© 2017 IJSRSET | Volume 3 | Issue 1 | Print ISSN: 2395-1990 | Online ISSN : 2394-4099
Themed Section: Engineering and Technology

Structure-Function Analysis of Toxin-like Protein of *Haliclona molitba* Associated with Symbiotic Bacteria from Indonesian Islands

Vivitri D. Prasasty*, Ritchie Rahardja, Rory A. Hutagalung

Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Indonesia

ABSTRACT

Tropical marine sponge, *Haliclona molitba* is collected from Indonesian archipelago and investigated for toxin-like protein activities. The aim of this research is to determine the structure-function relationships of the toxin-like protein, especially for hemolytic and hem agglutination activities which have wide range potentials as anticancer, antitumor, and antimicrobial, from two distinguished type of sponges: cultured (A03k) and uncultured (A03) *Haliclona molitba*. The crude toxin-like proteins were extracted from A03k and A03 at concentration 0.26 mg/mL and 0.23 mg/mL in aqueous extracts, respectively. On SDS-PAGE, the crude protein yielded five well-defined bands around 17, 24, 36, 96, and 120 kDa from A03 and four well-defined bands around 17, 37, 44 and 96 kDa from A03k. Hemolytic and hemagglunation assays were conducted by utilizing 8 weeks old of male Sprague Dawley rat erythrocyte. The results showed that hemolytic activity was found on protein extracted from A03k while hemagglutinating activities were not found in both cultured (A03k) and unculterd (A03) of *Haliclona molitba*. Symbiotic bacteria associated with *Haliclona molitba* are predicted as *Enterobacter* sp. This finding revealed that the marine sponge is a potential source of novel bioactive proteins.

Keywords: Toxin-Like Protein, *Haliclona molitba*, Hemolysis, Hemagglutination, Symbiotic Bacteria.

I. INTRODUCTION

Indonesia is known as one of the largest archipelago country in the world which is rich of marine biodiversity, including marine sponges [1]. The body shape and structure of the marine sponges are weak physically and easily damaged by predators and pathogenic organisms. To overcome natural attack, the various bioactive compounds are produced by sponges for their self-defense, and one of them is toxin-like protein compound [2]. Approximately 850 species of sponges have been found and identified which a 1/10 marine species that have been discovered in the world. Sponges produce bioactive compounds that could be used for many medical purposes such as anticancer, antitumor, antivirus, antibacteria and anti-inflammatory. Some examples like toxin compounds found in sponges are peptides, proteins, alkaloids, macrolides, and terpenoids. Moreover, proteins that have hemolytic and hemagglutination activities also found in sponges. The emergence of sponge toxin activities against red blood cells will open the possibility of their activity on other cells, such as cancer or tumor [3-6]. This finding opens the huge possibility in the discovery of new compounds that are unique and beneficial to humans [2, 7]. Investigation of various bioactive compounds from marine organisms becomes important to do, especially in developing various potential new drugs [8]. However, the popular biomedical applications from sponges trigger the overexploitation marine sponges in massive finding of novel bioactive compounds. To prevent larger exploitation that could cause marine sponge biota damaging, alternative way should be attempted. One of the best way is by cultivating marine sponges. This way is very popular due to easy to produce in large quantity in short period of time. Literally, changes in chemical composition sponge cultivation due to changes in environment condition effects need further analysis on the activity of bioactive compounds [8]. Therefore, the extraction results of bioactive protein from cultured and uncultured sponges need to be determined. The comparison of the crude protein extracts should be insvestigated include hemolytic and hemagglutination activities. Here, we report the comparison of bioactive

protein structure-function relationships from cultured and uncultured *Haliclona molitba* especially hemolytic and hemagglutination activities which could be considered as lead compound in combating cancer disease.

II. METHODS AND MATERIAL

Sponge *Haliclona molitba*

Haliclona molitba natural sponge samples (A03) have characteristics of black, speckled, and fleshy. A03 samples were taken from the sea around Thousand Islands, Indonesia with sixth-meter depth. The cultured sponge samples (A03k) were obtained from Pramuka Island, nearby Thousand Islands with characteristics similar with cultured one, collected from fifth-meter depth.

Sample Preparation and Protein Isolation

Preparation and isolation methods followed Sepcic *et al.* (2010) with modifications. All samples were lyophilized and dry mass was measured. After lyophilization, each sponge was cut with each mass reached 0.5 gram. Individual piece of sponge was added with 20 mL of deionized water. The extraction process lasted for six hours in conditions shaken at a constant speed, 140 rpm, at a temperature of 20^{o}C. The crude protein extract then centrifuged at 15,000 rpm with a temperature of 4°C for 15 minutes. After centrifugation, one mL of the supernatant was taken for protein concentration determination using Bradford method. Remaining supernatants were transferred into a 50 ml tube. All supernatants were frozen at -20°C. Supernatant that had been frozen then re-lyophilized and dry mass of crude protein was weighed. Protein concentration of cultured and uncultured sponges were measured. Bovine serum albumin (Fraction V) (Merck, Germany) was used as protein standard curve. The absorption is observed at a wavelength of 595 nm on spectrophotometer UV-Vis [9].

Determination of Protein Profile

Protein profiles were visualized by using Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE). Protein extracts were separated on 15% separating gel electrophoresis, 90 Volts for two hours.

Hemolytic assay

Hemolytic assay followed Sepcic *et al.* (2010) with modifications. Fresh blood from Sprague Dawley rats, 8 weeks old with a body mass of 270 grams, is used for hemolytic assay. The blood is centrifuged at 2,000 rpm for 4 min. at 4°C. blood plasma and serum were discarded while the pellet form of erythrocytes was kept and washed with a physiological saline 3 times before use. Physiological saline was used as much as nine times the mass of pellets. Then, the pellet was washed and then diluted with a buffer containing NaCl 0.13 M in Tris-HCl pH 7.4, 12.02 M until the concentration is 0.5% (w/v). Hemolytic activity is measured with microplate reader (BioRad). Each well was filled with 20 mL extract of a sponge that is dissolved in the buffer to form erythrocyte suspension 10 mg/mL. 100 µL of erythrocyte suspension was added to the microplates. 1% triton X-100 was used as positive control and buffer is added to the suspension of erythrocytes as negative control. Absorption value measured on microplate reader with a wavelength of 655 nm within the time range 0, 5, 10, 15 and 20 minutes. During time interval measurement, microplate shaken at moderate speed at 25°C.

Hemagglutination Assay

Hemagglutination assay also followed Sepcic *et al.* (2010) with modifications. 100 µL Erythrocytes suspension that has been made in a microplate U. Then, 20 µL of 30mg/mL sponge extracts were added. Hemagglutination activities can be seen visually after incubation at 25°C for 45 minutes and shaken at medium speed for 1 minute.

III. RESULTS AND DISCUSSION

A. Isolation of Bacterial Symbionts

Thirteen bacterial isolates were obtained from uncultured *Haliclona molitba* (A03). Three bacterial isolates were obtained from cultured *Haliclona molitba* (A03k) at NAM media in 10-1 dilution. Eight of thirteen bacteria isolates were grown in NAM media further proceed to bacterial symbiont characterizations. Bacterial isolates were challenged with protein extracts in agar plate to find bioactivities of protein extracts.

The result showed that protein extract from A03 has no inhibition against two isolates which indicates that both isolates are symbiont bacteria. However, five of six isolates were not symbiotic bacteria due to form satellite colonies. The characteristics of the isolates formed purple and violet colonies on MAC media, with diameter less than 0.5 mm in NAM media and formed yellow colonies on TCBS media. The symbiotic bacteria are predicted as Enterobacter sp. from A03 [14-16]. According to Boobathy *et al.* (2009), some bacteria are found as symbiontic bacteria in *Callyspongia diffusa* sponge. These bacteria are *Pseudomonas aeruginosa*, *Escherichia coli*, and *Vibrio parahaemolyticus*.

B. Toxin-Like Protein Isolation

Lyophilized toxin-like proteins from A03 was 2.78 g and A03k was 1.38 gr (Table 1). The concentration of toxin-like proteins from A03 and A03k were 0.23 mg/mL and 0.26 mg/mL, respectively. These results indicate that protein extract concentration was higher in cultured *Haliclona molitba* A03k than in uncultured one, A03. Protein profiles in 15% SDS-PAGE showed twenty bands obtained from A03 and A03k (Fig. 1). Five well-defined protein bands are found from A03 include size around 17, 24, 36, 96, and 120 kDa. In addition, four well-defined protein bands are found from A03k with size around 17, 37, 44 and 96 kDa.

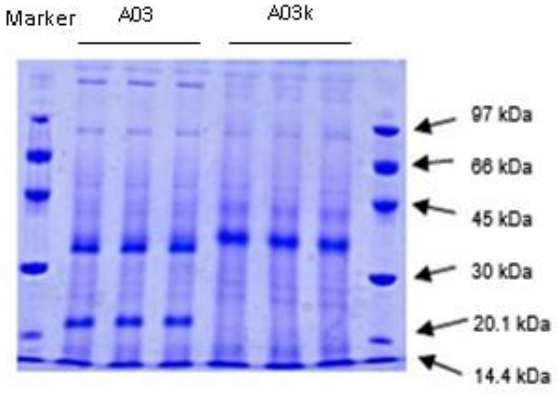

Figure 1. Toxin-like protein extract profiles on 15% SDS-PAGE

C. Hemolytic and hemagglutination activities

Hemolytic activities found higher compare to negative control in sample A03. Meanwhile, hemolytic activities found declining in sample A03k (Figure 2). These results indicate that activity of hemolytic from protein extract A03k can destroy red blood cells close to 50% of initial concentration.

Table 1. Toxin-like protein concentration summary

Sponge	Dry mass (g)	Volume (mL)	Protein Concentration (mg/mL)	Recovery (%)
A03	2.78	310	0.23	21.78
A03k	1.38	90	0.26	21.41

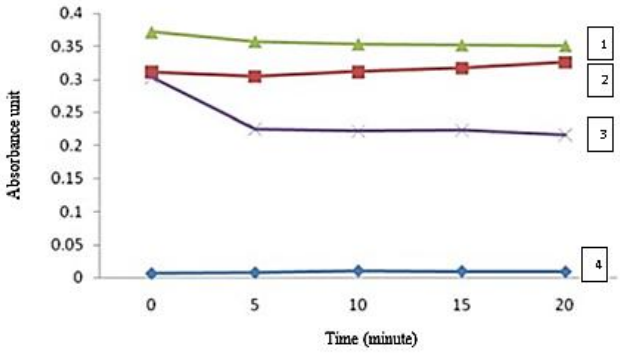

Figure 2. Hemolytic activities of protein extracts from A03 and A03k (1. A03; 2. negative control; 3. A03k; 4. positive control)

Protein extract from A03k showed hemolytic activity after 5 minutes to 20 minutes, by showing a decrease of absorption. The emergence of a compound having hemolytic activity showed cytotoxic activity as antineoplastic or anticancer agents [10]. Based on protein profiles upon SDS-PAGE, A03k has protein size at 24 kDa. According to Mangel *et al.* (1992), pore-forming proteins that have hemolytic activity have protein size 21 kDa, were found in sponge *Tethya lyncurium*. This might suggest the possibility protein that responsible in hemolytic activity is protein with size 24 kDa. The hemolytic mechanism to the cell membrane is depicted in Fig.3. Another possible result of hemolytic in *Haliclona molitba*, namely as alkylpyridinium compound, water-soluble alkaloid, which was first isolated from sponge *Reneira sarai* (genus *Haliclona*), is known to have hemolytic activity [12].

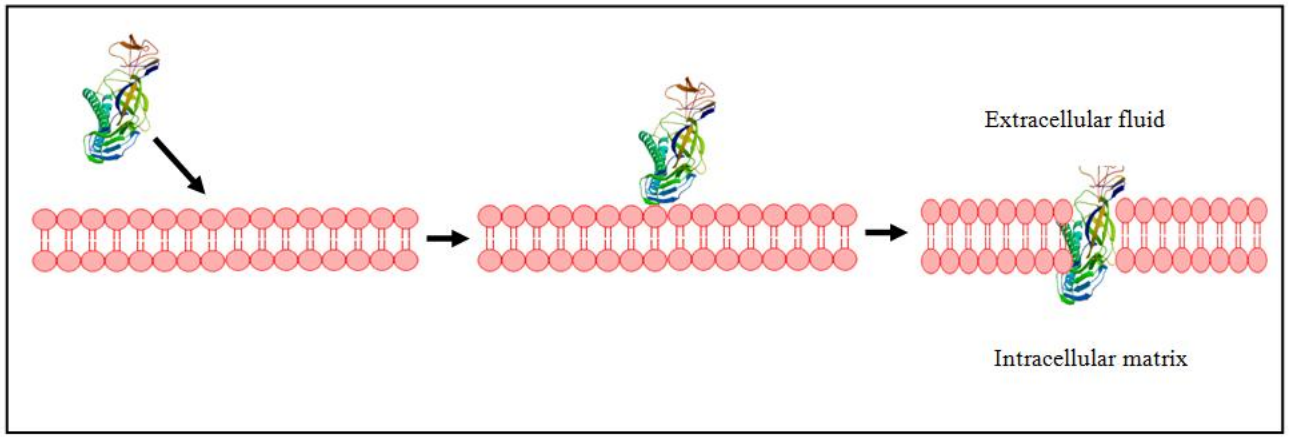

Figure 3. Hemolytic mechanism is performed by pore-forming protein also well-known as hemolysin

Meanwhile, protein extracts from A03 and A03k did not show hemagglutination activity (Fig. 4).

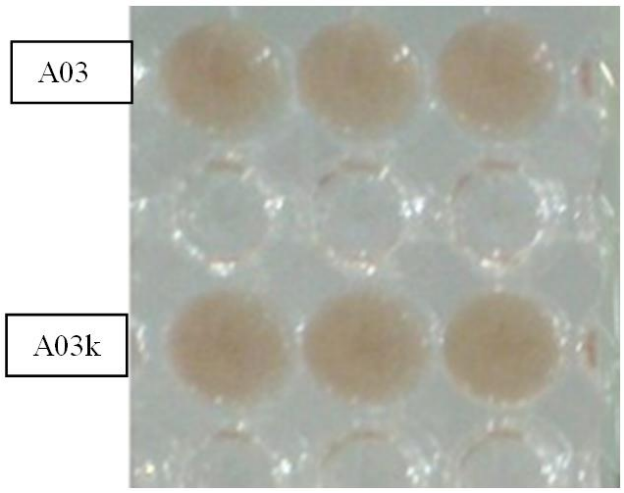

Figure 4. Hemagglutination assay upon rat's red blood cells

The production of bioactive compounds in sponge *Mycale peloruside* A may diverse compared to the cultured one. Sponges were cultured in the Gulf do not show the existence of *Mycale peloruside* A and it is assumed to be influenced by environmental conditions. However, other evidence indicates that the production of bioactive compounds in cultured sponge can be also equal with their natural condition [13]. According to Koopmans *et al.* (2009), some environmental factors can increase the concentration of bioactive compounds in sponge.

These factors are induced by the presence of predators, climate change, temperature, depth, microbial infections, type of food, and the emergence of wounds on the sponge body. The use of cultured sponge is necessary to obtain sufficient bioactive compounds from sponges as well. This effort will give huge impact on marine ecosystem by reducing the exploitation of marine sponges [17]. Optimizing of cultured sponge populations are desired to have decent amount of bioactive compound production for finding more bioactivities related medical purposes.

IV. CONCLUSION

The amount of symbiotic bacteria found in sponge A03 and A03k were thirteen and three bacterial isolates, respectively. The symbiotic bacteria are predicted as *Enterobacter* sp. from A03. The toxin-like protein concetration from A03k is higher than A03. Protein extract from sponge A03 did not have hemolytic activity. In contrast, protein extract sponge A03k showed hemolytic activity almost 50%. Both A03 and A03k did not have hemagglutination activity. Regarding hemolytic activity, A03k is potential to further investigate to other malignant cells, such as tumor cells to determine their activities. Thus, the cultured sponge is necessary developed to find more bioactive compound without massive exploit marine sponge ecosystems.

V. ACKNOWLEDGEMENT

This research is financially supported by Atma Jaya Catholic University Research Grant.

VI. REFERENCES

[1]. R. A. Hutagalung, Victor, M. Karjadidjaja, V. D. Prasasty, and N. Mulyono. 2014. Extraction and characterization of bioactive compounds from cultured and natural sponge, *Haliclona molitba* and Stylotella aurantium origin of Indonesia. International Journal of Bioscience, Biochemistry and Bioinformatics. 4(1): 14-18.

[2]. N. L. Thakur and W. E. G. Muller. 2004. Biotechnological potential of marine sponges. Curr. Sci. 86(11): 1506-1512.

[3]. T. Higa, J. Tanaka, A. Kitamura, T. Koyama, M. Takahashi, T. Uchida. 1994. Bioactive compound from marine sponges. Pure Appl. Chem. 66(10): 2227-2230.

[4]. S. Boobathy, T. T. A. Kumar, K. Kathiresan. 2009. Isolation of symbiotic bacteria and bioactive proteins from the marine sponge, Callyspongia diffusa. Indian J. Biotechnol. 8:275-277.

[5]. A. Duckworth and C. Battershill. 2003. Sponge aquaculture for the production of biologically active metabolites: the influence of farming protocols and environment. Aquac. 221: 311-329.

[6]. K. Sepcic, S. Kauferstein, D. Mebs, and T. Turk. 2010. Biological activities of aqueous and organic extracts from tropical marine sponges. Mar. Drugs. 8(5): 1550-1566.

[7]. I. M. D. Swantara, A. Supriyono, and M. Trinoviani. 2007. Isolasi dan identifikasi senyawa toksik pada spons dari perairan Gili Sulat-Lombok. J. Kimia. 1(1):67-79.

[8]. M. Koopmans, D. Martens, and R. H. Wijffels. 2009. Towards commercial production of sponge medicines. Marine Drugs. 7: 787–802.

[9]. J. M. Walker. 2002. The Protein Protocols. 2nd edition. New Jersey: Humana Pr.

[10]. P. Prahalathan, S. Bragadeeswaran, R. Sasikala, U. Kanagaraj, and P. Kumaravel. 2009. Antimicrobial and hemolytic activities of marine sponge-Halichondria panacea. J. Herbal Med. Toxicol. 3(2): 45-48.

[11]. A. Mangel, J. M. Leitao, R. Batel, H. Zimmermann, W. E. G. Muller, H. C. Schroder. 1992. Purification and characterization of a pore-forming protein from the marine sponge Tethya lyncurium. Eur. J. Biochem. 210:.499-507.

[12]. P. Malovrh, K. Sepcic, T. Turk, P. Macek P. 1999. Characterization of hemolytic activity of 3-alkylpyridinium polymers from the marine sponge Reniera sarai. Comparative Biochem. Physiol. 124: 221-226.

[13]. S. M. A. Kawsar, S. M. A. Mamun, M. S. Rahman, H. Yasumitsu, and Y. Ozeki. 2010. In vitro antibacterial and antifungal effects of a 30 kDa D-galactoside-spesific lectin from the demosponge, Halichondria okadai. Int. J. Biol. Life Sci. 6(1): 31-37.

[14]. J. E. L. Corry, G. D. W. Curtis, and R. M. Baird. 2003. Handbook of Culture Media for Food Microbiology: progress in industrial microbiology. Vol. 37. Amsterdam: Elsevier Science.

[15]. R. S. Horvath, and M. E. Ropp. 1974. Mechanism of action of eosin-methylene blue agar in the differentiation of Escherichia coli and Enterobacter aerogenes. Int. J. Syst. Bacteriol. 24(2): 221-224.

[16]. Y. K. Lee, J. H. Lee, and H. K. Lee. 2001. Microbial symbiosis in marine sponges. J. Microbiol 39(4): 254-264.

[17]. M. J. Page, P. T. Northcote, V. L. Webb, S. Mackey, and S. J. Handley. 2005. Aquaculture trials for the production of biologically active metabolites in the New Zealand sponge Mycalehentscheli (Demospongiae:Poecilosclerida). Aquac. 250: 256-269.