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Antioxidant Activity of Collagen Hydrolysates from Fish Skin with a Microbial Collagenase.

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

[1]Department of Fisheries Product Technology, Faculty of Agriculture, Sriwijaya University, Indralaya, South Sumatera, Indonesia
[2]Department of Food Science and Technology, Bogor Agricultural University, Darmaga Campus IPB Bogor 16002, Indonesia
[3]Agency for the Assessment and Application of Technology, Jakarta, Indonesia

ABSTRACT

Bioactive peptides from collagen was hydrolyzed using collagenase from *Bacillus licheniformis* F11.4 and the hydrolysates were evaluated for antioxidant activity. The degree of hydrolysis (DH), pattern protein by SDS-PAGE, DPPH radical-scavenging activity, and reducing power of the hydrolysates were investigated. Purified fraction of collagenase was further used to produce collagen hydrolysates with different time of hydrolysis. Within 90 min of hydrolysis, the maximum cleavage of peptide bonds occurred was found with DH 51.851%. Hydrolysates with time hydrolysis of 60 and 90 min exhibited the highest DPPH with collagenase activity are 0.014 U/mg, and 0.028 U/mg, respectively. Time of hydrolysis of 90 min and 60 min exhibited the highest reducing power with collagenase activity are 0.014 U/mg, and 0.028 U/mg, respectively.

Keywords: Antioxidant, collagen, collagenase, *Bacillus licheniformis* F11.4

Corresponding author

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INTRODUCTION

Collagen is one of the major proteins in the living body. Collagen and gelatin are widely used in the food, pharmaceutical, cosmetic, biomedical materials and leather industries. The main sources of industrial collagen are those from pig and bovine skin and bones. But the outbreak of mad cow disease have resulted in anxieties amongst users of cattle collagen. Besides, the collagen from pig's skin and bone is not allowed to use in some regions due to religious reasons. Fish offal, such as bones, scales, as well as skin is very rich in collagen. Collagen hydrolysate is a polypeptide composite made by further hydrolysis of denatured collagen.

Enzyme used to hydrolysed fish protein have at least one common characteristic: they have to be food grade and if they are of microbial origin, the producing organism has to be non-pathogenic [1]. The variety of food-grade proteolytic enzyme is wide and offers enzymologist good opportunity to produce collagen hydrolysate. The most common commercial protease reported used for the hydrolysis of fish protein from plant sources such as papain [2] or animal orgin such as pepsin [3]. Enzymes of microbial origin have been applied to the hydrolysis of fish protein. In comparison to animal or plant derived enzymes, microbial enzymes have other several advantages including a wide variety of available catalytic activites, greater pH and temperature stability [4].

Numerous peptides derived from hydrolyzed food proteins have been shown to have antioxidative activities. However, there is a little information regarding collagen hydrolysates from fish skin and their antioxidative activity. Therefore, this study aimed to produce a collagen hydrolysate from skin fish with different with different time of hydrolysis and activity of enzyme using purifed collagenase from *Bacillus licheniformis* F11.4 and to study their antioxidative activity.

MATERIALS AND METHOD

Microorganism

The *Bacillus licheniformis* F11 from Indonesian material (Palembang, South Sumatera). *Bacillus licheniformis* F11.4 displayed a rough colony morphology. By targeted deletion of the polyglutamate operon (*pga*) in *Bacillus licheniformis* F11, a derivative form, F11.1 (Δ*pga*), was obtained that, along with lacking polyglutamate (PGA) formation, displayed enhanced proteolytic activities. The phenotypic properties were maintained in a strain in which the *chiBA* operon was additionally deleted: F11.4 (Δ*chiBA* Δ*pga*) [5].

Assay of Collagenase Activity and Protein Determination

Collagenase activity was measured according to the Bergmeyer method [6] as substrate was collagen from fish skin. One activity unit (U) was defined as the number of 1µmol L tyrosin released as a result of the action of 1 mL culture filtrate containing collagenase on collagen for 1 min at 37°C and pH 7. Protein was estimated by Bradford's method [7]. The standard (bovine serum albumin) and the reagent were purchased from Bio-Rad laboratories

Protein pattern of collagen hydrolysate

Protein pattern of fish skin collagen hydrolysates were determined using SDS-PAGE [8] with 4.5% stacking gel and 20% separating gel according to method of Klompong *et al*. [9]. Hydrolysates were mixed with sample buffer (0.125 M Tris–HCl, pH 6.8 containing 4% SDS and 20% (v/v) glycerol) at a ratio of 1:1 (v/v). Proteins (15 µg) were loaded onto the gel. The electrophoresis was run at a constant current of 15 mA per gel by a Mini-Protean II Cell apparatus. The gels were fixed and stained with 0.05% (w/v) Coomassie Brilliant Blue R-250 in 15%methanol and 5% acetic acid and destained in 30% methanol and 10% acetic acid. Wide range molecular mass marker was used to estimate the molecular mass of hydrolysates.

DPPH radical scavenging activity

DPPH radical scavenging activity was measured based on methods described in Li *et al*. [10]. A 500 µl test sample was mixed with 500 µl of 99.5% ethanol and 125 µl of 99.5% ethanol containing 0.02% DPPH. This

mixture was kept in the dark at room temperature for 60 min before measuring for absorbance at 517 nm. Radical scavenging activity was calculated as follows:

$$\text{Radical scavenging activity (\%)} = \frac{\text{Control + Blank – Sample}}{\text{Control}} \times 100\%$$

where the control was the absorbance value of 500 µl of distilled water + 125 µll of ethanol including 0.02% DPPH + 500 µll of ethanol, the sample that for 500 µl of sample solution + 125 µl of ethanol including 0.02% DPPH + 500 µl of ethanol, and the blank that for 500 µl of sample solution + 125 µl of ethanol + 500 µl of ethanol.

Reducing power

Reducing power was determined by the method of Oyaiza [11]. The sample solution (0.5 ml, 40 mg protein/ml) was mixed with 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 C for 20 min. An aliquot (2.5 ml) of 10% trichoroacetic acid was added to the mixture, followed by centrifugation at 3000 rpm for 10 min. The upper layer of solution (2.5 ml) was mixed with 2.5 ml of distilled water and 2.5 ml of 0.1% ferric chloride and the absorbance was read at 700 nm. Increased absorbance of the reaction mixture indicates increasing reducing power.

RESULT AND DISCUSSION

Degree of hydrolysis

In the present study, fish skin collagen was hydrolyzed by purified collagenase, for the production of antioxidant peptides. The extent of collagen degradation by collagenase enzymes was estimated by assessing the degree of hydrolysis (DH) and it was observed increasing during hydrolysis time, reaching 51.85% (Fig. 1). The result was in accordance with Guarard *et al.* [12] who reported that DH of yelloefin tuna waste protein increased with increasing hydrolysis time.

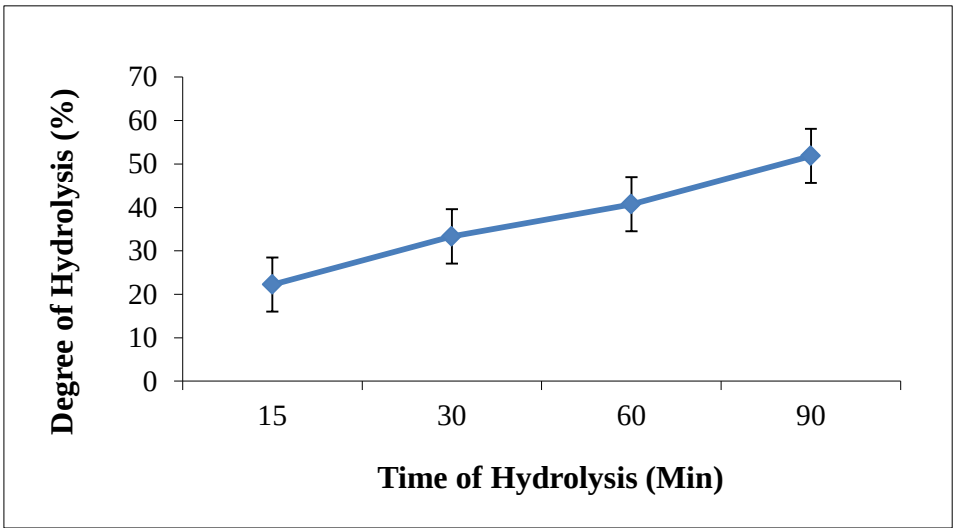

Figure 1. Degree of hydrolysis collagen hydrolysates by purified collagenase

Protein pattern of hydrolysate

The electrophorectic patterns of the hydrolysates were fractionated on the basis of their molecular weight using SDS-PAGE (Fig. 2). Standar MW markers (line 1) was used to determine the presence or absence of peptides of various molecular weights. Since enzyme hydrolysis breaks up collagen protein into a number of small peptides. During hydrolysis, enzymatic breakdown of protein involves a major structural change, in which the protein is slowly hydrolyzed in to smaller peptide units [9].

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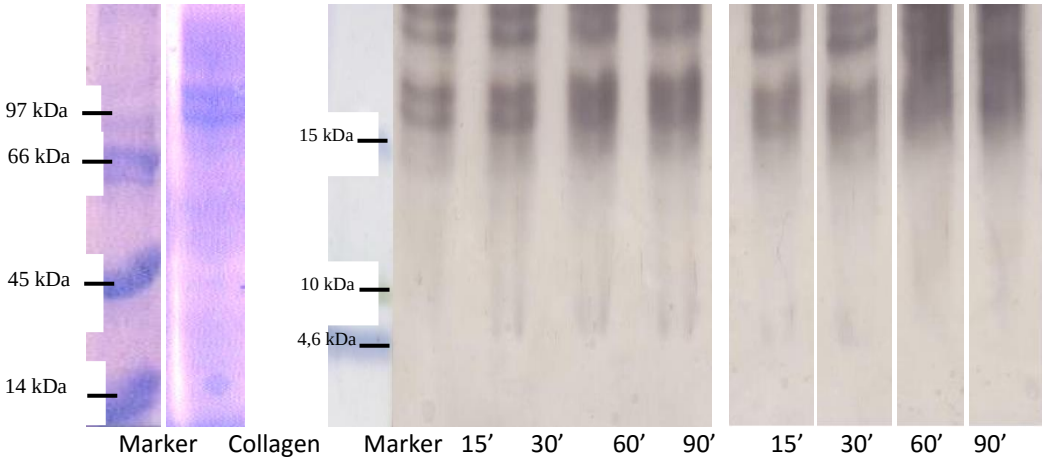

Figure 2. Pattern protein of collagen hydrolysates by purified collagenase from *Bacillus licheniformis* F11.4

The DPPH radical scavenging activity

The DPPH radical scavenging activities of collagen with different time of hydrolysis and activity of enzyme are depicted in Fig. 3. Collagen hydrolysate exhibited the highest DPPH radical scavenging activity at 60 min at 0.014 Unit/mg and 90 min at 0.028 U/mg. The DPPH radical scavenging activitiy of collagen at 0.014 U/ml activity of collagenase peaked after 60 of hydrolysis, decreasing thereafter and at 0.028 U/ml activity of collagenase peaked after 90 of hydrolysis (Fig. 3). The DPPH radical scavenging activity by BHT is greater than that of the hydrolysates by enzyme.

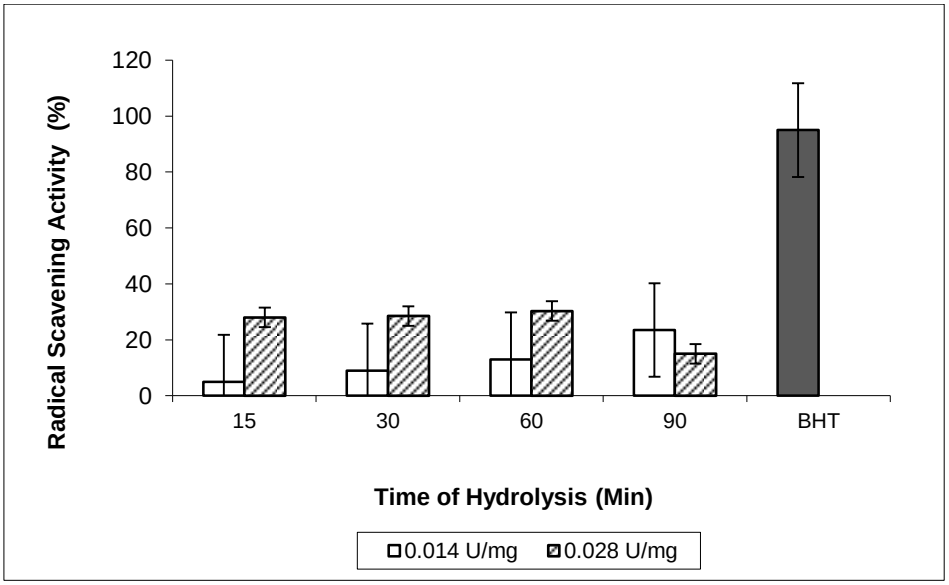

Figure 3. DPPH scavenging radical of collagen hydrolysates by purified collagenase

Diphenylpicrylhydrazyl (DPPH) is very commonly used by researchers to evaluate the radical scavenging ability of antioxidants. Antioxidants with higher DPPH inhibition are said to possess higher antioxidant efficacy [13]. DPPH is a stable free radical that shows maximal absorbance at 517 nm in ethanol. When DPPH encounters a proton-donating substance, such as an antioxidant, the radical is scavenged. The color is changed from purple to yellow and the absorbance is reduced [14]. Protein hydrolysates may also inhibit oxidation by their ability to chelate transition metal ions [15]. DPPH radical scavenging activities were found in protein hydrolysates derived from tuna backbone protein [16] and Alaska Pollack [17].

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Reducing power

The reducing power of collagen hydrolysete, as the time of hydrolysis increased, the reducing power increased. Collagen hydrolysate exhibited the highest reducing power at 90 min at 0.014 Unit/mg and 30 min at 0.028 U/mg. Reducing power of collagen at 0.014 U/ml activity of collagenase peaked after 90 of hydrolysis and at 0.028 U/ml activity of collagenase peaked after 30 of hydrolysis, decreasing thereafter (Fig. 4).

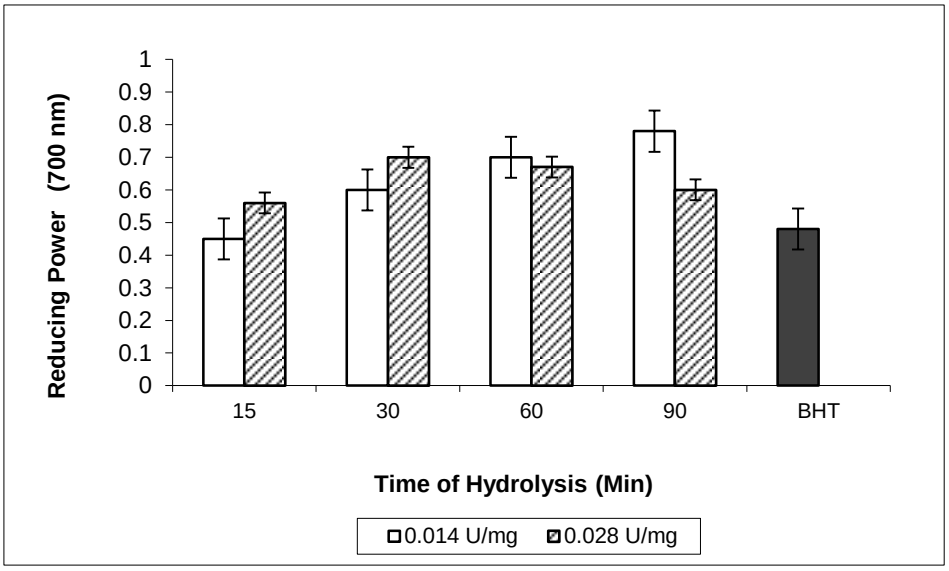

Figure 4. Reducing power of collagen hydrolysates by purified collagenase

For the reducing power assay, the presence of reductans (antioxidants) is tested samples results in reducing Fe^{3+} to ferrous (Fe^{2+}) form. The Fe^{3+} can therefore be monitored by measuring the formation of Perl's Prussian blue at 700 nm [19]. The results suggested than collagen hydrolyzed from fish skin as electron donors and could react with free radicals to form more stable products.

CONCLUSION

Bioactive peptides from collagen was hydrolyzed using collagenase from *Bacillus licheniformis* F11.4. Purified enzyme fraction used to produce collagen hydrolysates with different time of hydrolysis. Within 90 min of hydrolysis, the maximum cleavage of peptide bonds occurred was found with DH 51.85%. Hydrolysates with time hydrolysis of 60 and 90 min exhibited the highest DPPH radical scavenging activity. Time of hydrolysis of 90 min and 60 min exhibited the highest reducing power. Therefore, purified collagenase from *Bacillus licheniformis* F11.4 could be used to produce the collagen hydrolysates possessing antioxidant activity.

ACKNOWLEDGMENTS

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