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EXPRESSION OF A NOVEL GENE ENCODING PROTEASE INHIBITOR FROM METAGENOME OF SPONGE IN VIETNAM

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ABSTRACT

Marine sponge is known as a "gold mine" of natural products from marine environment. Many novel bioactive compounds have been isolated from marine sponges and sponge-associated microorganisms such as antibiotics, anti-cancer compounds, protease inhibitors, etc. In this study, we selected a gene encoding protease inhibitor from metagenome of a sponge collected in Quang Tri to express in *Escherichia coli* (*E. coli*) BL21(DE3). The gene PI-DN9 encoding protease inhibitor (1.3 kb) was cut off cloning vector pUC57/PI-DN9 containing gene PI-DN9 and inserted into expression vector pET-32a(+), the recombinant vector pET-32a(+)/PI-DN9 then was transformed and expressed in the *E. coli* strain BL21(DE3). Results showed that recombinant protein (50 kDa) was expressed successfully at 25°C, 1 mM of IPTG in 5 hours. The recombinant protein was purified using Ni-NTA affinity chromatography column. Western blot assay and bioactive assay showed good activity of the purified protein.

Keywords: *Escherichia coli*, expression vector, protease inhibitor, recombinant protein, sponge.

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INTRODUCTION

Proteases are important enzymes in host cells and responsible for many physiological functions, such as zymogene activation, coagulation, fibroblast fibrinolysis, hormone and bioactive peptide release, etc. However, the loss of control of proteolysis can cause cancer and cardiovascular diseases, inflammation, neuro degeneration as well as bacterial, viral and parasitic diseases. Nowadays, protease inhibitors are often used for treatment of diseases related to proteases. Therefore, discovery of novel protease inhibitors is still a special interest of scientists (Barrett et al., 1998; Agbowuro et al., 2017).

Metagenomics is new approach that allowsstudying genetic materials recovered directly from environment samples. To date, metagenomics has been used for finding novel bioactive compounds as well as investigating diversity of microorganisms from different environments. Particularly, studies focus on discovery of genes encoding potential bioactive compounds for bio-pharmaceutical field from marine microorganisms. Many protease inhibiors and other bioactive second metabolites have found from metagenome database (Baharum et al., 2010; Culligan et al., 2014; He et al., 2013).

In this study, we selected a novel gene encoding protease inhibitor from metagenome of marine sponge QT collected in Quang Tri and expressed in the *Escherichia coli* (*E. coli*) strain BL21(DE3) for finding and mining novel protease inhibitor.

MATERIALS AND METHODS

Cloning vector pUC57 (Genscript, USA) inserted gene PI-DN9 from metagenome of the sponge QT; expression vector pET-32a(+); strain *E. coli* Top10F'; strain *E. coli* BL21(DE3) (Invitrogen, USA); restriction enzyme *Eco*RI, *Not*I and DNA marker (Fermentas, USA); protein markers (Novagen, Netherlands; Sigma-Aldrich, USA, iNtRON Biotechnology, Korea; Affymetrix, USA); Thrombin (Novagen, The Netherlands); Skimmed milked (Difco, USA); trypsin, α-chymotrypsin, thermolysin, IPTG, NBT (Sigma-Aldrich, USA).

Construction of recombinant vector pET-32a(+)/PI-DN9

Metagenome of the sponge QT was sequenced and annotated using databases, such as CAZy (Cantarel et al., 2009), GO (Ashbumer et al., 2006), COG, SWISSPROT, KEGG (Tatusov et al., 2001) and tools such as dbCAN (Yin et al., 2112), Prodigal (Hyatt et al., 2010) and MetageneMark (Zhu et al., 2010) by BaseClear (Netherlands). The contig 00046 (prokka 10704) having similarity 50% with genes encoding protease inhibitor on GeneBank was selected for protein expression. The sequence of contig 00046 was designed to contain two restriction enzymes *Eco*RI and *Not*I (PI-DN9) and inserted in cloning vector pUC57. The gene PI-DN9 was cut from cloning vector pUC57/PI-DN9 using enzymes *Eco*RI và *Not*I. The expression vector pET-32a(+) was also cut using two above enzymes. Finally, the gene PI-DN9 was inserted into expression vector pET-32a(+) in order to create recombinant vector pET-32a(+)/PI-DN9.

Expression of the gene PI-DN9 in *E. coli* BL21(DE3)

The recombinant vector pET-32a(+)/PI-DN9 was transformed into *E.coli* BL21(DE3) competent cells by the heat shock method. The strain *E.coli* BL21(DE3) containing the recombinant vector was incubated for overnight in LB medium supplemented with 50 µg/mL of ampicillin (LBamp) at 37°C and shaked at 200 rpm. Culture broth was transferred into fresh Lbamp medium and incubated in the same above conditions until OD_{600} = 0.6, then added 1mM of Isopropylthio-ß-galactosida (IPTG) into culture broth and expressed at 25°C in 5 hours. The expressed protein was checked on 12.6% SDS-PAGE gel.

Western blot assay

In this study, Western blot assay was used to detect protease inhibitory protein. Protein was separated on 12.6% SDS-PAGE gel then transferred onto nitrocellulose or polyvinylidenedifluoride (PVDF) membrance by liquid blotting system (Invitrogen). The membrance was blocked with 5% milk in TBS for overnight and was incubated with antibody anti-TRx at 4°C (1:1000) for 2 hours. The membrance then was placed in TBS buffer and washed for 10 minutes. Next, the membrance was incubated with the secondary antibody solution for 1 hours at room temperature, then was placed in TBS and washed for 10 minutes. This step was repeated twice with fresh buffer. Finally, the NBT substrate was added onto membrance to visualize the protein activity.

Determination of protein in supernatant and pellet

Culture broth was centrifuged at 5000 rpm for 5 minutes and removed supernatant. The pellet was resuspended in TE buffer (20mM Tris, 10mM EDTA, 0.05mM PMF) and incubated at (-)80°C for 1 hour. The pellet was thawed at 50°C in 30 minutes and

disrupted by ultrasound. The solution was centrifuged at 13.000 rpm for 15 minutes. The supernatant was collected, and the pellet was resuspended again in TE buffer. Finally, SDS loading bufferwas added into culture broth before centrifugating. After centrifugating, supernatant and the pellet were deraturated at 100°C for 10 minutes. The expression protein was checked on 12.6% SDS-PAGE gel.

Purification of protein PI-DN9

The recombinant protein was purified by Invitrogen™ Novex™ Ni-NTA purification system according to manufacture's protocol. Then, the purified protein was then cut off *TRx-His* tagusing Thrombin Kit (Novagen, The Netherlands).

Primary screening of protease inhibitory activity of protein

Protease inhibitory activity of the recombinant protein was performed in skimmed milk agar plate (0.8% agar, 1% skimmed milked) with wells made in centre and periphery at a distance of 1 cm from the central well. Twenty microlitres of proteases (trypsin, or α-chymotrypsin, or thermolysin at 0.5mg/ml) were pipetted in the central well. The recombinant protein was added in one of the peripheral wells and sterile distilled water (negative control) in the other. The plate was incubated at 37°C. The inhibitory activity was detected by absence of clearance zone around the well containing the inhibitor (Sapna, 2013).

Protease inhibitory assay of the recombinant protein

The recombinant protein was added to a mixture of chloride, phosphate buffer and protease solutions (trypsin, or α-chymotrypsin, or thermolysin). After adjusting the pH to 7.5, BAPNA (N-α-benzoyl-DL-arginine-p-nitroanilide) was added into the mixture and incubated at 37°C for 20 minutes. Next, 5% TCA was added into the solution and incubated at room temperature for 20 minutes. Finally, the solution was filtered with Whatman no. 1 filter paper and the absorbance was measured at 280 nm. The inhibiting activity of protease inhibitor was calculated following formula:

Inhibiting activity (%)= C–T/C × 100

C was absorbance value of blank sample and T was absorbance value of samples with protease inhibitor (Karthik et al., 2014).

RESULTS AND DISCUSSION

Construction of recombinant vector pET-32a(+)/PI-DN9

The gene PI-DN9 was cut from plasmid pUC57/PI-DN9 using restriction enzymes *Eco*RI and *Not*I. The vector pET-32a(+) was also digested with *Eco*RI and *Not*I (Fig. 1A). The products were then purified and checked on 1% agarose gel (Fig. 1B). Agarose gel analysis showed that there was a band with size of 6 kb at lane 1 and lane 3 which was similar to the size of vector pET-32a(+). The band with size of 2.7 kb at lane 2 was similar to the size of vector pUC57 and the band in lane 4 with the size of 1.3 kb was similar to the size of gene PI-DN9.

The gene PI-DN9 was inserted into vector pET-32a(+) using T4 ligase, then transformed into strain *E. coli* Top10F' competent cells and incubated in LB medium containing 1mM ampicillin. Some colonies were randomly picked for plasmid extraction and then digested with *Eco*RI và *Not*I. The cutting products were checked on 1% agarose gel (Fig. 2).

The results showed that, the presenced bands with 1.3kb in size at lane 1 and 2 (Fig. 2) were similar to the size of the gene PI-DN9 (1.3kb). Besides, we detected other bands with 6kb in size at lane 1 and 2 were similar to the size of the vector pET-32a(+) without gene PI-DN9 at lane C. This result indicated that gene PI-DN9 may be inserted successfully into vector pET-32a(+). To

confirm this, we sequenced recombinant plasmid and result showed that the sequenced gene was similar 100% with gene PI-DN9 (data not shown). Therefore, the expression vector pET-32a(+)/PI-DN9 was successfully constructed.

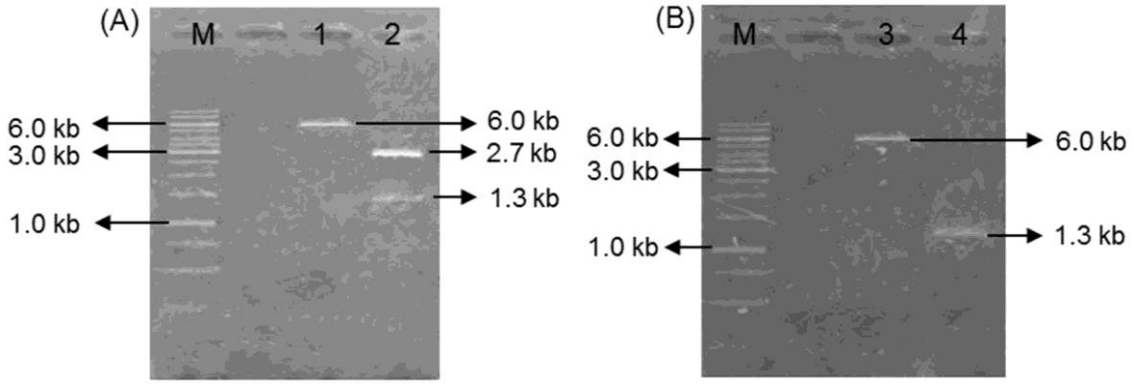

Figure 1. (A) Agarose gel analysis of vector pET-32a(+) and vector pUC57/PI-DN9 cut with *Eco*RI and*Not*I, (B) Purified products. Lane M: Maker 1 kb (Thermo), Lane 1(A): opened vector pET-32a(+) after cut with *Eco*RI and*Not*I, Lane 3: purified vector pET-32a(+), Lane 2(A): opened vector pUC57/PI-DN9 cut with *Eco*RI and *Not*I, Lane 4: purified gene PI-DN9

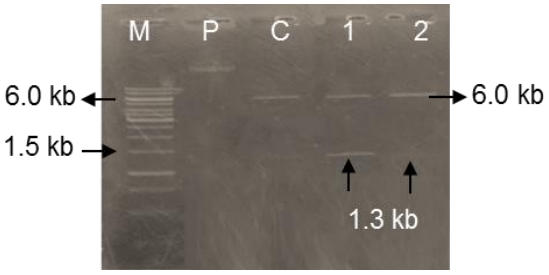

Figure 2. Agarose gel analysis of recombinant plasmid cut with *Eco*RI and *Not*I. lane M: marker 1 kb (Thermo); lane 1: plasmid pET-32a(+)/PI-DN9; lane 2, 3, 4: plasmid pET-32a(+)/PI-DN9 was cut by enzymes *Eco*RI/*Not*I; lane 5: vector pET-32a(+)

Expression of recombinant protein in *E. coli* BL21(DE3)

The recombinant vector pET-32a(+)/PI-DN9 was transformed into *E. coli* BL21(DE3) competent cells and incubated in LB medium with 1mM (amp). The presence of white colonies on plate indicated the recombinant vector may be transformed successfully in *E. coli* BL21(DE3). To confirm this, we picked randomly 3 colonies and expressed recombinant protein as described in the methods section (Fig. 3).

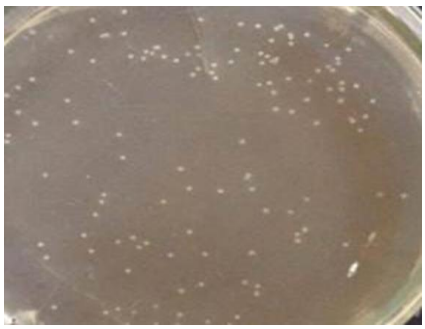

Figure 3. Colonies of *E. coli* BL21(DE3) containing recombinant vector pET-32a(+)/PI-DN9 on LBamp medium

Expressed recombinant protein was checked on SDS-PAGE gel and stained with Coomassie (Fig. 4A). The result showed that colonies induced with IPTG appeared an extra band with the size of 64 kDa, which is similar with the size of protein PI-DN9 attached *TRx-His* tag (Fig. 4A: lane 3,5,7). Lane 1 (only vector pET-32a(+) and lane 2, 4, 6 (colonies were not induced with IPTG) did not have this extra band. To confirm the expression abilities of recombinant protein, we selected one clone and performed Western blot assay with the specific anti-*TRx* antibody.

Western blot analysis showed a band with the size of 64 kDa, which was similar to the result of SDS-PAGE analysis. Therefore, we confirmed that the recombinant protein was expressed successfully.

In order to determine the fraction of expression protein, the recombinant protein in the supernatant and pellet fractions were denatured and checked on 12.6% SDS-PAGE gel. The SDS-PAGE analysis showed that the recombinant protein was expressed mainly in supernatant fraction (Fig. 4C, lane T).

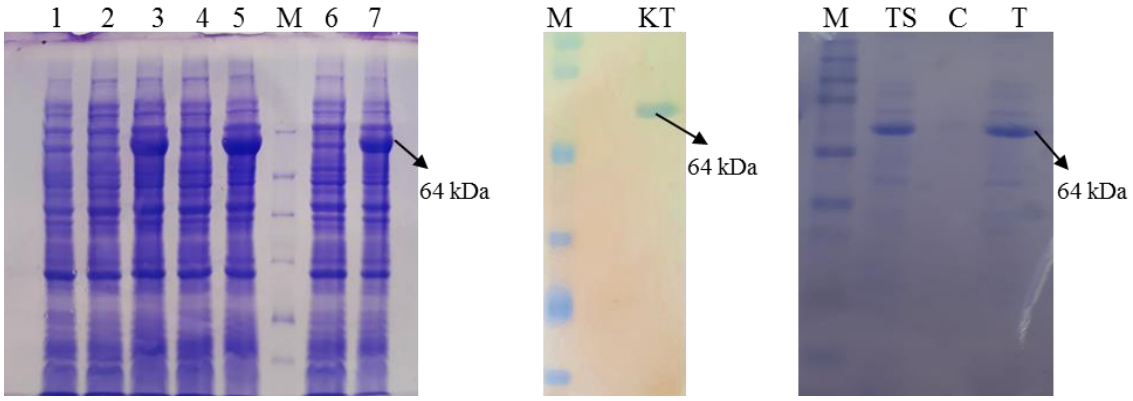

Figure 4. Analysis of protein expression on SDS-PAGE gel: (A) Protein expression PI-DN9 at 25°C and 1 mM of IPTG (Lane 1: clone without gen PI-DN9, Lane 2, 4, 6: clones containing Pet-32a(+)/PI-DN9 without induction with IPTG, Lane 3, 5, 7: clones containing pET-32a(+)/PI-DN9 induced with IPTG, Lane M: protein maker, (B) Western blot reaction of protein PI-DN9 with TRx (Land KT), (C) Protein expression in cultrure broth (TC), in pellet (C), in supernatant (T)

Purification of expressed protein and determination of its protease inhibitory activity

The vector pET-32a(+) contained the sequence of Hig-tag was used for protein expression, thereby the recombinant protein was purified by affinity chromatographic method using Ni-NTA purification system, then the purified protein fractions were checked on 12.6% SDS-PAGE gel. The result showed that, the recombinant protein PI-DN9 was in stage 1 and 2. However, amount of purified protein in stage 1was higher than in stage 2 (Fig. 5A).

In order to test protease inhibitory activity of the expressed protein, the sequence of *TRx-His* tag was removed from the purified protein using thrombin. The SDS-PAGE analysis of the protein treated with thrombin revealed the presence of two bands: one band had size of 50 kDa (size of protein PI-DN9) and one band with size of 14 kDa (size of *TRx-His* tag)(Fig. 5B). Therefore, we removed successfully *TRx-His* tag from the expressed protein.

Activity assay results of expressed protein against proteases (trypsin, α-chymotrypsin, thermolysin) using skimmed milked plate showed that protein PI-DN9 displayed inhibitory activity against trypsin (Fig. 6), but did not inhibit against α-chymotrypsin and thermolysin. Protease inhibitory assay indicated that inhibitory activity of protein against trypsin reached 83%.

There are some studies focused on discovery of protease inhibitory proteins from microorganisms associated with marine sponge; however, most of them use cultivation-dependent approaches (Wahyudi et al., 2010; Tabares et al., 2011; Pimentel-Elardo et al., 2011; Ramadan et al., 2012). With rapid development of advance sequencing technology, scientists begin discovering and mining novel natural products

based on metagenomics instead of traditional cultivation. For example, Jiang et al. (2011) cloned successfully a novel protease inhibition gene against serine (Spi1C) based on metagenome of marine microorganisms. The gene Spi1C had ORF with 642 bp and encoded for 214 amino acid with estimated size of 28.7 kDa. The protein Spi1C displayed inhibitory activity against serine proteases such as α-chymotrypsin và trypsin.

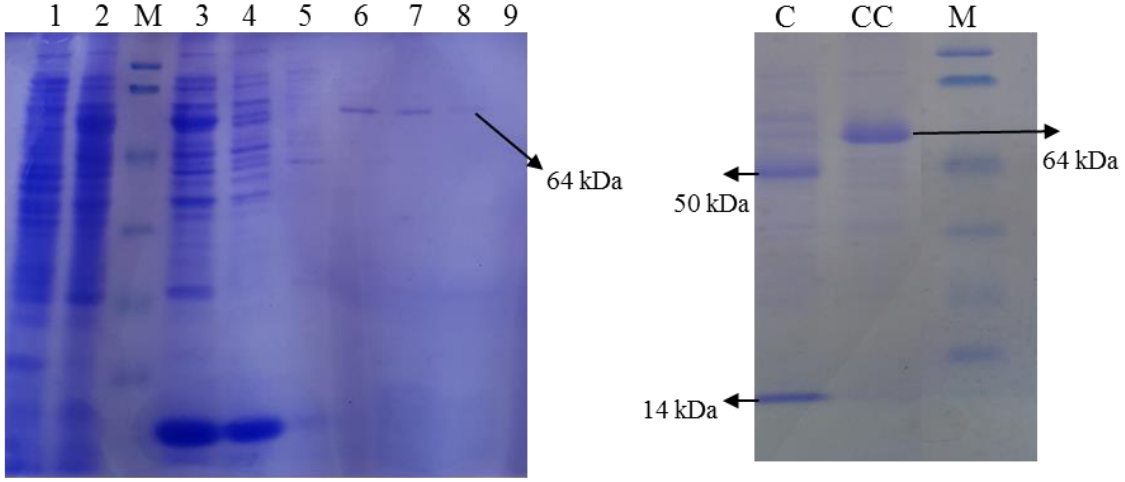

Figure 5. Analysis of the expressed protein after purifying on SDS-PAGE gel: (A) Fractions of expressed protein after purifying with Ni-NTA column (Lane M: protein marker (Bio-basic), Lane 1: protein without induced with IPTG, Lane 2: protein induced with IPTG, Lane 3: protein before purifying, Lane 4: protein after purifying with Ni-NTA column, Lane5: protein after column treated with imidazole 100 mM, Lane 6-10: proteine fractions after treating column with imidazole 250mM), (B) Protein expression after cut TRx-His (Land C), before cutting TRx-His (Lane CC) and protein maker of Bio-basic (M)

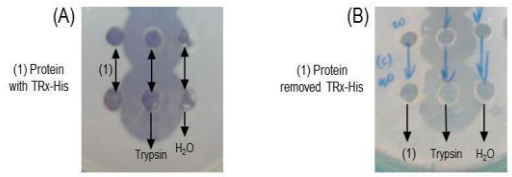

Figure 6. Protease inhibitor assay on plate (A) protein with TRx-His tag, (B) protein removed TRx-His tag

CONCLUSION

A novel gene encoding protease inhibitor PI-DN9 from metagenome of a sponge in Quang Tri was expressed successfully in *E. coli* BL21(DE3). The purified protein displayed inhibitory activity against trypsin with 83% inhibition activity. Our study showed that metagenomics is a potential tool for discovery and exploitation of novel protease inhibitor from uncultivable microorganisms.

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