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In vitro anti-tyrosinase and anti-elastase activity of collagen from sea cucumber (*Holothuria leucospilota*)

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Collagen is the main structural component of connective tissues in vertebral and invertebral animal tissues and organs. The collagen isolated from the body wall of sea cucumber (*Holothuria lecospilota*) can be used in the pharmaceutical field. The research aimed at isolating collagen from the sea cucumber body wall and testing the anti-tyrosinase and anti-elastase activity. This was done by isolation of collagen by soaking the body wall of sea cucumber using Tris-HCl-EDTA 0.1 M and dialyzing using 0.5 M acetic acid. The step was followed by characterization of collagen using FT-IR spectrophotometer and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The resulting collagen was tested for anti-tyrosinase and anti-elastase *in vitro*. The research showed that the resulting collagen was of the amide group with molecular weight of 166.43 and 138.35 kDa, showing α1 and α2 chains. Anti-tyrosinase activity test showed an IC_{50} value of 1.20 mg/mL, while anti-elastase activity test showed an IC_{50} value of 125 µg/mL. The collagen isolated from the sea cucumber has weak anti-tyrosinase and moderate anti-elastase activity.

Key words: Crude collagen, anti-tyrosinase, anti-elastase, sea cucumber (*Holothuria leucospilota*).

INTRODUCTION

Sea cucumber has important economic values. Besides its use for diet purpose, sea cucumber has promising marine-derived medicinal potentials (Bordbar et al., 2001). In addition, it can be used for its anti- cancer, bacterial and malarial activities (Janakiram et al., 2005; Layson et al., 2014; Liu et al., 2007). Generally, major sources of collagen are the skin and bone of pigs and cows. However, the occurrence of mad cow disease has resulted in unease among cattle gelatin users. Additionally, collagen obtained from pig bones cannot be used by many, due to religious constraints (Sadowska et al., 2003). Thus, there is a strong need to develop alternative collagen sources. Marine organisms have been recognized as potential alternative sources, due to their availability, lack of dietary restriction, lack of disease risk and high collagen yields (Liu et al., 2007).

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Figure 1. *Holothuria leucospilota* (black sea cucumber).

Collagen is a component that forms the sea cucumber body and account for 70% of its total protein content. Few studies have been conducted on sea cucumber from Indonesia, while collagen is widely used in pharmaceutical and cosmetic industries. Pharmacological activity of collagen is to scaffold mesenchymal stem cell and extracellular biometrical matrices to enhance cellular growth and development (Sadowska et al., 2003). In Indonesian waters, there are 53 species of sea cucumber, including *Holothuria, Actinopyga, Bohadschia, Labiodemas, Thelonata* and *Stichopus*. Among the species of sea cucumber, only 29 are internationally traded (Darsono, 2011), all of which belong to *Holoturiidae* and *Stichopodidae* families. One of them is *Holothuria lecospilota*, which is widely found in Pramuka Island, Kepulauan Seribu, Jakarta Bay area. The study aimed at isolating collagen from the body wall of sea cucumber and conduct *in vitro* test for anti-tyrosinase and anti-elastase activity in sea cucumber.

MATERIALS AND METHODS

Isolation of crude collagen

Two fresh samples of Black sea cucumber, *H. lecospilota* were taken from Pramuka Island, Kepulauan Seribu, Jakarta Bay area weighing between 700 and 800 g and were handpicked by the divers. The body wall of *H. leucospilota* was dissected for free adherent tissues, cut into small pieces (about 2 × 2 cm), and stored in a small container filled with phosphate buffer saline (PBS). The body wall of sea cucumber was soaked in destilled water for 1 h. The dissected body wall of sea cucumber was soaked in 0.1 M NaOH solution for 6 h, then soaked in 0.1 M pH 8.0 Tris-HCl for 24 h, washed using pH 7.0 double destilled water, and then stirred using 0.5 M acetic acid for 48 and 72 h. The filtrates were centrifuged under 8000 rpm speed for 20 min, salted out using 0.9 M NaCl for overnight, and then centrifuged under 8000 rpm speed for 20 min for obtaining deposits. The deposits were dialyzed by using a dialysis bag and 0.1 M acetic acid as medium under a temperature of 4°C for 12 h (Sadowska et al., 2003; Sionkowska et al., 2015; Zhong et al., 2015). Then, the medium was replaced with destilled water, and then dialyzed for a similar period. The resulting collaged was put in a freeze dryer.

Characterization of crude collagen

FT-IR spectrophotometer

FT-IR spectroscopy of freeze-dried sample of crude collagen was based on a Bio- Rad FT-IR –40, USA. Sample (10 mg) was mixed with 100 mg of dried potassium bromide (KBr) and compressed to make a salt disc (10 mm in diameter) for reading the spectrum further. Spectra were collected between wave numbers of 4000 and 500 cm^{-1}, which was compared with standard collagen (Sigma) (Sambrook and Russel, 2001; Kong and Yu, 2007).

SDS-PAGE electrophoresis

The molecular weight of the collagen was determined by SDS-PAGE and was compared with molecular marker–Sigma (Kim and Uyama, 2005).

Anti-tyrosinase activity test

The anti-tyrosinase activity of crude collagen was measured spectrophotometry. The enzymatic reaction was initiated by addition of a known amount of the enzyme to substrate solution containing dimethyl formamide (DMF and MBTH). DMF was added to the reaction mixture in order to keep the resulting colored complex in soluble state during the course of investigations. The progress of the reaction was followed by measuring the intensity of the resulting pink color at 505 nm. A typical reaction mixture with a total volume of 1.0 ml contained 100 µl enzyme solution (a), 500 µl substrate solution (b) and 400 µl phosphate buffer (pH 6.8). The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as, the concentrations of each sample that inhibited 50% of tyrosinase activity. The resulting data were expressed as a percentage of inhibition of tyrosinase activity (Kothary et al., 1984).

Anti-elastase activity test

The method of Kothary et al. (1984) was adapted to detect the production of elastase among test crude collagen. These activities were determined by colorimetric assay of Sachar et al. (1955) with some modifications, employing elastin Congo red (Sigma) as a substrate. Briefly, 250 µl each of 50 mM sodium borate buffer (pH 8.5) and elastin Congo red (20 mg ml^{-1} in 50 mM sodium borate buffer, pH 8.5) was mixed and vortexed at room temperature for 5 min. Next, 250 µl of test sample was added and incubated at 37°C for 3 h at 180 rpm.

Test sample was replaced by 250 µl of buffer as control. After incubation, 750 µl of 10% trichloroacetic acid was added to stop the reaction and kept for 30 min on ice. Insoluble material in the assay mixture was removed by centrifugation at 5000 rpm, 30 min and the absorbance was read at 495 nm using a double beam UV-vis spectrophotometer (Sachar et al., 1955).

Statistical analysis

All the experiments were performed three times with three replicates per experiment and data are expressed as mean ± standard deviation. Statistical significance of the differences was determined by the one way ANOVA test using Minitab (V.11.0 for Windows). Percentage reduction in elastase and tyrosinase activity in test in the presence of collagen was compared with untreated control by one way ANOVA using Duncan's method. P-values of ≤ 0.05 were considered as statistically significant.

RESULTS AND DISCUSSION

H. leucospilota morphologically has a round body cross section, relatively flat ventral side, and a round anus. It has dark skin with smooth and thick surface (Figure 1). The spicules found on dorsal area were of table, rosette and slab types. It is mostly found at the area with coarse sand substrate and the body is covered with smooth sand (Conand et al., 2013). Most parts of the sea cucumber are made of collagen and have been identified as type 1 collagen. Type 1 collagen is found in human skin; therefore, the collagen can be used for cosmetic products. Collagen extraction was conducted using a method proposed by Trotter et al. (1995) and Saito et al. (2002). The extraction did not obtain crude collagen extract because of too long soaking process in distilled water. Consequently, the resulting pellets were much fewer. Another study that combined the method proposed by Trotter et al. (1995) and Saito et al. (2002) used acetic acid and Tris HCl-EDTA. The research found collagen yield of 7.92% after soaking in acetic acid for 72 h. The calculation was based on crude weight. The resulting yields were higher than those found in the research conducted by Saito et al. (2002), in which the resulting crude collagen of the sea cucumber, *Stichopus japonicus* was only 3.3%.

Characterization of the collagen was conducted by using FT-IR spectrophotometer and SDS-PAGE. IR spectroscopic test was conducted to find out the functional groups or collagen-chitosan compound. FT-IR spectroscopy is a tool to measure radiation absorbance at infra-red area at various wavelengths. Qualitatively, FT-IR spectroscopy can be used to identify functional groups in a molecular structure. The data resulting from FT-IR spectrum are the characteristic spectral peaks, which are represented as transmittance curve (%) and wavenumber (cm^{-1}) of the tested sample and are analyzed. To analyze the data resulting from the infrared spectroscopic measurement, international conversion table, namely Handbook IR is necessary (Kong and Yu, 2007). The result of characterization using FT-IR spectrophotometer showed IR spectrum as presented in Figure 2.

The figure of FT-IR spectrum of sea cucumber collagen shows that the collagen resulting from isolation using 0.5 M acetic acid was amide A and 1. Amide A in the sea cucumber collagen was found at wavenumber of 3438.84 and 3433.06 cm^{-1}, respectively. The N-H group of peptide was influenced by hydrogen bonding whose position was shifted to a lower frequency. Amide A is an area, in which there is paired overextended NH group that formed hydrogen bonding and OH from hydroxyproline. Hydroxyproline is a specific amino acid in collagen. The peak of amide 1 in sea cucumber collagen was found at wavenumber of 1631.67 and 1629.74 cm^{-1}, respectively. Amide 1 has wavenumber range of 1690 to 1600 cm^{-1} and represents C=O group, which is secondary structure of protein. Amide 1 is related to stretching vibration of carbonyl group. It is a group with specific function to form collagen. The functional groups in sea cucumber (*H. leucospilota*) are different from other collagens, such as those found in *S. variegatus, Oreochromis niloticus, Thunnus albacares*, and puffer fish, namely amide A, B, I, II and II (Sionkowska et al., 2015; Zhong et al., 2015).

FT-IR absorbance spectrum of the collaged showed amide A at wavenumbers of 3438.84 and 3433.06 cm^{-1}, respectively. Peak amide 1 was found at wavenumbers of 1631.67 and 1629.74 cm^{-1}, respectively. The collagen has a specific peak at wavenumber of 3452.64 cm^{-1}, showing that it belongs to hydroxyl group (-OH); meanwhile, at wavenumber of 1657.57 cm^{-1}, amide 1 was found. Amide 1 is a crucial factor for understanding the secondary structure of protein (Vidal, 2013). Amide II was shown at wavenumber of 1560.47 cm^{-1}. Amide II represents a helix structure. Observation of the isolated collagen using SDS-PAGE was conducted to determine the protein band pattern and molecular weight of the protein. The collagen of sea cucumber (*H. leucospilota*) has 3 protein bands with molecular weight after soaking with 0.5 M acetic acid for 72 h (Figure 3).

Protein band after 72 h of treatment showed α_1 and α_2 chain at molecular weights of 166.43 and 138.35, respectively; the α chain showed molecular range of 112 to 199 kDa and β chain was shown at molecular weight of above 200 kDa. Characterization was conducted by using SDS PAGE based on the difference in the migration rate and molecular weight on a single electric field. The protein band pattern of the *H. leucospilota* collagen

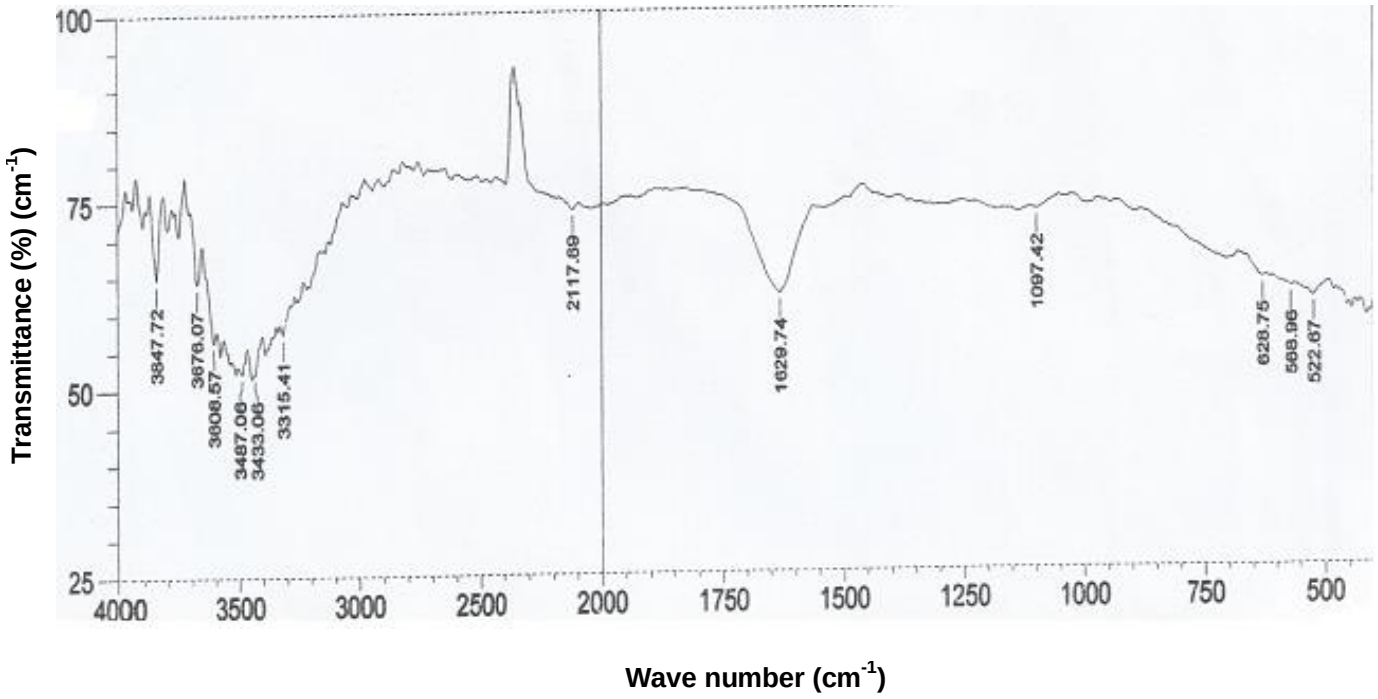

Figure 2. FT-IR spectrum of *H. Leucospilota* collagen.

consists of 3 protein bands, namely, 247.29 166.43 and 138.35 kDa. The protein bands showed α1 and α2 chains at molecular weights of 166.43 and 138.35 kDa, respectively, in which α chain was shown at molecular weight range of 112 to 199 kDa and β chain at molecular weight above 200 kDa. The research shows that protein band pattern of *S. Japonicus* collagen was 135 kdA as compared to 137 kDa in sea cucumber *Stichopus monotuberculatus* collagen (Cui et al., 2007). The primary structure of type 1 collagen consists of continuously repeated sequence Gly-X-Y (most of X is proline and Y is hydroxyproline). In a section that has more than a thousand of residues, every third residue is glycine. The repeated Gl-X-Y sequence in α1 chain plays an important role in forming the secondary structure of triple helix. Therefore, the collagen is type 1, which is mostly found in connective tissues, including those of tendon, bone and skin (Saito et al., 2002). Collagen is considered to be one of the most useful biomaterials because it has a wide range of industrial applications (Lafarga and Hayes, 2014).

Skin aging (whether extrinsic or intrinsic type) causes wrinkling, sagging, laxity, dyspigmentation, and telangiectasia. As the skin ages, the collagen (a major component of skin) and elastin in the dermis lose elasticity resulting in wrinkles. To prevent the skin from aging or wrinkles, natural phytochemical source is desirable. Elastase are known to be a major enzyme responsible for dehydration and wrinkle formation on the skin surface (Lafarga and Hayes, 2014). The results of *in*

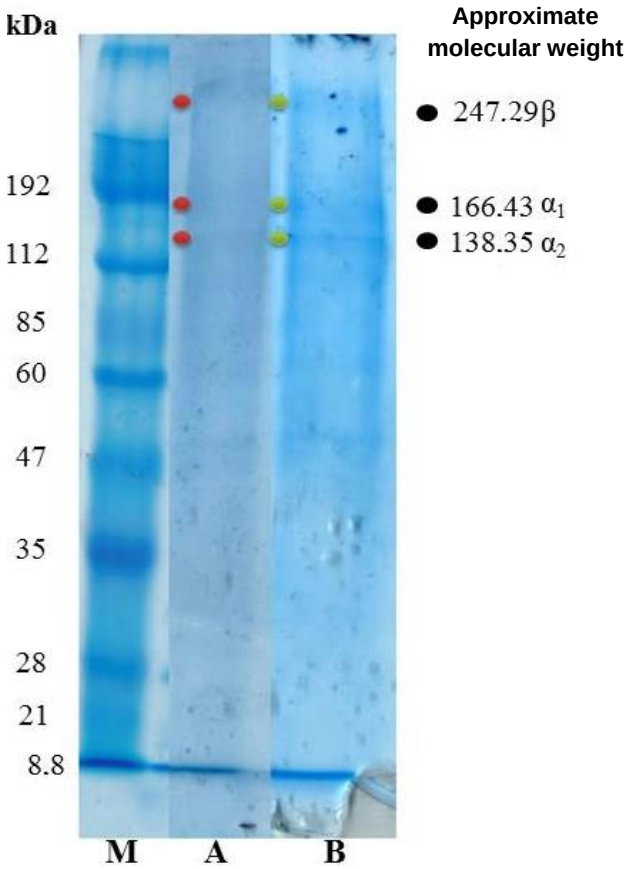

Figure 3. Protein band pattern of *H. leucospilota* collagen.

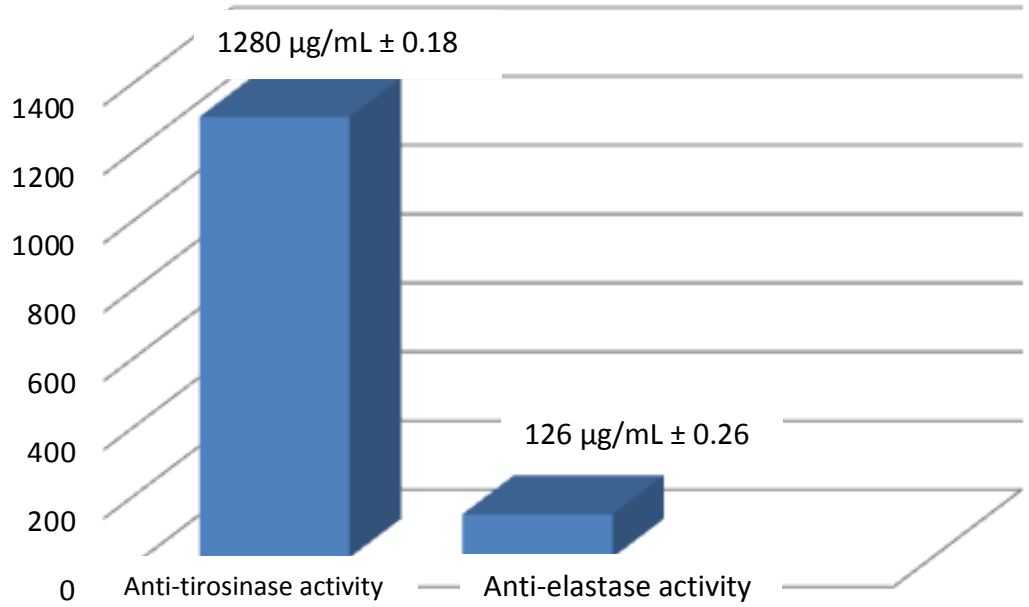

Figure 4. The results of measurement for anti- tyrosinase and elastase activity of the collagen.

vitro measurement for anti-tyrosinase and anti-elastase activity of the collagen are presented in Figure 4.

Measurement of anti-tyrosinase and anti-elastase activity of the collagen was conducted by varying the collagen concentrations from 50 to 2000 ppm. Then, the absorbance and percentage (%) inhibition were measured. The inhibitory effects of collagen on these two enzymes are shown in Figure 4. Measurement of anti-tyrosinase and anti-elastase activity of the collagen resulted in IC_{50} value of 1280 ± 0.98 and 126 ± 0.76 µg/mL. The study conducted by Choi et al. (2015) found that hydrolyzed collagen of *Todarodes pacificus* has anti-tyrosinase activity. On the other hand, this result corresponded to the results of Zhuang et al. (2009) that tyrosinase inhibitory activity of jellyfish (*Rhopilema esculentum*) collagen hydrolysate fraction was 47.2% at 5 mg/mL. Tyrosinase is a copper containing oxidase, widely distributed in micro-organisms, plants and animals, and a key enzyme in melanin biosynthesis.

Conclusion

In this study, the novel anti- tyrosinase and elastase activities of collagen from sea cucumber (*H. leucospilota*) showed an indication of the efficacy as potential anti-wrinkle. However, *in vivo* evaluations are needed for the development of natural source to formulate advanced skin care cosmetics.

Conflict of Interests

The authors have not declared any conflict of interests.

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