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EXPRESSION OF A NEW SERPIN GENE SCREENING FROM METAGENOMICS DATABASE IN *Pichia pastoris* AND SOME CHARACTERIZATION OF RECOMBINANT PROTEIN

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ABSTRACT

Currently, searching for new protease inhibitors is of interest to many scientists around the world because they play an important role in controlling harmful proteases. As terrestrial bioactive resources are becoming increasingly depleted, studies are aimed at finding other sources, for example, the ocean. Some recent reports indicate that sponge- associated microbes produce substances with high biological activity, such as anticancer and anti-inflammation, protease inhibition, etc. In this report, a new serpin gene screened from the metagenomics database of microorganism-associated *Spheciospongia vesparium* QT2 collected sea of Quang Tri province (Vietnam), was successful expressed in *Pichia pastoris* SMD1168. The obtained result showed that the maximum amount of recombinant protein secreted in the medium was received after 72 hours of induction with methanol. The PAGE electrophoresis supplemented with 0.1% casein and the Trypsin-sepharose 4B affinity chromatography column were performed, and the result was confirmed that the recombinant protein expressed in the *P. pastoris* SMD1168 (molecular weight about 50 kDa) was the target protein. The purified recombinant protein PI-QT exhibited inhibitor activity on trypsin, α-chymotrypsin and thermolysin and the inhibition was 88.7%, 69% and 43%, respectively. In addition, PI-QT protein is stable and has optimal activity in the pH range 7−9, the temperature is below 60 ^{o}C.

Keywords: *Pichia pastoris*, microorganisms, metagenomics, protease, protease inhibitors, sponge associated.

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INTRODUCTION

Proteases play an important role in the host cell, but they can also cause diseases such as emphysema, arthritis, pancreatitis, throbosis, high blood pressure, muscle dystrophy, cancer and AIDS when the organism has metabolic disorders (Wahyudi et al., 2010). Serine proteases constitute almost one-third of all proteases and play a pivotal role in the catalysis of intracellular and extracellular hydrolytic reactions, and if their activity is not controlled properly, they might be hazardous to living organisms, such as causing tissue damage, melanization, and inappropriate coagulation, among others. Thus, the activity of proteases must be properly and strictly controlled by inhibitors (Shakeel, 2020).

Due to the key roles in regulating the activities and specific interactions with protease, protease inhibitors (PIs) have become a valuable tool in medicine, agriculture and food (Shamsi et al., 2016). Therefore, finding and screening new PIs that are more effective in the control of protease activity becomes increasingly essential.

PIs have been found in a variety of sources such as microorganisms, plants and animals. The marine environment is extremely diverse, with huge variations in pressure and temperature. However, life, especially that of microorganisms, thrives in the entire marine biosphere and bacteria have adapted to different environmental conditions. These unique and extreme habitat conditions contribute to a huge diversity of marine microbes and novelty and value genes, among them are PIs (Baharum et al., 2010). Some studies have shown that it is possible to exploit PIs from the sponges and the microorganisms associated with them (Wahyudi et al., 2010; Abdelmohsen et al., 2012; Jiang et al., 2011; Karuppiah et al., 2017). Wahyudi (2010) had isolated three bacterial strains from the sponges *Jaspis* sp., which possessed inhibitory activity against serine protease (subtilisin), metalloprotease (thermolysin) and pathogenic bacteria *S. aureus* and *P. aeruginosa*. In another study, Abdelmohshen et al. (2012) isolated from sponge bacterial strain *Micromonospora* sp. RV115 with anti-tumor, antioxidant and protease inhibitory activity. In addition, a natural product, tetromycins 1-4 which inhibit some cysteine proteases, was extracted from the *Streptomyces axinellae* Po1001T associated with the sponge *Axinella polypoides* of the Mediterranean Sea (Pimentel-Elardo et al., 2011).

In the laboratory, only about 1% of microorganisms can be isolated and cultured using conventional methods, making the majority of microorganisms and their biochemical pathways inaccessible (Handelsman, 2004). Isolation and cultivation of marine microorganisms are more difficult, especially those associated with other organisms due to the quite complex interaction between them. Metagenomics is a powerful tool for assessing genetic information from uncultured microorganisms and this approach was used for investigations of the marine environment and many studies have shown that the oceans contain an unprecedented diversity of bacteria (Kennedy et al., 2010). Metagenomics techniques analyze an entire genetic material in a given marine habitat (Nguyen et al., 2013). From the metagenomics library, it is possible to screen gene encoding new bioactive compounds and this is really a breakthrough in the research and application of biotechnology.

In this study, a new gene coding for a serpin by screening from the metagenome of bacteria associated with *Spheciospongia vesparium* QT2 collected sea of Quang Tri province (Vietnam) was expressed in *Pichia pastoris* SMD 1168. Among recombinant protein expression systems, *Pichia pastoris* expression system is considered to be a suitable choice with many outstanding advantages such as high expression level, easy culture, rapid growth, secreted recombinant protein, and the recombinant strain is genetically stable without the need of using antibiotics for maintenance (Macauley-Patrick et al., 2005; Krainer et al., 2012). Comparing with *Saccharomyces cerevisiae* expression

system, a protein expressed in *Pichia pastoris* is not over saccharification and does not form a α-1,3 glycan bond, this bond is believed to induce an immune response when the protein is used in therapy (Darby et al., 2012).

MATERIALS AND METHODS

Materials

Cloning vector pUC57 (Genscript, USA) harboring *PI-QT* gene from the metagenome of the sponge *S. vesparium* QT2; expression vector pPIC9; *E. coli* Top10F'; strain *P. pastoris* SMD1168 (Invitrogen, USA); restriction enzymes *Eco*RI, *Not*I and DNA marker (Fermentas, USA); protein markers (Novagen, Netherlands; Sigma-Aldrich, USA, iNtRON Biotechnology, Korea; Affymetrix, USA); trypsin, α-chymotrypsin, thermolysin, BAPNA, sepharose 4B, Coomassie® Brilliant Blue R250, (Sigma-Aldrich); restriction enzymes); methanol; $(NH_4)_2SO_4$; chemicals for SDS-PAGE electrophoresis (Merck); dialysis bag (Thermofishe); filter membrane cut off 30 kDa (Microcon-30kDa Centrifugal Filter Unit).

Methods

Construction of recombinant plasmid pPIC9 carrying PI-QT gene

Plasmid pUC57 carrying *PI-QT* gene and plasmid pPIC9 both were cut by restriction enzymes *Eco*RI and *Not*I, then *PI-QT* gene was ligated into opening pPIC9 by T4 ligase (Thermofisher). Recombinant pPIC9 plasmid clones containing the target gene were re-examined by restriction reaction with *Eco*RI and *Not*I and sequencing.

Construction of recombinant Pichia pastoris SMD1168 harboring PI-QT gene

Construction of recombinant *P. pastoris* carrying recombinant pPIC9/*PI-QT* was performed according to the manufacture's instruction (*Pichia* expression kit- Invitrogen). In brief: recombinant pPIC9/*PI-QT* was electrical transformed into *P. pastoris* SMD1168 and recombinant yeast clone carrying target gene was screened by culturing transformed *P. pastoris* SMD1168 on MD medium (YNB 1.34%, biotin 4×10^{-5}%, dextrose 2%) without histidine as a selective factor.

Checking the genotype and phenotype of the transformed clones

The procedure was performed according to the manufacture's instruction (*Pichia* expression kit- Invitrogen). For genotype examination: total DNA of transformed yeast clones was extracted and PCR was carried out using AOX1 primers (5´AOX1: 5'-GACTGGTTCCAATTGACAA GC-3´; 3´AOX1: 5´-GCAAATGGCATT CTGACATCC-3´) (EasySelect™ Pichia Expression Kit of Invitrogen, USA). For phenotype checking: the transformed yeast clones were cultured on 2 media MD (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose) and MM (1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol) (EasySelect™ Pichia Expression Kit of Invitrogen, USA), incubated at 25 ^{o}C for 3−4 days and observed the growth of the colonies.

Expression of P. pastoris SMD1168/pPIC9/PI-QT and determination of the most appropriate expression conditions

The expression of recombinant *P. pastoris* SMD1168 was carried out according to the manufacture's instruction (*Pichia* expression kit-Invitrogen). Detection of the target protein using PAGE method.

PAGE method supplemented with casein 0.1%

Protease inhibitor detection: SDS-PAGE 12.6% with casein (0.1%) was performed, and at the end of the electrophoresis, the gel was treated with trypsin. If the protein was protease inhibitor, the band on the gel will return dark on a lighter gel base. This is a sensitive method, allowing direct detection of protease inhibitors with even though the presence of very small quantities of crude extract (< 20 µL) without the need for purification (Hoang Thu Ha & Pham Thi Tran Chau, 2008).

Affinity chromatography (Trypsin-Sepharore 4B column)

After 72 hours expression, recombinant protein in the culture medium was

precipitated with salt $(NH_4)_2SO_4$ and then the salt was removed by dialysis at 4 ^oC for 24 hours. Next step, the protein solution was subjected through a gel filtration chromatography (Sephacryl-S200 column). The sample was withdrawn by the FPLC automatic system at a flow rate of 0.5 mL/min, with Tris/HCl buffer 50 mM, pH 7.0; supplemented with sodium azide (NaN_3) 0.2%, and 2 mL of each fraction was collected. The presence of the target protein in the fractions was examined by SDS-PAGE electrophoresis and then the fractions containing the proteins of interest were combined and subjected through a 30 kDa cut-off filter (Microcon - 30 kDa Centrifugal Filter Unit). The protein fraction > 30 kDa was collected and subjected through the affinity chromatography (Trypsin-sepharose 4B column, Sigma) equilibrated with buffer A (Tris-HCl 20 mM, pH 7.5; KCl 50 mM) containing 2 mM $CaCl_2$. The non-absorbing and nonspecific binding proteins were withdrawn with 1 M NaCl solution in the same buffer A and a column-specific binding protein fraction was withdrawn with 0.001 M HCl solution containing 2 mM $CaCl_2$. The obtained protein was concentrated using a 30 kDa cut off filter (Phan Tuan Nghia & Dang Quang Hung, 2008; Sapna, 2013).

Determination of protease inhibitor activity of the recombinant PI-QT protein on agar plates

An agar plate with skimmed milk was prepared (0.8% agar, 1% Skimmed milk), some wells (1 cm diameter) were punched on the plate, one in the middle and the other around, each 1 cm apart. Add 20 µL protease (trypsin, α-chymotrypsin, or thermolysin, 0.5 mg/mL) into the middle well. The recombinant PI-QT protein was instilled in one of the adjacent wells and sterile water is added to the other. The agar plate was then incubated at 37 ^oC overnight (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 pH to 7.5, BAPNA (N-α-benzoyl-DL-arginine-p-nitroanilide) was added into the mixture and incubated at 37 ^oC for 20 mins. Next, 5% TCA was added into the solution and incubated at room temperature for 20 mins. 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 using following formula:

$$\text{Inhibiting activity (\%)} = \text{C-T/C} \times 100$$

Where: C - absorbance value of blank sample at 280 nm and T - absorbance value of samples with protease inhibitor at 280 nm (Karthik et al., 2014).

Effect of temperature and pH on inhibitor stability (Sapna, 2013)

The stability of the purified PI-QT was determined from 4 ^oC to 100 ^oC for 60 minutes. After incubation of PI-QT at desired temperature, the sample was incubated at 4 ^oC for 15 minutes before determination of amount and inhibitor activity of the protein.

The stability of the purified PI-QT was analyzed from pH 3−12 for 10 minutes and then pH was adjusted to 8 before determination of amount and inhibitor activity of the protein.

Determination of Protein concentration**:** by Bradford method (Sapna, 2013).

RESULTS AND DISCUSSION

Construction of recombinant vector pPIC9/*PI-QT*

The *PI-QT* gene was cut from plasmid pUC57/*PI-QT* using restriction enzymes *Eco*RI and *Not*I. The vector pPIC9 was also digested with *Eco*RI and *Not*I. The *PI-QT* gene was inserted into vector pPIC9 using T4 ligase, then transformed into competent cells *E.coli* Top10F' and the recombinant colonies were screened on LB medium containing 50 µg/mL ampicilin, supplementing with X-gal and IPTG. Some white colonies were randomly selected for checking the presence

of recombinant pPIC9/*PI-QT* by PCR using AOX1 primers. Transformed clones supposed to contain recombinant pPIC9/*PI-QT* (one band appeared on agarose gel about 1779 bp Figure 1b were used for recombinant plasmid isolation according to GeneJET Plasmid Miniprep Kit (Thermo) instruction. The isolated plasmid was digested with *Eco*RI and *Not*I, then subjected to electrophoresis. Two bands were visible on the agarose gel, one corresponding to pPIC9 vector (about 8 kb) and other to *PI-QT* gene (about 1.3 kb) (Fig. 1a). The sequencing result of pPIC9/*PI-QT* using AOX1 primers confirmed the success of the construction of recombinant pPIC9/*PI-QT*.

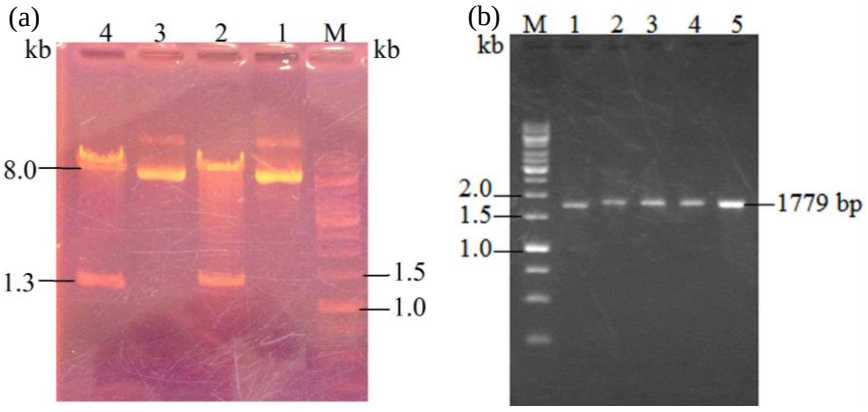

Figure 1. Recombinant plasmid pPIC9/*PI-QT* after digestion with restriction enzymes (a) and PCR product of recombinant *E. coli* using AOX1 primers (b). (a): Lanes 1 and 3- Plasmids isolated from white colonies of *E. coli* cutting with *Eco*RI and *Not*I. Lanes 2 & 4- Plasmids isolated from green colonies of *E.coli* cutting with *Eco*RI and *Not*I; (b): PCR products using AOX1 primers and total DNA from white colonies of *E. coli* as templates; M: DNA marker 1 kb (Thermo)

Expression of pPIC9/*PI-QT* in *P. Pastoris*

Recombinant vector pPIC9/*PI-QT* was transformed into competent cells *P. pastoris* SMD1168 by electroporation, then transformed yeast was spread on MD medium, cultured at 30 ^{o}C for 48 hours. Phenotype and genotype of the colonies were determined according to *Pichia* expression kit (Invitrogen) recommendation. The transformed yeast grew well on MD medium and poorly on MM medium, and this indicated that recombinant SMD1168 was Mut^{s} type. PCR product using AOX1 primers and total DNA of transformed SMD1168 confirmed the phenotype and genotype of recombinant SMD1168 by one band approximately 1779 bp on the gel Figure 2. Expression of PI-QT gene in the positive recombinant clone SMD1168 was carried out According to the manufacture's instruction for Muts type P. pastoris (Pichia expression kit, Invitrogen). The obtained result showed that for 24 hours, 48 hours, 72 hours and 96 hours of culture, a band about 50 kDa, corresponding to the molecular weight of PI-QT protein, appeared on the gel and the amount of the protein gradually increased by time (Fig. 3a, lanes 2−5). In contrast, this protein was not detectable in the culture of the yeast containing the parent vector pPIC9 (Fig. 3a, lane 1).

Protease inhibitor activity of crude extracts from SMD1168 clones harboring pPIC9/PI-QT was detected 24 hours after induction and increased steadily up to 88.7% (for trypsin), 69% (for α-chymotrypsin) and 43% (for thermolysin) after 72 hours induction and gradually decrease by 96 hours and 120 hours (Fig. 3b).

The crude extract of SMD1168 clone with parent vector pPIC9 did not inhibit these proteases (data not shown).

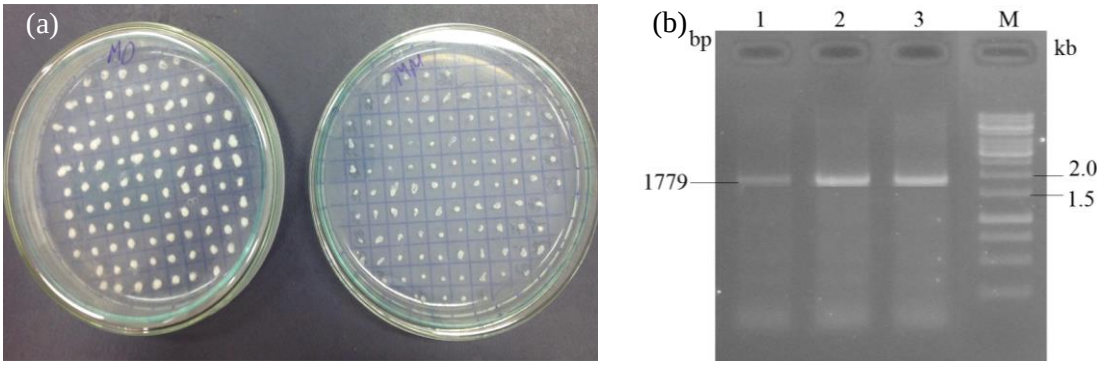

Figure 2. PCR products for confirmation Mut^s phenotype (a) and genotype of recombinant SMD1168 (b). M: DNA marker 1 kb (Thermo); Lanes 1, 2, 3: PCR products using AOX1 primers

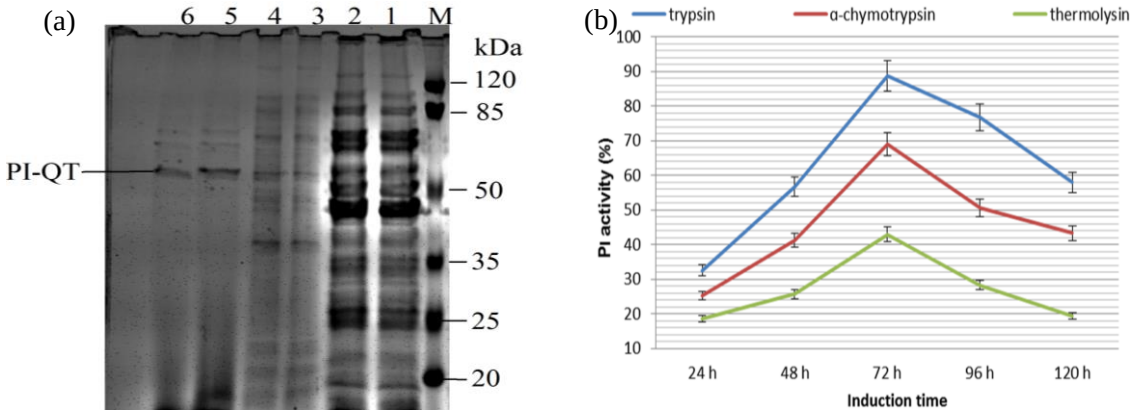

Figure 3. PI-QT gene expression in SMD1168 (a) and protease inhibitor activity (b). M: Protein marker (Bio-basic). Lane 1: SMD1168 carrying pPIC9. Lane 2: SMD1168 carrying pPIC9/*PI-QT* pre-induction. Lane 3: SMD1168 carrying pPIC9/*PI-QT* after 24 hours induction. Lane 4: SMD1168 carrying pPIC9/*PI-QT* after 48 hours induction. Lane 5: SMD1168 carrying pPIC9/*PI-QT* after 72 hours induction. Lane 6: SMD1168 carrying pPIC9/*PI-QT* after 96 hours induction

Purification of the recombinant PI-QT protein by Trypsin-Sepharore 4B affinity chromatography

Using PAGE method supplemented with casein 0.1% for detection of the recombinant protein. Figure 4 showed that the target protein was presented in the extract of SMD1168 clone with pPIC9/*PI-QT* vector due to a band of 50 kDa, similar to theoretical MW of PI-QT. But the extract from SMD1168 clone with pPIC9 vector produced no band on the PAGE gel Figure 4a.

The crude extract was precipitated with $(NH_4)_2SO_4$ and the precipitate after dialysis for removing the salt was subjected to filter membrane cut off 30 kDa, collect protein fraction > 30 kDa. SDS-PAGE 12.6% gel showed that proteins with MW < 30 kDa were extruded completely and proteins with MW of 50 kDa were recovered quite a lot (Fig. 4b, lane 1). Then recovered proteins were used for further purification by trypsin-sepharose affinity chromatography. SDS-PAGE indicated a single polypeptide at 50 kDa, likewise theoretical MW of PI-QT protein (Fig. 4c, lane 1). The purified protein was assessed for inhibitor activity against trypsin, α-chymotrypsin and thermolysin. The

obtained result documented inhibitor activity of the protein against 3 investigated proteases (Fig. 5). So, PI-QT protein expressed in *P. pastoris* SMD1168 was successfully purified.

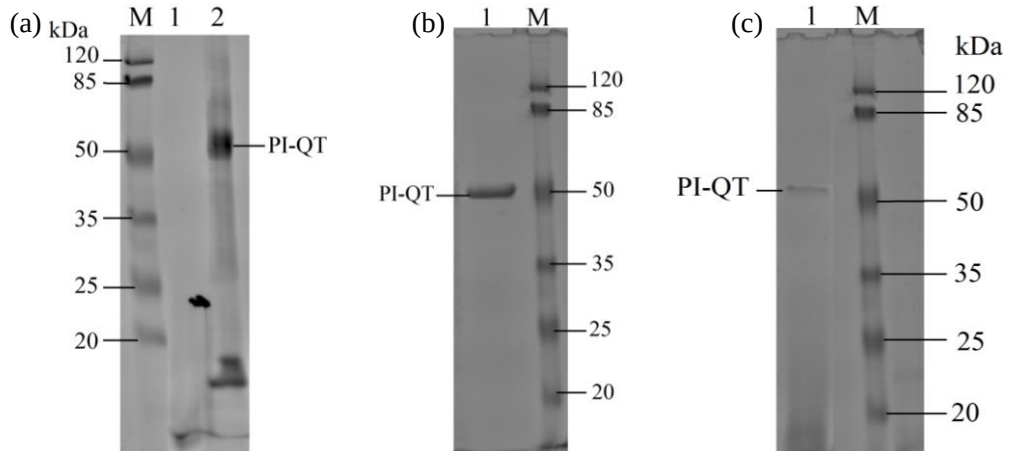

Figure 4. Detection of the target protein using PAGE method supplemented with casein 0.1% (a); Purified recombinant PI-QT protein (b, lane 1) and affinity chromatography (c, lane 1) M (a, b, c): Protein marker (Bio-basic). (a): Lane 1. Crude extract of SMD1168 clone harboring pPIC9 vector after 72 hours induction; Lane 2. Crude extract of SMD1168 clone harboring pPIC9/*PI-QT* vector after 72 hours induction

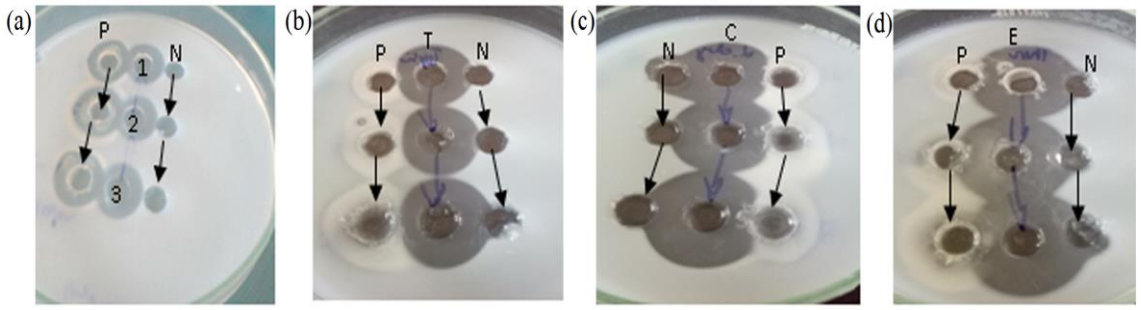

Figure 5. Inhibitor activity test of protein of SMD1168/pPIC9 (a) and purified recombinant PI-QT protein (b, c, d). (a): N- water, 1- trypsin, 2- α-chymotrypsin, 3- thermolysin, P- protein of SMD1168/pPIC9; (b), (c), (d): N- water, P- crude extract, T- trypsin, C- α-chymotrypsin, E- thermolysin

Characterization of the inhibitor

Effect of pH on inhibitor stability

PI-QT protein had stability over a wide range of pH as presented in Figure 6. The inhibitor activity was stable and reached a maximum in the pH range of 7–9, drastically decreases under high alkaline (pH > 10) and high acidic conditions (pH 3–4). Several reports have also shown that the inhibitor is maximally stable at pH 7, for example, inhibitor produced from the clone obtained from marine metagenome (Jiang et al., 2017), from soil streptomyce (Pandhare et al., 2002). According to Sapna (2013), under high alkaline (pH 11–12) and high acid (pH 2–3) conditions, the protease inhibitor is unstable (Sapna, 2013). The serine protease inhibitor has been reported to have high pH stability in the range of pH 2–12 and thermal stability (Tsybina et al., 2004).

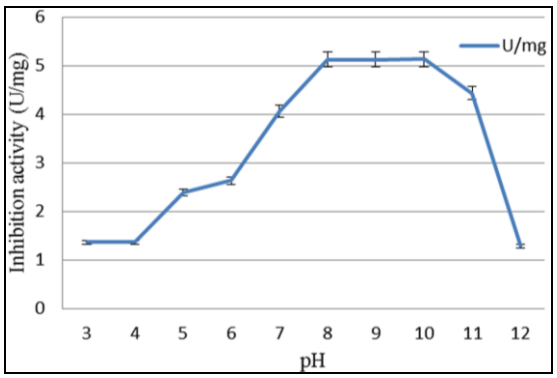

Figure 6. Effect of pH on inhibitor stability

Effect of temperature on inhibitor stability

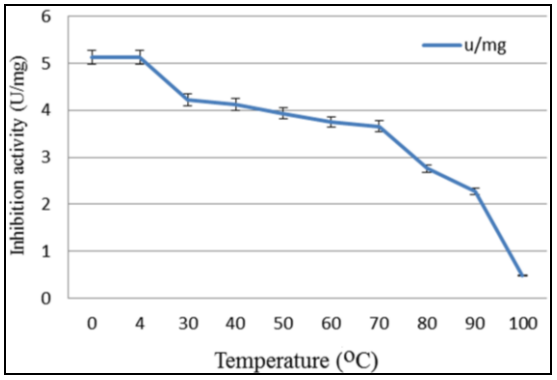

Figure 7. Effect of temperature on inhibitor stability

Thermostability of protease inhibitor PI-QT is presented in Figure 7. The inhibitor activity of PI-QT was stable at a temperature under 60 °C and above that temperature, both protein concentration and activity decreased sharply. The activity was lost after preincubation at 100 °C for 1 hour and achieved maximum at 4 °C and 60 °C, which shows similarity to the protease inhibitor in the report of Sapna (2013). Protease inhibitor isolated from *Hyptis suaveolens* L. seeds was stable over a wide temperature range, from 4–95 °C (Aguirre et al., 2004). Protease inhibitors from different sources exhibit different degrees of thermal and pH stability. Optimal activity of PI isolated from *Pseudomonas* sp. AU10 was at pH 8.0 and 40 °C (Fullana et al., 2017). Proteinaceous protease inhibitor from marine *Pseudomonas mendocina* was stable and active at pH 4–12 and also at 4–90 °C for 1 hour (Sapna et al., 2017). The majority of the protease inhibitors exhibiting high degree of thermal stability and PI-QT inhibitor confirmed this opinion.

Many PIs have been purified from various sources (plant, animal, and microorganisms) and act as natural antagonists of proteolytic enzymes. Although many drugs that can inhibit protease mechanisms are already available, researchers are continuing to find new kinds of protease inhibitors, natural or recombinant, with more effective application in medicine and other biotechnology fields. Numerous studies of protease inhibitors originating from bacteria were performed and documented their different biological activities such as antimalaria (Karthik et al., 2014), antiviral activity against Coxsackievirus B3 infection (El-Hadedy et al., 2015) and anticancer activity on *in-vitro* Hela and HepG2 cancer cell lines (Venkatachalam & Nadumane, 2019). But few studies are focusing on heterologous expression PIs, especially the exploration of novel PIs from uncultured microorganisms via genetic biotechnology.

CONCLUSION

The *PI-QT* gene (encoding for protease inhibitor protein) screening from the metagenome library of the bacteria associated marine sponge *Spheciospongia vesparium* (Quang Tri, Vietnam) was successful expressed in *P. pastoris* SMD1168. The level of extracellular expression of PI-QT protein is quite high. Purified recombinant PI-QT protein exhibited inhibitor activity against trypsin, α-chymotrypsin and thermolysin and the inhibition were 88.7%, 69% and 43%, respectively, after 72 hours induction with methanol. Recombinant PI-QT compound had optimal activity at 4 °C and 60 °C, pH 7–9. With the above properties, the recombinant PI-QT protease inhibitor has a high potential for application in different biotechnology fields.

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