The potency of protein Hydrolysate from Epiphytic bacteria associated with brown algae Sargassum sp. as anticancer agents

N Asmi1,†, A Ahmad2,*, M N Massi3 and H Natsir2

1Doctoral Program, Graduate School of Science, Faculty of Mathematics and Natural Sciences, Hasanuddin University, Perintis Kemerdekaan Street Km. 10 Tamalanrea, Makassar, 90245, Indonesia
2Chemistry Department, Mathematics and Natural Science Faculty, Hasanuddin University, Perintis Kemerdekaan Street Km. 10 Tamalanrea, Makassar 90245, Indonesia
3Department of Medicine, Faculty of Medicine, Hasanuddin University, Perintis Kemerdekaan Street Km. 10 Tamalanrea, Makassar 90245, Indonesia.

*Email: ahyrarahmad@gmail.com; †author with equal contribution

Abstract. The object of this study is the protein hydrolysates produced from the epiphytic bacteria associated with brown algae Sargassum sp. Hydrolysate protein for crude extract, F1, F2, F3 and F4 are obtained through hydrolysis using the trypsin enzyme. The conditions of reaction at pH 8.0, 37 °C, at an enzyme-substrate ratio of 1: 6 with various hydrolysis time (0, 1, 3, 5, 9 and 11 hours). The degree of hydrolysis was determined by using the TCA method and toxicity assay carried out by Brine Shrimp Lethality Test (BSLT) method using the shrimp larvae of Artemia salina Leach. The results of the study show the highest degree of hydrolysis protein was found on time of 9 hours and the highest activity was shown by the F4 with an LC50 value 27.91μg/mL. These findings suggest that hydrolysate proteins from epiphytic bacteria can be a potential source as anticancer against.

1. Introduction
The cancer rates surged to nearly 10 million deaths in 2018. A total of 18.1 million new cases were found and it is estimated that cancer will be the first cause of death in the last century [1]. Although the treatment of cancer has made significant progress, the prevalence of death from this disease is still very high and continues to increase. This is due to the resistance of cancer cells to the search and exploration of existing anticancer agents, so there is an urgent need for new anticancer agents that are able to fight and kill cancer [2].

Hydrolysate protein is more interest compound because from the research results these compounds have a positive impact on human health. Enzymatic hydrolysis is preferred because the process is easier to control and can break peptide bonds specifically from various proteases [3]. Hydrolysates are normally manufactured by incubating with proteolytic enzymes at elevated temperature (typically 37–40 °C). The degree of hydrolysis (DH) is an important characteristic and is defined as the percentage of peptide bonds cleaved [4]. DH is carefully monitored and enzyme action is stopped when the desired DH is reached, usually by heating.
Protein hydrolysates from marine products have interesting bioactive properties such as anticancer [5]. Nowadays, the exploration of protein hydrolysates from epiphytic bacteria associated with marine algae has not much been revealed. So that, the objective of this study was hydrolysis enzymatic of protein from epiphytic bacteria using trypsin with various hydrolysis time at 37°C and determine the degree of hydrolysis (DH) and value of toxicity assay.

2. Materials and methods

2.1 Materials

Materials used were included the brown alga _Sargassum_ sp., Brain Heart Infusion Broth (BHIB) medium, Buffer A (Tris (hydroxymethyl) aminomethane 0.1 M pH 8.3; NaCl 2 M; CaCl₂ 0.01 M; β-mercaptoethanol 1%; Triton X-100 0.5%), Buffer B (Tris(hydroxymethyl) aminomethane 0.1 M pH 8.3; NaCl 0.2 M; CaCl₂ 0.01 M), buffer C (Tris (hydroxymethyl) aminomethane 0.01 M pH 8.3; NaCl 0.2 M; CaCl₂ 0.01 M), trypsin enzyme (EC 3.4.21.4), distilled water, Ammonium sulphate, HCl 1 M, sea water and eggs of the shrimp _Artemia salina_ Leach.

2.2 Instruments

Instruments used were an analytical balance, fisher magnetic stirrer, micropipette (10-1000 μL), shaken incubator, magnifying glass, Eppendorf tubes, vials, 40-60 watt incandescent/neon lamp, refrigerator, petri dish, inoculation loop, BioDrop DUO UV-Vis spectrophotometer, Heraeus™ Biofuge™ Stratos™ Centrifuge Series, Bandelin SONOPULS HD 2070 ultrasonic homogenizer and glass tools commonly used in laboratories.

2.3 Procedures

2.3.1 Isolation of protein from epiphytic bacteria from brown algae _Sargassum_ sp. The isolate epiphytic bacteria from brown algae _Sargassum_ sp. was inoculated as many as 10% into the fermentation medium with the same composition as the medium inoculum. The isolate was then shaken at 180 rpm, 37 °C for 24 hours. Cell paste of bacteria is homogenized with 100 mL Buffer A solvent followed by cell lysis by sonication, freeze-thaw process and centrifuged at 5000 rpm for 30 minutes at 4 °C to obtain a crude extract [6,7]. The crude extract of protein was fractionated with ammonium sulphate at different levels of saturation and the protein fractions were dialysis.

2.3.2 Protein hydrolysis. The fractions of protein ware obtained through hydrolysis using the trypsin enzyme [8]. The conditions of reaction at pH 8.0, 37 °C, at an enzyme-substrate ratio of 1: 6 with various hydrolysis time (0, 1, 3, 5, 7, 9 and 11 hours).

2.3.3 Determination of Protein Content. The levels of protein in buffer A are determined based on the BioDrop UV-VIS spectrophotometry method [9], protein concentration was measured at wavelength 280 nm. The blank solution used was distilled water.

2.3.4 Degree of hydrolysis (DH). The degree of hydrolysis protein was determined with the method based by [10] slight modification with precipitation of trichloroacetic acid (TCA) 20% to producing 10% dissolved protein fraction and 10% insoluble fraction. Sample protein hydrolysate was thawed of room temperature and added 500 μl TCA 20%, homogenized and incubated at room temperature for 30 min. The solution was centrifuged of 3500 rpm for 20 min. The content of soluble protein and total protein was analyzed based on the BioDrop UV-VIS spectrophotometry method. It was calculated following the equation (1):

$$\text{DH (\%)} = \frac{10\% \text{ soluble protein of TCA} \times 100}{\text{Total protein}}$$  (1)
2.3.5 The anticancer activity test

The anticancer activity test with Brine Shrimp Lethality Test (BSLT) method [11]. Eggs of *Artemia Salina* Leach were inoculated with seawater and incubated at 30 °C with strong aeration with continuous incandescent light. After 24 hours of hatching eggs, 10 individuals were pipetted with micropipettes and placed in the small tube containing test extracts and controls (in different tubes). The number of dead individuals is calculated after 24 hours of exposure. The LC50 value was determined with probit analysis.

3. Result and discussion

3.1 Isolation of Protein

Isolation of protein started from the cultivation of bacteria from brown algae *Sargassum* sp. The cultivation of bacteria was carried out using BHIB medium under controlled laboratory conditions (shaken at 180 rpm, 37 °C for 24 hours). Cell paste of bacteria was homogenized with 100 mL Buffer A solvent followed by cell lysis by sonication, freeze-thaw process and centrifuged at 5000 rpm for 30 min and 4 °C to obtain a crude extract. It was fractionated with ammonium sulfate at a saturation level of 0-20% (F1), 20-40% (F2), 40-60% (F3) and 60-80% (F4). Fractionation aims to separate proteins based on differences in solubility in water which is the first step in the purification process. Dialysis was a further refining process. Dialysis was done using Buffer C. The dialysis method is a method often used to dissolve liquids used in membrane systems. Soluble molecules that are small out of the pores of the membrane, whereas larger molecules will remain in the membrane. After the dialysis process was complete, the protein content of dialysate was determined using BioDrop UV-VIS spectrophotometry method. The content of protein before hydrolysis shows in Figure 1.

3.2 Hydrolysis of Protein

Hydrolysis protein was carried out by enzymatic method using trypsin enzyme. After the hydrolysis process was complete, the hydrolyzate obtained was measured for protein content in each fraction. The protein content in each fraction was different before and after hydrolysis (Figure 1). This happens caused by proteins or long peptide chains during the hydrolysis process have been broken into smaller peptide fragment [12].

![Figure 1. The distinction of protein content in each fraction before and after hydrolysis.](image-url)
3.3 Degree of Hydrolysis (DH)

The anticancer capability can be determined from the degree of hydrolysis by influence the molecular size. The hydrolysis process was caused by peptide fragments into smaller because proteins or long peptide chains have been broken [5]. The successful of the hydrolysis process can be determined by result calculating the degree of hydrolysis of the sample. The percent DH that higher indicated that the hydrolysis process runs better as well as. The DH is proportion or total of peptide bonds that have been broken during the hydrolysis process and it was expressed in percent (%). It can also be defined as the percentage of peptide bonds of protein that have been broken during the enzymatic reaction processing [5]. In this study, the DH of protein was determined using the TCA method [10], in the most common variation, the volume of aqueous protein hydrolyzate solution is dissolved in the same volume of 20% TCA followed by centrifugation [13]. The amount of peptide in the TCA supernatant is then determined and expressed as a ratio of percent of total protein weight in the hydrolyzate [14].

The percentage of the degree of hydrolysis protein from epiphytic bacteria is presented in Figure 2. The result showed the highest DH of protein hydrolyzate on time 9 hours. It happens because the start of the hydrolysis process, this reaction was slow so that the cause of enzyme and substrate still not reacting. The DH to increase was caused by peptide and amino acid dissolved in TCA to increase so that peptide bonds breakdown during hydrolysis. The higher of DH so that solubility of the protein in water as well as [12].

![Figure 2. The percentage of the degree of hydrolysis from protein epiphytic bacteria](image)

3.4 Toxicity assay

The Determination of LC$_{50}$ values was conducted to determine the toxicity effects of the protein fraction hydrolysis by enzyme trypsin. The BSLT method is one method for screening compounds that have bioactivity as anticancer because it is cheaper, needs shorter time, easier to develop and there are no ethical rules in the use of test materials.

The activity of each fraction can be determined in LC$_{50}$ values using the probit-log concentration graph. Data resulting from the calculation of LC$_{50}$ values for *Artemia salina* Leach from protein hydrolysate fraction (Figure 3) shows that the protein hydrolysate fraction was toxic and the highest bioactive protein hydrolysate activity was shown at fraction F4 while the lowest bioactive protein hydrolysate activity was shown at fraction F2. These data indicated that protein hydrolysates from the protein of epiphytic bacteria were toxic and it can be used as a reference for the discovery of potential compounds as an anticancer agent.
Figure 3. The LC50 values of fractions protein hydrolysates from epiphytic bacteria

4. Conclusion
Bioactive protein hydrolysates from epiphytic bacteria associated with brown algae *Sargassum* sp. was obtained by enzymatic method using trypsin enzyme. The optimization of DH with the variation of time show the optimum DH is 9 hours. The toxicity activities of each fraction were evaluated and the highest activity of protein hydrolysate against *Artemia salina* Leach is the fraction F4 with LC50 is 27.91 μg/mL.

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