

Saponin Isolation as Main Ingredients of Insecticide and Collagen Type I From Crown of Thorn–Starfish (*Acanthaster planci*)

Anondho Wijanarko¹, Mikael Januardi Ginting², Muhamad Sahlan^{1*}, Imelda Krisanta Endah Savitri³, Yunita Florensia¹, Maria Regina Sudiarta¹, Satria Pastika¹, Fakhri Rafiki¹, Heri Hermansyah¹

¹Department of Chemical Engineering, Universitas Indonesia, Kampus Baru UI Depok-16424, Indonesia

²Department of Biology, Universitas Indonesia, Kampus Baru UI Depok-16424, Indonesia

³Department of Aquatic Product Technology, Faculty of Fishery, Pattimura University, Kampus Unpatti-Poka, Ambon, Indonesia 97233

Email: sahlan@che.ui.ac.id

Abstract. The outbreaks of crown of thorns starfish (*Acanthaster planci*) resulted in the severe destruction of coral reefs in a large number of Indonesia's marine ecosystem, especially in the western part. At the moment, control efforts are proven to be ineffective because of its high cost and labor intensive. Recent research found that *A. planci* contain saponins that act as cytotoxic compound and can be used as an environment-friendly insecticide to eradicate *Kalotermitidae* pest. Saponins extracted by maceration using ethanol 96.0% with a total yield of saponins 9.04% and 4.66% for two test. Purification of saponin was achieved by utilization of activated carbon with a mass of carbon:volume sample 1:2 (w/v) and stirred for 20 minutes. Sapogenin can be isolated by hydrolyzing using hydrochloric acid, and thus 168.4 mg sapogenin is obtained. In addition to saponins, *A. planci* also contains collagen Type I. Collagen isolation by multistage extraction began with extracting the collagen with alkaline solvent, with water, NaOH 0.1 M, and Ca(OH)₂ 0.2 M as the solvent variations. The second step is acid-enzymatic extraction by pepsin digestion in 0.5 M acetic acid. Collagen extract will be further purified by salting out and dialysis method to obtain pure collagen yield called Pepsin Solubilized Collagens (PSC). Characterization of PSC consists of quantitative and qualitative analysis such as Lowry method, gel electrophoresis, UV spectroscopy, amino acid composition analysis, and Scanning Electron Microscopy (SEM). The result shows Ca(OH)₂ 0.2 M as the best extraction solvent with 2.26% yield of PSC.

1. Introduction

Acanthaster planci is a species of starfish from Echinodermata phylum, which feeds on live corals and thus possesses a great threat to corals ecosystem. Commonly known as crown of thorns starfish in English or Oni Hitode (means ogre starfish) in Japanese, *A. planci* is famous for its thorns throughout its body surface. Adult crown-of-thorns starfish normally range in size from 25 to 35 cm and the



thorns are 5 to 6 cm long [1]. These starfish prefer to stay in more protected areas such as lagoons, and the deeper ocean along the reef front. As one of the main predators of coral reefs, *A. planci* can consume up to 5 – 6 m² corals in a year [1]. However, we recently discovered that the consumption rate could reach 5 – 13 m² per year [2]. Therefore, the population outbreak of *A. planci* will be resulted in corals destruction and thus give a negative impact on the ecosystem of the coastal region. Population outbreaks of *A. planci* are very dangerous because it damages coral reefs permanently, as well as disrupting the marine ecosystem since there are a lot of marine organisms live in the corals [1].

In Indonesia, *A. planci* is a species of starfish naturally found in the coral reefs in the Indo-Pacific Ocean. It acts as the control of coral reefs population because it is feeding on the corals, such as *Acropora* (small coral) [2]. However, in the last few years, there are many reports about *A. planci* population outbreaks, which damage coral reefs and marine ecosystem severely. Till now, *A. planci* population control in Indonesia, Japan, or Australia has been proven ineffective, such as manual removals, chemical injection into the starfish, provide fences alongside the corals, or cutting *A. planci* body into tiny pieces [2].

As an alternative of *A. planci*'s population control, an integrated research for utilizing *A. planci* contained bioactive should be conducted. *A. planci* has numbers of proteins in the spines venom of their surface body. It was reported there are at least three kinds of proteins/peptides in that venoms, Plancinins that act as an anticoagulant factor, Phospholipase A₂s (PLA₂s) as an antibacterial agent, and Placitoxins that is similar to mammalian deoxyribonucleases II [4]. Besides the spines venom inside its thorns, *A. planci* body contains many other substances such as lipid, saponin, collagen, carotenoids, calcium, phosphor, and other minerals, which haven't been utilized effectively.

Saponins are natural substances that can act as secondary metabolites. Saponins consist of polycyclic aglycones attached to one or more sugar side chains. The aglycone (glycoside-free) portions of the saponins also called sapogenins [5]. However, the term asterosaponin has come to represent any saponin isolated from Asteroidea. Asterosaponin is famous for its toxicity and highly lethal for another organism, such as fish, Annelida, Mollusca, arthropods, and vertebrates. Because of its toxicity, asterosaponin is being utilized as a cytotoxic agent, antineoplastic, hemolysis agent, antitumor, antibacterial, antiviral, antifungal, and anti-inflammatory. There are several types of asterosaponin from *A. planci*, namely thornasteroid A [6] and Acanthaglycoside A, B, C, D, E, F [7].

Saponins are phytochemicals which can be found in most vegetables, beans, and herbs, such as tea, asparagus, ginseng, *Phaleria macrocarpa*, *Yucca sp.*, *Polygala senega L.*, etc. As chemical compounds found abundantly in various plant species, saponins exhibit a variety of biological activities and have been investigated toward the development of new natural medicines because of their uses as an antibacterial agent, antiviral, anti-inflammatory, anticancer, and antitumor [8]. Besides its medical application, saponins are useful for pest control in agriculture or aquaculture because of its natural insecticide properties. In 2011, Agriculture Research Center in Bandung, studied the role of saponin from tea seed to exterminate shrimp pest. In 2007, Research and Development Center of Agriculture formulated a combination of tea saponin and *rerak* as a biopesticide for snail pest. Saponin utilization as a natural insecticide is highly potential because of its lethal effect on cold-blooded animals [9]. The deep experiment of this research and development center resulted that saponin utilization as an environmental safety pesticide is proven. Therefore, it's very beneficial to utilize saponins from *A. planci* which was known as a parasite and threat for corals and marine ecosystem. For increasing *A. planci*'s saponin utilization, preliminary research of its effectiveness against pest from *Kromoptera* family is conducted. This pest (termites) is very harmful to home appliances or furniture made of woods and bamboo. Termites booming caused financial loss up to Rp 250.000.000.000,- every year [10]. An easy and low-cost method for saponin extraction and purification must be performed as well as sapogenin isolation and in vitro test of its insecticide properties against termites.

As mentioned before, besides saponin, the whole body of *Acanthaster planci* highly contain type I collagen. Collagen is the most abundant protein in various connective tissues and was previously known only in animals. Therefore collagen for industrial purpose is mainly extracted from bovine,

porcine, and fowl origins. Also, collagens are proven to be higher in mammals compared to marine animals [11-12]. However, the outbreaks of bovine spongiform encephalopathy and foot-and-mouth disease have caused concern enhancement regarding bovine collagen application. Furthermore, halal issue and H5N1 disease (avian flu) also influenced concern enhancement regarding collagen originated from porcine and fowl [13]. On the other hand, plant-derived collagen, or phycollagen, would not be as effective because it acts only as a precursor to boost collagen synthesis. These factors have led to extensive study on marine collagen as an alternative to another source of collagen. Most of the present researches on marine collagen are extracted from fish, jellyfish, sea urchin, sea cucumber, and starfish [14-17]. Moreover, marine collagen has been recognized as potential alternative sources, due to their stability and high biocompatibility with human tissues [18], as well as their low denaturation temperature [19]. Besides its superior properties, marine collagen from the under-utilized *A. planci* has high potential because it would not compete with food necessity like it does with fish, sea urchin, sea cucumber, squids, etc. Hereinafter, increase the utilization of *A. planci* to get collagen extract by multistage extraction method is also be performed. Multistage extraction method for the industrial purpose has been well developed, and thus it is very effective and feasible. Afterward, it provides some insights on the physicochemical properties of *A. planci*'s collagen despite the lack of information. Finally, it provides hopefully an optimum extraction method to obtain high collagen yields as an alternative source of commercial collagen for food, cosmetic, biomaterial, and biomedical industries.

2. Materials and Methods

2.1 Saponin Isolation

2.1.1 Solvent Selection for Saponin Extraction. Crown of thorns starfish (*Acanthaster planci*) was captured from Ambon, Island, Moluccas prefecture in February 2014. The starfish was then frozen and stored at -20°C until used. As pretreatment, this starfish was washed thoroughly with distilled water to remove salt or other materials. After that, all thorns on *A. planci* body surface were removed manually, and then the body was cut into small pieces (less than 1 cm). The sample was further treated with hexane 1:1 (w/v) for 1×24 hour to remove the non-polar substances. The next step is to treat the sample with ethyl acetate 1:1 (w/v) for 1×24 hour to remove the semi-polar substances. Afterward, the saponin was extracted by organic solvent with four variations, such as distilled water, 96% ethanol, n-butanol, and isopropyl alcohol for 3×24 hour by changing the solution daily. The sample and solvent ratio was 1:3 (w/v). The mixture was filtered with Whatman 42 and further thickened (to remove the solvent) by increasing the temperature until the solvent boiling point was reached. As a result, a viscous solution was obtained and subjected to UV spectroscopy at $\lambda = 255\text{nm}$; $E1\% = 15$ [6] to quantify the amount of saponin. On the other hand, qualitative analysis of saponin extraction was carried out by Liebermann Burchard and Froth test. The solvent with the highest yield of saponin will be selected for the scale-up process.

2.1.2 Froth Test. 0.5 ml crude extract / 0.05-gram pasta from extraction was diluted with 1 ml of water at 80°C and then shook vigorously. If foaming happens, wait until 30 minutes then add 1 drop of HCL 2N [20-21].

2.1.3 Liebermann Burchard Test. Liebermann Burchard (LB) reagent consisted of anhydrous acetic acid and concentrated sulfuric acid. Sample identification was done by adding LB reagent with the ratio of 5:5:3 (sample: acetic acid: sulfuric acid) [27].

2.1.4 UV Spectroscopy. Quantitative analysis of saponin extraction was achieved by UV spectroscopy at $\lambda = 255\text{nm}$; $E_{1\text{cm}}^{1\%} = 15$ [6]. Saponin concentration in the extract could be measured by Lambert-Beer Law.

$$A = E_{1\text{cm}}^{1\%} \times b \times c \quad (1)$$

A = absorbance

$E_{1\text{cm}}^{1\%}$ = specific absorptivity coefficient

b = cuvette width (1 cm)

c = saponin concentration in the sample
(% or mg/100ml)

The absorption spectra were recorded by spectrophotometer Spectroquant Pharo 3000, Merck, Germany.

2.1.5 Scale up for Saponin Extraction. Scale up was achieved by using ethanol 96% as the extraction solvent (since it gave highest saponin yield from the selection process before). In scale-up, pretreatment was carried out similar to the pretreatment process in 2.1.1. Scale-up was carried out in duplicate, with the extract resulted from every step being filtered, thickened, and tested (Froth and Liebermann Burchard), as well as quantified by UV spectroscopy so that the daily profile can be obtained.

2.1.6 Saponin Purification with Activated Carbon. Saponin purification with activated carbon was repeated 3 times to find the optimum purification method. Commercial activated carbon with granular properties was used in this step. For every variation, the ratio of activated carbon mass and the sample solution is 1:1 or 1:2 (w/v). The selected solvent was ethanol 96% (sample:solution = 1:10 (v/v)) so that after purification sample can be further concentrated by evaporating the ethanol at 78.4°C . The process was carried out in batch for 10 or 20 minutes with continuous stirring and several variations as follow:

1. Activated carbon and sample ratio is 1:1(w/v), retention time 10 minutes, batch process, and no repetition.
2. Activated carbon and sample ratio is 1:1(w/v), retention time 10 minutes, batch process, and repeated for 5 times.
3. Activated carbon and sample ratio is 1:2(w/v), retention time 20 minutes, batch process, and no repetition.

2.1.7 Sapogenin Isolation. Saponin extraction from method 2.1.6 (c) was further treated with 150 ml ethanol 50% and 50 ml HCl 1.85N to isolate the sapogenin. After that, the mixture was refluxed for 7 hours at 78.37°C [22]. The precipitate was filtered and analyzed by UV spectroscopy to find the λ_{max} at EtOH=246.5 nm and $E_{1\text{cm}} = 16$ [6]. The precipitate resulted will be further analyzed with LC-MS at Forensic Laboratory Centre – Indonesian Police Headquarter.

2.1.8 Saponin and Sapogenin Characterization. Saponin and sapogenin characterization by LC-MS is carried out on the sample before and after sapogenin isolation treatment. LC-MS was also measured at Forensic Laboratory Centre – Indonesian Police Headquarter.

2.1.9 In Vitro Test of Saponin Role Insecticide. In vitro test of saponin role as biopesticide was carried out by exterminating termites from a guava tree. Saponin extract with a concentration of 20, 10, 1, and 2 mg/ml were applied against 5 – 10 termites.

The death rate was noted for every concentration, and thus the concentration with precise results will be selected for next step. The selected concentration will be further analyzed with statistical method. 100 termites were divided into 20 groups inside a $10 \times 10\text{cm}$ box. The first 10 groups were

sprayed with water, and the other 10 groups were sprayed with the selected concentration of saponin. The death rate for every box was noted and further analyzed to find the efficiency of saponin as insecticide

2.2 Collagen Isolation

Collagen extraction from the body of *A. planci* was performed according to the method of extraction of pepsin solubilized collagen from the body wall of crown-of-thorns starfish by [23], modified with biochemical characterization of collagen from the starfish *Asterias amurensis* by [24].

2.2.1 Collagen Extraction. All procedures were performed at 4°C. First, the thorns on *A. planci* body surface was removed entirely; then the entire body was cut into small pieces (2 2 cm) and washed with distilled water. 15 g of sample was soaked in distilled water at a sample-to-solution ratio of 1:10 (w/v) for two days to remove the non-collagenous materials. For solvent variation, the second and third sample was soaked in 0.1M NaOH and 0.2M Ca(OH)₂. The insoluble body tissue was collected and washed with 200 ml of distilled water for an hour with gentle stirring. The body was then demineralized and disaggregated with 250 ml of 0.05 M Tris-HCl (pH 8) containing 0.5 M ethylene diamine tetra-acetic acid disodium salt (EDTA- 2Na) for 3 days. The mixture was filtered and washed with 500 ml distilled water for 2 days by changing the distilled water every 24 h. The mixture was further centrifuged at 3200 g for 30 min to collect the collagen fibrils (crude extract).

For the second extraction process, the collagen fibril was suspended in 0.5 M acetic acid at a sample-to- solution ratio of 1:10 (w/v) and digested with pepsin at pepsin-to-collagen fibril ratio of 1:15 (w/w) for 2 days. The resultant viscous solution was filtered and centrifuged at 10000x g for 30 minutes. The resultant supernatant was then dialyzed against 0.02 M Na₂HPO₄ (pH 7.2) for 3 days by changing the solution daily.

2.2.2 Collagen Purification. The dialysate obtained from the previous step was further centrifuged at 10000x g for 30 h. The precipitate obtained was dissolved in 0.5M acetic acid. Salting out was then performed by adding NaCl to the mixture at a sample-to-solution ratio of 5:100 (w/v). The resultant precipitate was collected by centrifuging of the mixture at 20.000 × g for 30 h. The resultant precipitate was re-dissolved in 0.5M acetic acid before it was dialyzed against 0.1M acetic acid and distilled water, respectively. Dialysis against 0.1M acetic acid was performed for 2 days by changing the solution daily while the dialysis against distilled water was performed for 1 day along with the changed of the solution at every 5-6 h. This collagen obtained was referred to as pepsin-solubilized collagen (PSC).

2.2.3 Collagen Characterization. Collagen identification and characterization were carried out by UV spectroscopy, SDS Page, and amino acid composition analysis. Furthermore, the structure of pepsin-solubilized collagen (PSC) was compared to industrially manufactured collagen by Scanning Electron Microscopy (SEM) and quantified by Lowry method.

2.2.3.1 UV Spectroscopy. The collagen samples were dissolved in 0.5 M acetic acid solution with a sample/solution ratio of 1:10 (w/v) and the UV absorption spectra were recorded by a spectrophotometer (*Spectroquant Pharo 3000, Merck, Germany*) at room temperature.

2.2.3.2 Lowry Method. Lowry method was performed according to the methods of [25] with slight modification. Lowry method was carried out in duplicate. First, mix reagent A (1% KNaC₄H₄O₆·4H₂O, 8% Na₂CO₃ and 2% CuSO₄·5H₂O), reagent B (4% NaOH 1 M), and reagent C (1% Sodium Dodecyl Sulfate) with the ratio 3:1:1. Then prepare standards from 0.25 mg/ml bovine serum albumin (BSA) by adding 40-300 microliters of distilled water to bring volume to 350 microliters/tube. Add 400 microliters of the reagent, mix thoroughly, incubate at room temperature for 10 minutes. Add 200 microliters 0.2 N Folin reagent very quickly, and vortex

immediately. Complete mixing of the reagent must be accomplished quickly to avoid decomposition of the reagent before it reacts with protein. Incubate for 30 minutes more at room temperature. Use glass or polystyrene cuvettes to read the absorbance at 650 nm. BSA concentration and the absorbance can be plotted as a calibration curve to obtain a formula:

$$y = mx + c \quad (2)$$

Where y is the absorbance value, and x is the protein concentration in mg/ml. To find the protein concentration in the sample, we repeat the steps above by replacing BSA with the samples. But first, the PSC solution was centrifuged at $10,000 \times g$ for 2 min to remove the undissolved collagen fibrils. The supernatants were used in this analysis.

2.2.3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoretic patterns of collagens were analyzed according to the method of [26]. The SDS-PAGE was carried out using discontinuous Tris-glycine buffer (Tris-HCl 0.5 M pH 6.8, 2% SDS, 25% glycerol, 0.1% bromophenol blue, and 5% β -mercaptoethanol) with the ratio 1:1 (v/v). The mixtures were then heated at 95-100°C for 3 minutes. After that, the mixtures were centrifuged at 13,000 rpm for 3 minutes to remove the undissolved collagen fiber. A total of 30 μ g of each collagen sample was loaded on the gel (4% stacking gel and 10% resolving gel) and subjected to electrophoresis at a constant current of 20 mA/gel (Atto Mini Gel Electrophoresis, AE-6531, Japan). After SDS-PAGE, the gel was stained with staining solution containing 0.1% (w/v) Coomassie® brilliant blue R-250 in 40% (v/v) methanol and 10% (v/v) acetic acid for 12 h. The gel was then de-stained with a de-staining solution containing 40% (v/v) methanol and 10% (v/v) acetic acid.

2.2.3.4 Amino Acid Composition Analysis. Amino acid composition analysis was performed at PT. Saraswanti Indo Genetech (SIG Laboratory), Bogor, West Java and Province Health Laboratory of Jakarta.

2.2.3.5 Scanning Electron Microscopy (SEM). The morphological characteristics of the isolated collagen were observed by Scanning Electron Microscopy (SEM) at LIPI Biology Research Center, Bogor. First, the collagen sample was dehydrated and coated with gold ion by ion coater iB2 then it was ready to be observed.

3. Result and Discussion

3.1 Saponin Isolation

3.1.1. Solvent Selection for Saponin Extraction. This result showed that butanol gave the highest amount of crude extract (total mass of saponin) which means butanol is the best solvent for saponin extraction. However, UV spectroscopy analysis showed ethanol 96% as the best solvent. Qualitative analyses such as Liebermann Burchard and Froth test gave a positive result and proved the high saponin content in the extract. Purple coloration at Liebermann Burchard test showed that saponin contains aglikon/sapogenin triterpenoid [27].

Table 1. Crude extract with polar solvent variation.

Solvent	Sample (gram) (wet weight basis)	Evaporated weight (gram)	Crude Extract (%)
Butanol	25.04	15.74	62.86
Ethanol 96%	10.01	4.720	47.15
Water	25.01	11.56	46.24
Iso-propanol	10.05	1.120	11.14

Table 2. Yield of saponin from extraction with polar solvent.

Solvent	Weight Basis Initial Sample Weight (gram)	Total Mass of Saponin (mg)	Yield of Saponin (%)
Butanol	250,400	71,610	28,60
Ethanol	100,100	29,100	29,07
Water	250,100	4713	1,880
Iso-propanol	100,500	2,832	2,820

This result also showed that butanol gave the highest amount of crude extract (total mass of saponin) which means butanol is the best solvent for saponin extraction. However, UV spectroscopy analysis showed ethanol 96% as the best solvent. Qualitative analyses such as Liebermann Burchard (LB) and Froth test also gave a positive result and prove the high saponin content in the extract. Purple coloration at Liebermann Burchard test showed that saponin contains aglicone/sapogenin triterpenoid [27].

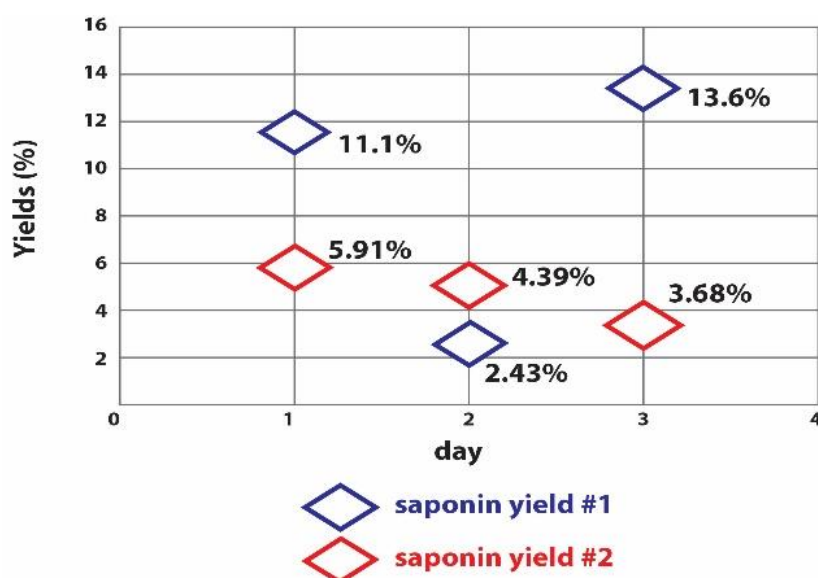
3.1.2. Scale up for Saponin Extraction

Scale up of saponins extraction using ethanol 96%, and obtained results yield saponins as shown in Fig. 1. It is seen that the second trial of saponin yield has lower than the first trial result (22.99%), and this second trial test yield (12.98%) is smaller than last solvent selection yield result. This is caused used starfish used at scale up, is different slice size, and it is provided this sample is not as small as solvent selection one (> 1 cm), so that reaction surface with ethanol 96% eigs reduced. This figure is shown extraction yield per day of saponins. It showed that a drop in yield saponins from day to day. However, in the first trial, yield has increased on the third day, and it caused of reaction time at the second day of extraction is happen only 12 hours while on the third day it took place 30 hours. The qualitative test result of scale-up extraction for proven of saponin existence is shown in Table 3.

Table 3. Froth and Liebermann Burchard Result.

Solvent		Froth Test	LB Test	Saponin Existence	Aglikon Type
Butanol		+	purple	+	Triterpenoid
Ethanol		+	purple	+	Triterpenoid
Water		+	purple	+	Triterpenoid
Iso-propanol		+	purple	+	Triterpenoid
Ethanol 1	day 1	+		+	Triterpenoid
	day 2	+		+	Triterpenoid
	day 3	+		+	Triterpenoid
Ethanol 2	day 1	+		+	Triterpenoid
	day 2	+		+	Triterpenoid
	day 3	+		+	Triterpenoid

Note: + shows positive result of Froth Test (foaming); and Liebermann – Burchard test (coloration)

**Figure. 1.** Daily profile of yield from saponin Extraction.

3.1.3. Saponin purification by active carbon. The purpose of purification is to remove impurity materials which unintentionally carried from the extraction process, as well as removing pigment and protein content of *A. planci*. From 3 variations that were mentioned before, the first and second test (2.1.6(a) and 2.1.6(b)), protein, pigment, and other impurities were successfully removed from the extract. Unfortunately, the saponin content also removed. In third test (2.1.6(c)) with carbon and sample ratio 1:2 (w/v), batch process and retention time 20 minutes, was successful to purified saponin extract. As a result, protein and other impurities, except pigment, were successfully removed from the extract.

Table 4. Saponin mass, before and after purification (t = 20 minutes and activated carbon: sample = 1:2 (w/v)) from purification process of saponin extract using ethanol II.

Number of Tests	Sample (gram) (dry weight basis)	Tested Sample (mg)	Absorbance	Total Mass of Saponin (mg) (dry weight basis)
1	11.62	67,5	0.198	11,366
2	11.62	67,5	0.183	10,505
3	11.62	67,5	0.205	11,768

This resulted extract from the third test was further purified. 11,213 mg saponin in 16 ml of sample solution was achieved from the utilization of ethanol II on the extraction process. The purified saponin was freeze-dried and subjected to UV spectroscopy analysis (Table 4).

3.1.4. Sapogenin Isolation. Characterization sapogenin extract contained in *A. planci* can be done, using first sapogenin isolation from saponins. To get genuine sapogenin without the sugar chain isolation, the process can be carried out by hydrolyzing saponins and sapogenin precipitate separated from the sugar chain. The process of hydrolysis and precipitation with hydrochloric acid carried by the reflux temperature of the solvent is ethanol at 73 °C for seven hours [22]. The sample used is a purified sample of the entire sample extract as much ethanol II 8:32 grams. From reflux process, it obtained brown precipitate that found from precipitated sediment at the bottom of the container after separation using filter paper and followed by centrifugation. As a result of this difficult separation process, it is obtained precipitate is only ± 200 mg (217.50 mg) sapogenin mixture. A part of the sample which is suspected contain a mixture of sapogenin, can be analyzed using a spectrophotometer UV at 246.5 nm = $\lambda_{\text{maxEtOH}} = 16$ [6]. One mg of the sample was tested quantitatively with spektrofotometer UV $\lambda_{\text{max}} = 246.5$ nm in EtOH = 16 and resulted that sapogenin mass obtained 168.33 mg. Remaining samples were obtained can be tested further for proof sapogenin molecules using LC-MS testing tool to get a molecular weight of sapogenin.

3.1.5 Characterization of Saponin and Sapogenin. Fig. 2 showed chromatogram results before and after isolation sapogenin. In this chromatogram, a retention time of before isolation samples at 0 to 2 seconds, 6-10 seconds, and 14-17 contained spectral peaks, but it disappeared after isolation samples. This suggests that in isolation process, compounds or substances that un-isolated, have a compound chain breakdown and it is broken by hydrolysis. Allegedly, saponins, sapogenin, and a sugar chain are contained spectral peaks on the results before isolation samples and after isolation through hydrolysis, the remaining isolates sapogenin only without the sugar chain, so that saponin spectral peaks are disappeared after isolation.

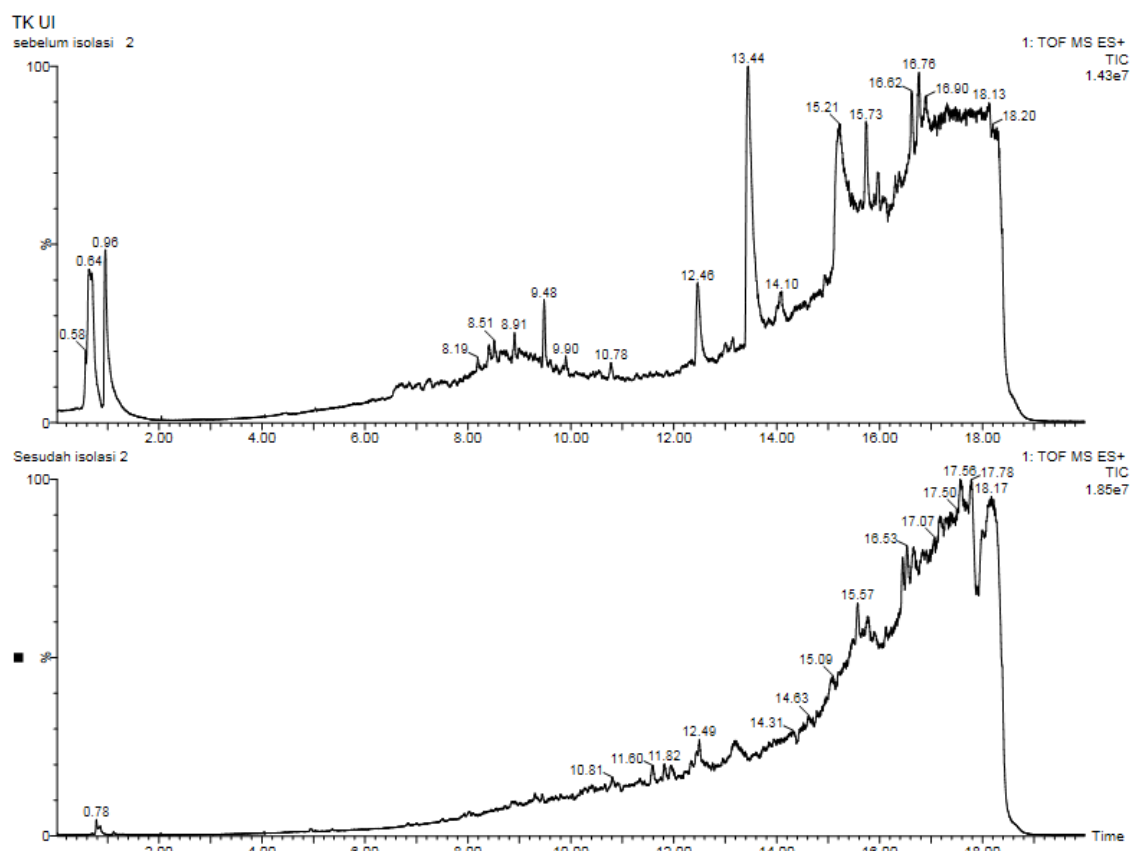


Figure. 2 Sample spectrum of saponin before and after isolation (documentation of PUSLABFOR).

In Fig. 3 are the results of *mass spectroscopy* (MS) of the sample either before or after isolation. In the results before isolation, LC-MS instrument at the Police H/Q Forensic Laboratory, searching peak - MS peak highest and search possibilities for the molecular formula based on molecular weight obtained. At spectrum with retention times above 11 seconds, which probably is the molecular formula molecule with a carbon chain under 10, there can be no saponins that have a carbon chain of at least 27 or 30 [3,28]. In the spectrum after isolation, the results of MS performed at a retention time over the time also shows the carbon chain molecules under 10, so either triterpenoid or steroidal sapogenin may not exist in the region. Although the results in the form of possibility MS develop a molecular formula that was alleged using computerization make uncertainty of a given molecular formula. Allegations that the region no saponin or sapogenin amplified by the shape of the spectrum is not clear where there are a lot of peaks and valleys also are attached to make the results contained in the area of retention time above 11 seconds is difficult to be analyzed and characterized by both computerized and manual with human vision. Therefore, saponin and sapogenin search centered on the spectrum with a retention time of under 11 seconds. Allegations that molecule can still be analyzed are those under 11 seconds, and precisely in the range 0 to 2 seconds because the peaks and valleys are also created on the curve very well. At the retention time range of 2 seconds both on the spectrum before and after isolation, there are many peaks of MS with a range of molecular weight of 200-800. Checking possibility of developing a molecular formula for each MS peaks, there is a molecule composed of a chain of the elements of carbon, oxygen, hydrogen, chloride, nitrogen and sulfur. According to [3,6-7] it does not only consist of saponin aglycone and sugar chains that are composed of chains of elemental carbon, hydrogen, and oxygen, but also bound with elements such as nitrogen, halogen, and sulfur. In the before isolation MS result, with suspected molecular formula of computer interpretation, there is a molecule that has a chain with

a carbon number between 27-50. While the after isolation MS results are molecules that have a carbon chain with a number between 26-40.

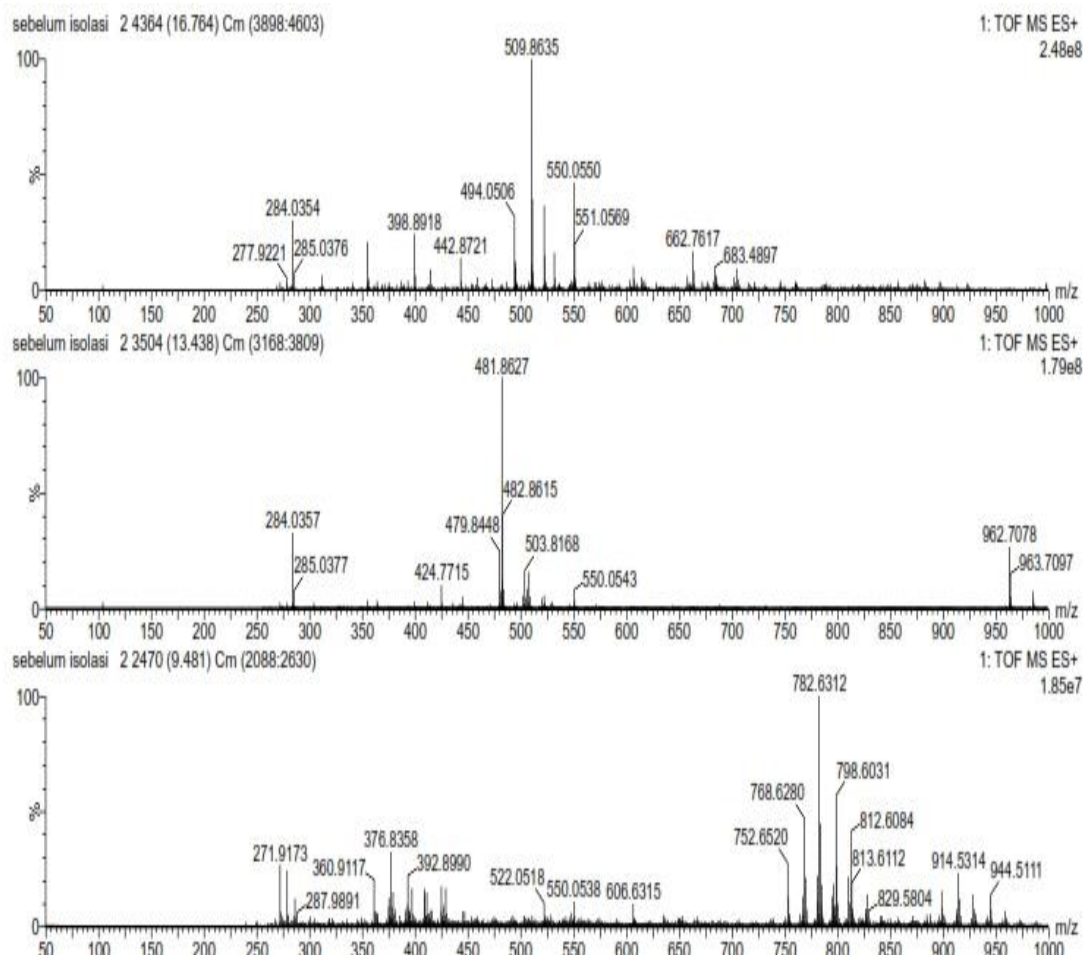


Figure. 3 MS Graph before the isolation on the retention time of 2.2470 - 2.436 seconds (result documentation from Forensic Laboratory Centre – Indonesian Police Headquarter).

Each MS peak with a chain that has a carbon number as enabled saponin or sapogenin, formulas tested one by one using the online search tool that is verified by the Royal Society of Chemistry, which was notified at www.chemspider.com. Unfortunately, the overall results of MS suspected saponin or sapogenin in the range of time under 11 seconds that shows no results on that website. Because of these conditions, to prove the presence or absence of saponin or sapogenin, it necessary to approach the LC-MS analysis of the other results. However, this analysis can only be done on the characterization of sapogenin while the characterization of saponins have been unable to do because of saponins can have a wide - range of species' sugar chain and it difficult to predict because of the diversity of possibilities molecular formula of sugar bound to the aglycone chain molecular weight also varies. Because of this, based on qualitative evidence sapogenin test using reagents Lieberman – Burchard, allegedly sapogenin present in *Acanthaster planci* is triterpenoids. This triterpenoids molecular weight based on open chemistry database *PubChem* is in the range of 424.7 ($C_{30}H_{48}O$ – Triterpenoids Taraxerone) to 752.9 ($C_{40}H_{64}O_{13}$ – Triterpenoids Cimisine B). In Fig. Four we can see that there is a range of molecular weights in the graph MS.

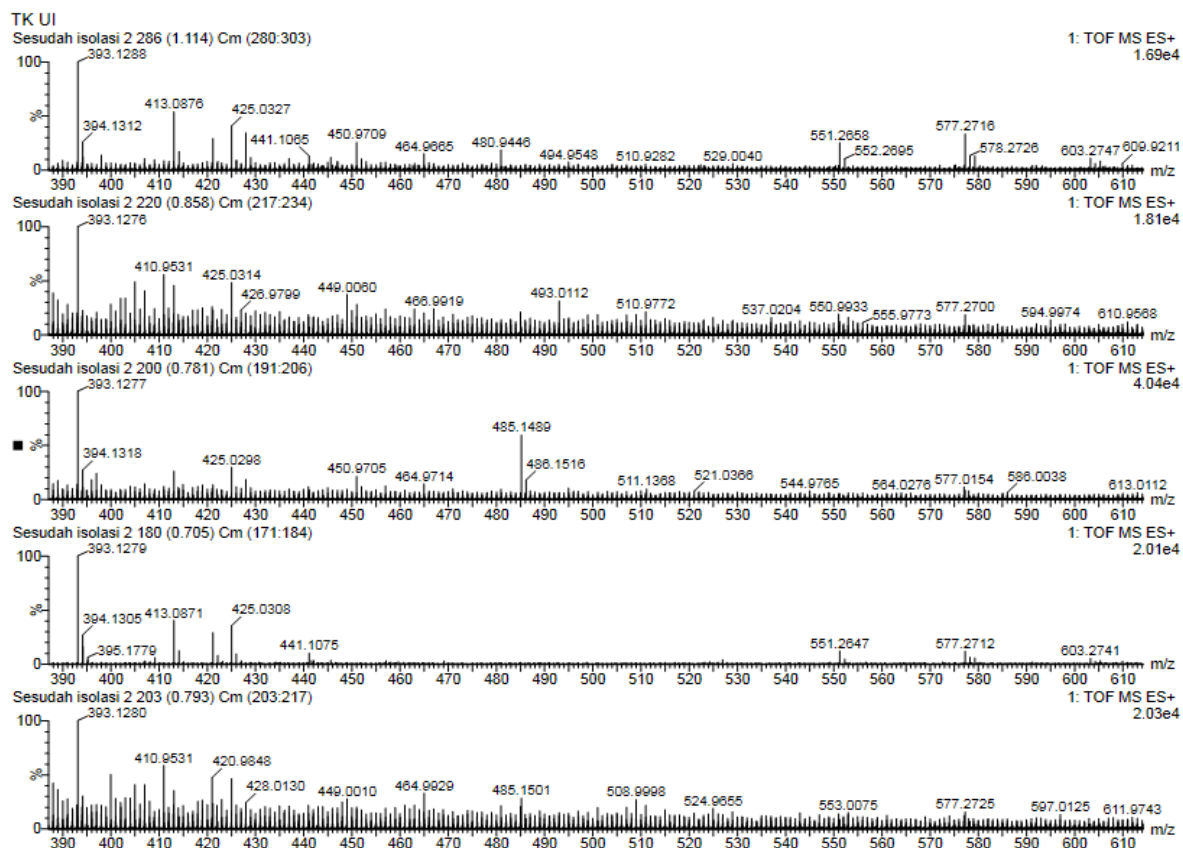


Figure. 4 MS Graph after isolation of the retention time of 0793-2286 seconds (result documentation from Forensic Laboratory Centre – Indonesian Police Headquarter).

Saponin extract with the concentration of 10, 20, 1, 2 mg/ml showed a good insecticide properties. However, sapogenin isolation by diluting the solid form with distilled water until the concentration reached 3 mg/ml was ineffective to exterminate termites. The average subsequent mortality using hypothesis testing, in which the pesticide is declared effective if the efficacy/pest mortality reached 70%. In a hypothesis test using the t distribution, with a level of significance of 5%, and the mean termite mortality assumption is 70% of the sample is 3.5, it was concluded that the concentration of saponin insecticide 2mg/mL does not effectively kill termites.

3.2 Collagen Isolation

3.2.1 Collagen Extraction. *Pepsin Solubilized Collagen* (PSC) was successfully isolated from *A. planci* body with multistage extraction method. The highest yield of PSC was 2.26% (wet weight basis) while the crude extract was 86.75%.

Table 5. The Result of Collagen Extraction

Solvent	Crude Extract (%)	Yield (%)
Distilled Water	64.07	1.23
NaOH 0.1M	63.18	2.15
Ca(OH) ₂ 0.2M	86.75	2.26

Higher quantities of PSC was recorded in the samples treated with Ca(OH)₂ 0.2M, followed by NaOH 0.1M, and distilled water. High values of protein from the samples treated with Ca(OH)₂

0.2M signifies the mild hydrolytic effect of calcium hydroxide solution on the peptide bonds of PSC when compared with NaOH and distilled water. This mild hydrolytic effect will decrease the risk of protein fraction or denaturation during the extraction process. Therefore calcium hydroxide at high concentrations appears to be the best solvent for alkaline hydrolysis [29].

3.2.2 Collagen Characterization

3.2.2.1 UV Spectroscopy. The UV absorption spectrums of PSC at wavelength range 190–400 nm were showed in Fig. 5. PSC extracted from *A. planci* showed maximum absorption at 215–245 nm which is hby the PSC from Edwards et al., which suggested that the polypeptides chains of collagen have the maximum absorbance at the wavelength ranges 190–230 nm. This is consistent with the previous researches which indicate that collagen commonly has a high amount of aliphatic amino acid, such as glycine, alanine, and valine, but the low amount of aromatic amino acids such as tryptophan, tyrosine, and phenylalanine. Due to the low content of tryptophan, tyrosine, and phenylalanine, there are no absorption bands between 250–290 nm [30]. This result is similar to those of collagens from sea cucumber (*Stichopus japonicus*) (220 nm), largefin longbarbel catfish (*Mystus macropterus*) (233 nm) and cobia skin (*Rachycentron canadum*) (221 nm).

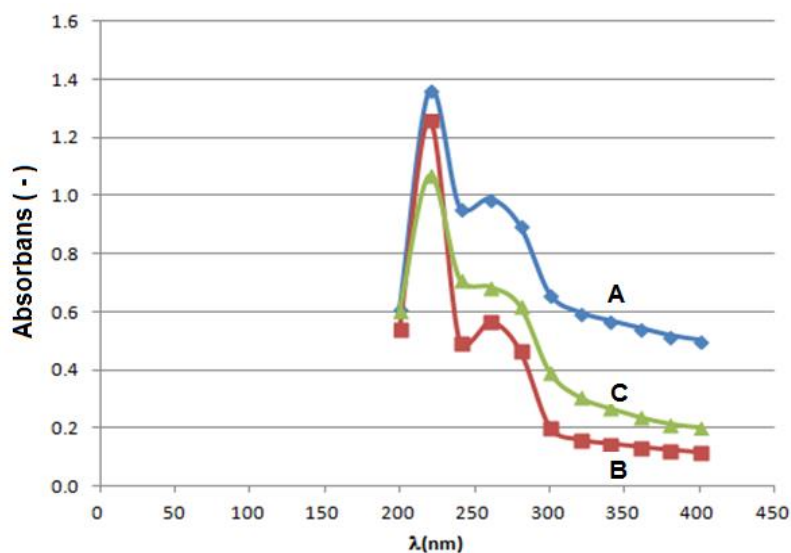


Figure 5. UV Absorption Spectrums of PSC with Solvent Variations: (a) Distilled Water, (b) NaOH 0.1 M, dan (c) $\text{Ca}(\text{OH})_2$ 0.2 M.

3.2.2.2 Lowry Method. Protein concentration in the PSC was quantified by Lowry method. The highest amount of protein content was present in samples treated with $\text{Ca}(\text{OH})_2$ 0.2M, followed by NaOH 0.1M, and distilled water.

Table 6. The Result of Lowry Method

Solvent	Absorbance	Concentration (mg/ml)	Protein Content (mg/ml)
Distilled Water	0.52	1.64	16.41
NaOH 0.1M	0.82	2.59	25.93
$\text{Ca}(\text{OH})_2$ 0.2M	1.14	3.53	35.36

3.2.2.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE). Electrophoretic pattern of the PSC from *A. planci* body is shown in Fig. 6. Previous studies indicate that collagen type I commonly consist of two α chains (Shoulders and Raines, 2009). The SDS-PAGE of PSC extracted from *A. planci* shows that PSC consisted of α and β (dimer) chains. However, the α chains were the major component of PSC, as evidenced by the increasing band intensity at 100-200 kDa. The β -component of PSC composed of β 11 and β 12 chains. The β 11 is a homodimer of α 1 chains whereas the β 12 chain is the heterodimer of α 1 and α 2 chains. These results suggested that PSC extracted from *A. planci* was most likely to be classified as type I collagen. Also, electrophoretic pattern of PSC was similar to those reported for collagen from crown of thorns starfish [23], sea cucumber [32], carp skin [32], blacktip and bamboo shark [17].

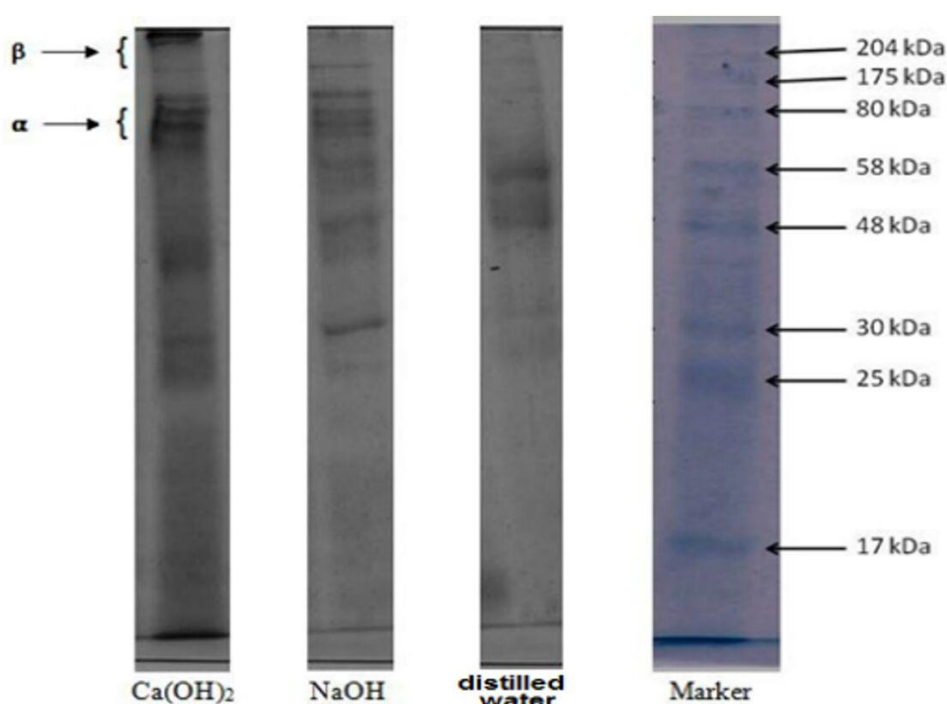


Figure. 6. SDS Page Pattern of PSC from the Body of Crown of Thorns Starfish with Variation of Solvent Extraction. Lane.

3.2.2.4 Amino Acid Composition Analysis. Glycine is the major amino acid in collagen because glycine could be found as being every third residue throughout the central region of the α chain. Also, collagen also characterized by its high contents of alanine, proline, and hydroxyproline. However, in previous studies, glycine can be replaced by alanine because of their properties and structure similarity.

In general, the amino acid composition of PSC extracted from *A. planci* body had similar amino acid profiles with sea cucumber (*Stichopus japonicus*) [33], catfish (*Mystus macropterus*) [34], bamboo shark (*Chiloscyllium punctatum*) and blacktip shark (*Carcharhinus limbatus*) [17] as well as sertacobia skin (*Rachycentron canadum*) [35], freshwater fish scales [36].

Table 7. Amino Acid Composition of PSC from Crown of Thorns Starfish

Amino Acid	Composition (per 1000 residue)	
	NaOH 0.1 M	Ca(OH) ₂ 0.2 M
Aspartic acid	n.d.	n.d.
Glutamic acid	n.d.	n.d.
Serine	166.29	79.63
Glycine	106.68	55.84
Histidine	n.d.	n.d.
Arginine	n.d.	n.d.
Threonine	n.d.	n.d.
Alanine	361.59	535.93
Proline	113.42	173.15
Valine	14.16	6.78
Methionine	n.d.	n.d.
Isoleucine	206.39	98.83
Leucine	8.29	3.97
Phenylalanine	18.79	9.00
Lysine HCl	77.01	36.88

n.d : not detected

3.2.2.5 Scanning Electron Microscopy (SEM). Morphological structures of the isolated collagen samples were observed under SEM micro- photography with lower and higher magnification. PSC had nodular like structures and tubular in nature, with irregular structures. Compared to PSC from other sources, such as the skin of sailfish (*Istiophorus platypterus*), PSC extracted from *A. planci* body are a thicker and largerze because it has not been lyophilized (Fig. 7). However, the PSC from *A. planci* showed a fibrillar structure with thick bundles and porous nature, which demonstrated that PSC from *A. planci* would have high wet ability and good fibril strength.

Pore size, porosity, and surface areas are widely recognized as important parameters for collagen to understand their biomedical importance. Other architectural features such as pore shape, pore wall morphology, and interconnectivity between pores of material have found to significantly influence cell behaviors such as cell adhesion, cell seeding, migration, growth, differentiation, mass transport, gene expression, and new tissue formation [37]. SEM microphotography with a magnification of $\times 3500$ (Fig. 8) showed massive and porous nature as the non-collagenous substances in the skin were partially dissolved by NaCl solution. Also, an almost uniform porous matrix of good interconnectivity was noticed. Based on the preceding account, PSC isolated from *A. planci* body could be used for biomaterial, cosmetic, biomedical application.

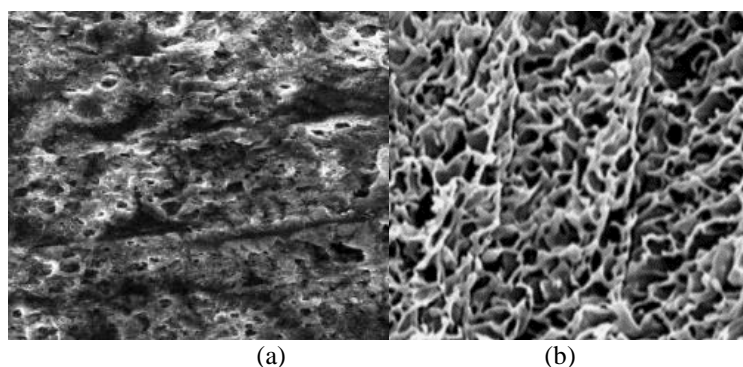


Figure. 7 Morphological Structures of PSC (Magnification $\times 1000$) from (a) Crown of Thorns Starfish and (b) Sail Fish [38].

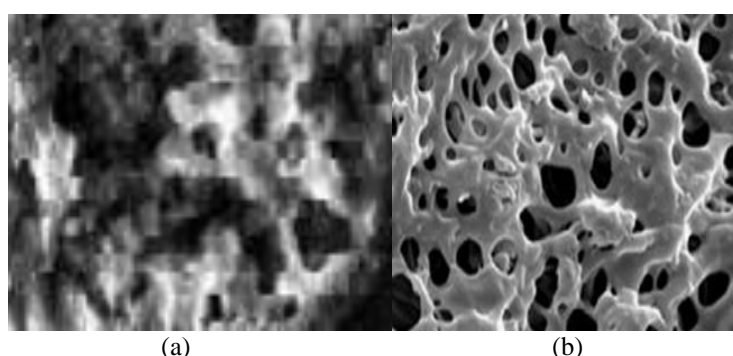


Figure. 8 Morphological Structures of PSC from: (a) Crown of Thorns Starfish (Magnification $\times 3500$) and (b) Sail Fish (Magnification $\times 2000$) [38].

3.2.3 Application and Further Development of Pepsin. Solubilized Collagen from Crown of Thorns Starfish (Collagen) has become a valuable and well-used component in biomedical, pharmacy, or cosmetic industry. Recent studies examined type I and III collagen content and distribution in the skin within the contexts of patient age [39]. As shown in Table 8, type I collagen content varied significantly among the four age groups. The result showed that total collagen declined with age, with the highest type I collagen content was noted in the youngest age group (adolescent).

Table 8. Type I Collagen Content in Normal Skin [39].

Age Group	Type I Collagen ($\mu\text{g/g}$)
Fetus	264.71 ± 5.88
Adolescent	279.12 ± 7.64
Adult	241.79 ± 8.23
Elderly	209.50 ± 14.31

Type I collagen is very fundamental in skin formation and repair, playing a crucial role in the maintenance of skin tensility and elasticity. Compared to collagen content in healthy human skin, collagen content in the PSC is very high (Table 10). Table 9 shown PSC isolated from the crown of thorns starfish.

Table 9. PSC isolated from Crown of Thorns Starfish [38].

Solvent	Collagen Content		
	$\mu\text{g/g}$	g/ml	% w/v
Distilled Water	7.847,50	0.016	1.64 %
NaOH 0.1M	13.561,03	0.026	2.59 %
Ca(OH) ₂ 0.2M	19.623,48	0.035	3.53 %

Based on commercial collagen evaluation by Peng [40], collagen content in PSC from *A. planci* is similar to commercial collagen such as Collasol® (Croda Chemicals Ltd, Humberside UK), Collagen CLR® (Chemisches Laboratorium Dr. Kurt Richter, Germany), and AteloHelogen® (Medical, North Ryde, Australia).

Table 10. Analysis Results on Three Samples of Collagen Suitable for Cosmetic Applications [40].

Test	AteloHelogen®	CLRCollagen®	Collasol®
Protein content (% w/v)	1.05%	0.28%	4.00%
pH	4.7	3.8	4.2
pI	>8.9	>8.9	<8.9
Arsenic	<1 ppm	<1 ppm	<1 ppm
Heavy metals	<5 ppm	<5 ppm	<5 ppm
Dry weight	1.15%	5.07%	5.25%
Conductivity	0.45 ms	19.0 ms	42.0 ms
Ash content	<0.1%	1.2%	1.1%
Hydration regain	21%	3%	7%

These results indicate that extraction and purification of collagen from *A. planci* should be further optimized because of its high potential in the biomedical and cosmetic industry.

4. Conclusion

The best result of saponin extraction was achieved by using ethanol 96%, compared to the other polar solvents, such as butanol, isopropyl alcohol, and distilled water. The highest yield obtained was 29.07%. Triterpenoid is the specific type of sapogenin found in the saponin extraction from *A. planci*. Scale-up with ethanol 96% as the extraction solvent gave the resulting yield obtained were 22.99% and 12.98%, respectively. Purification method for saponin extraction was achieved by using activated carbon with a mass of carbon: sample volume = 1:2 (w/v) for 20 minutes. Sapogenin isolation was carried out by acid hydrolysis using hydrochloric acid. As a result, 168.33 mg sapogenin were obtained from the total of 217.50 mg precipitate. Saponin extract with the concentration of 10, 20, 1, 2 mg/ml showed a good insecticide properties. However, sapogenin isolation by diluting the solid form with distilled water until the concentration reached 3 mg/ml was ineffective to exterminate termites. Saponin extract with the concentration of 2 mg/ml was able to exterminate termites within 6 minutes. Based on the t-distribution analysis with 5% level of significance, only 3 of 10 samples have insecticide efficacy (EI) $\leq 70\%$. Therefore it is not a

good/effective insecticide.

Type I collagen was successfully isolated from the body of the crown of thorns starfish with multistage extraction method. Three types of alkaline solvent were used in the extraction process. The best result was achieved by using Ca(OH)_2 0.2 M as the solvent, followed by NaOH 0.1 M and distilled water. The result shows Ca(OH)_2 0.2 M as the best extraction solvent because of its mild hydrolytic effect. Based on this method, 86.75% of crude extract and 2.26% of PSC yield was obtained. Characterization of PSC isolated from the body of the crown of thorns starfish was carried out by qualitative and quantitative analysis. Quantitative analyses consist of Lowry method and amino acid composition analysis. The result showed the highest protein content (37.19 mg/ml), was achieved by the utilization of Ca(OH)_2 0.2 M as the extraction solvent. Qualitative analyses consist of gel electrophoresis, UV spectroscopy, and Scanning Electron Microscopy (SEM). These analysis methods showed the structure and properties of type I collagen in the PSC, as well as its high potency for biomaterial, cosmetic, or biomedical application.

Acknowledgement

Deeply appreciation and gratefully thanks could be expressed to Directorate of Research and Community Engagement for PITTA research fund support.

References

- [1] Reichelt R.E., Bradbury R. H., Moran P. J. (1990). The crown-of-thorns starfish, *Acanthaster planci*, on the great barrier reef, Mathematical and Computer Modelling, 13 (6), pp. 45 – 60.
- Fraser, B. R. N 2000 *Best Practice Guide for Crown-of-Thorns Clean-ups*. Jakarta: Coastal Project.
- [2] Fraser, B. R. N 2000 *Best Practice Guide for Crown-of-Thorns Clean-ups*. Jakarta: Coastal Project.
- [3] Chaleb, I 2010 Saponins as Insecticides: a Review. *Tunisian Journal of Plant Protection*, 39-50.
- [4] Savitri I.K.E., M. Sahlan, F. Ibrahim, and A. Wijanarko 2012) Isolation and Characterization of Phospholipase A2 from Spines Venom of The Crown - of - Thorns Starfish Isolated from Papua Island. *International Journal of Pharma and Bio Sciences*, 3 (4), pp B603-B608.
- [5] Hostettmann K., Mastons, A 1995 *Saponins*.
- [6] Kitagawa I 1978 Saponin and Sapogenol. XXV. Steroidal Saponins from The Starfish *Acanthaster planci* L. (Crown of Thorn). Structure of Two Genuine Sapogenols, Thornasterol A and Thornasterol B, and Their Sulfates. *Chemical Pharmacy Bulletin*, 26(6), 1852-1863.
- [7] Komori, T 1997 Toxins from The Starfish *Acanthaster planci* and *Asterina pectinifera*. *Toxicon*, 35, 1537-1548.
- [8] Tamura Y 2012 Application of Saponin-Containing Plants in Foods and Cosmetics. *Intech*, 85-101.
- [9] *Budidaya Perikanan*. (2001, Maret). download November 12, 2013, dari Ristek Departemen Perikanan: www.ristek.go.id.
- [10] Suara Pembaruan. (2012, April 18). *Serangan Rayap Rugikan Indonesia Rp 250 miliar*. Retrieved Maret 13, 2014, Suara Pembaruan: <http://www.suarapembaruan.com/home/serangan-rayap-rugikan-indonesia-rp-250-miliar/19234>.
- [11] Brown, T.A., Nelson, D.E., Southon, J.R 1988 *Improved collagen extraction by modified Longin method*. *Radiocarbon* 30, 171-177.
- [12] Zhang, Y., Liu, W., Li, G., Shi, B., Miao, Y. and Wu, X 2007 *Isolation and partial characterization of pepsin-soluble collagen from the skin of grass carp (Ctenopharyngodon idella)*. *Food Chemistry* 103: 906-912.
- [13] Sadowska M, Kołodziejka I, and Niecikowska C 2003 *Isolation of Collagen from the*

- Skins of Baltic Cod (Gadus morhua)*. Food Chemistry, 81, 257-262.
- [14] Kimura S, Omura Y, Ishida M, and Shirai H 1993 *Molecular Characterization of Fibrillar Collagen from the Body Wall of Starfish Asterias amurensis*. Comp Biochem Physiol B 104, 663-668.
- [15] Mizuta S, Hwang J, and Yoshinaka R 2002 *Molecular Species of Collagen from Wing Muscle of Skate (Raja kenoi)*. Food Chemistry, 76, 53-58
- [16] Nagai T and Suzuki N 2000 *Partial Characterization of Collagen from Purple Sea Urchin (Anthocidaris crassispina) Test*, International Journal Food Science and Technology. 35, 497-501.
- [17] Kittiphattanabawon, P. Benjakul S, Visessanguan W, Shahidi F 2010 *Isolation and Characterization of Collagen from the Cartilages of Brownbanded Bamboo Shark (Chiloscyllium punctatum) and Blacktip Shark (Carcharhinus limbatus)*. LWT - Food Science and Technology, 43(5), pp.792-800
- [18] Rigby, B.J 1968 *Amino-acid Composition and Thermal Stability of The Skin Collagen of The Antarctic Ice-Fish*. Nature, 219, 166-167
- [19] Li, C.M., Zhong, Z.H., Wan, Q.H., Zhao, H., Gu, H.F. and Xiong, S.B 2006 *Preparation and Thermal Stability of Collagen from Scales of Grass Carp (Ctenopharyngodon idellus)*. European Food Research and Technology 223 (3-4), 236-241
- [20] Astuti, S. M 2011 *Skrining Fitokimia dan Uji Aktivitas Antibiotika Ekstrak Etanol, Daun, Batang, Bunga, dan Umbi Tanaman Binahong (Anredera cordifolia X Steenis)*. Bulletin Balai Besar Pengujian Mutu dan Sertifikasi Obat Hewan (BBPMSOH). 19 : 1-13 (<http://www.scribd.com/doc/217213144/Skrining-Fitokimia-Dan-Uji-Aktifitas-Antibiotika-Ekstrak-Etanol-Daun-Batang-Bunga-Dan-Umbi-Tanaman-Binahong-Sri-Murni>)
- [21] Sari, A. D. (2012). *Aktivitas Antioksidan dan Komponen Bioaktif Bintang Laut Culcita sp.* Bogor: Repository IPB
- [22] A. D., Paton D., Krista Ballantyne K., Aubin, A.A 2002 Process for recovery and purification of saponins and sapogenins from quinoa (Chenopodium quinoa), US6355249 B2
- [23] Tan, C.C., Karim, A. A., Latiff, A. A., Gan, C.Y. and Ghazali, F. C. 2013. *Extraction and Characterization of Pepsin Solubilized Collagen from the Body Wall of Crown of Thorns Starfish (Acanthaster planci)*. International Food Research Journal, 20(6), 3013-3020.
- [24] Lee, K 2009 *Biochemical Characterization of Collagen from the Starfish Asterias amurensis*. Journal of the Korean Society for Applied Biological Chemistry, 52(3), 221-226
- [25] Komsa-Penkova, R.; Spirova, R. and Bechev, B 1996 Modification of Lowry's method for collagen concentration measurement. *J. Biochem. Biophys. Meth.*, 32, 33-43.
- [26] Laemmli, U. K 1970 *Cleavage of structural proteins during the assembly of the head of bacteriophage T4*. Nature 227: 680-685.
- [27] Webb, L. J 1995 A Preliminary Phytochemical Survey of Papua - New Guinea. In L. J. Webb, *Pacific Science* (pp. 431 - 441).
- [28] Suparjo. 2010 *Saponin: Peran dan Pengaruhnya Bagi Ternak dan Manusia*. Jambi: Fakultas Peternakan Universitas Jambi, Jambi (<http://jajo66.files.wordpress.com/2008/06/saponin.pdf>).
- [29] Usman, L.A. Ibiyemi, S.A., Oluwaniyi O.O., Ameen, O. M 2003 *Effect of acid and alkaline hydrolysis on the concentrations of albumin and globulin in Thevetia peruviana seed cake protein extract*, Afrika Journal of Biotechnology Vol. 8 (22), pp. 6374-6378.
- [30] Lin, S.-H., Chen, C.-N. and Juang, R.-S 2006 *Extraction equilibria and separation of phenylalanine and aspartic acid from water with di(2-ethylhexyl)phosphoric acid*. J. Chemical. Technology and Biotechnology, 81: 406-412.
- [31] Adibzadeh, N., Aminzadeh, S., and Jamili, S 2014 *Purification and Characterization of Pepsin- Solubilized Collagen from Skin of Sea Cucumber Holothuria parva.*, Applied

- Biochemistry and Biotechnology, 173, pp.143–154.
- [32] Jia, Y.J., Wang, H.B, Wang, H.Y., Li Y.Y., Wang M., Zhou J 2012 *Biochemical Properties of Skin Collagens Isolated from Black Carp (Mylopharyngodon piceus)*. Food Science and Biotechnology, 21(6), pp.1585–1592
- [33] Cui, F., Xue, C., Li, Z., Zhang, Y., Dong, P., Fu, X., Gao, X 2007 *Characterization and Subunit Composition of Collagen from the body Wall of Sea Cucumber Stichopus japonicas*. Food Chemistry, 100, pp.1120–1125.
- [34] Zhang, M., Liu, W., and Li, G 2009 *Isolation and Characterization of Collagens from the Skin of Largefin Longbarbel Catfish (Mystus macropterus)*. Food Chemistry, 115, 826-831
- [35] Zeng, S. Yin J, Yang S, Zhang C, Yang P, Wu W 2012 *Structure and Characteristics of Acid and Pepsin-Solubilized Collagens From the Skin of Cobia (Rachycentron canadum)*. Food Chemistry, 135(3), pp.1975–1984.
- [36] Zhang, F.X., Wang A.N, Li Z.H., He S.W., Shao L.J 2011 *Preparation and Characterisation of Collagen from Freshwater Fish Scales*. Food and Nutrition Sciences, 02(08), 818–823.
- [37] Yang, S. F., Leong, K. F., Du, Z. H., & Chua, C. K 2001 *The design of scaffolds for use in tissue engineering*. Part 1. Traditional factors. Tissue Engineering, 7(6), 679–689
- [38] Tamilmozhi, S., Veeruraj, A. and Arumugam, M. 2013. *Isolation and Characterization of Acid and Pepsin- Solubilized Collagen from the Skin of Sail Fish (Istiophorus platypterus)*. Food Research International, 54(2), pp.1499– 1505.
- [39] Wang C., Rong Y.H., Ning F.G Zhang G.A 2011 *The Content and Ratio of Type I and III Collagen in Skin Differ with Age and Injury*. African Journal of Biotechnology, 10(13), pp.2524– 2529.
- [40] Peng, Y., Glattauer V, Werkmeister JA, Ramshaw JA 2004 *Evaluation for Collagen Products for Cosmetic Application*. Journal of Cosmetic Science, 55(4), pp.327–41