

Exploration of the collagen of non commercial sea cucumber *Holothuria atra* and commercial sea cucumber *Stichopus vastus* in the Karimunjawa Islands, Indonesia

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Abstract. Yuniati R, Sulardiono B. 2019. Exploration of the collagen of non commercial sea cucumber *Holothuria atra* and commercial sea cucumber *Stichopus vastus* in the Karimunjawa Islands, Indonesia. *Ocean Life 3*: 18-23. The commercial value of sea cucumber needs to be explored because of the availability of bioactive compounds that play an important role in biological activities, including collagen. However, based on the content of compounds, each species has its own variety of values. This study aims to determine the partial characteristics of collagen content of sea cucumber *H. atra* as a non-commercial sea cucumber and of sea cucumber *S. vastus* as commercial sea cucumber in Karimunjawa, Central Java Province. The preparation stage includes the stages of sampling of sea cucumbers *H. atra* and *S. vastus*, followed by the cleaning and washing, and soaking in distilled water, in alcohol, in a solution of Tris-HCl and ethylenediaminetetraacetate (EDTA) and, at last, in sodium hydroxide (NaOH) solution. It was continued by the extraction with immersion of 0.5 M acetic acid solution, and the isolation by precipitation in NaCl and dialysis solutions. In conclusion, the yield extract of *H. atra* and *S. vastus* both had no different values, namely 0.88% and 0.92% and it was classified as low. The whiteness degree of *H. atra* collagen was $61.60 \pm 0.57\%$ and it was higher than the degree of sea cucumber *S. vastus* which was $20 \pm 1.00\%$. The functional group of sea cucumber *H. atra* consists of an amide with wave number of 3460.96 cm^{-1} and alkene with a wave number of 1636.77 cm^{-1} , while in the functional collagen group, sea cucumber *S. vastus* contains amide with wave absorption number of 3434.28 cm^{-1} and alkene with wave absorption number of 1639.46 cm^{-1} and the strong absorption peak at 1033.85 cm^{-1} indicating the presence of free (C = O) bonds. The data and information obtained is used for the biological resource management plan in Karimunjawa.

Keywords: Collagen, *Holothuria atra*, Karimunjawa, *Stichopus vastus*

Abbreviations: FTIR: Foutier Transform Infra Red, EDTA: Trishcl Ethylenediamine Tetra Acetate, NaCl: Sodium chloride, NaOH: Sodium Hydroxide, KBr: Potassium Bromide, HCl: Hydrochloric Acid, TEMED: Tetra Methyl Ethylene Diamine, APS: Ammonium Persulfat, CBB: coomassie brilliant blue, BME: Betamercaptoethanol, PDA: Photodioidide Array Detector, pH: The degree of acidity.

INTRODUCTION

Sea cucumber are benthic that live in coral reef ecosystems and their associations in shallow waters to the deep sea, move slowly (Friedman et al. 2008) and have a relatively long life span (average 5-10 years) (Purcell 2009). Indonesia has 23 species of them that have been identified (Sendih and Gunawan 2006), and the ones which have the highest economic values were found in the genera *Holothuria*, *Muelleria*, and *Stichopus*. Captured sea cucumbers are generally included in the family of aspidochirotidids having 7 genera (Bruckner et al. 2003), inter alia, *S. vastus* and *H. atra*. Species *S. vastus* belong to genera *Stichopus* (Setyastuti and Purwati 2015), and it is classified as a commercial species (Purcell et al. 2012), while *H. atra* species belong to *Holothurian* genera that live on shallow coastal of coral reef ecosystems and muddy sand habitat. This species found in Karimunjawa are not used optimally and are classified as non-commercial. The species are also found in Palau, and in Palau, they are not

used economically and belong to the group of non-commercial sea cucumbers (Pakoa et al. 2014).

Sea cucumbers are known to contain bioactive compounds that have benefits for health and pharmaceutical production. Various bioactive compounds from sea cucumbers that have been explored from marine waters are widely developed, including collagen compounds. However, the development of integrated bioactive compounds as resource conservation and as promising economic benefit has not been widely developed, especially in the development of non-commercial sea cucumbers in Karimunjawa, Central Java Province. Sea cucumber of *S. vastus* is exploited as a commercial sea cucumber because this species has useful bioactive compounds for health and pharmaceutical products, one of which is collagen compounds. On the other hand, Karimunjawa sea cucumber *H. atra* is not exploited optimally even though Sea Cucumber (including *H. atra*) contains 80% collagen (Lubis et al. 2016). This has a consequence, namely, the decreasing of the commercial sea cucumber catch target. Therefore, it is

necessary to develop non-commercial sea cucumbers, in which one type of non-commercial sea cucumber in Karimunjawa is *H. atra*. In this study, the function of sea cucumber *H. atra* is as a non-commercial sea cucumber while *S. vastus* functions as a commercial sea cucumber in Karimunjawa, so that the goal of sustainable management and conservation of resources can be achieved.

Collagen is a fibrous protein and is a major component of connective tissue (Lodish et al. 2000), which has the chemical structure $C_{102}H_{149}N_{31}O_{38}$ (Ogawa et al. 2004) and is a structural protein found in many marine resources (Silva et al. 2014). Sea cucumber collagen has the potential as a substitute for collagen from mammalian sources (Siddiqui et al. 2013), which is known to be harmful to humans due to the effects of infected diseases. Based on this, a preliminary study is needed to find out the qualitative characteristics of sea cucumber collagen *H. atra* as a non-commercial sea cucumber and, as a comparative study, also the characteristics of *S. vastus* which were found in Karimunjawa. Several variables are used to see the partial characteristics of sea cucumber collagen, including pH, whiteness degree (color description), and functional groups. The study aimed to obtain information about the quality characteristics of sea cucumber collagen *H. atra* as a non-commercial group and sea cucumber *S. vastus* as a commercial group in Karimunjawa. The results of the study were utilized as a database in the management of sea cucumber resources.

MATERIALS AND METHODS

Materials and equipment

The material used in the study was sea cucumber *H. atra* and *S. vastus* taken from Karimunjawa waters. Sixty individuals of *H. atra* (non-commercial species) and 35 individuals of *S. vastus* (commercial species) were caught by fishermen from Karimunjawa waters. Equipment for the extraction and isolation of collagen were cutting boards, knives, scales, beaker glasses, magnetic stirrers, freeze dryers, refrigerated showcases and refrigerated centrifuges, while for collagen testing / characterization were oven, furnace, kjeltec, soxhlet, electrophoresis, FTIR (fourier transform infra red) spectrophotometers from Perkin Elmer, Spectrum One, pH meters, and Color Flex EZ Hunter Lab.

Analysis of collagen extract

Sea cucumber samples were taken from Karimunjawa waters then dissected and then the visceral and body walls were separated. The body walls were taken, cleaned and thawed, then inserted into the coolbox, to be taken to the laboratory. Stages of extraction follow the method of Fawzya et al. (2012). Extraction was carried out by immersing the sample in 10 parts volume of 0.5 M acetic acid for 2 days, and then, filtered using calico cloth. To precipitate the filtrate containing collagen was by adding NaCl salt to the final concentration of 1 M and stored overnight, then centrifuged for 60 minutes. Centrifuged pellets were dissolved in 0.5 M acetic acid and to remove

salts in the process of preparation and extraction of collagen, dialysis was carried out with 0.1 M acetate buffer for 1 night, which was replaced every 4 hours, in which at the last replacement, distilled water was used. By replacing it, the salt molecules diffuse out of the dialysis bag so that the collagen protein obtained was purer, and was then dried, through centrifugation. The next step, the measurements were done on collagen extract yield, pH, and whiteness degrees, and analysis of amino acid functional groups. The measurement of extract yield percentage was intended to determine collagen levels based on the weight of collagen produced, by comparing the weight (gram) and dry weight of sea cucumber samples (grams). The percentage of extract yield was achieved by comparing the final results or the results of drying the frozen extract (after freeze drying) with the initial weight of sea cucumber samples (%). The degree of acidity (pH) of the collagen solution was measured using a pH meter

Collagen isolation

Collagen isolation was carried out following the modification method by Fawzya et al. (2012). All extraction stages were carried out at 4°C. The steps were washing the sea cucumber, meat taking, then cutting into small pieces and soaking in aquades (1: 10 b / v) for 2 times while stirring using a stirrer for 30 minutes, to clean sea cucumber meat from the remains of stomach contents and other impurities. Next was the replacement of distilled water with 50% alcohol (1: 2), stirred for 30 minutes to remove fat; then the washing of it with distilled water until the pH was neutral. Subsequent immersion used 10 parts of the mixed volume of Tris-HCl 0.1 M and 4 mM EDTA, for 1 night. Soaking was intended to maintain pH stability and to reduce minerals. After washing with distilled water, the marinade solution was replaced with 10 parts volume of 0.1 M NaOH for 2 days, to eliminate non-collagen proteins, and then was washed again with distilled water. Soaking with NaOH was done until the solution marinade contained no protein, which was checked using the Biuret test.

The whiteness degree (color description) was measured through digital colorimetry using ColorFlex EZ Hunter Lab, so that the values of L, a, and b (%) were obtained. The whiteness degree classification (color description) used the CIE-L * a * b system, where the L * value showed the brightness of the color, where L * = 0 for black and L * = 100 for white. The CIE_a * dimension showed the type of green - red, where negative numbers a * indicated green and vice versa CIE_a * positive indicated red, the CIE_b * for blue - yellow, where negative numbers b * indicated blue and vice versa CIE_b * positive indicated yellow (Hutchings 1999). To analyze the color differences in both collagen *H. atra* and *S. vastus* extracts, the following equation was used: $\Delta E = \sqrt{([\Delta L])^2 + ([\Delta a])^2 + ([\Delta b])^2}$.

Characterize of collagen functional groups used the Fourier Transform Infra Red (FTIR) method (Munyonga et al. 2004), with a spectrophotometer Perkin Elmer, spectrum one. The sample was added by KBr (1: 100), then smoothed until evenly mixed. The next step was pressing

with a vacuum pump for 15 minutes, and reading the absorbance at wave numbers 500-3000 cm^{-1} . Based on the formed curve, the type of bond and its functional group were determined based on FTIR references.

RESULTS AND DISCUSSION

Identification of sea cucumber

The test sea cucumber sample was *H. atra* (Echinoderm: holothuridea). Based on the result of observations in the field, it is showed that this type of sea cucumber spreading on the shores of Karimunjawa waters morphologically has a cylindrical body shape. In general, sea cucumbers in Karimunjawa have a black body which turns to red when they are held by hand in living conditions and the walls are relatively thin. In Karimunjawa, sea cucumber *H. atra* is not classified as economically important and is commercially relatively inexpensive, so it is classified into non-commercial sea cucumbers. Meanwhile, the observation of sea cucumber *S. vastus* (Echinoderm: stichopodidae) has a soft body, blackish white dorsal parts, wide papilla spread and irregular folds formation. The tip of the papilla protrusion is clear white. The stomach part of the skin is white and there are neatly arranged brown tube legs in 3 rows. When the sea cucumber of *S. vastus* is touched and / or disturbed, the sea cucumber removes all the contents of its innards being thrown out. Sea cucumbers *S. vastus* have been captured by many local people, because the price is quite good, so sea cucumber *S. vastus* is grouped into commercial sea cucumbers in Karimunjawa, and is known as *gametes*.

Extract analysis

The result of the extract analysis showed that the extract yield of *H. atra* was 0.88% with moisture content of 0.12%, and with the appearance of blackish white. On the other hand, the yield analysis of *S. vastus* extract obtained value of 0.92% with moisture content of 0.17%. The color appearance of *H. atra* is blackish white, and *S. vastus* is dull grayish white. The measurement result of acidity degree (pH) of collagen extract in both species showed the same value, which was equal to 6.00. The results of the study can be seen in Table 1.

The whiteness degree

The results of the analysis of the degree of whiteness (color description) from collagen were $61.60 \pm 0.57\%$ in *H. atra* and $20 \pm 1.00\%$ in *S. vastus* (Table 2). The difference of whiteness degree between the two collagen extracts can be seen through the equation of $\Delta E = \sqrt{[(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]}$, so that the value of $\Delta E = 55.87$. Based on this equation, it could be seen that the difference in color of collagen extract of *H. atra* and *S. vastus* is 55.87. The results of the analysis showed that *H. atra* collagen ($61.60 \pm 0.57\%$) was brighter than *S. vastus* collagen ($20.00 \pm 1.00\%$).

Table 1. Yield content, Moisture content, pH, and color appearance of *H. atra* and *S. vastus* from Karimunjawa, Central Java Province, Indonesia

Economic value	Species name	Extract yield content (%)	Moisture content (%)	pH	Color of extract appearance
Non commercial	<i>H. atra</i>	0.88	0.12	6.00	Blackish white
Commercial	<i>S. vastus</i>	0.92	0.17	6.00	Dull grayish white

Table 2. The whiteness degree (color description) of *H. atra* and *S. vastus* collagen from Karimunjawa, Central Java, Indonesia

Sampel	Value (%)		
	L	a	b
<i>H. atra</i>	62.00	-16	17.0
	62.00	-15	16.0
	61.00	-15	16.0
Average	61.60	15.3	16.3
SD	0.57		
<i>S. vastus</i>	21.00	-7	15.0
	20.00	-7	13.0
	19.00	-7	13.0
Average	20.00	-7	13.6
SD	1.00		

Note: SD: Standard deviation

Functional group of collagen

The result of partial characterization of sea cucumber collagen compounds can be seen in Figure 1 for sea cucumber *H. atra* and Figure 2 for sea cucumber *S. vastus*. The interpretation of the data showed that the functional compound group of *H. atra* collagen consists of an amide group (N=H bonds) with a wave absorption number of 3460.96 cm^{-1} , and an alkene group (C=C bonds) with a wave absorption number of 1636.77 cm^{-1} .

Figure 2 indicated that *S. vastus* functional compound group consists of an amide group (N = H bonds) with wave absorption number of 3434.28 cm^{-1} and alkene groups (C=C) bonds with wave absorption number of 1639.46 cm^{-1} and the strong absorption peak at 1033.85 cm^{-1} indicated the presence of C=O bonds.

From the results of the spectrum that appeared in both species, sea cucumbers have groups that are in accordance with the chemical structure of collagen in general which is composed of amino acid sequences. When viewed from the first and second absorption peaks, collagen from the two types of sea cucumbers appears to have a similar functional compound group, but there is a noticeable difference in the fourth absorption area which is a fingerprint, so these two compounds are actually not identical (Skoog 1998).

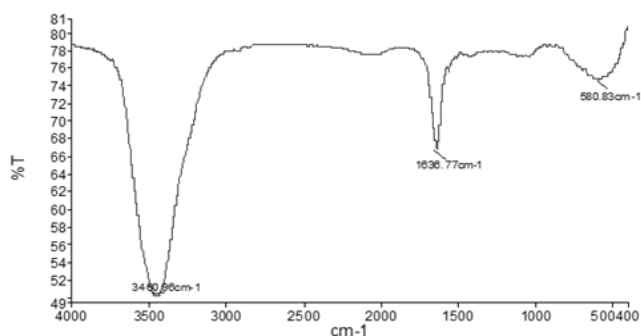


Figure 1. Graph of infrared spectrum of collagen of sea cucumber *H. atra*

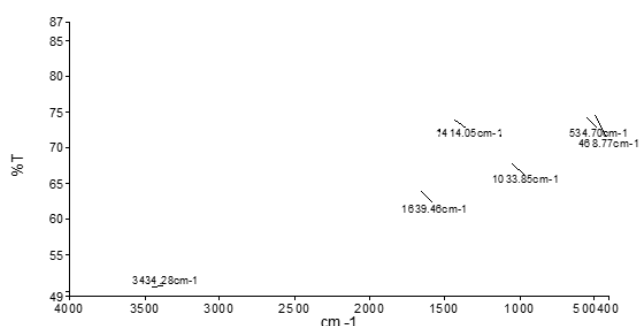


Figure 2. Graph of infrared spectrum of collagen sea cucumber *S. vastus*

Discussion

Collagen contained in the body of sea cucumbers reaches 80% (Fawzya et al 2016) and many of them are in integumentum (Abeidin et al. 2015). The quality of sea cucumber extract collagen can be used as an indicator of standard charts for utilization of pharmaceutical and health products. Some of the used indicators are yield, pH, whiteness degree and functional compound groups. Some of the roles of collagen are homeostasis, interactions with platelets, interactions with fibronectin, increasing fluid exudation, enhancing cellular components, increasing growth factors and promoting fibroplasia and sometimes epidermal proliferation (Triyono, 2005). The extract yield is an important parameter in the process of isolating sea cucumber collagen produced by the body tissue of sea cucumbers. Body wall collagen extract of sea cucumber *H. atra* is dull grayish white, while the *S. vastus* is rather blackish white. The drying process of sea cucumber collagen extract is done by freeze dryer. Collagen extract yield was obtained by comparing the total powder of collagen extract resulting from drying with the initial weight of the sample before isolating the collagen in percentage.

The results of the calculation of the extract yield percentage of *H. atra* and *S. vastus* collagen were 0.88% and 0.92% respectively. The value of *S. vastus* collagen in this research was lower than that of *S. variagatus* which was 1.50% (Alhana et al., 2015), but higher than the yield value of *S. hermannii* which was 0.66% (Safithri et al.

2018). The content of non commercial sea cucumber *H. atra* collagen was included as low category (Lubis 2015). Increased yield can be influenced by water content, in which extracts with low water content will produce high yield values. In this research, the Moisture content of the extract of non-commercial sea cucumber *H. atra* was 0.12% resulting in a yield of 0.88%, while the Moisture content of the extract of *S. vastus* was 0.17% resulting in a yield of 0.92%.

The results of pH measurement of *H. atra* and *S. vastus* extract were both 6.00. This pH value is lower than the value issued by the National Standardization Agency, which ranges from 6.5 to 8 (National Standardization Agency 2014). If the pH value of the research results is compared with the pH value of the results of Alhana et al (2015) in the study of sea cucumber *S. variagatus* (pH value: 7.37), the results of this study are low pH value but they are same when compared to sea cucumber *S. hermannii* of 6.91 (Safithri et al 2018). Although the material used to extract collagen is same, namely acetic acid, but the concentration and extraction temperature applied in this study are different so it produces different pH characteristics of collagen. According to Peng et al. (2004), commercial collagen used for cosmetics is at pH 3.8-4, and the pH value is related to the salt / mineral content which functions as a buffer for collagen solution. Based on this, if both sea cucumbers are applied to commercial cosmetic products, then both species need to be developed

The degree of whiteness (color description) can be used as an indicator of collagen quality based on the physical properties of collagen. Collagen can be said to be of good quality, if it has a white base color with the whiteness degrees close to 100% (Alhana et al. 2015). The result of the whiteness degree analysis (color description) on non-commercial sea cucumber *H. atra* collagen was $61.60 \pm 0.57\%$. As a comparison, the result of the analysis of whiteness degree in the commercial sea cucumber *S. vastus* collagen was $20.0 \pm 1.00\%$. Based on the analysis results, the degree of the whiteness degree of non-commercial sea cucumber *H. atra* collagen was brighter than the collagen of commercial sea cucumber *S. vastus*, so that it could be said that the collagen of non-commercial sea cucumber *H. atra* had better quality than the one of commercial sea cucumber *S. vastus*. The whiteness degree of *H. atra* was not much different from the whiteness degree of *S. variagatus* which was 69.01% (Alhana et al. 2015). The difference in the whiteness degree (color description) of sea cucumber collagen between species can be caused by the utilization of the extraction method, which affects the effectiveness of extraction in reducing pigment and increasing the whiteness degree. Thus, based on the value of the quality of the whiteness degree, the extract of sea cucumber *H. atra* can be said to be still in the acceptable quality category for collagen products.

The functional group is the reaction of reactive part of the organic molecule in which the results can be predicted. Functional groups contain atoms other than carbon atoms and have free electron pairs on these atoms. Based on the graph of the infrared spectrum of sea cucumber *H. atra* collagen, the amide group (N-H bonds) occurs at the wave

absorption number of 3460.96 cm^{-1} , and the alkene group (C=C bonds) is at the wave absorption number of 1636.77 cm^{-1} , while in the analysis of infrared spectrum from sea cucumber *S. vastus* collagen, it can be seen that the presence of amide group (N=H bonds) is on the number of wave absorption of 3434.28 cm^{-1} and alkene groups (C=C) bonds with wave absorption number of 1639.46 cm^{-1} and the strong absorption peak is at 1033.85 cm^{-1} . According to Singh et al. (2011), a peak absorption strength at 1033.85 cm^{-1} indicates the presence of free (C = O) bonds, and (N=H) bond vibrations. Of the two types of sea cucumbers, it generally can be said that collagen composition is structurally composed of amino acids.

Based on the result of measurement of pH values and the degree of leucorrhoea of non-commercial sea cucumber *H. atra* collagen and commercial sea cucumber *S. vastus* collagen, these two species had quite good quality to be developed as a commercial product. Based on the results of the assessment of the values of the two types of sea cucumber, the value of the percentage of collagen is low. This indicates the extraction quality in the sea cucumber *H. atra* and *S. vastus* in Karimunjawa is quite good. Based on analysis of functional group on the sea cucumber *H. atra* and *S. vastus*, it can be seen that they are generally composed of amino acids with amide (N-H) and alkene (C-C) bonds.

Collagen from these two species of sea cucumber appears to have a similar functional group, based on the first and second absorption peaks, but there are noticeable differences in the area namely fingerprinting that show no identical on them (Skoog, 1998). Given that, in this study, the character of functional groups is carried out qualitatively, so the results cannot be used in commercial product applications, and quantitative analysis is needed. Various functional groups contain atoms other than carbon atoms and have free electron pairs on these atoms. A functional group reference is a certain atom which is bound in a certain arrangement that gives certain physical and chemical properties to a compound. According to Saraswati et al, (2016), functional groups are a group of atoms that are responsible for the characteristic reactions of compounds, wherein these functional groups play an important role in controlling organic reactions.

As is known before, sea cucumber *H. atra* is rare, and sea cucumber *S. vastus* is not a catch target of Karimunjawa fishermen. This is supported by Abedin et al. (2015) who stated that sea cucumbers (*S. vastus*) are underutilized species in Malaysia, so the benefits of their collagen are to wasted only. However, after the rare sea cucumber *H. scabra* was found, the catch target of Karimunjawa fishermen switch to several other species that still had relatively good selling prices, among them was *S. vastus*. On the other hand, in the waters of Karimunjawa, there were abundant species of *H. atra* which had not been utilized optimally by local fishermen. Therefore, if the non-commercial species *H. atra* can be developed, it can assist in the efforts to conserve these resources (*H. atra* and *S. vastus*) in order to remain sustainable. Collagen is one of the compounds possessed by sea cucumbers which can be used commercially. This is because the benefits of sea

cucumber collagen are very strategic for health products, especially in the use of cosmetic product (Siahaan et al 2017) and the other benefit is the use of them in hydrolyzing collagen as a functional ingredient in food and nutraceutical products (Abeidin et al 2015). To reach this goal, the first step is the importance of spreading information about the quality of sea cucumbers, especially in *H. atra* and *S. vastus* species in Karimunjawa, although the quality of this collagen cannot be used as a comparison in its application. At least this step is the first step that can be used as a database before quantitative analysis is carried out.

It can be concluded that the whiteness degree analysis (color description) of *H. atra* collagen was $61.60 \pm 0.57\%$, meanwhile the *S. vastus* was $20.0 \pm 1.00\%$. It can be said that the quality of the whiteness in *H. atra* was better than that of the sea cucumber *S. vastus*. The FTIR spectrum of functional groups in the sea cucumber *H. atra* shows the presence of amides (N = H bonds) and alkene (C = C bonds), meanwhile, in sea cucumber *S. vastus*, it shows the presence of amides (N = H bonds) and alkene (C = C bonds) and strong absorption at the peak of 1033.85 cm^{-1} which indicates a free bond (C = O). The whiteness degree analysis (color description) on collagen of non-commercial sea cucumber *H. atra* was $61.60 \pm 0.57\%$, while the analysis of whiteness degree on the collagen of sea cucumber commercial *S. vastus* was $20.0 \pm 1.00\%$, so that it can be said that the degree of whiteness in *H. atra* is better than that of sea cucumber *S. vastus*. The FTIR spectrum of functional groups contained in sea cucumber *H. atra* shows the presence of amides (N = H bonds) and alkene (C = C bonds), meanwhile, in sea cucumber *S. vastus*, it shows the presence of amides (N = H bonds) and alkene (C = C bonds) and strong absorption at the peak of 1033.85 cm^{-1} which indicates a free bond (C = O).

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