plants, meat, milk (Korhonen and Pihlanto-Leppala 2003, 2006), and fish (Shahidi 1995; Senevirathne and Kim 2012). An example of anti-oxidative BP is lunasin, which is identified in soybean and other plants (Gálvez and de Lumen 1999). This peptide is already commercialized in the US and reported to decrease low density lipoprotein (LDL) and cholesterol in the blood (Gálvez 2012).

Recent studies have shown that anti-oxidative BP can be released from casein through enzymatic hydrolysis or during fermentation of milk using protease-producing lactic acid bacteria (Korhonen and Pihlanto-Leppala 2003). The anti-oxidative BP displays free radical scavenging activities and inhibits enzymatic and non-enzymatic lipid peroxidation, most likely for being a preferred target over fatty acid free radicals (Rival *et al.* 2001). Consumption of anti-oxidative BP derived from goat is reported to show anti-atherogenicity by prolonging resistance of the lipoprotein fraction to oxidation (Kullisaar *et al.* 2006).

Snakehead murrel is one of the freshwater fishes from *Channidae* family with high collagen content in their skin. The skin of the fish has been considered as waste in the fish processing. In this study, we had extracted collagen from fish skin by acid treatment and found that the peptides hydrolysate derived from collagen was able to inhibit the activity of HMG-CoA reductase, the key enzyme for cholesterol biosynthesis. We also detected anti-oxidant activities with this peptides hydrolysate. The peptides were produced by proteolytic digestion using collagenases from *B. licheniformis* F11.4, a mutant

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of *B. licheniformis* from Indonesia. This bacterium has previously been shown as to demonstrate high proteolytic and collagenolytic activities (Waldeck *et al.* 2006), secreting collagenases of 124 kDa and 26 kDa when grown in the presence of water-soluble collagen derived from milkfish skin (Baehaki *et al.* 2012; Baehaki *et al.* 2014). Our present study revealed the potential use of the peptides hydrolysate derived from the snakehead murrel skin acid soluble collagen as anti-cholesterol and anti-oxidant agents. This is the first report on the use of bioactive peptides hydrolysate derived from collagen of snakehead skin. Also, while most of marine bioactive peptides are developed as anti-hypertension agent, this report explored the possibility of application of marine bioactive peptides as anti-cholesterol and anti-oxidant instead. This report paves foundation for further identification of the bioactive peptides and structural-function study of the enzymes.

2. Materials and Methods

2.1. Organisms and Materials

Bacillus licheniformis F11.4 was kindly provided by the Indonesian government agency for assessment and application of technology (BPPT). The bacterial mutant is derived from *B. licheniformis* F.11, which was discovered during research collaboration between BPPT and Munster University-Germany under the Indo-German Biotechnology scheme. Collagen from snakehead fish skin was prepared according to Singh *et al.* (2011). Chemicals were purchased from Merck, Sigma, or Oxoid, through local distributors, except when specifically mentioned.

2.2. Production and Partial Purification of Collagenases

B. licheniformis F11.4 was grown in a medium containing 1% NaCl, 0.5% tryptone, 0.25% yeast extract, and 5% collagen, for 24 hours at 37°C with agitation speed of 120 rpm. Collagenase secreted into the medium was harvested by cold centrifugation (4°C) for 15 minutes at 7,500 g prior to ammonium sulphate precipitation at 50% saturation (w/v). The protein precipitate was dissolved in 0.02 M phosphate buffer, pH 8.0, and dialyzed (MWCO 10 kDa) overnight against 0.01 M phosphate buffer, pH 8.0, at 4°C. The dialyzed sample, designated as the crude enzyme, was applied to DEAE-Sepharose anion exchanger column (GE Healthcare Life Science, Pittsburg–PA, USA) that had previously been equilibrated with 0.02 M phosphate buffer, pH 8.0. The column was eluted with a gradient of 0-1 M NaCl in 0.02 M phosphate buffer, pH 8.0, at a flow rate of 0.5 ml/min. The purification was performed on Äkta purifier system (GE Healthcare Life Science, Pittsburg–PA, USA) at room temperature (25°C).

2.3. Collagenase Assay and Protein Concentration Determination

Protease activity was measured using 5% collagen as the substrate (Bergmeyer *et al.* 1983). Briefly, 50 µl of enzyme was mixed with 250 µl of substrate. The mixture was incubated for ten minutes at 37°C. The enzymatic hydrolysis was stopped by addition of 1,250 µl of 0.2 M trichloroacetic acid (TCA), followed by cold centrifugation at 4,000 g for ten minutes. The supernatant was mixed with 0.4 M Na_2CO_3, followed by addition of Follin reagent at a ratio of 1:2. The mixture was further incubated at 37°C for 20 minutes. The amount of amino acid produced was measured at 578 nm. One unit of enzyme activity (U) is defined as the amount of enzyme required to produce 1 µmol of amino acid per minute under specific conditions. Protein concentration was determined according to Bradford (1976) using bovine serum albumin (BSA) as the standard.

2.4. SDS PAGE and Zymogram

Molecular weight of proteins was estimated using SDS-PAGE (Laemmli 1970). Enzymes activity in situ was demonstrated in a zymogram gel (Choi *et al.* 2000), using 8% non-denaturing PA gel containing 0.1% (w/v) collagen.

2.5. Preparation of Bioactive Peptides Hydrolysate

One ml of partially purified enzymes (0.4-0.7 mg/ml) was added into 50 ml collagen solution (0.5-1.0 mg/ml) and mixed with 40 ml phosphate buffer 500 mM. The mixture was incubated for 120 minutes at 40°C. The enzymatic hydrolysis was stopped by an addition of 250 mM TCA. The peptide solution was recovered after centrifugation at 4,000 g in 4°C and stored at -20°C. For inhibition studies, similar reaction was conducted at small scale (one tenth) in the presence of 20 µl of 5 mM ethylenediaminetetraacetatic acid (EDTA) or phenylmethanesulfonylfluoride (PMSF).

2.6. HMG-CoA Reductase Assay

HMG-CoA reductase assay was performed according to Perchellet *et al.* 2009. Prior to the assay, a spectrophotometer was conditioned at 37°C and the measurement was carried out at 340 nm with and interval reading of 15 seconds for five minutes. The reaction mixture contained 5 µl Pravastatin or peptides hydrolysate (the assay buffer as the blank), NADPH, HMG-CoA substrate, and HMG-CoA Reductase (HMGR) (the assay buffer for the negative control and blank). The reaction mixture was thoroughly mixed prior to measurement in the spectrometer. The specific activity of enzyme was calculated according to the following equation:

$$\text{Units/mgP} = \frac{(\Delta A340/\text{min sample} - \Delta A340/\text{min blank}) \times TV)}{12.44 \times V \times 0.6 \times LP}$$

Where:

12.44 : ε^{mM}-the extinction coefficient for NADPH at 340 nm is 6.22 $mM^{-1}cm^{-1}$. 12.44 represents the 2 NADPH consumed in the reaction
TV : total volume of the reaction in ml (ml)
V : volume of enzyme used (ml)
0.6 : enzyme concentration (mg/ml)
LP : Light path in cm (1 cm)

One unit of enzyme is defined as the amount of enzyme required to convert one milli mole of NADPH to $NADP^+$ per minute at 37°C.

2.7. 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Activity Assay

The anti-oxidant measurement was performed according to Li *et al.* 2007. Briefly, 500 µl of peptide solution was mixed with 500 µl of 99.5% ethanol and 125 µl 0.02% DPPH in 99.5% ethanol. The mixture was kept in the dark for one hour at room temperature. Degradation of DPPH was measured at 517 nm. Radical scavenging activity (RSA) was calculated as a percentage of the activity of the control (absence of antioxidant).

$$\text{RSA} = \frac{\text{Control} + (\text{Blank} - \text{sample})}{\text{Control}} \times 100\%$$

3. Results

3.1. Partial Purification and Characterization of Collagenases

Fractions collected during elution of DEAE anion exchanger column with NaCl at a gradient concentration of 0-1 M, showed the presence of at least seven collagenases (b1-b7) secreted by *B. licheniformis* F11.4 (Figure 1). SDS-PAGE present in Figure 1 shows that is C is crude collagenase and D is collagenase after ammonium sulphate addition. The collagenases were classified by means of protease inhibitors such as, PMSF (superscript P) for serine protease and EDTA (superscript E) for metallo-protease (Figure 2).

Interestingly, the inhibition profile identified serine protease (b1), metallo-protease (b2), and neither of both (b3-b7). The zymography assay may indicate the presence of possible dimer, trimer, or other oligomeric enzyme states, which are undetectable in SDS PAGE under denaturing conditions. Further, b7 was discovered to actually contain likely two species, which are neither serine nor metallo-protease (fraction 27) and a serine protease (40 and 50). The latter was designated as fraction F while fractions 26-34 were combined and designated as fraction D. Henceforth, the collagenase samples used were addressed as the fraction D and F. The b7 species (Figure 1) was probably a very small enzyme or an enzyme fragment that still contains the active site of collagenase. Unfortunately, this molecular information cannot yet be disclosed. Nevertheless, the characterization of partially purified enzyme indicated the presence of more than one collagenase with different characteristics.

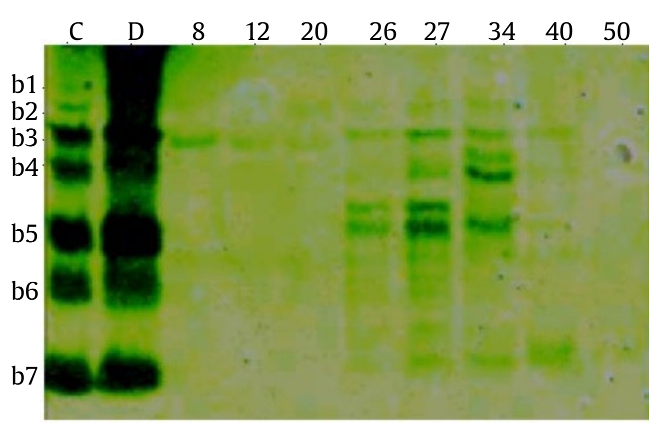

Figure 1. SDS-PAGE profile of the enzymes after purification with ammonium sulphate added and anion exchanger column. C: crude enzyme, D: after ammonium sulphate fractionation, 8-50 are fractions of enzymes

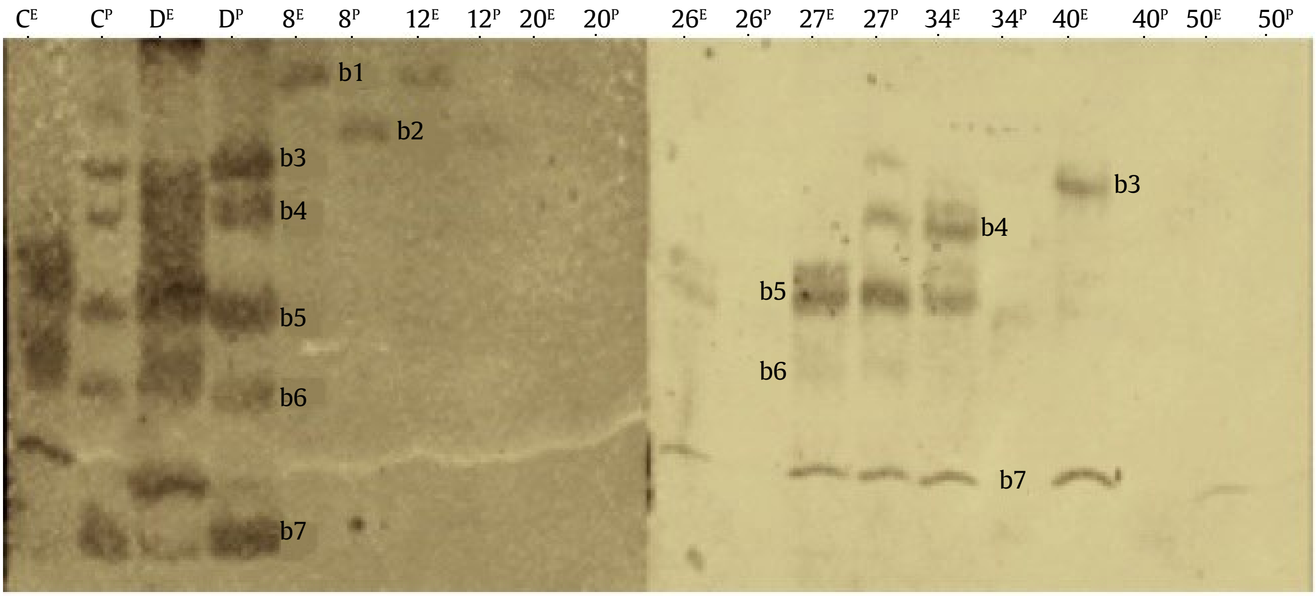

Figure 2. Zymography analysis of enzyme upon challenged with proteases inhibitors. C is crude enzyme, D is after ammonium sulphate fractionation, and 8-50 are fractions numbers of enzyme. Notation E and P (superscript) S refers to EDTA and PMSF, respectively

3.2. Anti-cholesterol Activity

Anti-cholesterol potential of the peptides hydrolysate generated from the snakehead skin collagen by the collagenases of *B. licheniformis* F11.4 was evaluated by means of inhibition of the activity of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) (0.5-0.7 mgP/ml) using the well-known anti-cholesterol drug pravastatin as the reference. Samples employed were collagen treated with enzymes of fraction D or F (in the presence and absence of 5 mM PMSF or 5 mM EDTA) and non-hydrolyzed snakehead skin collagen (for the basal activity). The result is presented in Figure 3. Interestingly, peptides hydrolysate generated by fraction D displayed similar inhibition power of HMGR activity to that of pravastatin. Further, treatment of fraction D with PMSF and/or EDTA brought down the inhibition to the basal level. On the other hand, peptides produced by fraction F demonstrated only 50% inhibition to HMG-CoA activity in comparison to pravastatin. However, the activity of fraction F appeared to be sustained upon treatment by both EDTA and PMSF. Fraction D is enigmatic because inhibition of one or few of its enzyme components by EDTA and/or PMSF resulted in impaired bioactive peptides production. Corresponding to the characteristics of collagenases

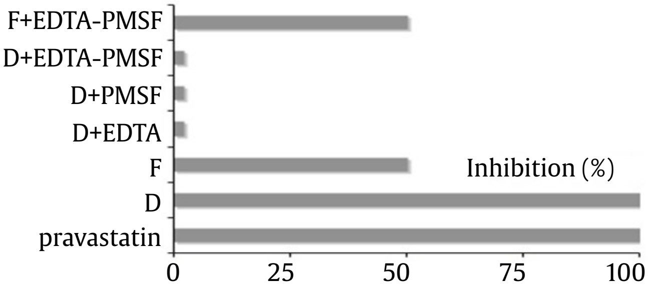

Figure 3. Inhibition of HMG-CoA reductase

in fraction D, this result suggested that the bioactive peptides were produced by the mixture of serine and/or metallo-proteases, thus b1 and/or b2.

3.3. Anti-oxidant Activity

Anti-oxidant activity was evaluated by measuring reduction of DPPH. Vitamin C (2 mM), vitamin E (2 mM), and BHT (2 mM) were employed as the references whilst non-hydrolyzed snakehead fish collagen as the basal. The result of radical scavenging challenge assay (Figure 4) suggested that the peptides hydrolysate generated by fractions D demonstrated no or negligible anti-oxidant activity, while peptides generated by fractions F showed up to 10% scavenging activity. Interestingly, the mixture of peptides hydrolysate generated by mixture of fraction D and F showed up to 20% scavenging

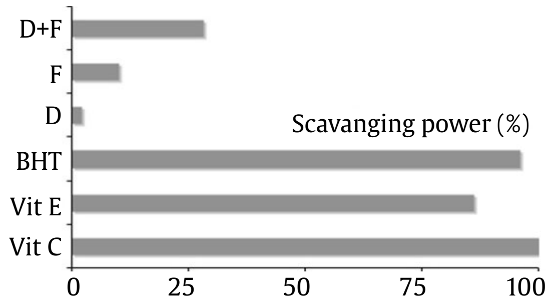

Figure 4. Antioxidant activity

activity. This suggests that components of the two enzyme fractions operate synergic.

4. Discussion

Snakehead fish (*Channa striata*) has been exploited for application in protein therapy, such as wound healing (Baie and Sheikh 2000). The skin of the fish contains high amount of proteins in a form of collagen (17% w/w) that can be used, for example, to prepare gelatin (See *et al.* 2010). The skin part is mostly discarded after the fish processing (Shahidi 1995), thus employing it as the collagen for generation of bioactive peptides is advantageous.

Peptides hydrolysate from snakehead fish skin collagen was generated through enzymatic hydrolysis using collagenases from *B. licheniformis* F11.4, a mutant of *B. licheniformis* 11 from Indonesia (Waldeck *et al.* 2006). Although SDS PAGE and zymography analysis were unable to clearly classify all collagenase fractions secreted, the inhibition study clearly shown the presence of serine-, metallo-, and neither of both types of proteases. The previous study using collagen from the skin of milkfish (*Chanos chanos*) indicated secretion of only two metallo-collagenases with apparent molecular weight of 26 kDa and 124 kDa (Baehaki *et al.* 2012; Baehaki *et al.* 2014). Unfortunately, this study could not clearly identify the molecular weight of each collagenase species because the various bands appeared in both SDS PAGE and zymography analysis but they are not entirely correlated. We could not yet exclude the presence of various oligomeric or even partial degradation forms of the enzymes. This issue would be solved in a peptide-mass finger printing analysis, which would be the next experiments to do. In particular, species b3 to b6 demonstrated similar bioactivity during the collagenase assay *in situ*, during which all were identified as neither serine nor metallo-protease. This finding is interesting because most of collagenases from *Bacillus* are characterized as metallo-protease (Liu *et al.* 2010; Baehaki *et al.* 2012; Baehaki *et al.* 2014), serine protease (Nagano and To 1999), or Ca^{2+}-dependent and with disulfide bonds collagenase (Wu *et al.* 2009). Also, the finding of species F is very interesting because the enzyme is significantly smaller than the reported collagenases from other strains of *Bacillus*, e.g. from *B. cereus* MBL13 (~38 kDa), *B. circulans* (~39 kDa), *B. cereus* Soc67 (~88kDa), *B. cereus* non-haemolytic enterotoxin (Nhe) (~105 kDa), and *B. substilis* FS-2 (~125 kDa) (Maäkinen and Maäkinen 1987; Lund and Granum 1999; Nagano and To 1999; Rao *et al.* 2008; Liu *et al.* 2010). This small enzyme species could have been a product of autolysis of the larger collagenase and still contains the active site (Vasilyeva 2002). Similar situation has been reported for an esterase from *Emericella nidulans* and *Taralomyces emersonii* (~1.6 kDa), which is called microenzyme (Fan and Mattey 1999). Further characterization of the enzyme in fraction F would be very interesting for future application of microenzymes.

Fractions D and F were challenged to produce the snakehead fish skin collagen peptides hydrolysate for inhibition of HMGR activities and radical scavenging. HMGR is responsible for synthesis of mevalonate, and considered as the key enzyme for biosynthesis of cholesterol and other non-steroidal isoprenoid compounds (Arnaud 2005). Therefore, controlling the HMGR activity may lead to control the synthesis of cholesterol. Peptides hydrolysate produced by both fractions D and F demonstrated full and 50% inhibition to HMGR activity, respectively. However, fraction D was unable to produce the peptides hydrolysate in the presence of EDTA and/or PMSF. On the other hands, fraction F still hydrolyzed the collagen to produce bioactive peptides hydrolysate in the presence of the protease inhibitors. Combining the fractions D and F to produce bioactive peptides hydrolysate with anti-cholesterol functionality could be an intelligent option. This option may coincide with the finding of higher anti-oxidant activity of peptides hydrolysate recovered from hydrolysis of collagen by the combined fractions D and F. Thus, combined fractions D and F produced bioactive peptides hydrolysate with high anti-cholesterol activity accompanied by anti-oxidant activity, which leads to lower cholesterol level as well as prevention of radical formation in the blood. This circumstance would be ideal to combat development of cardiovascular diseases.

Conflict of Interest

The authors declare no conflict of interest on the publication of this article.

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