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Extraction and Characterization of Collagen from Sand Sea Cucumber (*Holothuria scabra*)

(Ekstraksi dan Karakterisasi Kolagen dari Teripang Pasir (*Holothuria scabra*))

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ABSTRACT

Sand sea cucumber (*Holothuria scabra*) is an aquatic product that belongs to Echinodermata, a habitant in almost all Indonesian seas. The main component of the sea cucumber is protein, one of which is collagen. This study aimed to extract and characterize collagen from the species using the acid-base extraction method. The characterization of sea cucumber collagen includes molecular weight, amino acid components, Fourier transform infrared spectrophotometry, and scanning electron microscopy analysis. This study has successfully extracted collagen from the sample using an extraction system: NaOH 0.1 M; CH_3COOH 0.1 M; and distilled water under 45°C treatments, gave 6% yield. The collagen has a molecular weight 110–130 kDa. Based on the infrared spectra, the specific functional groups of the collagen are amide A (3379.29 cm^{-1}), amide B (2924.09 cm^{-1}), amide I (1681.93 cm^{-1}), amide II (1560.41 cm^{-1}), and amide III (1249.87 cm^{-1}). The collagen falls into type I. We suggest an alternative resource of collagen from sand sea cucumber, other than poultry and mammals.

Keywords: characterization, collagen, extraction, fishery, sand sea cucumber

ABSTRAK

Teripang pasir (*Holoturia scabra*) termasuk jenis Echnodermata dari perairan yang memiliki habitat di hampir seluruh perairan Indonesia. Penelitian ini bertujuan mengekstraksi dan mencirikan kolagen dari species tersebut dengan menggunakan metode ekstraksi asam–basa. Karakterisasi kolagen teripang meliputi: bobot molekul, analisis komponen asam amino, spektofotometri inframerah Fouries, dan mikroskopi payaran elektron. Penelitian ini telah berhasil mengekstraksi kolagen dari sampel menggunakan sistem ekstraksi: NaOH 0,1 M; CH_3COOH 0,1 M; dan air distilasi 45°C, menghasilkan rendemen 6%. Kolagen yang diperoleh memiliki bobot molekul sekitar 110–130 kDa. Berdasarkan data hasil analisis spektrometri inframerah, gugus fungsi khas dari kolagen yang diperoleh adalah amida A (3379,29 cm^{-1}), amida B (2924,09 cm^{-1}), amida I (1681,93 cm^{-1}), amida II (1560,41 cm^{-1}), dan amida III (1249,87 cm^{-1}). Hasil tersebut menunjukkan bahwa kolagen teripang pasir (*H. scabra*) memiliki karakteristik kolagen tipe I. Temuan ini mengisyaratkan sumber alternatif kolagen dari teripang, selain dari unggas dan mamalia.

Kata kunci: ekstraksi, karakterisasi, kolagen, perikanan, teripang pasir

INTRODUCTION

At the time being, the demand for collagen was derived from poultry farms and the mammals' wastes. Commercial collagen generally comes from cows and pigs. Many diseases are found in poultry and mammals recently, such as avian influenza and mad cow. Thus, it is necessary to search for alternative raw materials for collagen production, such as aquatic products. Collagen from the aquatic products has advantages, such as poultry and mammals-related disease-free, relatively high collagen, and halal raw materials. It can use in the food and drug industry (Senadheera *et al.* 2020). The aquatic products include cork and patin fish (Hardyanti 2014; Wulandari 2016) and sea cucumbers (Abedin *et al.* 2014; Safithri *et al.* 2018; Siahaan *et al.* 2017; Siddiqui *et al.* 2013a). In the last decade, growing interest among researchers because of their nutritional value, health benefits, and potential medical therapy. Collagen derived from fishery products has a more thermostable superiority and a more tightly collagen structure (Senadheera *et al.* 2020).

Sea cucumber is an aquatic product belongs to Echinodermata, a habitant in almost all Indonesian seas. The main component of the sea cucumber is protein, including collagen. Collagen from some sea cucumber species in Indonesia has been successfully extracted and characterized, as reported by Alhana *et al*. (2015) and Fawziya *et al*. (2016) on the type of gamma sea cucumber (*Stichopus variegatus*), Gianto *et al*. (2018) on the type of golden cucumber (*S.*

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horrens), as well as Nauli (2019) on the type of *Paracaudina australis*. However, collagen derived from sand sea cucumber (*Holothuria scabra*) has not been much revealing before.

This study was intended to extract and characterize the collagen from sand sea cucumbers using an acid-base solvent extraction method. The collagen was characterized by molecular weight, amino acid constituents, Fourier transform infrared spectrophotometry, and scanning electron microscopy analysis. The finding would be beneficial to find an alternative resource of collagen from sea cucumber, other than poultry and mammals. The collagen could be applied as the raw material for the food and health industry directly or after processing the collagen hydrolysate that have a short structure (< 5 kDa).

MATERIAL AND METHODS

Sample Preparation

The sand sea cucumber with a minimum 200-g weight was collected and cultivated from the Marine Bio-Industrial Centers, Research Center for Oceanography, LIPI Lombok. The samples were cleaned from the outer skin and separated between the flesh and belly contents. Afterward, the flesh was washed, cut into dice-size, and dried at 60°C. The dried material was subsequently ground into powder-sized up to 100 mesh and stored in a desiccator until used in the following experiment.

Collagen Extraction

The dried powder was extracted for collagen after solvent optimization to obtain the highest extracted yield. This system was based on acid-base extraction with three replicates, as presented in Table 1. The extracted collagen was then freeze-dried.

Molecular Weight Analysis

The molecular weight of the collagen was determined using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The extract solution was prepared by dissolving the freeze-dried material from each dilution step (D1-D3) of each extraction system (M1-M4) (Table 1) into 60°C distilled water to a final concentration of 10 mg/mL (Gómez-Guillén *et al*. 2002; Khiari *et al*. 2011; Khiari *et al*. 2013). The pellet residue (P) solution from each extraction system (M1−M4) and catfish skin collagen (KI), and commercial tilapia fish collagen peptide (KK) as controls were also prepared in the same way as the preparation of the collagen extract solution. The solution was further diluted with Laemmli sample buffer (Biorad) containing β-mercaptoethanol (Sigma) and heated in boiling water for 10 min. The sample and protein marker (Precision Plus Protein™ Dual Color Standard; Biorad) were electrophoresed in the 4% stacking gel and 5% resolving gel (Laemmli, 1970) at a 90 V constant voltage. Then the gel was stained with the Coomassie Brilliant Blue R-250 and destained with the destaining solution I (40% (v/v) methanol, 7% (v/v) acetic acid, and 53% (v/v) distilled water, and solution II (5% (v/v) methanol, 7% (v/v) acetic acid, and 88% (v/v) distilled water.

Amino Acid Composition Analysis

The amino acid composition analysis was carried out using ultraperformance liquid chromatography (UPLC) Waters quantitatively on the freeze-dried extracted intact collagen. The columns used were ACCQ-Taq Ultra C-18, photodiode array detector (PDA), temperature 49°C with a flow rate of 0.5 mL per min, at a wavelength of 260 nm, and injection volume of 1 µL. The amino acids analyzed consisted of 15 amino acids: histidine, threonine, proline, tyrosine, leucine, lysine, aspartic acid, glycine, arginine, alanine,

Table 1 Various acid-base systems for the sand sea cucumber collagen

Solvent system	System I (M1)	System II (M2)	System III (M3)	System IV (M4)
Solvent I (D1)	0.1 M NaOH (1:10 v/v) Homogenization for 6 h at room temperature	0.1 M NaOH (1:10 v/v) Homogenization for 6 h at room temperature	EDTA pH: 7.4 (1: 10 v/v) Homogenization for 6 h at room temperature	0.1 M NaOH (1:10 v/v) Homogenization for 24 h at room temperature
Solvent II (D2)	EDTA pH: 7.4 (1: 10 v/v) Homogenization for 6 h at room temperature	0.5 M CH_3COOH (1:10 v/v) Homogenization for 24 h at room temperature	NaOH 0,1 M (1:10 v/v) Homogenization for 6 h at room temperature	0.5 M CH_3COOH (1:10 v/v) Homogenization for 24 h at room temperature
Solvent III (D3)	0.5 M CH_3COOH (1:10 v/v) Homogenization for 24 h at room temperature	45ºC aquadest (1:5 v/v) Homogenization for 6 h at room temperature	0.5 M CH_3COOH (1:10 v/v) Homogenization for 24 h at room temperature	45ºC aquadest (1:5 v/v) Homogenization for 6 h at room temperature
Yield	6%	3.2%	3.5%	4.8%

Description: Before each solvent replacement, the solution was centrifuged at 20,000xg at 4ºC for 10 min. The resulted pellet was then dissolved in the subsequent solvent, whereas the filtrate was further analyzed.

valine, isoleucine, phenylalanine, serin, and glutamic acid.

Functional Group Analysis

The freeze-dried extracted functional group analysis was performed using the Fourier transform infrared (FTIR) Perkin Elmer L1600107 spectrophotometer at a wavenumber of 4000–6500 cm^{-1}, by Attenuated Total Reflectance (ATR).

Collagen Physical Structure Analysis

The collagen structure was observed using a scanning electron microscope (SEM) Thermo Scientific Quattro S, on the freeze-dried extracted intact collagen. The installation using specimen stub and coated with Au metal using Coater Ion. The observation was carried out at 2,000× and 20,000× magnification.

RESULTS AND DISCUSSIONS

Collagen Extract

Collagen from aquatic products has been investigated from jellyfish (*Rhopilema esculentum*), sea urchins (*Paracentrotus lividus*), and starfish (*Acanthaster planci*) (Benedetto *et al*. 2014; Bermueller *et al*. 2013; Cheng *et al*. 2017; Tan *et al*. 2013). Besides, some types of fish have also been successfully used as the source of collagen, including salmon (*Salmo salar*), tuna (*Thunnus albacares*), and sardines (*Sardinella longiceps*) (Alves *et al*. 2017; Muthumari *et al*. 2016; Woo *et al*. 2008). Further research proves the presence of specific biological activity of the collagen that can be beneficial in food and health. Collagen derived from aquatic products is an alternative source for collagen's essential ingredient, commonly derived from pig and cow. Moreover, there is proof of some diseases derived from cow and pig, such as bovine spongiform encephalopathy (BSE), transmissible spongiform encephalopathy (TSE), and foot and mouth disease (FMD), causing many explorations trying to find new sources of collagen, including from aquatic products.

In general, extraction in this experiment was carried out under a maceration system using three solvents (Table I) and subsequent collagen separation employing protein purification technique. This study reported that system IV gave the highest collagen yield from the sand sea cucumber, i.e., 6% dry weight. The comparison of this yield with previous studies was shown in Table 2. Specifically, this result was lower than the yield from gamma sea cucumber (*S. variegatus*) (Khirzin *et al*. 2016). Nevertheless, this result was higher than the average yield from gold sea cucumber (*S. hermanii*) by Safithri *et al*. (2018). This result was also higher than the extracted collagen yield

Table 2 The comparison of the extracted collagen yield in this research with previous studies

Source of collagen	Extraction system	Yield (% dry weight)		Reference
		Collagen	PDC	
Gold sea cucumber (*Stichopus hermanii*)	Immersion using 0.1 M NaOH Acid-soluble extraction using 0.5 M CH_3COOH	0.66 ± 0.14	ND	Safithri *et al*. (2018)
Gamma sea cucumber (*S. variegatus*)	Concentration using 0.3% NaOH and 0.1% CH_3COOH	1.5	ND	Alhana *et al*. (2015)
Sea cucumber (*S. monotuberculatus*)	Acid dissolution using 0.5 M CH_3COOH	2.63	ND	Zhong *et al*. (2015)
Sand sea cucumber (*H. scabra*)	System IV (Table 1)	6	ND	This study
Gamma sea cucumber (*S. variegates*)	Disaggregation using 0.1 M Tris-HCl pH 8 and 4 mM EDTA Immersion using 0.1 M NaOH Acid-soluble extraction using 0.5 M CH_3COOH	16.40	ND	Khirzin *et al*. (2016)
Sea cucumber (*Acaudina leucoprocta*)	Demineralization pretreatment using 0.2 M EDTA Collagen extraction based proteolysis	ND	43,66 ± 0.65	Lin *et al*. (2017)
Sea cucumber (*S. monotuberculatus*)	Dissolution using 0.5 M CH_3COOH Collagen extraction based on proteolytic of pepsin	ND	61.93	Zhong *et al*. (2015)
Sea cucumber (*Bohadschia bivitatta*)	Pretreatment using 4 mM EDTA and 0.1 M Tris-HCl pH 8.0 Collagen extraction using 0.5 M CH_3COOH containing pepsin	ND	65	Siddiqui *et al*. (2013)
Sea cucumber (*H. cinerascens*)	Decomposition using 0.5 N NaCl, 50 mM EDTA, 0.2 N β-134 mercaptoethanol, and 0.1 N Tris-HCl pH 8 Collagen extraction based on pepsin proteolysis with 0.5 N CH_3COOH	ND	72.2	Li *et al*. (2019)

Description: PDC = Porcine dermal collagen and ND = Not determined.

from gamma sea cucumber with the best treatment of various treatments by Alhana *et al*. (2015). This result was even higher than the yield from sea cucumber (*S. monotuberculatus*) by Zhong *et al*. (2015). Further, several other studies had successfully extracted pepsin-dissolved collagen (PDC) from various types of sea cucumber, such as *Bohadschia bivitatta* (Siddiqui *et al*. 2013b), *S. monotuberculatus* (Zhong *et al*. 2015), *Acaudina leucoprocta* (high purity; Lin *et al*. 2017), and *H. cinerascens* (Li *et al*. 2019), with a higher yield than the result from this investigation.

The collagen extraction from the sand sea cucumber in this research generally has a relatively similar process to the commonly known collagen extraction steps, in which pretreatment is followed by extraction. The pretreatment step in this study used 0.1 M NaOH, intending to remove proteins other than collagen, pigment, and fat, which could interfere with the collagen extraction (Nagai *et al*. 2002). Furthermore, the extraction was carried out through maceration with 0.5 M CH_3COOH and 45°C distilled water. The maceration step with 0.1 M CH_3COOH was aimed to dissolve the acid-dissolved collagen, and with an increase of up to 0.5 M CH_3COOH showed an increase in the extracted yield (Sadowska *et al*. 2003). The maceration step with the distilled water helps to cleave the peptide bonds in the collagen, thereby easing the subsequent extraction process. The result of the freeze-dried extracted collagen of the sand sea cucumber is shown in Figure 1.

Molecular Weight Analysis

The purpose of SDS-PAGE analysis was to determine the molecular weight of the collagen. Khiari *et al*. (2014) reported that characteristic bands, i.e., α1 and α2 monomeric chains, on the SDS-PAGE analysis indicate that collagen is classified into type I. Zhong *et al*. (2015) also showed that the sea cucumber collagen is a type I collagen with a triple-helix structure formed by three chains of α1 homologous (trimer) with a molecular weight of ~135–138 kDa, in addition to the α2 chain. The result of the SDS-PAGE analysis (Figure 2) showed that among the four systems of the collagen extraction (M1-M4) with gradual dilution using different solvent 1–3 (D1-D3), (Table 1), the extraction system IV (M4) provided better result which showed by the collagen bands at the molecular weight of ~110–130 kDa after gradual dilution D3 compared to the bands of the catfish skin collagen (KI) as a control. Whereas there were no collagen bands from the extraction system I-III (M1-M3) at each gradual dilution (D1-D3) nor in the remaining pellet (P) of each system. This result indicated that the sand sea collagen extracted using system IV (M4) in this study has the α1 and α2 chain bands, characteristics of the type I collagen, with a molecular weight ~130 kDa and ~110 kDa, respectively. This pattern corresponds to the previous results of the collagen from the body wall of *H. scabra,* which was found to be type I collagen. It contains three homologous α1 chains formed α1 and α2 chains the triple helix as (α1)3 with a molecular weight of 133.2 kDa (Ram 2017). On the other hand, the extraction through pepsin-solubilized collagen (PSC) from the skin of sea cucumber *H. parva* has a molecular

Figure 1 The freeze-dried extracted collagen of the sand sea cucumber (*H. scabra*).

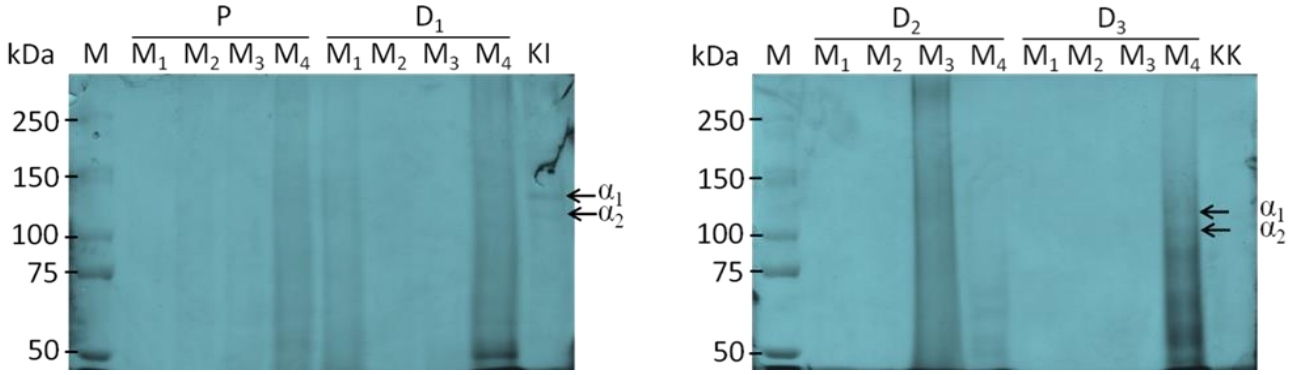

Figure 2 SDS-PAGE profiles of the *H. scabra* collagen. Among the four systems of the collagen extraction used in this research, system IV (M4) provided the best result which showed the collagen band at the molecular weight of ~110–130 kDa. M = protein marker, P = pellet residue of each extraction system, D_{1-3} = dilution results of solvent I-III (Table 1), M_{1-4} = extraction systems I-IV (Table 1), KI = catfish skin collagen, and KK = commercial tilapia fish collagen peptide.

structure of (α1)3 with a relative molecular mass of 130 kDa (Adibzadeh *et al.* 2014). The α1 chain SDS-PAGE pattern of the extracted collagen of the gamma sea cucumber also exhibited type I collagen-containing the main component of the α1 chain with a molecular weight of ~130.33 kDa (Khirzin *et al*. 2016). Besides, this pattern also corresponds to the results of the PDC extraction from the several types of sea cucumber, such as *S. japonicus* (Cui *et al*. 2007), *S. monotuberculatus* (Zhong *et al*. 2015), and *A. leucoprocta* (Lin *et al*. 2017). Moreover, Saito *et al*. (2002) showed that the collagen of the *S. japonicus* was successfully extracted and characterized as a type I collagen which has two distinct subunits, α1 and α2, and forms a triple-helix heterologous (α1)2α2. Whereas, Abdillah *et al*. (2017) showed that the collagen from *H. leucospilota* was identified as type I collagen, which has α1 and α2 chains with higher molecular weight the protein bands of the SDS-PAGE result, i.e., 166.43 and 138.35 kDa, respectively. Li *et al*. (2019) showed that the SDS-PAGE profile of the PDC of *H. cinerascens* belongs to the type I collagen, which contains three chains of α1 chain with smaller molecular weight, i.e., ~ 80-90 kDa. The presence of these SDS-PAGE bands showed that the (α1)3 triple-helix structure of the collagen is maintained.

Furthermore, the presence of the other protein bands in the SDS-PAGE indicates a smaller size than the collagen band size, especially in the extract solution from system IV (M4) after gradual dilution D2 and D3, that might represent other structural proteins of the sand sea cucumber body wall that were also extracted during the extraction. Wang *et al*. (2020) reported that the body wall of *Apostichopus japonicus* contains structural proteins, which were divided into three divisions, i.e., extracellular matrix (ECM) proteins, muscle proteins, and proteases confirmed by using a proteomics approach. The ECM proteins consist of collagens, proteoglycans, and glycoproteins. Meanwhile, those included in muscle proteins are myosins, actins, troponins, tropomyosin, paramyosin, actin-binding proteins, myosin-binding proteins, titins, obscurins, and twitchins. The last type is proteases consisting of aspartic peptidases, cysteine peptidases, metallopeptidases, serine peptidases, and threonine peptidase. Further, according to Cui *et al*. (2007), the addition of mercaptoethanol to the SDS-PAGE analysis, which does not affect the band pattern of the α-chain, indicates that the *H. scabra* collagen does not have disulfide bonds.

Functional Groups Analysis

The FTIR analysis for collagen is helpful to show the specific functional groups in the collagen, such as an amide group. Also, the triple-helix structure of the collagen can be indicated by the infrared spectra (Zhong *et al*. 2015). The functional groups of the sand sea cucumber collagen in the FTIR spectra were detected as amide A, amide B, amide I, amide II, and amide III (Figure 3). The amide A and amide B peaks were observed at a wavenumber of 3379.29 cm^{-1} and 2924.09 cm^{-1}, respectively (Table 3). Amide A resulted from the vibration of NH, while amide B was formed from the CH_2 asymmetry vibration (Coates 2000; Doyle *et al*. 1975).

Moreover, the absorption of the amide I, which shows the vibration of C=O, was observed at a wavenumber of 1681.93 cm^{-1}. The amide I is composed of α-helix, β-sheet, and coil (Kong & Yu 2007). There were also the amide II and amide III,

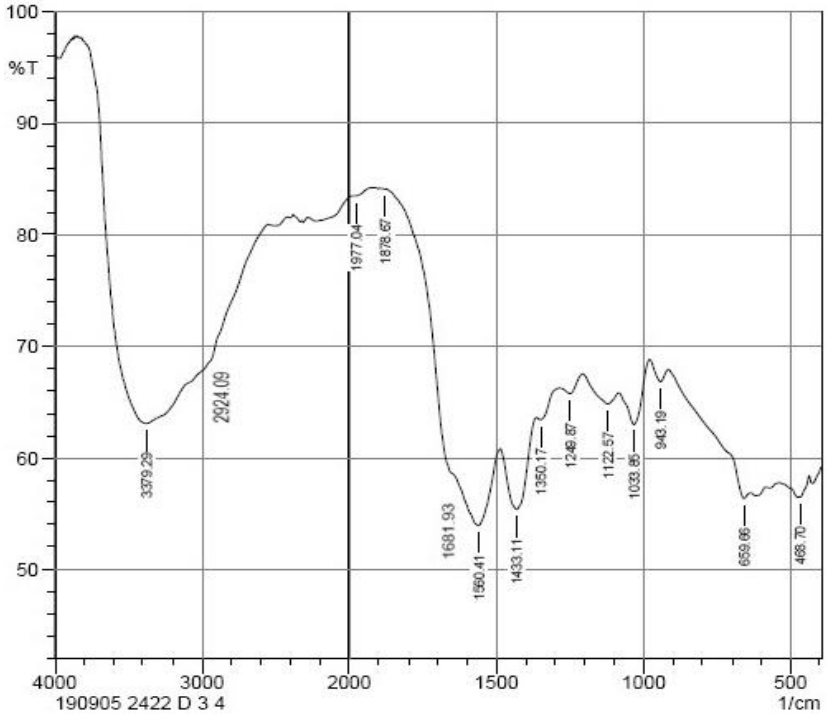

Figure 3 The Fourier-Transform Infrared Spectroscopy (FTIR) spectrum of the *H. scabra* collagen.

which show CN stretching and NH bending, respectively, at a wavenumber of 1560.41 cm^{-1} and 1249.87 cm^{-1}, respectively (Kong & Yu 2007). The extracted *H. scabra* collagen had a maintained-intact triple-helix structure characterized by the ratio of the peak spectrum of the amide III, which was close to 1.0 (Matmaroh *et al*. 2011). Based on these FTIR spectra, the extraction method used did not damage the triple-helix structure of the *H. scabra* collagen. These spectra were also similar to the results of the extracted collagen of the gamma sea cucumber (Alhana *et al*. 2015), sea cucumber (*A. leucoprocta*) (Lin *et al*. 2017), and golden sea cucumber (*S. hermanii*) (Safithri *et al*. 2018).

Collagen Physical Structure Analysis

The physical structure analysis of the sand sea cucumber collagen using SEM was aimed to observe the structure based on the image of the sample surface scanned with a focused-electrons beam. Based on the results of the microscope capture, it was clear that the collagen extracted shows a clot structure that contains the triple-helix structure of the polypeptide of the collagen component (Figure 4). Based on the SEM results, the extraction process of the collagen did not damage the collagen structure.

Amino Acid Composition Analysis

The determination of the amino acid composition was aimed to evaluate the properties of the collagen, especially in the properties of hydrophobic and hydrophilic amino acids. The amino acids composition of this particular collagen is presented in Table 4. The predominant amino acid composition was glycine, followed by glutamic acid and arginine with a concentration of approximately 557.26, 295.82, and 276.48 ppm, respectively. The amino acid composition with the dominant content of the three amino acids was consistent with the result shown in the collagen extracted from the golden sea cucumber (Safithri *et al.* 2018). Moreover, glycine and glutamic acid as the two primary amino acids contained in the sand sea cucumber collagen, corresponded to the result from the *S. japonicus* (Cui *et al*. 2007), sea cucumber (*S. monotuberculatus*) (Zhong *et al*. 2015), and gamma sea cucumber (Khirzin *et al*. 2016). Besides, glycine as the most dominant amino acid in the *H. scabra* collagen residue corresponded to the collagen extracted from the gamma sea cucumber (Alhana *et al*. 2015), sea

Table 4 The composition of the amino acid of the *H. scabra* collagen

Amino acid	Concentration (ppm)
Histidine	4.09
Threonine	105.16
Proline	221.57
Tyrosine	6.69
Leucine	66.31
Lysine	46.11
Aspartic acid	146.28
Glycine	557.26
Arginine	276.48
Alanine	216.42
Valine	69.95
Isoleucine	40.89
Phenylalanine	3.52
Serine	95.58
Glutamic acid	295.82

Table 3 The peaks of infrared spectra attributed to the amides of the *H. scabra* collagen

Amide	Wavenumber (cm^{-1})		Functional group absorption
	The standard area of absorption	Absorption of *H. scabra* collagen	
Amide A	3440–3400 (Doyle *et al*. 1975)	3379.29	NH vibration
Amide B	2935–2915 (Coates 2000)	2924.09	CH_2 asymmetric vibration
Amide I	1690–1600 (Kong & Yu 2007)	1681.93	C=O vibration
Amide II	1575–1480 (Kong & Yu 2007)	1560.41	CN stretching
Amide III	1301–1229 (Kong & Yu 2007)	1249.87	NH bending

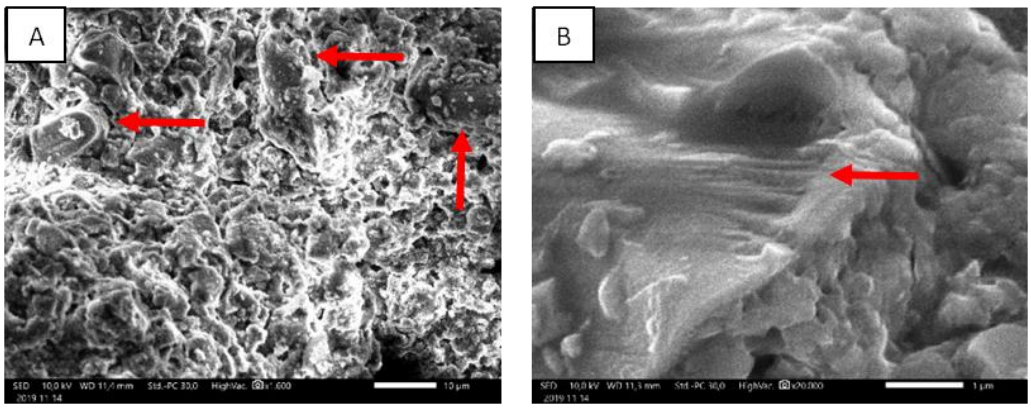

Figure 4 The Scanning Electron Microscopy (SEM) analysis results of the *H. scabra* collagen structure. A) 2000xmagnification and B) 20000xmagnification.

cucumber *A. leucoprocta* (Lin *et al*. 2017), and sea cucumber *H. cinerascens* (Li *et al*. 2019). The collagen molecule is a triple-helix polypeptide that forms the Gly-X-Y sequence, wherein Gly is glycine, X is proline, and Y is hydroxyproline (Friess, 1998). The abundance of glycine amino acids in *H. scabra* collagen triggers hydrogen bonds in the helix collagen strand (Fontaine-Vive *et al.* 2009).

CONCLUSIONS

The collagen of the sand sea cucumber has been extracted, generating a collagen yield of 6% from dry weight by extraction using maceration with 0.1 M NaOH, followed by 0.5 M CH_3COOH, and a final maceration in 45°C distilled water. The collagen has been characterized to have a molecular weight of ~110–130 kDa (type I collagen) based on the SDS-PAGE analysis. Moreover, characterization with FTIR generated spectral peaks in amide A (3379.29 cm^{-1}), amide B (2924.09 cm-1), amide I (1681.93 cm-1), amide II (1560.41 cm-1), and amide III (1249.87 cm-1). Besides, the SEM analysis result of the sand sea collagen showed that the extraction process did not damage the polypeptide triple-helix collagen structure.

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