Symbiont bacteria cultures from the red algae *Eucheuma spinosum*, isolation of bioactive proteins and their anticancer potential test

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Abstract. Red algae *Eucheuma spinosum* is one of marine organisms which have the potential bioactive protein. This research aimed to determine the protein concentration of red algae *Eucheuma spinosum* and to discover its potential as an anticancer agent. Protein was isolated from bacterial symbiont of red algae *Eucheuma spinosum* by buffer A with pH value 8.3. Protein crude extract was fractionated by adding ammonium sulphate with a saturation level of 0-20%, 20-40%, 40-60%, and 60-80%. The result was dialyzed using cellophane membrane. Lowry method with Bovine Serum Albumin (BSA) was used as the standard to determine the protein level. An anticancer preliminary test was conducted using Brine Shrimp Lethality Test (BSLT) method. The result showed that the protein concentration from crude extract of red algae *Eucheuma spinosum* was 33.325 mg/mL. The highest concentration that was obtained at fraction 0-20% is 32.145 mg/mL. The result of toxicity test using Brine Shrimp Lethality Test (BSLT) method at protein fraction of 20-40% has a very low LC₅₀ value at <1000 μg/mL. Red algae protein fraction is potential to be developed as anticancer agent.

1. Introduction
One of the main leading causes of death globally is cancer diseases. In 2018, 9.6 million deaths are recorded [1]. The prevalence of cancer of all Indonesians was 1.4% or 347,792 populations. Cancer is caused by various factors that include genetics, carcinogen, and lifestyle [2].

So far there have been several ways of treatment for cancer including through surgery, chemotherapy, and radiotherapy. Some of these methods do not provide maximum results and can cause side effects in the short and long term [3,4,5]. The method of treatment with a surgical pathway has a disadvantage that is that cancer cells that have spread cannot be treated, as well as chemotherapy...
and radiotherapy methods have a weakness that can affect healthy cells so that it can cause other side effects [4].

The resistance of cancer cells to cancer drugs is currently causing the development and search for anticancer compounds with new ways of working and specific targets and fewer side effects [6,7]. Potential agents as anticancer compounds have been proposed as bioactive peptides because they have high specificity and selectivity to cancer cells [8].

The use of bioactive peptide to cure cancer can be prescribed in many ways as injected drugs, hormones, vaccines, and carriers for cytotoxic drugs [9]. Protein enzymatic hydrolysis could produce bioactive peptides [10,11].

Protein can be obtained from marine organisms such as seaweeds (algae) which contain high and varied protein. It was reported that the value of protein in algae is 47% of its dry weight [10]. One of the marine algae is red algae. The active compound of algae has various function [12], it is feared that it can threaten algae populations, therefore, alternative use of algae is needed.

Algae is one of the marine organisms with symbiont bacteria [13]. Symbiont bacteria from algae have a similar bioactive compound with its host which is the potential to produce bioactive peptides [14] and can be easily cultured and cultivated for scale-up production.

Research on bioactive peptides from symbionts of algae marine biota as an anticancer agent so far is still lacking. The use of bioactive peptides as raw materials for drugs is the best alternative to minimalize the problems resulted from conventional chemotherapy [9]. The result of isolation and purification of the protein of symbiont bacteria red algae showed a high protein level and potential to be developed as an anticancer agents.

2. Materials and Methods

2.1 Materials

Materials of this research were red algae *Eucheuma spinosum*, buffer A (Tris–HCl 0.1 M pH 8.3, Triton X-100 0.5%, β-mercaptoethanol 1%, CaCl$_2$ 0.01 M, NaCl 2 M), buffer B (Tris–HCl 0.1 M pH 8.3, CaCl$_2$ 0.01 M, NaCl 0.2 M), buffer C (Tris–HCl 0.01 M pH 8.3, CaCl$_2$ 0.01 M, NaCl 0.2 M), distilled water, BSA (Bovine Serum Albumin), ammonium sulphate, Lowry A (Follin ciocalteus, phosphotungstat phosphomolybdat acid solution with distilled water 1 : 1), Lowry B (Na$_2$CO$_3$ 2%; NaOH 0.1 N, CuSO$_4$·5H$_2$O 1%, sodium potassium tartrate 2%), HCl 1 M, sterile sea water, egg shrimp *Artemia salina* Leach, cellophane bags, and filter paper.

2.2 Instruments

Instruments of this research were analytical balance, centrifuge, fisher magnetic stirrer, knife, blender, micropipette (200-5000 μL), loop, vial, incandescent/fluorescent 40-60 watts, 20D’ electronic, spray bottle and glassware commonly used in laboratories.

2.3 Methods

2.3.1 Protein Isolation

Intracellular of debris cells from red algae was added buffer of A solvent (0.1 M Tris–HCl pH 8.3; potassium chloride 0.01 M, sodium chloride 0.2 M 1% β-mercaptoethanol; 0.5% Triton X-100), and then centrifuged at 5000 rpm for 30 minute [15].

2.3.2 Fractionation

Crude extract was fractionated by using ammonium sulphate salt powder at saturation level of 0-20%, 20-40%, 40-60% and 60-80% [16].
2.3.3 Dialysis
Protein fraction was inserted into the cellophane membrane and soaked into buffer C (Tris–HCl 0.01 M pH 8.3; potassium chloride 0.01 M, sodium chloride 0.2 M) [16].

2.3.4 Determining Protein Level
The protein level of each fraction was determined using the Lowry method and Bovine Serum Albumin (BSA) as standard solution. Protein level was calculated using the following formula [17]:

\[
\text{Protein level (mg/mL)} = \frac{\text{Absorbance} \times \text{Slope}}{\text{Intercept}}
\]  

(1)

2.3.5 Toxicity Test Using by Brine Shrimp Lethality Test (BSLT) Method
Toxicity test was performed by using Brine Shrimp Lethality Test (BSLT) with shrimp larvae and protein fraction as the test solution. The test solution was made at 1, 10 and 100 ppm concentration which was lighted for 24 hours. The number of dead and living larvae was calculated with its LC50 value was determined using Probit analysis. The percentage of larvae mortality was determined by using Abbot Formula [18]:

\[
\% \text{Mortality} = \frac{\Sigma \text{test larvae were dead} - \Sigma \text{control larvae dead}}{\Sigma \text{test larvae}} \times 100\%
\]  

(2)

3. Result and Discussion
3.1 Protein Isolation and Purification
The extraction and isolation process of bioactive proteins from symbiont bacteria red algae Eucheuma spinosum followed a modified previous method [15]. Both processes were performed at low temperature (0-4ºC) by using Buffer A. The processes were performed under such condition due to the high influence of the environment on protein. Therefore, protein purification was performed by using a certain buffer and at low temperature.

Protein sediment of each protein fraction (F1, F2, F3, and F4) were dissolved by using buffer B. The volume of each fraction is almost equal. The sample was soaked into Buffer C which was previously inserted into the semipermeable membrane (cellophane bags). The protein content of each fraction, from intracellular protein, after the dialysis process is provided in Table 1.

Table 1. Determination results of protein concentration on crude extract and protein fractions at various ammonium sulphate saturation levels of red algae Eucheuma spinosum.

| Protein fraction | Volume of each fraction (mL) | Protein Concentration (mg/mL) |
|------------------|-------------------------------|-----------------------------|
| Crude extracts   | 50                            | 33.325                      |
| 0-20 %           | 4.5                           | 32.145                      |
| 20-40 %          | 3.5                           | 7.995                       |
| 40-60 %          | 5.0                           | 25.775                      |
| 60-80 %          | 5.0                           | 14.68                       |

Table 1 showed that the protein concentration value of crude extract was 33.325 mg/mL, whereas the highest concentration of protein fraction was obtained at fraction 0-20% of 32.145 mg/mL and the lowest concentration at fraction of 20-40% about 7.995 mg/mL. Difference concentration on each
fraction indicated different protein sedimentation and protein saturation. The more saturated protein left less protein sedimentation, and vice versa [19].

3.2 Anticancer Activity Test Using by Brine Shrimp Lethality Test (BSLT) Method
Toxicity test was performed on *Artemia salina* Leach shrimp larvae by using bioactive proteins of red algae *Eucheuma spinosum*. Table 2 showed that bioactive proteins of crude extract and protein fraction of red algae *Eucheuma spinosum* has a very high toxicity value. Fraction F1 (fraction 0-20%), and F4 (60-80%) were categorized as non-toxic, whilst F0 (crude extract), F2 (20-40%), and F3 (40-60%) were toxic. Protein fraction with 60-80% saturation showed non-toxic response on *Artemia salina* Leach. shrimp larvae with the highest LC$_{50}$ value of 163,267.6 μg/mL. Protein fraction with saturation 20-40% (F2) showed toxic response on *Artemia salina* Leach with the lowest LC$_{50}$ value of 8.65 mg/L.

Table 2. Value of LC$_{50}$ calculation results on shrimp larvae *Artemia salina* Leach from crude extract and protein fractions of red algae *Eucheuma spinosum*.

| Test protein compound | LC$_{50}$ (μg/mL) | The level of toxicity |
|-----------------------|-------------------|----------------------|
| Crude extract         | 18.80             | Very Toxic           |
| 0-20%                 | >1000             | Not Toxic            |
| 20-40%                | 8.65              | Very Toxic           |
| 40-60%                | 990.83            | Very Toxic           |
| 60-80%                | >1000             | Not Toxic            |

4. Conclusion
It can be concluded that the highest of the obtained protein level from red algae *Eucheuma spinosum* was 32.145 mg/mL from protein fraction of 0-20%, and the potential anticancer agent was fraction of 20-40%, and 40-60% with LC$_{50}$ value about 8.65 μg/mL, and 990.83 μg/mL respectively.

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