

Purification and gene cloning of a novel antibacterial phospholipase A2 from the sponge *Agelas clathroides* in Kapoposang Island Indonesia terrestrial

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Abstract: A new phospholipase A2 enzyme (PLA2) has been purified from the sponge of *Agelas clathroides* by using ammonium sulphate fractionation, column chromatography and reversed-phase HPLC. It 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 PLA2. It consists of 474 nucleotides encoding 157 amino acid residues including a putative initiation Met. To obtain it in large amounts, the coding sequence of PLA2 was cloned into pGEX-2TK vector and expressed as a PLA2 fusion protein in *Escherichia coli* BL21 strain. The soluble fusion protein collected from the supernatant of the cell lysate with induction by 50 μ M isopropyl- β -D-thiogalactopyranoside (IPTG) was purified in a single-step on glutathione agarose bead chromatography. The purified native PLA2 protein and recombinant PLA2 fusion protein were determined for novel antibacterial activity. Recombinant PLA2 fusion protein exhibited a similar antibacterial activity to the native PLA2. The recombinant PLA2 had stronger antibacterial activity toward *Salmonella typhi* and *Staphylococcus aureus* (G⁺) with the inhibition zone diameters of 2.0 times higher than that toward *Escherichia coli* and *Vibrio cholerae* (G⁻). These works might provide a significant foundation for following research on the antibacterial action of PLA2 protein from marine sponges.

Keywords: Sponge, *Agelas clathroides*, Phospholipase A2, Recombinant Protein, Antibacterial Activity

1. Introduction

The evolution of antibiotic-resistant bacteria has stimulated the search for potent antibacterial agents from natural products. While natural products have traditionally been harvested from terrestrial sources, reports showed that more than 15,000 marine natural products have been isolated in the period from 1965 to 2005^[1]. A major contributing factor to this development is the fact that modern biotechnology has made easier to gain access to the great biodiversity of life found in the oceans^[2]. The marine environment offers a great biodiversity for the isolation of pharmacologically active compounds^[3]. Efficient high throughput screening methods have been developed to exploit the marine biodiversity

including marine sponges (porifera) in drug discovery programs^[3,4]. Since the early days of marine natural product discovery, *Porifera* (sponges) and *Chordata* have dominated as the major contributing phyla of novel bioactive compounds^[1].

Phospholipase A2 (PLA2) catalyses the hydrolysis of the sn-2 acyl ester bond of phospholipids in a reaction resulting in the release of a free fatty acid and lysophospholipid. PLA2s are present as membrane-associated and soluble enzymes in almost all cell types and play important roles in the biosynthesis of eicosanoids, turnover of membrane phospholipids, cellular signalling and protection of membranes against peroxidation damage^[5]. PLA2s are essential components of snake and other venoms where the various forms of the enzyme have haemolytic, myotoxic,

neurotoxic, procoagulant and anticoagulant functions^[6]. In humans, PLA2s are associated with numerous clinical inflammatory processes^[7]. In addition, the human and mouse PLA2s are highly conserved in their amino acid sequence and tertiary structure. An important property of secretory PLA2s is their capability to kill bacteria *in vitro*^[8] and *in vivo*^[9].

The functional role of PLA2 in eicosanoid-mediated disorders makes this enzyme a potentially important therapeutic target^[10]. The snake venom and mammalian PLA2s have been studied extensively and in detail; there are only few studies on the PLA2s of marine invertebrates^[11]. In addition, distinct PLA2s have been characterised and cloned in a number of other animal species, including invertebrates^[12] and fish^[13] as well as in plants^[14]. To our knowledge, PLA2 has not been purified and cloned from sponge, especially from Indonesia terrestrial such as Kapoposang island. Indonesia, known as a maritime country with ocean area of 75% covering the country, has abundant source of marine biota, among others are a variety of sponges species. Some species have been reported to contain bioactive compounds that have been widely applied in pharmaceutical industries^[15]. However, research on the exploration of certain groups of protein compounds derived from sponges as a whole material for medicines designated to human and animal has not been published so far.

In this study, we report for the first time, the purification and characterization of PLA2 from *Agelas clathroides* of marine sponges. Based on partial amino acid sequencing, cDNA encoding PLA2 was cloned and the amino acid sequence and structure was deduced, resulting high homology with cnidaria PLA2 (79%). Finally, the recombinant gene encoding PLA2 was expressed in *E. coli* BL21 strain to produce recombinant GST-PLA2 fusion protein and its antibacterial activity was compared with the native PLA2 protein. The results of this work indicated that recombinant PLA2 had stronger antibacterial activity toward *S. typhi* and *S. aureus* with the inhibition zones diameters of 2.0 times higher than that toward the other Gram negative bacterial test. Regarding the two strains of Gram negative *V. cholerae* and *E. coli*, the inhibition zones diameters were almost the same using lysozyme protein as positive control. Furthermore, this research is the first report on the purification and gene cloning of a novel antibacterial PLA2 of the marine sponge. These works might provide a significant foundation for following intensive and detailed research on the structural and action of molecular mechanism of PLA2 protein from marine sponges in drug discovery programs.

2. Materials and Methods

2.1. Materials

Materials used in this research were sponge *Agelas clathroides* (from Kapoposang island, South Sulawesi Province, Indonesia terrestrial), bacterial test of *E. coli*, *S. aureus*, *S. typhi*, and *V. cholerae*, aquades, MHA (Muller Hinton Agar) media, pGEX-2TK vector, CM-cellulose,

Sephadex G-75, *E. coli* BL-21 competent cells, Trizol reagent kit, 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), GST (Glutathion S-transferase), and lysozyme protein.

2.2. Extraction and Isolation of Sponge PLA2 Protein

Extraction and isolation of sponge PLA2 protein were conducted using previous methods as follows^[16, 17], 500 g of fresh sample sponge *Agelas clathroides*, homogenized with waring blender in 500 mL of 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 %), filtered with buchner. The filtrate obtained was frozen and thawed between 2 or 3 times, and then centrifuged at 12,000 rpm and 4°C for 30 minutes. The supernatant obtained was stored in a refrigerator until tested for antibacterial activity and further purification steps.

2.3. Fractionation and Dialysis of Protein

The supernatant (whole extracts) containing protein and having anti bacterial activities was then fractionated using ammonium sulphate at saturated levels of 0 – 20 %, 20 – 40 %, 40 – 60 %, and 60 – 80 %, respectively.

The precipitates obtained after fractionation at optimum saturation level of ammonium sulphate was then resuspended in buffer B (Tris-HCl 0.1 M pH 8.3, NaCl 0.2 M, CaCl₂ 0.01 M), and then dialysed in buffer C (Tris-HCl 0.01 M pH 8.3, NaCl 0.2 M, CaCl₂ 0.01 M) using selophan pocket (Sigma) until colorless. After dialysis, protein fraction was subjected to antibacterial testing similar to the previous testing on the prepare of whole extract protein.

2.4. Purification of Native PLA2 Protein

The protein fraction of ammonium sulphate with maximum antibacterial activity (100 mg) of *Agelas clathroides* was dissolved in 5 mL of 50 mM Tris-HCl buffer (pH 8.3) and centrifuged (15,000 rpm, 5 min). The supernatant were loaded onto a carboxymethyl cellulose (CM, Sigma-Aldrich) cation-exchange column (3 X 20 cm) which had been equilibrated with 20 mM Tris-HCl buffer (pH 8.3). After the unbound proteins had been eluted, bound proteins with antibacterial activity were eluted using a gradient from 20 to 1500 mM Tris-HCl buffer (pH 8.3) at a flow rate of 0.5 mL/min and 60 fractions were collected. Protein fractions were lyophilized and then redissolved in 50 mM Tris-HCl buffer (pH 8.3) for antibacterial activity assay. Pooled active fractions were loaded onto a Sephadex G-75 gel filtration column (1 X 30 cm, Amersham Pharmacia Biotech, Sweden). After the unbound proteins had been eluted, bound proteins with antibacterial activity were eluted with the same buffer solution at a flow rate of 0.5 mL/min. The elution proteins was monitored at 280 nm by UV Spectrometer. The fractions were lyophilized and redissolved in 50 mM Tris-HCl buffer (pH 8.3) for antibacterial activity assay. Fractions showing antibacterial activity were subjected to reverse-phase high-performance

liquid chromatography (RP-HPLC) on a C8 column (4.6 X 100 mm, Amersham Pharmacia Biotech, Sweden) under a shallower gradient from 30 to 60% acetonitrile in 0.1% TFA over 60 min, at a flow rate of 0.5 mL/min. The elution proteins was monitored at 214 nm by UV Spectrometer. After each step, the protein profiles of the active fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% separating gel. Proteins were detected by Coomassie Brilliant Blue R staining.

2.5. Protein Concentration Quantitative

The calculation of protein concentration at different purification steps was determined based on Lowry method^[18] using Bovine Serum Albumine (BSA) as a standard.

2.6. Determination of Partial Amino Acid Sequences of PLA2

The purified native PLA2 (20-30 µg) was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% separating gel. Proteins were detected by Coomassie Brilliant Blue R staining. A single band corresponding to the PLA2 protein was extracted from gel, and the high purity protein was treated with lysyl-endopeptidase enzyme in 100 µL of 50 mM Tris-HCl buffer (pH 8.3), at PLA2 protein ratio of 1:20 by weight for 10 h at 30 °C. Acetonitrile was added to the reaction mixture at a final concentration of 5% (v/v) followed by sonication for 5 min. The mixture was centrifuged at 15,000 rpm for 1 min at 4°C, and the supernatant (peptides) was applied to a protein sequencer (Applied Biosystems Division, Model 473A, Perkin-Elmer).

2.7. Preparation of the Total RNA from Sponge *Agelas Clathroides*

The sponge *Agelas clathroides* was cut into small peices and homogenized by adding Trizol reagent (Gibco BRL) immediately after removal to new eppendorf tube. Total RNA was extracted according to the manufacturer's protocol (Gibco BRL).

2.8. cDNA Synthesis and Molecular Cloning of the Gene Encoding PLA2 Protein

Based on the known partial amino acid sequence and homogenous analysis by Protein Sequencing and BLASTN program^[19], two primers P1 and P2 were designed (P1: 5'-CTTGCTTTGGCTGATCAAGAAAATAAG-3', P2: 5'-GTAAAATGATTCCTCATGAAACACTT-3'). RT-PCR kit (Takara, Japan) was used to amplify the 3' end of cDNA, in which primers P1 and P2, Oligo dT-Adaptor primer M13 and primer M4 were used according to the manufacturer's protocol (Takara, Japan). After that, primer P3 (5'-TCCCGGCCTGCAGAGACTTAGC-3') was designed near the 3' end of cDNA. RT-PCR was performed to obtain the encoded region of cDNA by using primer P1 and P3. Next, cDNA product encoding full length of PLA2 protein was cloning into pGEX-2TK vector to yield pGEX-2TK PLA2 cDNA plasmid.

2.9. cDNA Sequencing and Analysis

The nucleotide sequence was analyzed by the dideoxy chain-termination method using DNA sequencer machinery (Applied Biosystems ABI Prism 310). The cDNA sequence and deduced amino acid sequence were compared with sequences in the GenBank database through BLASTN, BLASTP, and Bioedit 7.9.1 software^[19].

2.10. Expression of Recombinant GST-PLA2 Fusion Protein in *E.coli*

The GST Gene Fusion System (Pharmacia Biotech Inc.) was used to express PLA2 following the procedure of Ahmad, *et al.*, 1999^[20]. An overnight culture of *E. coli* BL21 containing pGEX-2T PLA2 cDNA was diluted 1:10 in 400 mL of LB medium supplemented with 200 µg/mL ampicillin, and next grown at 37°C to an optical density (OD)₆₀₀ of 1.0 and induced with 50 µM IPTG for 3 h at 37°C. The culture (400 mL) was centrifuged and the pellet suspended in 10 ml of lysis buffer (Tris-HCl 0.1 M pH 8.3, NaCl 2 M, CaCl₂ 0.01 M, β-mercaptoetanol 1 %, Triton X- 100 0.5 %), containing 0.1% (v/v) phenylbenzosulfonyl fluoride and 1 mg/mL lysozyme. Following a 15-min incubation on ice, dithiothreitol and Sarkosyl were added to 5 mM and 1.5% final volumes, respectively. The sample was sonicated for 2 min on ice in a water bath sonicator, centrifuged, and Triton-X 100 (2% final volume) was added to the supernatant. Next, glutathione agarose beads were added to the supernatant and incubated for 20 min at 4°C with gentle rotation. The beads were collected by centrifugation at 3,500 rpm for 2 min and washed five times with cold PBS buffer. The fusion protein (GST-PLA2) were eluted with 4 mL of 20 mM glutathione in 50 mM Tris-HCl pH 8.3, and the resultant eluate was concentrated with a Millipore membrane, followed by the addition of glycerol to a final concentration of 20%. The samples obtained were resolved by 10% SDS-PAGE, as described^[20]. The recombinant GST-PLA2 fusion protein concentrations were determined by Lowry method^[18] using Bovine Serum Albumine (BSA) as a standard. The antibacterial activity of the recombinant GST-PLA2 fusion protein together with the native PLA2 protein was determined as later described.

2.11. Antibacterial Activity Assays

Antibacterial activity assays against *E. coli*, *S. aureus*, *S. typhi*, and *V. cholerae* was conducted using diffusion method^[16, 17]. All about 20 µL of samples (whole extract and protein fractions obtained at each purification step, approximately 4 µg), were applied on sterile paper disc (diameter 6 mm) and put on the agar surface of the bacterial test culture. After one day (24 h) incubation at 37°C, the inhibition zone diameter was determined in millimeter. The same procedure was applied to 20 µL GST alone (approximately 4 µg) and 20 µL lysozyme (approximately 4 µg) as negative and positive controls, respectively. The assay was conducted in duplicate and repeated three times to produce representative experimental data.

3. Results

3.1. Purification of Native PLA2 Protein

Protein fraction from ammonium sulphate fractionation was applied on a CM-cellulose cation-exchange column (3 X 20 cm), and the proteins bound to the CM were eluted using a gradient from 20 to 1500 mM Tris-HCl pH 8.3 at a flow rate of 0.5 ml/min and 60 fractions were collected up to 600 min. The fractions eluting between 0.6 and 1.2 M Tris-HCl demonstrated antibacterial activity (Figure 1A). The protein fraction from CM-Cellulose cation-exchange column that

demonstrated antibacterial activity were applied to gel filtration chromatography on Sephadex-G75. Nine peaks were collected out of which only peak 9 showed antibacterial activity (Figure 1 B). For protein sequencing, peak 9 was further purified by reversed-phase HPLC on a C8 column using a shallower acetonitrile gradient, resulting in single protein purity (Figure 1 C). Based on SDS-PAGE (10%), the molecular weight of the purified PLA2 protein was estimated to be around 39 kDa (Figure 1 D). The result was similar to the one calculation based on Bioedit 7.9.1 software which showed that the molecular weight was 38.979 kDa (result not shown).

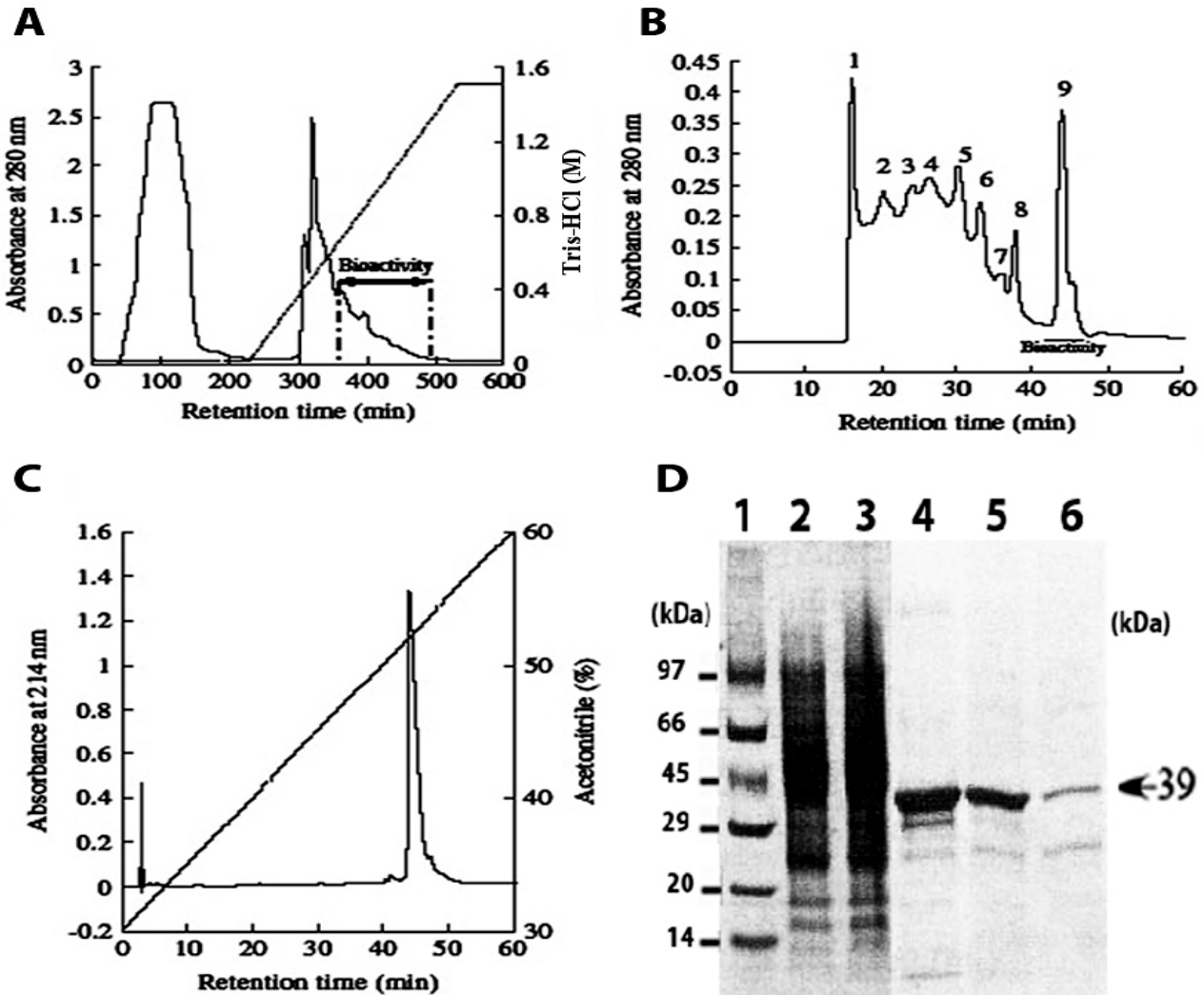


Figure 1. Isolation and purification steps of a novel antibacterial PLA2 protein from the sponge *Agelas clathroides*.

As shown in Figure 1, (A) Cation-exchange chromatography of protein fraction on CM-Cellulose. (B) Gel filtration chromatography of pooled active fractions from the cation-exchange column on a Sephadex G-75 column (C) Pure protein of peak 9 from gel filtration chromatography in (B) was purified by reversed-phase HPLC on a C8 column. (D) SDS-PAGE (10%) analysis of the active fractions obtained at each purification step. Lane 1, Protein marker in kDa; lane 2, whole extract materials; lane 3, protein fraction from ammonium sulphate fractionation; lane 4, pooled active

fractions from CM cation-exchange chromatography; lane 5, pooled active fractions (peak 9) from gel filtration chromatography; lane 6, single peak from the reversed-phase HPLC.

3.2. Amino Acid Sequencing and Gene Cloning Encoding PLA2 Protein

At first, 11 residues amino acids (LALADQENKSL) and 12 residues amino acids (VKCFMRNHFNNK) were determined by direct N-terminal amino acid sequencing of

PLA2 protein using Protein Sequencer. In order to obtain the complete amino acid sequence of the mature PLA2 protein, RT-PCR and cDNA sequencing were further performed. The primers for RT-PCR were designed based on the partial amino acid sequence above, and a full length cDNA fragment of about 541 bp, including untranslation regions was obtained. The fragment DNA was cloned into the pGEX-2TK vector to yield pGEX-2TK PLA2 cDNA plasmid (Figure 2) and then sequenced. The complete amino acid sequence was then deduced from the cDNA sequence. It shows the mature PLA2 protein that contains an open reading frame (ORF) of 610 nucleotides, including the untranslation regions poly A⁺ ending and encoded 157 amino acid residues including initiation Met (Figure 3). Based on Bioedit 7.9.1 software, the amino acids sequence revealed high homology with the PLA2 protein cnidaria (78%), but low homology with the PLA2 protein Echinodermata (12%), and very low homology with the PLA2 protein chordata (6%) (Figure 4). This results indicated that the fragment DNA isolated from sponge *Agelas clathroides* from Kapoposang island, South Sulawesi Province, Indonesia was a gene encoding PLA2 protein, and also this gene could be amplified using the generated primer pairs from the partial amino acid sequence of native PLA2 protein. Future intensive research is needed to trace the existence of this gene and it's product, especially for determination of enzymatic activity to strengthen the structural and functional analysis of PLA2 protein.

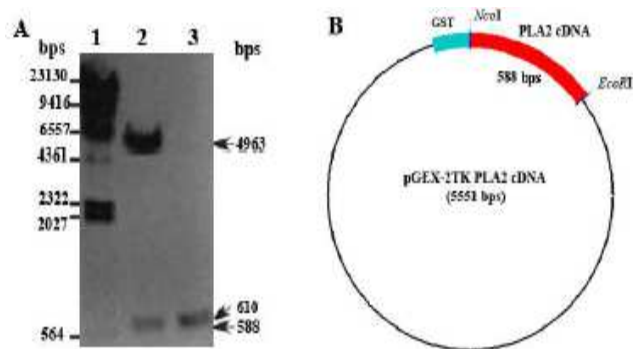


Figure 2. Schematic representation of the PLA2 gene synthesis by the RT-PCR method and the construction of pGEX-2TK fusion vector.

As shown in Figure 2, A) Agarose gel electrophoresis of DNA fragment of RT-PCR product. Lane 1, DNA lambda/*Hind*III marker; lane 2, two fragments DNA containing pGEX-2TK vector (4963 bps) and insert of PLA2 DNA minus 5' untranslation region (588 bps) digested by *Nco*I/*Eco*RI restriction enzymes of recombinant plasmid in B; and lane 3, RT-PCR product of PLA2 DNA (610 bps). B) Restriction map of recombinant pGEX-2TK PLA2 cDNA plasmid.

The nucleotide sequence was analyzed by the dideoxy chain-termination method. As shown in Figure 3, the result of N-terminal amino acid sequencing obtained from Protein Sequencing is indicated in box and the two primers P1 and P2 obtained based on partial amino acids sequencing are underlined.

| | | | |
|-----|--|------------------------|-----|
| | | ccaacgatatccgtgcgcacct | -22 |
| 1 | atg cag ctt tat acc gtt ttt ttc acc gcg tct gtt gtg gta att | 45 | |
| 1 | Met Gln Leu Tyr Thr Val Phe Phe Thr Ala Ser Val Val Val Ile | 15 | |
| 46 | <u>ctt gct ttg gct gat caa gaa aat aag tca tta gac ttc atc tcg</u> | 90 | |
| 16 | <u>Leu Ala Leu Ala Asp Asp Gln Glu Asn Lys Ser Leu</u> Asp Phe Ile Ser | 30 | |
| 91 | gaa ggt gga att gcc ggq cga ggg gat ttc cag cac agt gcc cta | 135 | |
| 31 | Glu Gly Gly Ile Ala Gly Arg Gly Asp Phe Gln Asp Ser Ala Leu | 45 | |
| 136 | atc aaa aaa tat acg gga cgc aat oca ttg gat ccg tgg gat tat | 180 | |
| 46 | Ile Lys Lys Tyr Thr Gly Arg Asn Pro Leu Asp Pro Trp Asp Tyr | 60 | |
| 181 | ggc tgc tgg tgt gga cta ggt ggc gat gga acg tac gtt gat gtc | 225 | |
| 61 | Gly Cys Trp Cys Phe Gly Leu Gly Asp Gly Lys Thr Val Asp Val | 75 | |
| 226 | gta gcg tgg tgc gac tac cat cac gac atg ccc ttc aat agt ggt | 270 | |
| 76 | Val Ala Trp Cys Asp Tyr His His Asp Met Pro Phe Asn Ser Gly | 90 | |
| 271 | tac caa ggg gaa cgc cca atc tgc agc tct aac gca cct gtc cat | 315 | |
| 91 | Tyr Gln Gly Glu Arg Pro Thr Cys Ser Ser Asn Ala Pro Val His | 105 | |
| 316 | aag aac tac tat ttc agc ggt ctc aaa tgt tcg aca ggt tgg ccc | 360 | |
| 106 | Lys Asn Tyr Tyr Phe Ser Gly Leu Lys Cys Ser Thr Gly Trp Pro | 120 | |
| 361 | aca tgc aag tgt gga cga tgc att tgt gct tgt gcg att gct cag | 405 | |
| 121 | Thr Cys Lys Cys Gly Arg Cys Ile Cys Ala Cys Ala Ile Ala Gln | 135 | |
| 406 | gta aag tgt ttc atg agg aat cat ttt aac aat aaa tac cag tca | 450 | |
| 136 | Val Lys Cys Phe Met Arg Asn His Phe Asn Asn Lys Tyr Gln Ser | 150 | |
| 451 | tac gac aaa aat atc tgt tgc tag tgaaaaatcgctgataactgaattaa | 501 | |
| 151 | Tyr Asp Lys Asn Ile Cys Cys End | 157 | |
| 502 | atatagaaaatcatgtataacaataaacagaaacttacagatctcgagtgcgattacc | 560 | |
| 561 | ctgtgtttacatctgaaaaaaaaaaaaa | 580 | |

Figure 3. The cDNA and deduced amino acid sequence of PLA2 protein. Fragment achieved by RT-PCR amplification of total sponge *Agelas clathroides* cDNAs was inserted into pGEX-2TK vector.

The complete amino acid sequences of four proteins of marine invertebrate are shown in Figure 4. The positions of the amino acid sequence are indicated at the ends. Amino acid residues that are identical in two are marked with : and three are marked with . and analogous amino acids in all these proteins are indicated by bold letter.

| | 10 | 20 | 30 | 40 | 50 |
|--------------------|-------------|------------|----------------|-----------------|------------|
| Ac PLA2 | MDLYTVFFTTA | SVVVILALAD | QENKSIDFTS | EGGIAGRGRDF | QDSALIKKYT |
| Cn PLA2 | MDLYTYFFFTF | SLVLILALAD | QENKSIDFTQ | EGGIAGRGRDF | QFSYLIRKYT |
| Ec PLA2 | MKLLILVLAA | TVAVVNSDVS | SVYQFGKFTS | CYGGAG---- | FFD |
| Ch-PLA2 | -----MRTLWI | VAVMLTGVGE | DLSGDTGDMIN | KKTGTFG----- | ----- |
| Clustal Con | | . : . . . | . : . : | : | |
| | | | | | |
| | 60 | 70 | 80 | 90 | 100 |
| Ac PLA2 | GRNFLDPWDY | GCWCGLGGDG | TYVDVVAWCD | YHDMFPNFSG | YQGERPTCSS |
| Cn PLA2 | GRNFLDWDYGY | GCWCGLGGGK | TFVDGVWDVC | YHDMCFNFSI | YQGERPTCSK |
| Ec PLA2 | ---LDYNGY | GCYCYGGGK | PLDWDTRCC | LVEDNCKYKA | TI--AEDCGS |
| Ch-PLA2 | ---LFSYIYY | GCYCGWGGK | KPQDADRRC | FVHDCCYGSV | N-GCDPKLST |
| Clustal Con | : . Y | GC:CG GG.G | . D . C | HD : . . | |
| | | | | | |
| | 110 | 120 | 130 | 140 | 150 |
| Ac PLA2 | NAPVHKNYTF | SGLKCSGT-- | -----WPTC--KCG | RCICACATAA | |
| Cn PLA2 | NAPVHKNYTF | SGLKCSGT-- | -----WLTG--KCG | RAICACDIAA | |
| Ec PLA2 | WDPIYIVDYG | EQTTDASGNC | VIKCKKAADY | SWYSTNFCER | EFMCECDRAG |
| Ch-PLA2 | YS-----YSF | QNGDIVCG-- | -----DDDPCL | RAVCECDRVA | |
| Clustal Con | Y : . | G | | C | : . C C . |
| | | | | | |
| | 160 | 170 | 180 | Identity | |
| Ac PLA2 | VKCFMRN--H | FNFKYQSYDK | NICCK----- | 100% | |
| Cn PLA2 | VKCFMRN--H | FNFKYQNYKK | NICX----- | 78% | |
| Ec PLA2 | ACQCFEKRPT | YNQAYESYDK | DSCK----- | 12% | |
| Ch-PLA2 | AICSGENMNT | YDKKTMLYSL | FDCKEESKCK X | 6% | |
| Clustal Con | C . : . | ::: Y Y . | C | | |

Figure 4. Comparison of the aligned amino acid sequences of PLA2 of sponge *Agelas clathroides* (ac_PLA2) with chordata (ch_PLA2), echinodermata(ec_PLA2), and cnidaria (cn_PLA2).

3.3. Expression and Purification of the Recombinant PLA2 Protein

The coding sequence of cDNA PLA2 was expressed as part of the pGEX-2T fusion protein, GST-PLA2. The single-step purification procedure as described in Ahmad, *et al.*, 1999^[20], was used to improve solubility of the GST-PLA2 fusion protein. Based on SDS-PAGE (10%) in Figure 5, the molecular mass of the GST-PLA2 fusion protein product was

≈ 65 kDa (fusion of the molecular mass of the PLA2 alone was ≈ 39 kDa plus the GST was ≈ 26 kDa) was dramatically accumulated in *E. coli* BL 21 cell containing the pGEX-2TK PLA2 plasmid with induction by 50 μM IPTG and could be purified to more than 95% homogeneity, using glutathione agarose beads (see lane 4 in Figure 5).

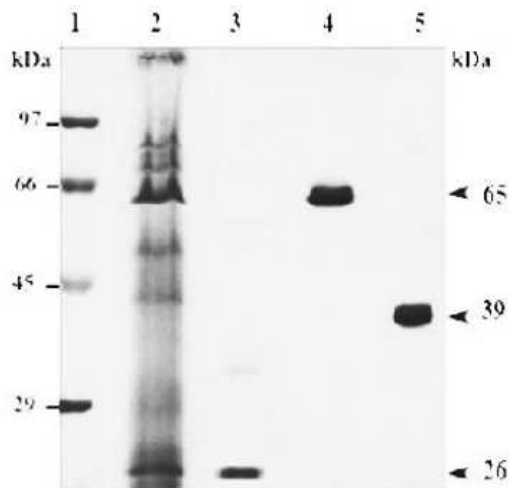


Figure 5. Electrophoresis SDS-PAGE (10%) analysis of the recombinant GST-PLA2 fusion protein and native PLA2 protein.

As shown in Figure 5, Lane 1, Protein marker; Lane 2, Whole extract protein in *E. coli* cell containing pGEX-2TK PLA2 cDNA; Lane 3, GST protein; Lane 4, GST-PLA2 fusion protein; and Lane 5, native PLA2 protein.

3.4. Antibacterial Activity of the Recombinant GST-PLA2 Fusion Protein

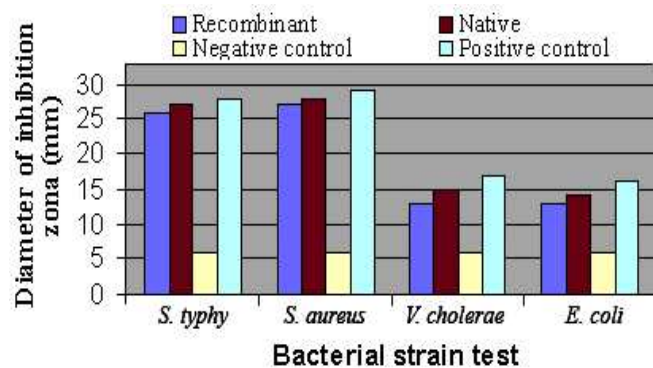


Figure 6. The diameters of inhibition zone resulting from the expression of the native PLA2 and recombinant of GST-PLA2 fusion protein (approximately 4 μg), negative control (GST, approximately 4 μg), and positive control (lysozyme, approximately 4 μg) against different bacterial strain test.

The antibacterial activity of the recombinant GST-PLA2 fusion protein against different bacterial tests was performed by agar diffusion assay. The recombinant PLA2 displayed antibacterial activity not only against *S. typhi* and *S. aureus* (Gram positive) but also against strains of *E. coli* and *V. cholerae* (Gram negative). The inhibitory effect of the recombinant PLA2 was observed towards *S. typhi*, *S. aureus*, *V. cholerae* and *E. coli* strains with the inhibition zone

diameters of 24 mm, 23 mm, 13 mm, and 13 mm respectively, similar to native PLA2. The negative control of GST protein in the same concentration had no corresponding antibacterial activity on any of the bacterial strain test. The recombinant PLA2 had stronger antibacterial activity toward *S. typhi* and *S. aureus* with the inhibition zones diameters of 2.0 times higher than that toward other Gram negative bacteria. Regarding the two strains of Gram negative *V. cholerae* and *E. coli* the inhibition zones diameters were almost the same using lysozyme (approximately 4 μg) as positive control (Figure 6).

4. Discussion

In the current study, we purified and cloned a novel antibacterial PLA2 protein from a sponge *Agelas clathroides*, belonging to the phylum porifera. Recently Nevalainen *et al.* 2004^[17], showed the wide distribution of PLA2 in porifera and it's possible functional role in the defense system of marine sponges. In general PLA2, a hydrolytic enzyme, catalyzes the cleavage of the sn-2 acyl ester bond of phospholipases, resulting in the release of a free fatty acid and lysophospholipid. Sessile organisms like sponges are dependent on chemical defense and protective mechanisms other than active defense including migration, hiding, and physical attack. Depending on habitat changes, the appearance of a predator or pathogen, and other stress factors they are exposed to, sessile animals follow different strategies. The first-line defense against sudden stress factors is the transformation of inactive precursor compounds stored in the tissue of the affected organism, thereby yielding transformation products that exhibit a pronounced defensive activity. PLA2 is a ubiquitous defense enzyme found in snake and bee venoms and distributed throughout the plant and animal kingdom^[21]. Recent studies suggested that varying levels of PLA2 were found in marine invertebrates including Porifera^[17], Cnidaria^[22], and Echinodermata^[12]. It has been reported that the cell proliferation of marine sponges is mediated by PLA2^[23]. But purification and cloning of PLA2 from sponge, especially from Indonesia terrestrial such as Kapoposang island has not been reported so far. On the other hand, the bactericidal activity of a full set of recombinant human and murine sPLA2s was tested against *L. monocytogenes*, *S. aureus* and *E. coli*^[8]. All our preliminary results indicated that recombinant PLA2 protein was a potential antibacterial protein with possible effect on the host immune response.

Many antibacterial screening studies have shown that Gram-positive bacteria are more sensitive than Gram-negative bacteria to extracts of sponges and ascidians^[24, 25]. In fact, out of 777 species of sponges collected from the Caribbean Sea, 35% had activity against Gram-positive bacteria, 15% had activity against Gram-negative bacteria. This is in agreement with our results, where sponge *Agelas clathroides* PLA2 protein extracted by polar solvent (Tris-HCl buffer) tested showed stronger activity (active at a concentration of 0.2 μg/μL, approximately 4 μg) against Gram-positive than Gram-negative bacteria strains. Further investigation should

be conducted to confirm whether recombinant PLA2 protein is really involved in invertebrates immune response mechanisms, emphasizing the protein's dynamic change before and after microbe challenge, with antibodies to the recombinant PLA2 protein.

5. Conclusions

In this research, a novel antibacterial PLA2 with molecular weight of 39 kDa from sponge *Agelas clathroides* was characterized, and its cDNA with 474 nucleotides including initiation and stop codon was cloned and expressed in *E. coli* as recombinant GST-PLA2 fusion protein. Our results indicate that PLA2 from sponge *Agelas clathroides* acts primarily on Gram-positive bacteria.

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