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Collagen Peptides from Fish Skin with Angiotensin I-Converting Enzyme (ACE) Inhibitor and Cancer Antiproliferative Activity.

Ace Baehaki[1]*, Maggy T. Suhartono[2], Sukarno[2], Dahrul Syah[2], and Siswa Setyahadi[3].

[1]Department of Fisheries Product Technology, Faculty of Agriculture, Sriwijaya University, Indralaya, Ogan Ilir, South Sumatera, Indonesia.
[2]Department of Food Science and Technology, Faculty of Agricultural Technology, Bogor Agricultural University (IPB), Bogor, West Java, Indonesia.
[3]Agency for the Assessment and Application of Technology (BPPT), Republic of Indonesia, Jakarta, Indonesia.

ABSTRACT

Bioactive peptide from fish skin collagen was produced using collagenase from *Bacillus licheniformis* F11.4 of Indonesia origin. Bioactive peptide from skin fish collagen using collagenase from *Bacillus licheniformis* F11.4. Ammonium sulphate precipitated enzyme were applied on 0.54 g/ml collagen. The peptides were evaluated for Angiotensin I-Converting Enzyme Inhibitor and anti-cancer activity. Collagen peptides exhibited the highest activity of Angiotensin I-Converting Enzyme Inhibitor at 60 min at 0.032 Unit/ml and 0.016 U/ml of enzyme activity. Collagen peptides exhibited the highest activity at 60 min towards HeLa cells, and the highest activity at 30 min towards HCT-166 cells. Therefore, collagenase from *Bacillus licheniformis* F11.4 could be used to produce the collagen peptides possessing Angiotensin I-converting Enzyme Inhibitor and anti-cancer activity.

Keywords: Angiotensin I-Converting Enzyme; Inhibitor; anti-cancer; collagen; *Bacillus licheniformis* F11.4.

Corresponding author

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INTRODUCTION

Hydrolysates can be defined as protein that are chemically or biologically broken down peptides of varying size. Chemical hydrolysis used more commonly in industrial practice, but biochemical hydrolysis holds the most promises for the future because it results in food grade products of high functional and nutritive value. Biochemical hydrolysis is performed by utilizing enzyme to hydrolyze peptide bonds that can be done via proteolytic enzyme already present in bacteria or by adding enzyme from other sources. The process of using enzymes offers many advantages because it allowed good control of the hydrolysis at a low cost with good properties of resulting products. By applying enzyme technology, it may be possible to produce a board spectrum of food ingredient or industrial products for a wide range of applications [1]

Representatives of the genus *Bacillus* constitute the major bacterial workhorses for industrial enzyme production. Among these, *Bacillus licheniformis* (regarded to GRAS) plays the most prominent role since it is known to produce high enzyme protein. Collagenase is an enzyme that is highly specific for both native and denatured collagen. It is widely used in medical industries, molecular biology experiments, as well as in the food industry [2]. There had been several *B. licheniformis* strains collected in our laboratory which were known to produce protease and collagenase [3]. Collagenase extracted from viscera organ of fish or other animals [4] is usually not cost-effective. *Bacillus* producing collagenase such as: *Bacillus licheniformis* N22 [5], *Bacillus subtilis* FS-2 [6], *Bacillus* sp. MO-1 [7], *Bacillus subtilis* CN2 [8], and *Bacillus* pumilus COI-J [9] have been reported.

Peptides derived from hydrolyzed proteins have been shown to have ACE inhibitor activities. Fish protein hydrolysate such as peptide from tuna [10], sardine [11], and seaweed pipefish [12] have been reported to exhibit angiotensin I-converting enzyme (ACE) inhibitor activities. Morever, preliminary data suggest that hydrolysated fish protein could represent an interesting source of anticancer peptides [13, 14], anti anemia agent [15], and antioxidant [16-18].

In search of the protease producing microorganisms which can be applied in deproteination process for chitin extraction from the shrimp waste, we screened and found isolate *Bacillus licheniformis* F11. Two mutants of *B. licheniformis* F11 were found : *B. licheniformis* F11.1 which lack of gene encoding *ChiA*, and *B. licheniformis* F11.4 which lack of gene encoding *ChiA* and *B* [19]. Therefore, this study aimed to produce a collagen peptide from skin milk fish using collagenase from *Bacillus licheniformis* F11.4 and to study their angiotensin I-converting enzyme inhibitors and cancer antiproliferative activity.

MATERIALS AND METHOD

Microorganism

The *Bacillus licheniformis* F11 was screened from a total of 109 isolates originated from Palembang South Sumatera during exploration of chitinase and protease producing indigenous microorganisms.

Assay of Collagenase Activity and Protein Determination

Collagenase activity was measured according to the Bergmeyer method (20] with collagen from fish skin (5%) as the substrate. As much as 50 µl enzyme filtrate was mixed with 250 µl substrate and incubated for 10 minute at 37 ^{0}C . Trichoracetic acid (TCA) 0.2 M was added and incubated at 37 ^{0}C for 10 minutes, followed by centrifuged at 4000 g 10 minutes. The supernatant was mixed with Na_2CO_3 0.4 M, followed by addition of Folin Ciocalteau reagent (1:2) and incubation furthur at 37 ^{0}C for 20 minutes. The reaction products was measured at λ 578 nm. Substrate solution without enzyme was used as control. One unit (U) of enzyme activity was defined as enzyme which produce 1 µ mole of tyrosine per min.

Protein concentration was analysed by Bradford's method [21] using reagents consisted of 100 mg comassie brilliant blue (CBB) G-250 in 50 ml ethanol 95 % and 100 ml phosphate acid 85 % in 1 liter. Bovine serum albumin was used as the protein standard. Triplicate experiments were conducted for each measurements.

Preparation of Collagen from Fish Skin

Collagen was made from Milk Fish (*Chanos chanos*) skin to the following method. To remove non-collagenous proteins, the prepared skin fish was mixed with 0.1 mol/L NaOH at a solid (fish skin) to alkali solution (NaOH) ratio of 1:10 (w/v), followed by continuous stirring for 6 h using an overhead stirrer. The alkali solution was changed every 2 h. Pretreated fish skin was soaked in 1.5% acetic acid with a solid to solvent ratio of 1:2 (w/v) for 24 h. Skin was wash with cold water until neutral pH, followed by extraction with aquades with a solid to solvent ratio of 2:1 (w/v) for 3 h at 50^0 C. The Result of extraction was collagen at protein 0.54 mg/ml.

Effect of ammonium sulphate enzyme and hydrolysis time on Degree of Hydrolysis (DH)

Collagen solutions were incubated at optimal temperature for proteolytic activity of each species for 10 min. Ammonium Sulphate (AS) fraction of collagenase from *Bacillus licheniformis* F11.4 was added into the mixtures. At hydrolysis time designated (0, 15, 30, 60, and 90 minutes), 1 mL of sample was taken and mixed with 1 mL of 1% SDS solution (90 °C) before placing in a water bath at 90 °C for 10 min. The degree of hydrolysis (DH) of gelatin hydrolysate was analyzed according to the method of Benjakul and Morrissey [22]. The samples (125 µL) were added with 2.0 mL of 0.2 M phosphate buffer, pH 8.2 and 1.0 mL of 0.01% TNBS solution. The solution was mixed thoroughly and placed in a water bath at 50 °C for 30 min in dark. To terminate the reaction, 2.0 mL of 0.1 M sodium sulfite was added. The mixtures were cooled for 15 min at room temperature. The absorbance was measured at 420 nm and α- amino acid content was expressed in terms of L-leucine. DH was calculated as follows:

$$DH= [(L_t-L_0)/(L_{max}-L_0)] \times 100$$

where Lt is the amount of α-amino acid released at time t. L0 is the amount of α-amino acid in original gelatin solution. Lmax is the total α-amino acid in original gelatin solution obtained after acid hydrolysis with 6 N HCl at 100 °C for 24 h.

Protein pattern of collagen hydrolysate

Protein pattern of fish skin collagen hydrolysates were determined using SDS-PAGE with 8 % polyacrylamide gels [23]. The standard moleculer weight markers were phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa) and lysozyme (14.3 kDa).

Angiotensin I-Converting Enzyme (ACE)Inhibitory Activity

The ACE (Angiotensin I-Converting Enzyme) inhibitory activity was assayed by modification of the method of Cushman and Cheung [24]. A sample solution (50 µl) with 50 µl of the ACE solution (25 mU/ml) was pre-incubated at 37^oC for 5 min, then incubated with 150 µl of the substrate (8.3 mM Hip-His-Leu in a 50 mM sodium borate buffer containing 0.5M NaCl at pH 8.3) at 37 ^oC for 60 min. The reaction was stopped by the addition of 250 µl of 1M HCl. The resulting hippuric acid was extracted with 1.5 ml of ethylacetate. After centrifugation (800 × *g*, 15 min), 1 ml of the upper layer was transferred into a glass tube, and evaporated at room temperature for 2 h under vacuum. The hippuric acid was dissolved in 3.0 ml of distilled water. The absorbance was measured at 228 nm using Spectrophotometer.

Cancer Antiproliferation Activity

Effects of collagen peptides on cell cancer (HeLa and HCT-116) proliferation were measured using MTT assay (Roche Applied Science, Indianapolis, IN). This assay was based on the reduction of a tetrazolium salt (MTT) into yellow formazan salt by active mitochondria. For proliferation tests, cells were transferred into a 96-well microtitre plate at a density of 7000 cells by well in 100 ll of medium (DMEM, 4.5 g/l glucose supplemented with 10% of fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml of streptomycin) and in the presence or in the absence of fish collagen peptide at 1 mg/ml concentrations. After 48 h at 37 ^{0}C, 5% CO2 atmosphere, cells were incubated in the presence of MTT for 4 h. Absorbance (490 nm,

against 655 nm reference) in each well was measured in a microplate reader spectrophotometer (Benchmark, Biorad Laboratories, Hercules, CA). Results were expressed as percentages of basal growth activity.

RESULT AND DISCUSSION

Degree of hydrolysis

Enzymatic hydrolysis of protein is one of the technique to improve the functionalities of protein. These in turn are dependent on the nature of the protein and the specificity of the enzyme used, as well as on the hydrolysis conditions, particular pH and temperature. In the current study, the biological activities of collagen hydrolysates were investigated, on which there are relatively few studies in the literature.The progression in DH during the hydrolysis of by collagenase from *Bacillus licheniformis* F11.4 is shown in Fig. 1.

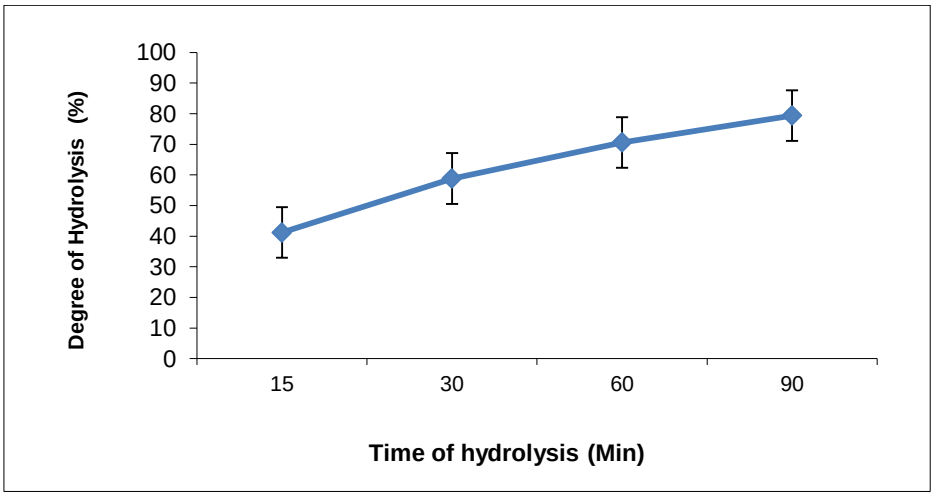

Figure 1: Degree of hydrolysis collagen peptide by collagenase *B. licheniformis* F11.4

The DH value increased during hydrolysis time, reaching 79.41% in 90 min. Degree of hydrolysis collagen from skin milk fish (*Chanos chanos*) treated with collagenase increased linearly with the increment of incubation time. The result was in agreement with previous studies. Guerard *et al.* [25] found that DH increased as incubation time increased on the hydolysis of yellow fin tuna by alcalase and umamizyme. Norma *et al*. [26] reported the same conclusion for threadfin bream hydrolysis by alcalase.

Protein pattern of collagen hydrolysate

Protein patterns of collagen is shown in Fig. 2. There is difference in protein pattern of collagen with time hydrolysis and different activity of enzyme (0.032 U/ml and 0.016 U/ml). When fish skin collagen was hydrolyzed to different activity of enzyme, the lower molecular mass proteins or peptides were obtained with increasing activity of enzyme. No β-chain and α-chain were remained in hydrolysates with activity of enzyme is 0.032 U/ml.

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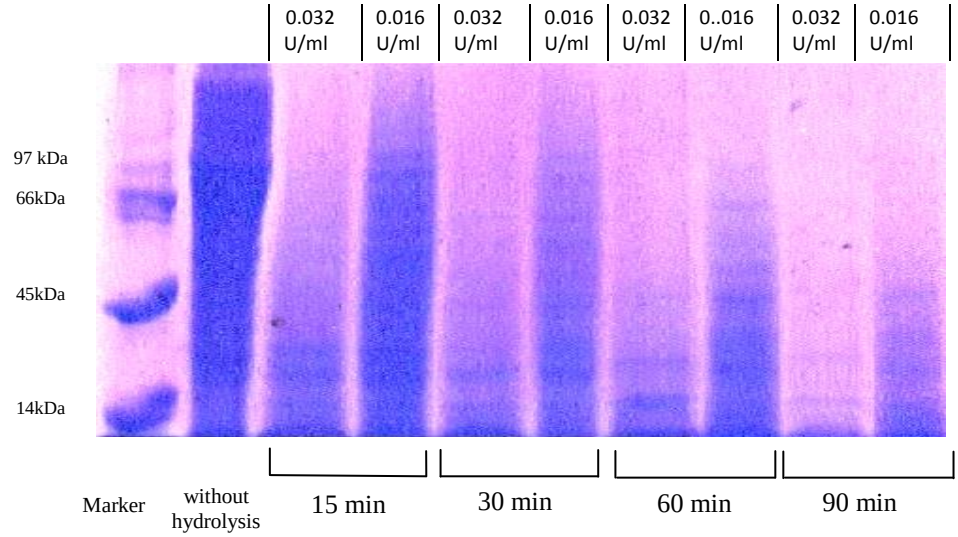

Figure 2: Pattern protein of collagen peptide by collagenase *B. licheniformis* F11.4

In general, the proportion of high molecular mass peptides in cod byproduct [27] and salmon byproducts [28] decreased with increasing DHs. During hydrolysis, enzymatic breakdown of protein involves a major structural change, in which the protein is slowly hydrolyzed into smaller peptide units [29].

Angiotensin I-Converting Enzyme (ACE) Inhibitory Activity

Fig. 3 shows the Angiotensin I-Converting Enzyme (ACE) inhibitory activity of the collagen peptides. The results indicated that collagenase from *B.licheniformis* F11.4 generated ACE inhibitory from the collagen peptides. ACE inhibitory activity of the collagen peptides from application of collagenase with different time of hydrolysis and activity of enzyme are depicted in Fig. 3. The collagen peptides exhibited the highest activity at 60 min incubation with 0.032 Unit/ml and 0.016 U/ml of enzyme activity. ACE inhibitory activity peaked after 60 min of hydrolysis, and decreasing thereafter.

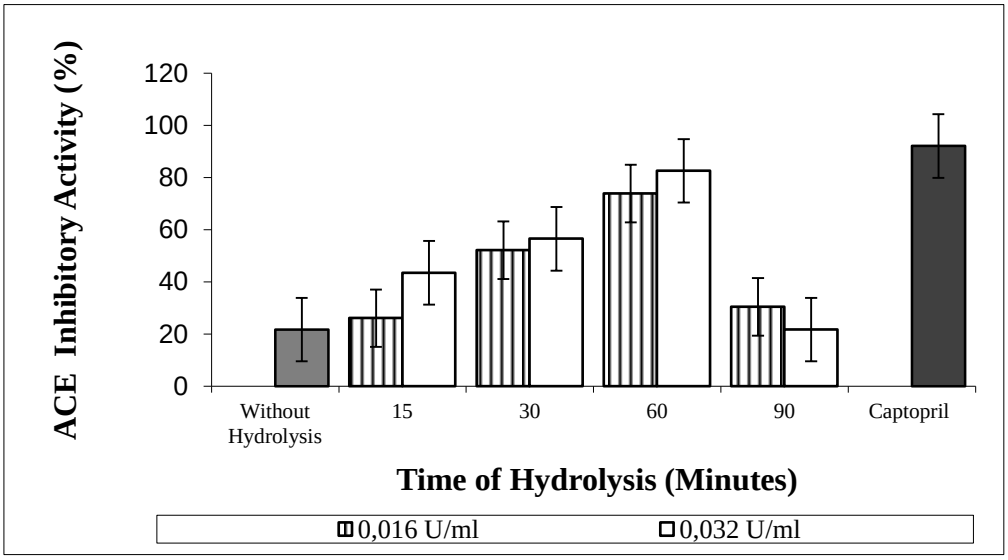

Figure 3: ACE inhibitory activity of collagen peptide. Bars represent the standar deviation from triplicate determinations.

The collagen peptides has ACE inhibitory activity may relate with it's proline at C-terminal part Proline or aromatic amino acids existing at the C-terminal are suitable for peptide binding to ACE [30]. Other studies

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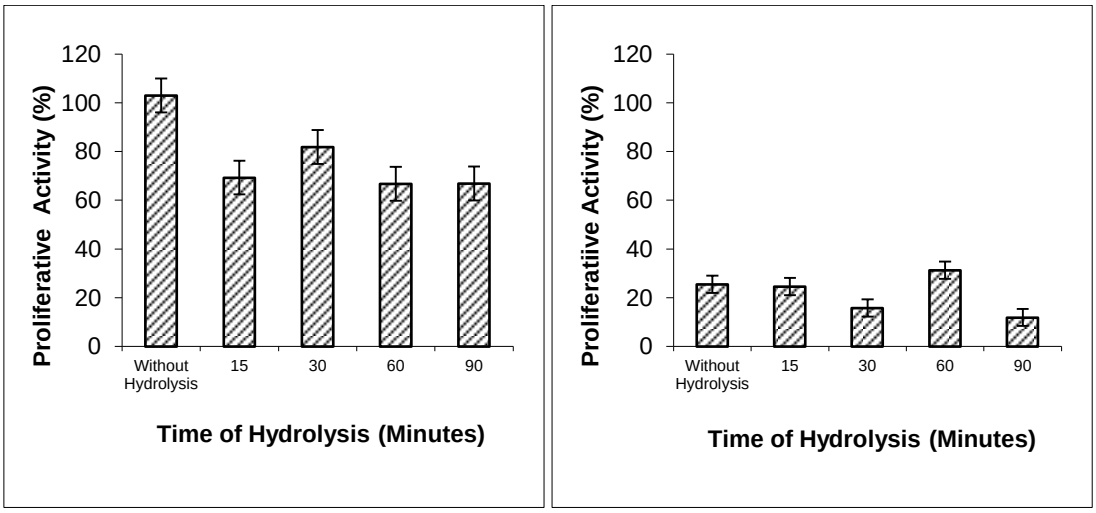
demonstrated that Pro at the antepenultimate position in the peptide sequence also enhanced binding to the ACE enzyme [31]. Most commercial inhibitors (Captopril and Enalapril) also bear Pro residue in their structure [32]. In this research captropil ($C_9H_{15}NO_3S$) was used 1 mg/ml.

Cancer Antiproliferative activity

Antiproliferative activity was considered to be the effect produced when the hydrolysates were incorporated before cell growth started. The collagen peptides prepared from fish collagen affected the viability of both cell lines depending on the time of hydrolysis used (Fig. 4A, B). Cell viability was determined after 48 h with a change of culture medium and the addition of 1 mg/mL concentration of the collagen peptide.



A B

Figure 4: Antiproliferative activity of collagen peptide with concentration is 1 mg/mL. Bars represent the standar deviation from triplicate determinations. (A=HeLa cell, B= HCT-116 cell).

Antiproliferative activity of collagen with different time of hydrolysis and activity of enzyme are depicted in Fig. 4. Collagen peptide exhibited the highest activity at 60 min towards HeLa cells (Fig. 4A), and the highest activity at 30 min towards HCT-166 cells (Fig. 4B).

Picot et al (2006) evaluated the antiproliferative activity of 18 fish protein hydrolysates and found the highest effect on MCF-7 cell lines to be around 40% for a cod hydrolysate obtained with Protamex and Alcalase. At the same concentration (1 mg/mL) the collagen peptide caused a similar effect on HeLa cells (33.3 % of growthinhibition), however on HCT-166 cell had a much stronger effect (81.1% of inhibition).

Three peptides from a fish source have been described as having antitumor activity: (i) a 440.9 Da anchovy hydrophobic peptide was able to induce apoptosis in human U937 lymphoma cells by increasing caspase-3 and caspase- 8 activity [14], (ii) tilapia hepcidin TH2-3 that manifested significant antitumor activity in human fibrosarcoma cells [33] and (iii) epinecidin-1, a peptide from fish (*Epinephelus coioides*) which had an antitumor effect similar to lytic peptides in human fibrosarcoma cells [34]. However none of these peptides originate from fish gelatin/collagen.

CONCLUSION

Bioactive peptide from skin fish collagen was produced using collagenase from *Bacillus licheniformis* F11.4 of Indonesia origin. Collagen peptides exhibited the highest activity of Angiotensin I-Converting Enzyme Inhibitorat 60 min at 0.032 Unit/ml and 0.016 U/ml of enzyme activity. Collagen peptides exhibited the highest activity at 60 min towards HeLa cells, and the highest activity at 30 min towards HCT-166 cells

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