Research Article

PURIFICATION AND GENE CLONING OF A NOVEL ANTIBACTERIAL L-ASPARAGINASE OF THE ENDOPHYTIC MICROSymbiont ALGAE *SARGASSUM* SP. FROM CIKOANG ISLAND, INDONESIAN TERRITORY

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ABSTRACT

Endophytic bacterial symbiont algae are an environmentally potential source of various bioactive compounds that can be used as raw material for drugs. A new anti-bacterial L-asparaginase (L-ASNase) has been purified from the endophytic bacterial symbiont algae *Sargassum* sp. by using ammonium sulphate fractionation, followed by dialysis, column chromatography and reversed-phase HPLC, and behaves as a single-band on SDS-PAGE with molecular weight of 39 kDa. Based on amino acids partial sequence, we cloned and sequenced cDNA encoding L-ASNase. The cDNA of L-ASNase gene consists of 843 nucleotides encoding 280 amino acid residues including a putative initiation Met. To obtain it in large amounts, the coding sequence of L-ASNase was cloned into pGEX-2TK vector to yield recombinant plasmid pGEX-2TK-L-ASNase cDNA and expressed as an L-ASNase fusion protein in *Escherichia coli* BL21 strain. The soluble fusion protein collected from the supernatant of the cell lysate after lysis by ultrasonication was purified by a single-step chromatography on glutathione agarose beads. The purified native L-ASNase protein and recombinant L-ASNase fusion protein was determined for a novel anti-bacterial activity. Recombinant L-ASNase fusion protein exhibited a similar anti-bacterial activity to the native L-ASNase. The recombinant L-ASNase had stronger anti-bacterial activity toward *Staphylococcus aureus* (G⁺) with the diameters of inhibition zone 2.5 times more than that from *Vibrio cholerae* (G⁻) with the same protein product. This research might provide a significant supporting for the following research on the anti-bacterial action and mechanism in cellular and molecular level of L-ASNase protein from endophytic bacterial symbiont algae *Sargassum* sp.

Keywords: *Sargassum* sp, bacterial symbiont, L-ASNase, Recombinant protein, Anti-bacterial activity

INTRODUCTION

Indonesia has been widely known as an archipelagic country, most of its area is ocean and has the longest coastline in the world which is ± 80,791.42 Km. In the ocean there are various living things in the form of water plants and aquatic animals. The great potential for marine biota inspires scientists and producers of the world's antibiotic compounds begin to look at the sea as a source of potential antibiotics and anticancer substances. This may be because most of the natural resources in the sea have not been maximally exploited. In addition the needs for new types of antibiotics in the current world is are increasingly urgent, because many pathogenic bacteria are starting to be resistant to the antibiotics, rendering first generation standard antibioticsless effective. The high number of cases of infection and infectious diseases, both endemic and epidemic, as well as the continuous use of drugs without the supervision of a doctor is suspected to be the cause of resistance. Infection is a big problem and very serious because it is among the ten highest causes of death in the world. Therefore, the search for new, effective and affordable anti-bacterial drugs is needed to overcome infectious diseases. In the

last two decades it is estimated that 2500 secondary metabolite compounds from marine biota and symbionts have been isolated and identified, and as many as 353 of them are thought to have potential as new anti-bacterial ingredients. Extracts of secondary metabolites from marine algae contain bioactive compounds which are known to have pharmacological properties such as: antibacterial¹ antimalarials and anti-tuberculosis^{2,3,4,5}. So far there has been very little or no research data that explores groups of protein compounds from marine algae and symbiotic bacteria as raw material for anti-bacterial drugs, although there have been many reported antimicrobial agents of protein and peptide compounds from other organisms^{6,7,8}.

Some marine biota such as sponges and algae have been researched for their non-protein compounds, explored and developed for use as a source of medicine raw materials in the pharmaceutical industry. Exploration and research on marine biota for pharmaceutical purposes has grown rapidly in the last 30-40 years. This is accelerated by the increasing awareness of domestic and foreign drug and drug industry players to prioritize the use of drugs from natural ingredients because they have

relatively no side effects, which is known as "back to nature"^{9,10}. Preliminary studies of non-polar compounds from several macroalgae species are reported to have antibiotic, antibacterial and antitumor potential¹¹. For example, the research on *Thalassia hemprichii* macroalgae species which later became known to produce antibacterial compounds. After purification, the extracted macroalgae is dripped on sterile filter paper and the process of testing the minimum resistance level is carried out. At this stage it is known that the purification components have inhibitory activity against bacterial growth of clinical isolates, although the type of bioactive compounds is unknown¹¹. The ability of crude extracts of several species of marine algae to exert antibacterial activity against *S. aureus* has been investigated¹². This study specifically found that *Caulerpa taylori* macroalgae species, *Halimeda discoidea*, *Ulva rugida* C. Agard, *Dictyota*, sp, and *Osmundea hybrida* were effective in inhibiting the growth of *S. aureus* bacteria with an inhibitory diameter of approximately 14 mm. Some of the recent studies indicated antimicrobial activities of whole extract of red algae, brown algae and green algae sampled from Iran waters, Turkey and England^{13,14,15}.

So far there has not been much research data exploring groups of marine algae bioactive compounds from the waters of South Sulawesi as raw materials for drugs in infectious and cancerous diseases. Preliminary studies that have been carried out on the *Laurencia cartilaginea* Yamada protein fraction of the Barang Lompo island showed anti-bacterial activity, especially against *S. aureus* and *E. coli*¹⁶. In addition to the island waters of Barang Lompo, in South Sulawesi there is the island of Cikoang in Takalar district and Kapoposan island in Pangkep regency which have waters with algae populations that are quite abundant with various species. Previous research shows that red algae *Gelidium amansii* and green algae *Turbinaria decurrens* from Cikoang waters, Takalar Regency has been proven to contain antibacterial compounds, although the extract type of compounds is unknown, which is able to inhibit the growth of pathogenic bacteria against *S. aureus* and *S. typhi*. The use of bioactive compounds will be better if it can be obtained from abundant sources of algae, and the types of algae that are abundant in Indonesian waters, especially in South Sulawesi are brown algae. On the basis of the above thinking, further research will be conducted on the isolation, purification and identification of bioactive compounds that have the potential as anti-bacteria from endophytic bacteria which are symbiotic with brown algae *Sargassum* sp. and continued with cloning and expression of genes that encode potential bioactive proteins as antimicrobial agents. The use of bacteria is better because the growth is easily controlled, can be purified and cultured on a laboratory scale. One of the enzymes that is used in the medical world is L-ASNase. L-ASNase provides great benefits in cancer therapy, especially in acute lymphoblastic leukemia¹⁷. L-ASNase is produced in large numbers by several microbes including *E.coli*, *Erwinia cartova*, *Enterobacter aerogenes*, *Corynebacterium glutamicum*, *C. utilis*¹⁸ and¹⁹ published that *Bacillus* sp bacteria can be used to produce L-ASNase. *E.agglomerans* and *S. aureus* bacteria have been isolated from *Sargassum* sp. from the island of Lae-Lae, Spermonde islands, Makassar. From the results of this study it better knowledge and understanding of the components of bioactive compounds of red algae and brown algae that can be used as new medicinal raw materials, which are highly effective and highly sensitivity to local germs, are expected to emerge. Consequently, these compounds can then be developed and utilized by the domestic drug industry to manufacture environmentally anti-bacterial drug products that will encourage national independence in drugs production based on local raw materials derived from marine algae symbionts in the waters of the Spermonde (Cikoang) Islands in South Sulawesi.

MATERIALS AND METHODS

Isolation of Bioactive Proteins from Marine Algae Symbiont Bacteria

Extraction and isolation of bioactive proteins from brown algae symbiont bacteria using previously reported method²⁰ which modified²¹ as follows. Pure isolates with the highest activity from the results of previous study (Ahmad, *et al.*, in submitted) were inoculated on the production media and incubated at 37 °C on a shaker incubator for 7 days. Bacterial growth is characterized by the formation of small or large granules, as well as changes in the color and thickness of the media. Next, the culture was centrifuged at 5000 rpm and 4°C for 30 minutes. The cell pellet was added 300 mL buffers A then the cell suspended obtained were frozen/diluted 2-3 times then centrifuged at 12,000 rpm, temperature 4°C for 30 minutes. Furthermore, the supernatant is stored in the refrigerator before the anti-bacterial tests and subsequent purification process with the ammonium sulphate fractionation method at saturation levels of 0-20%, 20-40%, 40-60% and 60-80% and followed by dialysis then preparations for protein fractions were stored at 4°C before further analysis. The levels of bioactive protein in 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%) was determined based on the Lowry method using bovine serum albumine (BSA) as a standard protein solution.

Purification and characterization of bioactive protein from marine algae symbiont bacteria

Protein fraction from fractionation at saturation levels 0-20%, 20-40%, 40-60% and 60-80%, then purified by CM-cellulose and Sephadex G-75 column chromatography and reverse phase HPLC chromatography in column mono S which was balanced with 0.1% trifluoro acetate acid (TFA) in water. After the column was washed for 5 minutes, protein samples were eluted linearly with acetonitrile (0-80%, v / v) in 0.1% TFA at a drop rate of 1 ml / minute. After the protein fraction is collected, it is dried with a freeze dryer then resuspended in a buffer of 10 mM Tris-HCl (pH 8.5).

Analysis of bioactive protein purity

Bioactive protein obtained in 0.1 M pH 8.3 Tris-HCl buffer extract and purification resulting from ammonium sulphate fractionation, CM-cellulose, Sephadex G-75, and reverse-phase HPLC chromatography (RP-HPLC) were tested for purity with gel electrophoresis 14% SDS-PAGE.

Purification of bioactive protein and determination of its amino acids sequence

Bioactive protein purification is done by cutting protein bands from gel electrophoresis, then the protein is eluted from the gel using an elution kit (biorad) protein tool. The eluted protein is then dialyzed using C buffer and followed by distilled water for 24 hours each. Protein samples were reduced using 50 mM DDT for 3 hours in a buffer solution of 100 mM Tris-HCl (pH 8.5), 1 mM EDTA, and 6 M guanidine-HCl, then pyridylethylation with 1 □L 4-vinylpyridine for 2 hours. The pyridylethylated protein is then digested with trypsin or peptidase C enzyme, then the peptides obtained are separated by Smart System HPLC (Amersham Pharmacia Biotech), then the amino acid sequence of each peptide fragment is determined using Automated Edman Degradation with protein sequencer (model 473A; Applied Biosystem).

Cloning and Expression of Recombinant Bioactive Protein in *E. coli* cells

Total RNA isolation from marine algae symbiotic bacteria from the species with the highest bioactivity was carried out using the guanidium thiocyanate method²². The total RNA obtained was dissolved in sterile distilled water and extracted with phenol, precipitated with ethanol, and finally dissolved with sterile distilled water. mRNA isolation (poly A⁺ RNA) was performed using oligo dT chromatography column (Takara). To obtain cDNA encoding bioactive protein, amplification was done by RT-PCR method using a pair of primers designed from amino acid sequences from previous experiments. cDNA samples obtained from RT-PCR were then cloned into the pGEX-2TK expression vector (Pharmacia), then the pGEX-2TK expression vector containing genes encoding bioactive proteins (recombinant vectors) was transformed in competent of *E. coli* bacteria (which is easy infected with recombinant vectors), then the recombinant bioactive protein is produced in *E. coli* BL-21 bacteria²³. To compare the recombinant bioactive protein obtained with natural

bioactive protein (native protein) it will be tested for its bioactivity as anti-microbial as in the following test method.

Determination of *in vitro* antibacterial activities

Bacterial culture (*S. aureus* and *V. cholerae*) used as bioindicators were rejuvenated on TSA media in a tilted tube for 2 x 24 hours at 37° C. Deep-grown colonies to be slanted were taken one ounce, homogenized with 9 mL saline solution, and incubated on 10 cm petri dishes containing Mueller Hinton Agar (MHA) medium. Anti-bacterial test was carried out by agar diffusion method^[21] using a sterile 6 mm Whatman No.1 paper placed on the surface of the MHA medium. Furthermore, as many as 250 μ L of protein compounds from recombinant and native L-ASNase protein were dripped, then incubated for 2 x 24 hours at 37°C. The activity test of bioactive compounds is carried out by measuring the zone of inhibition on the culture of the test bacteria using a slide rule stated in mm.

Table 1: Extracellular and intracellular protein level of each fractions

No	Protein Type	Protein Fraction	Fraction Volume (mL)	Protein Concentration (mg/mL)	Total protein (mg)
1	Extracellular protein	0-20 %	9.0	1.002	9.018
		20-40 %	9.0	0.387	3.483
		40-60 %	8.5	0.437	3.715
		60-80 %	8.5	0.693	5.891
2	Intracellular protein	0-20 %	10.5	2.705	28.424
		20-40 %	9.0	1.083	9.747
		40-60 %	9.0	5.365	48.285
		60-80 %	8.5	6.530	55.505

Figure 1: Antibacterial protein purification from Microsymbiont *E. aerogenes* strain SB 5 (1). using a series of chromatography tools. A. CM-selulose column chromatography, B. Sephadex G-75 column chromatography, C. RP-HPLC in mono S, D. Protein purity test at each stage with SDS-PAGE electrophoresis (14%)

Figure 2: Cloning and sequencing of synthesis L-ASNase protein using RT-PCR. A) electrophoresis of agarose DNA fragments from RT-PCR synthesis using primers designed from bioactive protein amino acid sequences, and B) DNA insertion of RT-PCR results on pGEX-2TK plasmid produced pGEX-2TK L-ASNase recombinant plasmid.

1	ATG GGT TTC AAC ATC AAA GGT CTC ACC GTC GCC GCC CTG GCG GCG	45
1	Met Gly Phe Asn Ile Lys Gly Leu Thr Val Ala Ala Leu Ala Ala	15
46	CTG GGC CAT GCC TCC CCG CTC TAT TCC CGG GCC GAT GCC AAT GTC	90
16	Leu Gly His <u>Ala Ser Pro Leu Tyr Ser Arg Ala</u> Asp Ala Asn Val	30
91	ACG TAT GTC TTT ACC AAT GCT CAC GGC CTC AAC TTC ACG CAG ATG	135
31	Thr Tyr Val Phe Thr Asn Ala His Gly Leu Asn Phe Thr Gln Met	45
136	AAC ACC ACC CTG CCG AAT GTT ACC ATC CTG GCA ACC GGC GGC ACC	180
46	Asn Thr Thr Leu Pro Asn Val Thr Ile Leu Ala Thr Gly Gly Thr	60
181	ATT GCC GGC TCC AGT GCC GAC AAC ACG GCC ACG ACG GGC TAC AAG	225
61	Ile Ala Gly Ser Ser Ala Asp Asn Thr Ala Thr Thr Gly Tyr Lys	75
226	GCG GGT GCG ATT GGG ATC CAA CAG CTG ATC GAT GCG GTG CCG GAG	270
76	Ala Gly Ala Ile Gly Ile Gln Gln Leu Ile Asp Ala Val Pro Glu	90
271	ATG CTG AAC GTG GCC AAC GTG GCC GGC GTG CAG GTC ACC AAC GTG	315
91	Met Leu Asn Val Ala Asn Val Ala Gly Val Gln Val Thr Asn Val	105
316	GGC AGC CCG GAC GTG ACG TCG CAC ATC CTG TTA GAC ATG GTG CGC	360
106	Gly Ser Pro Asp Val Thr Ser His Ile Leu Leu Asp Met Val Arg	120
361	ATG CTG GAC GAG CTG GTG TGC CAG GAC GAG ACC ATG GCC GGC GCG	405
121	Met Leu Asp Glu Leu Val Cys Gln Asp Glu Thr Met Ala Gly Ala	135
406	GTG ATC ACG CAC GGC ACC GAC ACG CTC GAG GAG ACG GCT TTC TTC	450
136	Val Ile Thr His Gly Thr Asp Thr Leu Glu Glu Thr Ala Phe Phe	150
451	CTC GAC GCC ACG ATG TCC TGC CGC AAG CCC GTC GTC GTC GTC GGC	495
151	Leu Asp Ala Thr Met Ser Cys Arg Lys Pro Val Val Val Val Gly	165
496	GCC ATG CGC CCC TCG ACC GCC ATC TCC GCC GAC GGG CCC TTC AAC	540
166	Ala Met Arg Pro Ser Thr Ala Ile Ser Ala Asp Gly Pro Phe Asn	180
541	CTG CTG CAG TCG GTG ACG GTG GCG GCA ACG CCG GCC GCG CGC GAT	585
181	Leu Leu Gln Ser Val Thr Val Ala Ala Thr Pro Ala Ala Arg Asp	195
586	CGC GGC ACC CTC GTC GTG CTC AAC GAC CGT GTC CTG TCG GCC TTC	630
196	Arg Gly Thr Leu Val Val Leu Asn Asp Arg Val Leu Ser Ala Phe	210
631	TAC ACC TCC AAG ACC AAC GCC AAC ACC ATG GAC ACC TTC AAG GCG	675
211	Tyr Thr Ser Lys Thr Asn Ala Asn Thr Met Asp Thr Phe Lys Ala	225
676	ATC GAG ATG GGC GCC CTC GCC GCC ATC GTC TCC AAC AAG CCG TAT	720
226	Ile Glu Met Gly Ala Leu Ala Ala Ile Val Ser Asn Lys Pro Tyr	240
721	CTC TAT TAC CCG CCG GTG CAG CCG ACG GGG CAT GAG TTC TTT GAC	765
241	Leu Tyr Tyr Pro <u>Pro Val Gln Pro Thr Gly His Glu Phe</u> Phe Asp	255
766	GTC CGC AAA GTC TCC GCC CTG CCC CGA GTT GAC ATC CTG TAC GCT	810
256	Val Arg Lys Val Ser Ala Leu Pro Arg Val Asp Ile Leu Tyr Ala	270
811	TAT CAG GAC CTG CAG AGT GAC ACA CTA GTC TAG	843
271	Tyr Gln Asp Leu Gln Ser Asp Thr Leu Val End	280

Figure 3: The sequence of nucleotides and the prediction of amino acids from the L-ASNase cDNA-protein. The position of nucleotides is numbered starting from the first nucleotide of the L-ASNase cDNA-protein which encodes as many as 280 amino acids in its open reading frame (ORF)

Figure 4: Homology level of the L-ASNase amino acid sequence from several microbes compared with amino acid sequences in L-ASNase protein from *A. terreus* L-ASNase, from *A. perfigatu* L-ASNase and *C. graminic* L-ASNase.

Figure 5: The pattern of the results of SDS-PAGE electrophoresis analysis (14%) of the natural L-ASNase and recombinant L-ASNase protein. Column 1, Protein marker; Column 2, total crude extract protein from *E. coli* cells containing pGEX-2TK L-ASNase cDNA; Column 3, GST alone; Column 4, recombinant GST-L-ASNase fusion protein; and Column 5, natural L-ASNase protein.

RESULTS AND DISCUSSION

Extraction and Isolation of Bioactive Protein

Extraction and isolation of this protein is carried out at low temperatures (0-4 °C) with the help of buffer A. This is because protein is a group of very heterogeneous biomacromolecules. When outside the cell, protein is very unstable and can be denatured at high temperatures. In addition protein is also sensitive to high concentration of acids or bases, so that bacterial purification is carried out at low temperatures in certain buffers and pH24. Crude extracellular and intracellular protein extracts were fractionated with ammonium sulphate at saturation rates 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. Addition of ammonium sulphate salt at low concentrations to high concentrations at each fractionation level, resulted in different types of proteins settling in each fraction. Protein deposits from each fraction F1, F2, F3, and F4 were dissolved with buffers B with the same volume of each fraction, then put into a cellophane bag. Cellophane bag which has been filled with protein fraction is put into a beaker which contains buffer C solution and then stirred with stirrer magnetic stirrer to accelerate the process of protein purification. The dialysis process aims to remove small compounds and excess ammonium sulphate salts which also precipitate along with protein. The protein content of each fraction of both extracellular and intracellular proteins can be seen in Table 1. The table shows that the protein levels in each fraction are different. This occurs because of differences in the solubility of proteins in water so that the proteins that settle are also different. Proteins with less solubility in water precipitate compared to proteins with higher solubility in water. The highest level of extracellular protein was found in the 0-20% fraction of 1.002 mg/mL. Whereas intracellular protein was found in 60-80% fraction of 6.530 mg/mL. From these data it can be expected that extracellular protein is a type of protein that has low solubility in water while intracellular proteins have high solubility in water.

Antimicrobial test for each Protein Fraction of *E. aerogenes* SB (51)

Testing of bioactive protein inhibitory power on bacterial test growth was carried out using agar diffusion method. This method is a relatively easy method, low cost and very suitable for bacteria with fast growth. The bacteria used consisted of gram-negative bacteria namely *E. coli* and gram-positive bacteria, *S. aureus*. Before testing the inhibitory power, bacteria are first inoculated

Figure 6: Growth inhibition zone diameter of recombinant L-ASNase protein fraction (4 µg), natural L-ASNase protein (4 µg), GST alone as negative control (4 µg) and 30 ppm Ampicillin as positive control against *S. aureus* and *V. cholerae* test bacteria

on oblique NA media in a test tube for 1 x 24 hours. Inoculation aims to rejuvenate pure bacterial culture so that growth in the test media is optimal. Rejuvenated bacteria were suspended in sterile 0.9% NaCl to maintain the physiological conditions of the test bacteria. Then the turbidity was measured by using a digital 20 electronic so that 25% transmittance was obtained. This is so that the number of cells from bacteria in the suspension are the same so that the growth in the test media is evenly distributed. In testing the inhibitory power, positive control and negative control are used. The positive control used is ampicillin, which serves as a comparison of the antimicrobial activity of the sample. While the negative control used is BSA, which is a pure protein that is known to have no antimicrobial activity so that it can be used to determine whether the inhibitory response actually originated from the sample and is not caused by technical factors of treatment. Ampicillin was used as a positive control because it is a broad-spectrum antimicrobial compound that can be used for gram-positive bacteria and gram-negative bacteria. Ampicillin works by inhibiting bacterial protein synthesis, particularly by inhibiting the enzyme which acts as a catalyst to form peptide bonds in the process of protein synthesis in bacteria¹¹. The protein fraction that has been dialyzed is then tested for antimicrobial activity. The antimicrobial activity of each protein fraction is indicated by the presence of a clear zone around the paper disc.

Purification of Bioactive Protein from Microsymbiont of *E. aerogenes* Strain SB5(1) and Determination of Partial Amino Acid Sequence

A total of 100 mg of protein fraction from microsymbiont *E. aerogenes* SB 5 (1) strain which has a maximum activity at 40-60% ammonium sulphate saturation level was further purified by CM-cellulose column chromatography with a column size of 3 X 20 cm. The protein bound to the CM resin was eluted in a gradient with Tris-HCl buffer pH 8.3 at a concentration range of 0.1 to 0.5 M at a drop velocity of 1 mL / minute for 600 minutes. Each fraction obtained was tested for protein at a wavelength of 280 nm, showing a protein separation pattern as shown in Figure 1A. After antibacterial activity measurements of all fractions were obtained, the fractions 24 to 28 had the highest antibacterial activity (Figure 1A). Furthermore, fraction 24 until fraction 28 was put together and filtered and then purified with G-75 sephadex column chromatography with a column size of 1.0 X 30 cm. After protein is eluted with the same buffer, detection at wavelength of 280 nm shows a pattern of protein separation with 2 peaks, but only the second peak has antibacterial activity (Figure 1B). For the purposes of reading the partial amino acid sequence, the protein fraction which had the last antibacterial

activity was purified by RP-HPLC in column mono S using acetonitrile as the elution solution, so that a protein peak was obtained as shown in Figure 1C. All the protein fractions obtained at each stage of purification were checked for purity by SDS-PAGE electrophoresis (14%), indicating the level of protein purity as shown in Figure 1D. In crude extracts and ammonium sulphate fractions there are still many impurity proteins found (columns 2 and 3 in Figure 1D), while the impurity began to decrease with CM-cellulose and Sephadex G-75 column chromatography, and the last purification with RP-HPLC obtained one band of pure protein with a molecular weight of about 39 kDa. A total of 150 µg of the protein purified from microsymbiont *E. aerogenes* SB 5 (1) strain was analysed with SDS-PAGE electrophoresis, then the gel was painted with CBB. Purification of bioactive protein by cutting the protein spot from SDS-PAGE gel, followed by removed from the gel can be used to determine the sequence of amino acids. The determination of amino acid sequences using methods such as those mentioned in the Materials and Methods section, showed that the partial amino acid sequences of bioactive proteins are as follows: fragment 1: Ala-Ser-Pro-Leu-Tyr-Ser-Arg-Ala-Asp-Ala and fragment 2: Pro-Val-Gln-Pro-Ala-Thr-Gly-His-Glu-Phe. To get the cDNA encoding the microsymbiont *E. aerogenes* SB 5 (1) strain bioactive protein, RT-PCR was carried out using primers designed from partial amino acid sequences of bioactive protein and mRNA isolated from the highest bioactivity type in the crude extract, namely *E. aerogenes* microsymbiont type. The amino acid sequence used for the primary design is Ala-Ser-Pro-Leu-Tyr-Ser-Arg-Ala-Asp-Ala and Pro-Val-Gln-Pro-Ala-Thr-Gly-His-Glu-Phe from each fragment 1 and 2 to yield primer pairs 5'-GCCTCCCCGCTCTATTCCCGGGCC-3' and P2 and 5'-ACTGATGCCCCGACGGCTGCACCGG-3'. The results of the analysis using BLASTP and Bioedit 7.9 methods show that the sequence is an amino acid sequence of L-ASNase protein which is a class of hydrolase enzymes. This is supported by the high homology between the amino acid sequences of L-ASNase protein in some marine invertebrates (see Figure 2).

RNA Isolation, RT-PCR, and cloning of gen encoding inactive protein

RNA isolation from the type of microsymbiont *E. aerogenes* of SB 5 strain (1) was carried out using the guanidium thiocyanate method with Trizol reagent Kit as previously mentioned¹². The RNA solution obtained is dissolved in sterile water and extracted with phenol, precipitated (precipitation) with absolute ethanol, and finally dissolved in sterile water. mRNA isolation (poly A⁺ RNA) was carried out using an oligodT (Takara) column according to the method provided. To obtain cDNA as a gene probe that encodes the bioactive protein, RT-PCR analysis was carried out using primers designed from partial amino acid sequences from purification of bioactive protein *E. aerogenes* strain SB 5 (1). The result of the reaction, cDNA, was then cloned on the pGEX-2TK plasmid (Figure 2) and the sequence of the genetic code (sequencing) was determined. To obtain cDNA encoding microsymbiont algae bioactive protein, RT-PCR was carried out using primers designed from partial amino acid sequences of microsymbiont bioactive protein *E. aerogenes* SB 5 (1) and mRNA strains isolated from algae *Sargassum* sp. The amino acid sequence used for the primary design is Ala-Ser-Pro-Leu-Tyr-Ser-Arg-Ala-Asp-Ala and Pro-Val-Gln-Pro-Ala-Thr-Gly-His-Glu-Phe from each fragment 1 and 2 to yield primer pairs 5'-GCCTCCCCGCTCTATTCCCGGGCC-3' and P2 and 5'-ACTGATGCCCCGACGGCTGCACCGG-3'.

From both amino acid sequences, primer pairs can be derived 5'-GCCTCCCCGCTCTATTCCCGGGCC-3' and P2 and 5'-ACTGATGCCCCGACGGCTGCACCGG-3'. RT-PCR results showed a DNA band of 853 bps (Figure 2A column 3) which was smaller than the predicted size because it was probably not a

complete L-ASNase gene. DNA fragments that encode bioactive proteins from microsymbiont *E. aerogenes* strain SB 5 (1), were, labeled with ATP containing P-32 radioactive substances. The DNA fragments that have been labeled are used to detect cDNA (gene library in ZAP II cDNA library) which encodes a full-size L-ASNase gene, then is subcloned on pGEX-2TK (Pharmacia Biotech Inc.) plasmid as an expression vector in *E. coli* BL21 bacterial cells. Results Sequence of the genetic code of the bioactive protein cDNA-protein L-ASNase fragment from Microsymbiont *E. aerogenes* strain SB 5 (1) was performed using a DNA sequencer (Applied Biosystems ABI 310 Model). Figure 3 shows the sequence of full / complete Microsymbiont *E. aerogenes* strain SB 5 (1) bioactive proteins that have codon initiation and stop codons. The size of the bioactive cDNA-protein is 843 bps (Figures 3 and 4), starting from the amino acid methionine (M). The box above the nucleotide sequence shows the primary place that is constructed (design) of the partial amino acid sequence. The result of sequence analysis of the L-ASNase cDNA-protein genetic code shows that the cDNA has a high (homology) similarity to the L-ASNase gene from L-ASNase protein from *A. terreus* L-ASNase which is around 95%, while the similarity with the L-ASNase gene from *A. fumigatus* L-ASNase and *C. graminis* L-ASNase were about 25% and 29%, respectively (Figure 4).

Expression of Recombinant Bioactive Protein L-ASNase in E.coli Cells

The L-ASNase gene sequence cloned into the pGEX-2TK plasmid produced recombinant pGEX-2TK L-ASNase cDNA plasmid (Figure 2B), expressed into *E. coli* BL21 bacterial cells as a combined protein (GST-L-ASNase fusion protein) induced by 60 µM IPTG. The [purification of recombinant L-ASNase protein from impregnated protein in *E. coli* cells using glutathion agarose beads, obtained a protein band with a molecular weight of about 65 kDa as a combined GST-L-ASNase protein (Figure 5).

Antibacterial bioactivity of recombinant L-ASNase protein

Antibacterial bioactivity from L-ASNase recombinant protein products against two pathogenic bacteria was tested by agar diffusion method (Figure 6), indicating that the recombinant protein had bioactivity on two test bacteria in both gram positive and gram negative bacteria. The highest antibacterial bioactivity was found in *S. aureus* bacteria, where the bioactivity was 30 mm. respectively. Whereas in *V. cholerae* the bioactivity is 12 mm. From the results of this study it can be concluded that the antibacterial bioactive protein that was successfully purified and cloned from Microsymbiont *E. aerogenes* SB 5 (1) strain was a type of L-ASNase protein classified as glycoprotein, which has high antibacterial bioactivity against gram-positive bacteria. especially against *S. aureus* bacteria.

CONCLUSION

Intracellular protein fraction of brown algae microsymbionts *E. aerogenes* SB 5 (1) shows higher activity than extracellular protein fractions. The highest antimicrobial activity against *S. aureus* was observed in the fraction of 40-60% with inhibitory power of 17.40 mm. Protein purification using three successive column chromatography phases, namely CM-cellulose, Sephadex G-75, and RP-HPLC in mono S column produce one band pure protein which is known as L-ASNase protein with a molecular weight of about 39 kDa. Based on the partial amino acid sequences of L-ASNase namely Pro-Leu-Gln-Gly-Arg-Ser-Gln-Lys-Thr-Glu and Pro-Ser-His-Ser-Asp-Trp-Al-Gly-Gly-Gln, gene that encodes the L-ASNase protein in microsymbiont *E. aerogenes* strain SB 5 (1) was successfully cloned and expressed in *E. coli* cells as a recombinant L-ASNase protein which has

antibacterial activity that is relatively similar to the natural L-ASNase protein (native L-ASNase)

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REFERENCES

- Muniarsih, T. dan Rachmaniar, R. 1999. Identifikasi Senyawa Antimikroba dari Spons *Aaptos aaptos* dari Kepulauan Spermonde dengan Spektroskopi Massa. Puslitbang Oseonologi LIPI. Jakarta.
- Copp, B. R. and Pearce, A.N. 2007. Natural product growth inhibitors of *Mycobacterium tuberculosis*. Nat Prod Rep., 24(2): 278-297
- Tasdemir D., Topaloglu B., Perozzo R., Brun R., O'Neill R., Carballeira N.M., Zhang X., Tonge P.J., Linden A. and Rüedi P. 2007. Marine natural products from the Turkish sponge *Agelas clatroides* that inhibit the enoylreductases from *Plasmodium falciparum*, *Mycobacterium tuberculosis* and *Escherichia coli*. Bioorg Med Chem. 15, 6834-6845
- Azevedo, L. G. et al., 2008. Comparative cytotoxic and anti-tuberculosis activity of *Aplysinacaissara* marine sponge crude extracts. Comp Biochem Physiol C Toxicol. Pharmacol. 147(1):36-42.
- Abdel-Aal, W.S., Hassan, H. Y., Aboul-Fadl T, Youssef, A.F. 2010. Pharmacophoric model building for antituberculosis activity of the individual Schiff bases of small combinatorial library. Eur J Med Chem. 45(3):1098-1106.
- Spavieri J, Allmendinger A, Kaiser M. et al. 2010. Antimycobacterial and cytotoxic potential of some British green algae. Phytother. Res., in press, doi: 10.1002/ptr.3208
- Kamimoto, K. 1956. Antibacterial substances extracted from seaweeds. Effects of extracts from seaweeds on the growth of some acid-fast bacteria. Nippon Saikingaku Zasshi, 11, 307-313
- Tyagi, A., Tuknait, A., Anand, P., Gupta, S., Sharma, M., et al., 2015, Cancer PPD: a database of anticancer peptides and proteins, Nucleic Acid Res. Vol. 43, Database issue D837-D843
- Rismana, E. 2001. Biota Laut Berpotensi Besar jadi Sumber Bahan Baku Obat dari Industri Farmasi, (On line), (file://A:\Biota%20Laut%20Berpotensi%20Besar.htm, diakses 25 Januari 2018)
- Anonymous. 2006. "Rumput laut alternatif yang menguntungkan. Situs Kedai Iptek PKT – BPPT.
- Soriano F., Huelves L., Naves P., Rodríguez-Cerrato V., del Prado G., Ruiz V., and Ponte C. 2009. *In vitro* activity of ciprofloxacin, moxifloxacin, vancomycin and erythromycin against planktonic and biofilm forms of *Corynebacterium urealyticum*. J. Antimicrob Chemother. 63, 353-356
- Wiwin, D., 2004, Penemu Senyawa Antimikroba dari Rumput Laut, (<http://roemahherba.net/index.php?option=content&task=view&id=261&Itemid=2&phpshop=ce6bf18f144216158f0291793a6a02e0>), diakses 6 Desember 2007
- Val, A. G., Platas, G., Basilio, A., Cabello, A., Gorrochategui, J., Suay, I., Vicente, F., Portillo, E., Rio, M. J., Reina, G. G., Palaez, F., 2001, Screening of Antimicrobial Activities in Red, Green, and Brown Macroalgae from Gran Canaria, Spanyol, (Online), (<http://www.im.microbios.org>, diakses September 2011)
- Allmendinger A, Spavieri J, Kaiser M. et al. 2010, Antiprotozoal, Antimycobacterial and Cytotoxic Potential of Twenty-Three British and Irish Red Algae Phytother. Res., 24: 1099-110
- Spavieri J, Allmendinger A, Kaiser M et al. 2010. Antimycobacterial and cytotoxic potential of some British green algae. Phytother. Res., in press, doi: 10.1002/ptr.3208
- Selçuk, S. S., Meriçli, A. H., Güven, K.C., et al., 2011, Antiprotozoal, antimycobacterial and cytotoxic potential of some British red algae. Phytother. Res., 25, 778-783
- Ahmad, A., Prastawa, B. dan Salama, D. 2006. Bioaktivitas antimikroba dan antikanker fraksi protein yang diisolasi dari beberapa spesies makro alga di pulau Barang Lompo Sulawesi Selatan. Prosiding Seminar Nasional Research Grant TPSDP Batch II, Bali.
- Ahmad, A., Natsir H., and Patta, A. M. 2013. Purification and Immobilization of L-Asparaginase Enzyme from the Thermophilic Bacteria *Bacillus Licheniformis* Strain Hsa3-1a, Internat. J. of Pharma and Bio Sciences, 4 (4): 278-280.
- El-Bessoumy, AA, Sarhan, M., and Mansour, J., 2004, Production, Isolation, and Purification of L - Asparaginase from *Pseudomonas aeruginosa* 50071 Using Solid state Fermentation, Journal of Biochemistry and Molecular Biology, Vol. 37, 387-393.
- E-Moharram, M., Gamal-Eldeen, AM, and El-Sayed, ST, 2010, Production, immobilization and Anti-Tumor Activity of L - Asparaginase of *Bacillus* sp R36, American Journal of Science, Vol. 6, 131-140.
- Schroder, H.C. Ushijima, H. Krasko, A., Gamulin, V., Thakur, N.L. Diehl-Seifert, B., Muller, I.M. and Muller, W.E.G. 2003. Emergence and Disappearance of an immune molecule, an antimicrobial Lectin in basal metazoan. J. Biol. Chem., 278, 32810-32817.
- Ely, R., Supriya, T. and Naik, C.G. 2004, Antimicrobial Activity of Marine Organisms Collected of The Coast of South East India J. of Exp. Marine Biol. Eco., 21, 1-7.
- Ahmad, A., Kikuchi, H., Takami, Y. and Nakayama, T. 2005. Different Roles of N-terminal and C-terminal Halves of HIRA in Transcription Regulation of Cell Cycle-related Genes That Contribute to Control of Vertebrate Cell Growth, J. Biol. Chem., 280, 32090-32100.
- Ahmad, A., Takami, Y. and Nakayama, T. 1999. WD repeats of the p48 sub unit of chicken chromatin assembly factor-1 required for in vitro interaction with chicken histone deacetylase-2. J. Biol. Chem, 274, 16646-16653.
- Voet, D. and Voet, J. D. 2010. Biochemistry, 4th Edition, John Wiley & Sons, Inc. 123-127.

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