Isolation and identification of bioactive proteins from the brown algae *Sargassum*, Sp. and their potential as anticancer agents

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Abstract. A study was conducted on the bioactivity as anticancer agents of protein fractions isolated from the brown algae *Sargassum*, sp. collected from Laikang Island, Takalar, South Sulawesi, Indonesia. The proteins were isolated using buffer Tris-HCl pH 8.3 containing 0.2 M NaCl; 0.01 M CaCl2; 1% β-mercaptoethanol; and 0.5% Triton X-100. Fractionation of bioactive proteins from crude extract used the salting out method with the addition of a (NH4)2SO4 salt powder at percent saturation rates of 0-20%, 20-40%, 40-60%, and 60-80%. Pre-purification of the proteins through dialysis was carried out in cellophane bags. Protein concentration was determined by the Lowry method using BSA (Bovine Serum Albumin) as a standard solution. The anticancer activity test used the Brine Shrimp Lethality Test (BSLT) preliminary test method, with further confirmation from antimitotic testing using urchin zygote cells. The protein concentration of the crude extract of brown alga *Sargassum* was 2.89 mg/mL. The 60-80% saturation fraction (F4) had the highest protein concentration (2.545 mg/mL). The 0-20% saturation protein fraction (F1) showed the highest activity in the anticancer tests, with an LC50 value of 55.62 µg/mL and IC50 value of 53.80 µg/mL. The 0-20% saturation protein fraction (F1) showed potential for development as an alternative anticancer agents in the future.

1. Introduction

Indonesia is an archipelagic country, with a sea area larger than the land area. The territory of Indonesia to the 12 km territorial water limit covers 5 million km² comprising 1.9 million km² of land, 2.8 million km² of archipelagic waters, and 0.3 million km² of territorial seas. This means that the total area of the Indonesian seas is 3.1 million km², or about 65% of the entire territory of Indonesia [1]. As
Indonesia is predominantly ocean, our future will be largely determined by our ability to utilize marine biota. These seas are home to a wide diversity of marine organisms, including animals and aquatic plants, like sponges, algae, plankton, fish and so on. This exceptional marine diversity is a potential source of wealth for Indonesia. It is a challenge for us to explore the seas, and seek the many opportunities offered by aquatic animals and plants. Algae are one potential natural resource in Indonesia territorial. The term algae include both macroalgae and microalgae. In Indonesia, some types of macroalgae, such as *Eucheuma*, *H. macrobola*, *Sargassum*, *Chlorella*, and *Spirulina*, have a high economic value and have been the object of extensive research [2]. The number of studies on active components from algae has grown in line with the success of natural compounds isolated. Such compounds are reported by researchers or scientist to possess antibacterial, anthelmintic, anticancer, and antiulcer properties, to be used in lowering blood pressure, lowering cholesterol, and preventing strokes, as well as in the treatment of goiter, iron or essential metal ions deficiency and blood deficiency or leukemia diseases [3].

Cancer is the uncontrolled and unregulated growth and spread of cells. It can affect almost any part of the body. The growths often invade surrounding tissue and can metastasize to distant sites. Metastasis is a major cause of cancer-related death. Cancers can be cured by surgery, immunotherapy, radiotherapy or chemotherapy, especially if they are detected early in the first phase. Cancer is a leading cause of death worldwide, accounting for 7.9 million deaths (around 15% of all deaths) in 2009. Lung, stomach, liver, brain, gland, colon and breast cancer cause the most cancer deaths each year. About 30% of cancer deaths are considered to be due to five leading behavioural and dietary risks: high body mass index, low fruit, fiber/cellulose and vegetable intake, lack of physical activity or sport, tobacco use, and alcohol abuse. Deaths from cancer worldwide are projected to continue rising, with an estimated 13.5 million deaths in 2030 [3].

The high number of cancer deaths has drawn the attention of researchers or scientist in the world toward the search for potential compounds as anticancer agents/treatments. In recent years, many researchers and scientist have focused on natural compounds as target test materials; besides their abundant presence in nature, the use of natural products can minimize side effects [4]. Agents with anticancer properties isolated from macroalgae include sesquiterpenoids from *Caulerpa taxifolia*, kahalalide F from *Bryopsis*, peptide compounds from *C. vulgaris*, β-carotene from *R. palmata*, Eucheuma Serra Agglutinin (ESA) which is a lectin compound from *E. serra*, protein fractions from macroalgae *G. amansii*, *H. macrobola*, and *T. decurrens*, and protein compounds from the endophytic fungus *Xylariapsidii* KT30 isolated from the brown alga *K. alvarezi* [5, 6]. So far there has been little research to explore the bioactive protein compounds of brown algae, as anticancer drug raw materials. Therefore it was considered necessary to extend such exploration towards the potential of brown algae. This study was conducted to isolate, purify, and identify bioactive protein substances from the brown alga *Sargassum*, sp. The bioactive proteins were isolated and extracted from brown alga through a series of processes of isolation, fractionation, dialysis, and purification. The toxicity of the obtained protein fractions would be tested using BSLT (Brine Shrimp Lethality Test) methods against the shrimp larvae *A. salina* Leach, to be confirmed by antimitotic testing using urchin *T. gratilla*, Linn zygote cells.

2. Materials and methods
The study was conducted from February to June 2019 in the Biochemistry and Biotechnology laboratory, Department of Chemistry, Faculty of Mathematics and Natural Sciences, Hasanuddin University, and the Microbiology Laboratory, Faculty of Pharmacy, Hasanuddin University, Makassar, Indonesia.

2.1. Materials
The materials used in this research included the brown alga *Sargassum*, sp. Buffer A (Tris –HCl 0.1 M pH 8.3; NaCl 2 M; CaCl2 0.01 M; β-mercaptoethanol 1%; Triton X-100 0.5%), Buffer B (Tris –HCl 0.1 M pH 8.3; NaCl 0.2 M; CaCl2 0.01 M), buffer C (Tris –HCl 0.01 M pH 8.3; NaCl 0.2 M; CaCl2
0.01 M), distilled water, BSA (Bovine Serum Albumin), (NH₄)₂SO₄, Lowry A (Folin-Ciocalteu’s/phosphotungstate-phosphomolybdate acid solution) with sterile distilled H₂O 1:1, Lowry B (Na₂CO₃ 2%; NaOH 0.1 N; CuSO₄.5H₂O 1%, Na.K tartrate 2%), HCl 1 M, sterile sea water, eggs of the shrimp Artemia salina Leach, Vincristine drug, male and female urchins (Tripneustes gratilla Linn.), potassium chloride 10%, cellophane bags (sigma), Whatman filter paper no. 42, and filter cloth.

2.2. Instruments
Instruments used in this study included an analytical balance, centrifuge, fisher magnetic stirrer, knife, homogenizer blender, micropipette (10-1000 µL), magnifying glass, Eppendorf tubes, vials, 40-60 watt incandescent/neon lamp, refrigerator, syringes, microscope, Spectronic 20D spectrophotometer, spray bottles, and glass tools commonly used in Biochemistry or Microbiology laboratories.

2.3. Procedures
2.3.1. Sample Preparation. The isolation of bioactive proteins from macroalgae used a procedure modified from previous methods [6] as follows. The selected macroalgae were cut into small pieces and a weighed to provide samples of 500 g fresh weight. The samples were pulverized in a blender with 1000 mL buffer A solvent (0.1 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl₂ 1% β-mercaptoethanol; 0.5% Triton X-100), and then filtered through a fine filter cloth. The filtrate was freeze-thawed 2-3 times and centrifuged (6000 rpm at 4 °C) for 20 minutes to obtain a crude extract.

2.3.2. Fractionation. The protein in crude extract was fractionated using (NH₄)₂SO₄ salt at rates of 0-20%, 20-40%, 40-60% and 60-80% saturation.

2.3.3. Dialysis. The precipitates obtained after fractionation with each (NH₄)₂SO₄ saturation level were dissolved in buffer B (0.1 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl₂) and then dialyzed in buffer C (0.01 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl₂). The protein fraction was placed in a cellophane bag after ensuring that there was no leakage or damage. The cellophane bag filled with protein fractions was put into an Erlenmeyer containing buffer C solution and stirred with a magnetic stirrer. The dialysis was continued until the buffer solution became colourless.

2.3.4. Determination of Protein. The protein concentration of each fraction was determined by the Lowry method [7] using BSA protein as a standard solution.

2.4. Toxicity Test using Shrimp Larvae of A. salina, Leach by the BSLT Method
2.4.1. Preparation of Shrimp Larvae. The shrimp eggs of A. salina, Leach were placed in bottles filled with sterile sea water containing 0.06 % (w/v) dry yeast for hatching, and then aerated with O₂ aerator under 40-60 watt incandescent/neon lamps. The hatching temperature was maintained in the range of 25-30 °C with lighting for 48 hours. After the eggs hatched, shrimp larvae were taken to be tested [8].

2.4.2. Implementation Test. The Brine Shrimp Lethality Test (BSLT) method used in the toxicity tests followed procedures modified from previous research [9]. The test compounds were prepared in concentrations of 1, 10, and 100 µg/mL, and were transfer to 3 new vials. About ten shrimp larvae were placed in each test compound, then sterile sea water was added to give a volume of 5 mL, and the vial was stored under illumination for 20-24 hours. The same treatment was carried out using buffer B (0.1 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl₂) without a protein extract as a negative control and Vincristine drug as a positive control. Next, the dead and live shrimp larvae of A. salina, Leach were observed and counted. The LC₅₀ value was determined through Probit-log concentration graph or table of Probit analysis. The percentage mortality of shrimp larvae of A. salina, Leach could be determined from the Abbott formula as follow [7].
2.5. Antimitotic Test of Urchin Zygote Cells

2.5.1. Urchin Egg and Sperm Cells Preparation. The induction of male and female urchin spawning was administrated by injecting 5 mL of 10% potassium chloride into the gonad section. The milky sperm and golden yellow egg cells of *A. salina*, Leach were stored in different Erlenmeyers. A total of 1 mL sperm and 5 mL of egg cells of *A. salina*, Leach were placed in a Erlenmeyers with 50 mL protozoa-free seawater for the fertilization process to occur.

2.5.2. Test Sample Preparation. The brown alga *Sargassum*, sp. fraction with high toxicity algae in the BSLT test was prepared in concentrations variation of 0, 1, 10, and 100 µg/mL. The negative control was prepared using buffer B (0.1 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl$_2$) and a positive control was made using Vincristine drug with a concentrations variation of 0, 1, 10, and 100 µg/mL.

2.5.3. Test Implementation. The samples were placed in an Eppendorff tube to which seawater was added according to calculations to bring the final volume to 1 mL. At 10 minutes post-fertilization, 100 µL of zygotes were added to the tube. Each protein extract (sample) test and control positive or negative were repeated a further 2 times, then the samples were stored at a temperature of 15-20°C with interspersed shaking. The cells were observed through a microscope after two hours of incubation. The IC$_{50}$ values were calculated using a Probit-log concentration graph or table of Probit. The percentage inhibition of urchin *T. gratilla*, Linn zygote cells could be calculated from the following formula [8].

\[
\frac{\text{% mortality}}{} = \frac{\sum \text{test larvae were dead} - \sum \text{control larvae were dead}}{\sum \text{test larvae}} \times 100\% \tag{1}
\]

3. Results and Discussion

3.1. Isolation of Proteins from macroalgae

The samples of brown algae *Sargassum*, sp. used in this study were obtained from Laikang Island, Takalar, Indonesia. The process of cell lysis was done through pulverization with the use of Buffer A solvent. The lysis aimed to break the algal cell walls so that the proteins contained in the cell could be dissolved in the buffer A (0.1 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl$_2$ 1% β-mercaptoethanol; 0.5% Triton X-100). The presence of Triton X-100 0.5% in buffer A served to help the process of cell lysis by tensing the plasma chemically so that in the presence of physical friction the cell would be split. The freeze-thaw process was used to enhance cell lysis. In the frozen state, the cavity would be filled with water molecules so that the volume would increase, while in the liquid state, the water molecules would move resulting in declining volume. Thus the frozen water in the cells would make it easier to rupture these cells. The precipitate and supernatant were separated through centrifugation. The principle of using a centrifuge to separate different substances is based on the molecular density, asunder centrifugal force the heavier substances would go to the bottom, while the lighter substances would rise to the top level [10].

\[
\frac{\text{% inhibition}}{} = \frac{\sum \text{cell not dividing in sample} - \sum \text{cell not dividing in control}}{\sum \text{cell total}} \times 100\% \tag{2}
\]
Isolation of proteins from the macroalgae was carried out at low temperatures of 0-4 °C in Buffer A (0.1 M Tris–HCl pH 8.3; 0.2 M NaCl; 0.01 M CaCl₂ 1% β-mercaptoethanol; 0.5% Triton X-100), because the proteins are strongly influenced by environmental conditions. Therefore, the temperature and pH of the solution should be maintained so as to damage the proteins. Pre-purification of the isolated proteins was carried out using (NH₄)₂SO₄ fractionation followed by dialysis using a cellophane bag (Sigma). The fractionation aimed to isolate proteins based on their different solubility in water. The protein fractionation process was carried out through the addition of (NH₄)₂SO₄ salt at saturation levels of 0-20%, 20-40%, 40-60% and 60-80%. The protein precipitation used the principle of salting out, where water binds with (NH₄)₂SO₄ salt. The addition of (NH₄)₂SO₄ salt from low to high concentrations led to the build-up of different types of protein. According to [11], several types of protein have different solubility in water so that the addition of salt at a certain concentration will lead to the precipitation of a particular protein. The (NH₄)₂SO₄ fractionation produced proteins with high salt content, therefore (NH₄)₂SO₄ salts remaining in the precipitation process were separated through dialysis in buffer C solution using a semipermeable membrane (cellophane bag). The dialysis method is the best known method to eliminate confounding molecules, such as salt or other small ions and monomers. The dialysis process was performed at 4˚C to prevent damage to the protein compounds. The proteins produced by the dialysis process were pure proteins, free from (NH₄)₂SO₄ salt and monomers or other small ions.

3.2. Determination of Protein Contents
The determination of protein contents was done using the Lowry method with BSA as standard solution, which is based on the reaction of proteins with phosphotungstate-phosphomolybdate acid in an alkaline medium and would produce a blue coloration where the colour intensity depended on the contents of the protein. The absorbance and or transmittance measurements were performed using a Spectronic 20D⁺ spectrophotometer.

Based on the measurements made, the concentration and the total proteins in brown alga Sargassum, sp. crude extract and in protein fractions at different levels of saturation of (NH₄)₂SO₄ fractionation can be seen in Table 1. Table 1 shows that the protein concentration in the crude extract was 2.89 mg/mL with a total protein yield of 661.81 mg from 229.0 mL crude extract. The highest protein concentration (2.545 mg/mL) was found in the 60-80% fraction, and the 20-40% fraction had the lowest protein concentration (0.29 mg/mL). The different protein concentrations show that different types of protein accumulated in each fraction. Some proteins have different properties and solubility in water. The higher the solubility, the less protein was accumulated, and vice versa.

Table 1. Distribution of protein contents and total proteins in the crude extract and protein fractions from the brown alga Sargassum, sp. at different levels of (NH₄)₂SO₄ saturation

| Protein fraction | Volume of each fraction (mL) | Protein concentration (mg/mL) | Total Protein (mg) |
|------------------|-----------------------------|--------------------------------|--------------------|
| Crude extract    | 229.0                       | 2.890                          | 661.8100           |
| 0 – 20 %         | 5.0                         | 0.555                          | 3.2745             |
| 20 – 40 %        | 3.5                         | 0.290                          | 1.1890             |
| 40 – 60 %        | 4.0                         | 0.470                          | 1.8800             |
| 60 – 80 %        | 18.0                        | 2.545                          | 36.9025            |

3.3. Anticancer Activity Test of the Protein Fraction from Brown algae Sargassum, sp.
The anticancer activity test of the protein fraction from brown algae Sargassum, sp. was performed using the Brine Shrimp Lethality Test (BSLT) against shrimp larvae of A. salina Leach as a preliminary test, with results confirmed by an antimitotic test on zygote cell of the urchin T. gratilla Linn.
3.3.1. Toxicity Test against A. salina Leach. The determination of LC$_{50}$ values was conducted to determine the toxic effects of the crude extract and protein fraction in the brown algae Sargassum, sp. Observations and preliminary test were made after one day by counting the number of live and dead shrimp larvae of A. salina Leach in order to obtain shrimp mortality. LC$_{50}$ values were then determined using a probit-log concentration graph. The LC$_{50}$ values indicate the sample concentration level that could lead to 50% death of test shrimp larvae of A. salina Leach. The LC$_{50}$ value of each protein fraction in the brown algae Sargassum, sp. and Vincristine drug can be seen in Table 2. The LC$_{50}$ values obtained (Table 2) show that the crude extract and protein fractions brown alga from Sargassum, sp. were toxic. According to the previously research [7], a substance can be considered active or toxic if its LC$_{50}$ value is< 1.000 mg/mL for the crude extract and < 30 mg/mL for a pure substance. Based on Figure 1, the 0-20% saturation protein fraction had the highest toxicity with an LC$_{50}$ value of 55.62 µg/mL, whereas the 40-60% saturation protein fraction had the lowest toxicity with a LC$_{50}$ value of 173.82 µg/mL.

Table 2. The calculated LC$_{50}$ values of some brown alga Sargassum, sp. protein fractions and Vincristine drug against shrimp larvae (A. salina Leach).

| Test compound | LC$_{50}$ value (µg/mL) | Toxicity |
|---------------|------------------------|----------|
| Crude extract | 129.63 | Toxic |
| 0-20% | 55.62 | Toxic |
| 20-40% | 145.95 | Toxic |
| 40-60% | 173.82 | Toxic |
| 60-80% | 124.51 | Toxic |
| Vincristine | 0.23 | Toxic |

Figure 1. LC$_{50}$ values of some brown algae Sargassum, sp. protein fractions and Vincristine drug tested against larvae of the shrimp larvae A. salina, Leach
3.3.2. Preparation of Antimitotic Activity Test. The most toxic protein fractions were further tested for antimitotic activity against urchin zygote cells using Vincristine drug as a comparison (positive control). After two hours of incubation, the divided and undivided urchin *T. gratilla* Linn zygote cells were counted. The inhibition of division in urchin *T. gratilla*. Linn zygote cells can be seen in Figure 2. The total numbers of divided and undivided zygote cells were counted and percentage inhibition calculated. The calculated % inhibition of cell division in *T. gratilla* Linn zygotes exposed to 0-20% saturation protein fraction at different concentrations is shown in Table 3. Based on Table 3, the inhibition value of the 0-20% saturation protein fraction at a concentration of 1 µg/mL was 18.16% and at a concentration of 10 µg/mL was 36.0%. There were more divided cells than undivided cells, which suggest that the inhibition of cell division was below 50% due to low concentration. The higher the concentration used, the higher the inhibition. The 50% value was exceeded at a concentration of 100 µg/mL with cell division inhibition reaching 55.0%.

**Table 3.** Calculated percentage inhibition of cell division in *T. gratilla*, Linn zygotes exposed to the 0-20% saturation protein fraction

| Protein fraction, F1 (µg/mL) | % Inhibition |
|-----------------------------|--------------|
| 1                           | 18.16        |
| 10                          | 36.0         |
| 100                         | 55.0         |

**Figure 2.** The division of urchin *Tripneustes gratilla* Linn. zygote cells using the antimitotic test method: (a) protein fraction 0-20% at 1 µg/mL, (b) protein fraction 0-20% at 10 µg/mL, (c) protein fraction 0-20% at 100 µg/mL, (d) Solvent (control -), and (e) Vincristine drug (control +).
Table 4. The calculated IC$_{50}$ values for inhibition of division in *Tripneustes gratilla* Linn zygote cells

| Test compounds     | IC$_{50}$ value (µg/mL) |
|--------------------|-------------------------|
| Protein fraction 0-20% | 53.80                   |
| Vincristine         | 0.36                    |

In this procedure, the cell division inhibitory activity (% inhibition) was evaluated based on the IC$_{50}$, the concentration that could inhibit cell division by 50%. The IC$_{50}$ values were obtained using the Probit-log concentration graph or Probit table method. The IC$_{50}$ values of the 0-20% saturation protein fraction and Vincristine drug as a positive control can be seen in Table 4.

Figure 3 shows that the IC$_{50}$ value of the protein fraction of 0-20% saturation was 53.80 µg/mL. According to previous study [14] the level of cytotoxicity of an extract can be based on its IC$_{50}$ values: < 10 µg/mL (very strong), 10-100 µg/mL (strong), 100-500 µg/mL (moderate). This indicates that the 0-20% protein fraction has the potential as an anticancer agent. If it was viewed from the similarities of nature with Vincristine drug which had IC$_{50}$ value of 0.36 µg/mL, the 0-20% saturation protein fraction could have the potential for development as an alternative anticancer agent in the future.

4. Conclusion

Bioactive protein compounds can be isolated from the brown alga *Sargassum*, sp. through a series of processes (extraction, isolation, fractionation and purification by dialysis). The protein concentration of the crude extract obtained brown alga *Sargassum*, sp. was 2.89 mg/mL. The 0-20% protein fraction from the brown alga *Sargassum*, sp. exhibited the strongest activity brown algae with an LC$_{50}$ value of 55.62 µg/mL against larvae of the shrimp *A. salina* Leach, and an IC$_{50}$ value of 53.80 µg/mL against zygote cells of the urchin *T. gratilla* Linn. This fraction showed the potential for development as an alternative anticancer agent. The purification and sequencing of the amino acids residue contained in the proteins isolated during this study should be performed. In addition, further work is needed to test the specific anticancer activity of the protein fraction against cancer cell line such as P-388 and HeLa cells.
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