

Screening and Characterization of Protease Inhibitors from Marine Bacteria Associated with Sponge *Jaspis* sp.

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Three isolates among 138 sponge-associated bacteria were isolated from Waigeo Island, Raja Ampat West Papua Province, Indonesia, have been shown protease inhibitory activity against subtilisin (serine protease), thermolysin (metalloprotease), and crude extract from pathogenic bacteria (*Escherichia coli enteropathogenic*/EPEC K.1.1, *Staphylococcus aureus*, and *Pseudomonas aeruginosa*). Those three isolates were designated as sponge associated bacteria SAB S-12, SAB S-21, and SAB S-17. A simple casein and Sea Water Complete (SWC) double layer agar method was used to screen the bacteria against pathogenic bacteria producing protease, i.e. EPEC K.1.1, *S. aureus*, and *P. aeruginosa*. Among them, SAB S-12 isolate showed no inhibitory zone indicated. The isolate had the highest inhibitory activity against subtilisin and crude extract enzyme of pathogenic bacteria, the inhibitory activity was 91.6 and 98.9%, respectively. In addition, the SAB S-21 isolate had the highest inhibitory activity against thermolysin, it was 70.4%. The optimum pH and temperature for protease inhibition of the three isolates was at pH 7.0-8.0 and 40-50 °C respectively. Based on 16S rRNA gene sequence, the closest related with SAB S-12, SAB-17, and SAB-21 isolates was *Providencia* sp. (92% identity), *Paracoccus* sp. (86% identity), and *Bacillus* sp. (100% identity), respectively.

Key words: sponge-associated bacteria, protease inhibitor, pathogenic bacteria, screening, 16S rRNA

INTRODUCTION

Enzyme inhibitors have been pay attention as useful tools, not only for the study of enzyme structures and reaction mechanisms but also for potential utilization in pharmacology (Bode & Huber 1992) and agriculture (Terashita *et al.* 1980). It is useful in the diagnosis and therapy of inflammation and pancreatitis (Delston *et al.* 2003). It is also useful for fruit-body formation of mushroom cultivation (Astuti *et al.* 2002). Specific and selective protease inhibitors are potentially powerful tools for inactivating target proteases in the pathogenic processes of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular dystrophy, cancer, and AIDS (Demuth 1990).

For the last 10 years, attention on protease as the drugs target has increased slightly due to their role on illness mechanism of virus, such as influenza and HIV, malaria, cancer, and even degenerative illnesses, such as Alzheimer. Although many drugs that can inhibit protease mechanisms are already available, researches has being done to find natural protease inhibitor from many kinds of source, such as virus, bacteria, fungi, and also other organisms, such as sponges and tunicate (Imada 2004). Mayer and Lehmann (2000) reported that among sea organisms, sponge was the most potential producer of bioactive agents including enzyme inhibitor components

(Lee *et al.* 2001). Sponge-associated bacteria are also produce bioactive components (Webster *et al.* 2001). Bacterial and cyanobacterial symbions of sponge, especially *Aplysina aerophobia*, could reach up to 40% of total sponge biomass (Ahn *et al.* 2003). The symbiotic association with the sponge can be either intranucleus symbiotic, intracellular symbiotic, or extracellularr exosymbiotic. This symbiotic can happen due to sponge as a filter feeder organism. Lee *et al.* (2001) reported that bioactive components of the sponge originally come from biosynthetic products of its symbions. And bacteria was found the most in sponge, it was more than 3×10^4 CFU/g (Nurhayati *et al.* 2006).

Protease inhibitors can keep a protease from working and splitting a protein into peptides, and It can blocked protease enzyme activity. However, there is a few number protease inhibitor had been found from marine environment, especially from sponge-associated bacteria. Therefore, this study was to screen bacteria producing protease inhibitor isolated from sponge *Jaspis* sp. and to analyze inhibitory activity against subtilisin, thermolysin, and crude extract from pathogenic bacteria.

MATERIALS AND METHODS

Bacterial Strains, Protease Substrates, and Growth Media. 138 bacteria isolated from *Jaspis* sp. were used in this study. Pathogenic bacteria, such as Enteropathogenic *Escherichia coli* (EPEC) K.1.1 (obtained from Dr. Sri Budiarti), *Pseudomonas aeruginosa*, and *Staphylococcus*

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aureus (obtained from PPSHB, IPB) were also used throughout this research, and routinely maintained in Luria Broth (LB; Oxoid). The following proteases were used in this research, Subtilisin from *Bacillus licheniformis* (SIGMA) and Thermolysin from *Bacillus thermoproteolyticus rokko* (SIGMA).

Screening of Sponge-Associated Bacteria Producing Protease Inhibitor. As many as 138 bacteria isolated from *Jaspis* sp. were determined their inhibitory protease activity using double layer medium of Imada's (1985a) method. This method used sea water complete (SWC) as the lower layer medium and mixture of NA and SM medium as the upper layer medium, and the bacterial isolates were applied on the lower layer of medium and incubated for 24 hours at 37 °C. After 24 hours of incubation, bacterial colony was removed, then the medium was filled up with the upper layer medium. The pathogenic bacteria was subsequently inoculated on the upper layer medium and incubated for 24 hours at 37 °C. Inhibitory protease activity was indicated by no or reducing of proteolytic zone around the bacterial colony.

Protease Activity Assay. Three of pathogenic bacterial strains i.e. Enteropathogenic *Escherichia coli* (EPEC) K.1.1, *P. aeruginosa*, and *S. aureus* were inoculated on LB medium and pre-cultured at 37 °C on a 100 rpm rotary shaker until the optical density (OD) of 0.8 was achieved. Then, 10% (v/v) of the pre-culture was inoculated in 150 ml of the same medium and incubated for 56 hours at 37 °C. The culture was harvested by centrifugation at 3000 rpm and the supernatant was used for the assay of inhibitory activity. Protease activity was measured by Walter's method (1984) with slight modification.

Protease Inhibition Assay. Inhibition activity was measured using method according to Imada *et al.* (1985b) with slight modification. Twenty-five µl of subtilisin, thermolysin, and crude extract of pathogenic bacteria (EPEC K.1.1, *S. aureus*, and *P. aeruginosa*) in 0.5 ml of 30 mM Tris-HCl buffer pH 8.0, was pre-incubated with 0.5 ml of culture supernatant for 12 minutes at 30 °C. The substrate which used were subtilisin (91 mg protein/ml) as one of the serine protease group, thermolysin (42 units/mg solid; 64 units/mg protein) as one of the metalloprotease group, and EPEC K.1.1, *P. aeruginosa*, and *S. aureus* which were produced based on Baehaki (2004). EPEC K.1.1 (0.013 U/ml) for isolate SAB S-12, *S. aureus* (0.04 U/ml) for isolate SAB S-21, and *P. aeruginosa* (0.25 U/ml) for isolate SAB S-17.

One ml of 1% (w/v) Hammarsten casein (MERCK) in the same buffer was added to pre-incubated mixture. After incubation of the mixture for 12 minutes at 30 °C, 2 ml of 5% (w/v) trichloroacetic acid (TCA) was added to terminate the enzyme reaction and mixture was kept for 20 minutes at 30 °C. After that, the solutions were centrifuged at 3000 rpm for 10 minutes. The optical density of filtrate was measured by turbidimetric measurement at 280 nm in a GeneQuant pro 81670 type spectrophotometer (Cambridge Ltd, England). Simultaneously, the optical density of the blank and control (without inhibitor solution) were also determined. The percentage of inhibition was calculated as $= (1-B/A) \times 100\%$, wherein A was the

increased optical density without the inhibitor after 12 minutes reaction, and B was the increased optical density with the inhibitor after 12 minutes. One unit of inhibitor activity was defined as the amount by which enzyme activity was reduced by 50% under the above condition.

Characterization of Inhibitor Protease on Various Temperatures and pH. The culture supernatant solution containing protease inhibitor was adjusted to various temperatures from 10 to 90 °C for 10 minutes and the residual inhibitory activity was measured. The solution containing culture supernatant was adjusted to various pH levels from 3 to 9 for 10 minutes and incubated for 10 minutes at each optimum temperature. After incubation, pH was readjusted to assay pH (8.0) and the residual inhibitory activity was determined. Citrate buffer and Tris-(hydroxymethyl)-aminomethane (Tris) buffer were used as the buffer solutions (0.03 M).

Characterization and Identification of Bacteria Producing Protease Inhibitor. Characterization morphologically for potential bacteria which were extracted from *Jaspis* sp. producing protease inhibitor was done by Gram staining (Cappucino & Sherman 1983). The shape of the bacteria was observed by microscope (Olympus Type CH20BIMF200) in 1000 magnification. Molecular identification of three strains of bacteria producing inhibitor protease were identified based on 16S rRNA gene sequence, by three steps, (i) isolation of the DNA (Sambrook & Russell 2001), (ii) amplification of 16S rRNA gene by PCR using amplification machine (Gene Amp PCR system 2400 Perkin Elmer), and (iii) sequencing of the 16S rRNA gene. The primer used to amplify the 16S rRNA were forward primer 63f (5'-CAG GCC TAA CAC ATG CAA GTC) and 1387r (5'-GGG CCG WGT GTA CAA GGC) (Marchesi *et al.* 1998) using method described by Bahri *et al.* (2009). The DNA fragments were sequenced by PT Charoen Pokphan Indonesia Jakarta. A comparison between the 16S rRNA gene sequence of sponge-associated bacteria producing protease inhibitor to that of 16S rRNA gene data found in the GenBank database was done by connecting to the BLASTN (basic local alignment search tool for nucleotide) program at <http://www.ncbi.nlm.nih.gov>. By that software, the sequencing of the DNA would be gotten in high homology with the DNA sequence of SAB S-12. All DNA sequences alignment was arranged in FASTA3 format and then similarity analysis was done using ClustalW program from the website www.ebi.ac.uk/clustalW. The result was saved on TreeConW software, and by using this software, the phylogenetic tree was made in Phylip format with bootstrap replication 100X. In the making of phylogenetic tree, *Alteromonas* (FN661963.1), *Passiflora rubra* (AY261639.1), *P. piscicida* (X82215.1), *Idiomarina zobellii* (GQ131629.1), *Pseudoalteromonas bacteriolytica* (AF173962.1), and *Algalicola sagamiensis* (AB063324.1) were used as the reference strains.

RESULTS

Selected Sponge-Associated Bacteria Producing Protease Inhibitor. Among 138 bacterial isolates which

were isolated from marine sponge *Jaspis* sp., three strains produced protease inhibitor, they were designated as SAB S-12, SAB S-21, and SAB S-17, respectively. Those isolates were able to reduce or inhibit production of protease produced by pathogenic bacteria (EPEC K.1.1, *P. aeruginosa*, and *S. aureus*) indicated by no proteolytic zone or reducing of proteolytic zone surrounding bacterial colony. SAB S-12 showed the highest capability to produce protease inhibitor, as there was no proteolytic zone around its growth zone, while both SAB S-21 and SAB S-17 showed capability to produce protease inhibitor, but their capability was less than SAB S-12 (Figure 1).

Protease Activity. The period of the optimum protease production of EPEC K.1.1, *S. aureus*, and *P. aeruginosa* were gained after 24, 16, and 40 hours of incubation, respectively. The highest protease activity of EPEC K.1.1 was 0.01 U/ml at 16 hours. Its lag phase was within 8 hours, followed by an exponential phase up to 32 hours with maximum OD of 1.86. This result indicated that EPEC K.1.1 had optimum protease production period at 24 hours, where the protease activity on this time was the highest (0.01 U/ml), therefore the harvest time of EPEC K.1.1 was 24 hours. Protease was produced along with the cell growth and it reached the highest protease activity on its nearly stationer phase. The highest protease activity of *S. aureus* was 0.04 U/ml at 16 hours. The lag phase of *S. aureus* was within 8 hours, followed by an exponential phase up to 40 hours within maximum OD₆₀₀ of 1.97. The highest protease activity of *P. aeruginosa* was 0.25 U/ml at 32 hours. Its lag phase was within 16 hours, followed by an exponential phase up to 32 hours with maximum OD₆₀₀ of 1.98.

Protease Inhibition Activity. The highest inhibitory activity of isolates SAB S-12, SAB S-21, and SAB S-17 in subtilisin substrate were 62.06% at 28 hours, 27.58% at 28 hours, and 20.68% at 28 hours, respectively. In addition, the highest inhibitory activity of isolate SAB S-12, SAB S-21, and SAB S-17 in thermolysin substrate were 6.25% at 8 hours, 14.58% at 28 hours, and 12.50% at 28 hours, respectively. This result means that the optimum protease inhibitor activity of SAB S-12, SAB S-21, and SAB S-17 in subtilisin substrate were at 28 hours of cultivation, while SAB S-12 was at 8 hours of cultivation in thermolysin substrate. The highest inhibitory activity of isolate SAB S-12 in EPEC K.1.1's enzyme extract substrate was 98.84% at 24 hours cultivation. The highest inhibitory activity of isolate SAB S-21 in *S. aureus*'s enzyme extract substrate was 51.29% at 24 hours cultivation. The highest inhibitory activity of isolate SAB S-17 in *P. aeruginosa*'s enzyme extract was 23.52% at 20 hours cultivation. The results showed that SAB S-12, SAB S-21, and SAB S-17 needed different time to reach protease inhibition capability. Each isolates reach its optimum condition after that their protease inhibition decreased slightly.

Characteristics of Inhibitor Protease. The highest inhibitory activity of isolate SAB S-12, SAB S-21, and SAB S-17 in subtilisin substrate were 88.45% at 40 °C, 44.67% at 50 °C, and 21.53% at 40 °C, respectively. The highest inhibitory activity of isolate SAB S-12, SAB S-21, and SAB S-17 in thermolysin substrate were 59.47% at 40 °C, 47.52% at 40 °C, and 50.52% at 40 °C, respectively. This result indicated that the optimum protease inhibitor activity of SAB S-12, SAB S-21, and SAB S-17 in subtilisin substrate were 40, 50, and 40 °C, while for the protease

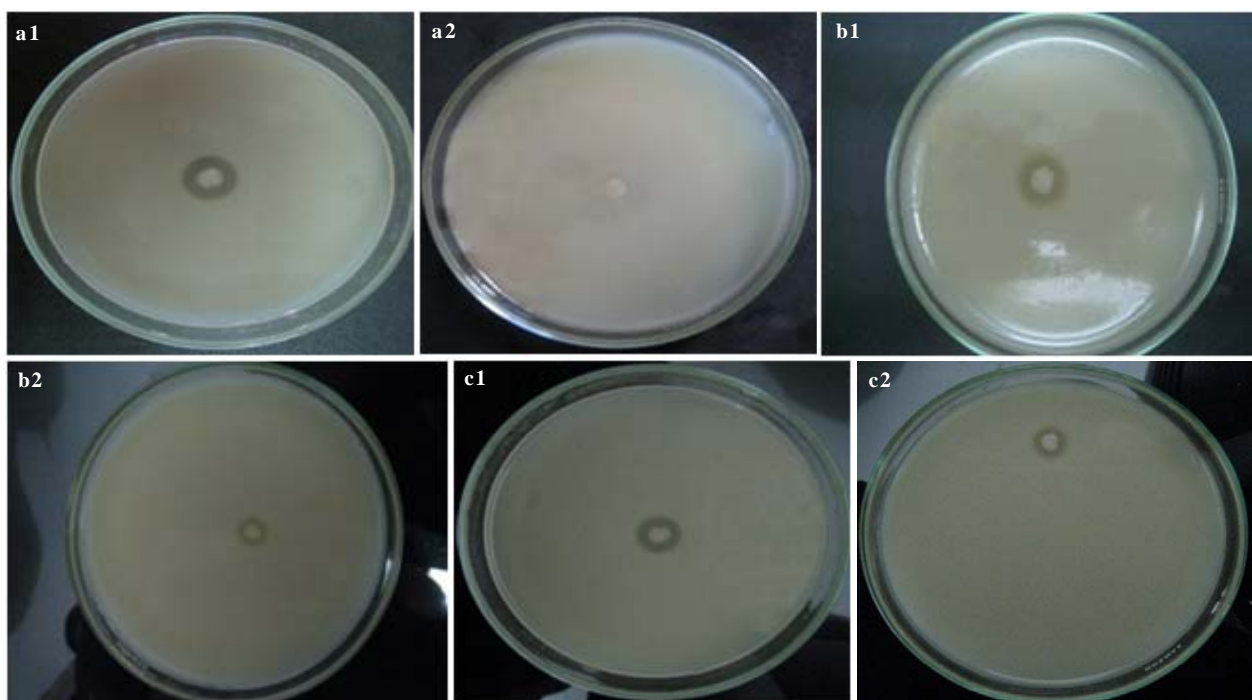


Figure 1. Comparison between (a1) control (EPEC K.1.1) and (a2) application of SAB S-12 against EPEC K.1.1, (b1) control (*S. aureus*) and (b2) application of SAB S-21 against *S. aureus*, (c1) control (*P.aeruginosa*) and (c2) application of SAB S-17 on *P. aeruginosa* (scale of picture 1:8).

inhibitor activity of the three of isolates mentioned above in thermolysin substrate were 40 °C (Figure 2). Furthermore, the highest inhibitory activity of isolate SAB S-12, SAB S-21, and SAB S-17 in thermolysin substrate was 50.43% at pH 8.0, 70.37% at pH 8.0, and 49.89% at pH 8.0 condition, respectively. The highest inhibitory activity of isolate SAB S-12, SAB S-21, and SAB S-17 in subtilisin substrate was 91.57% at pH 7.0, 57.23% at pH 8.0 condition, and 30.78% at pH 7.5, respectively (Figure 3).

Identification of Bacteria Producing Protease Inhibitors. The result of PCR of 16S rRNA genes of isolates SAB S-12, SAB S-17, and SAB S-21 were successfully amplified, i.e. 1300 bp in size (data not shown). Sequence analysis of the 16S rRNA gene sequence of three bacterial isolates producing protease inhibitor are shown in Table 1. SAB S-12 was identified as *Providencia* sp. (92% identity), SAB S-17 was identified as *Paracoccus* (86% identity), and SAB S-21 was identified as *Bacillus* sp. (100% identity). The phylogenetic tree of of three isolates, SAB S-12, SAB S-21, and SAB S-17 are shown in Figure 4.

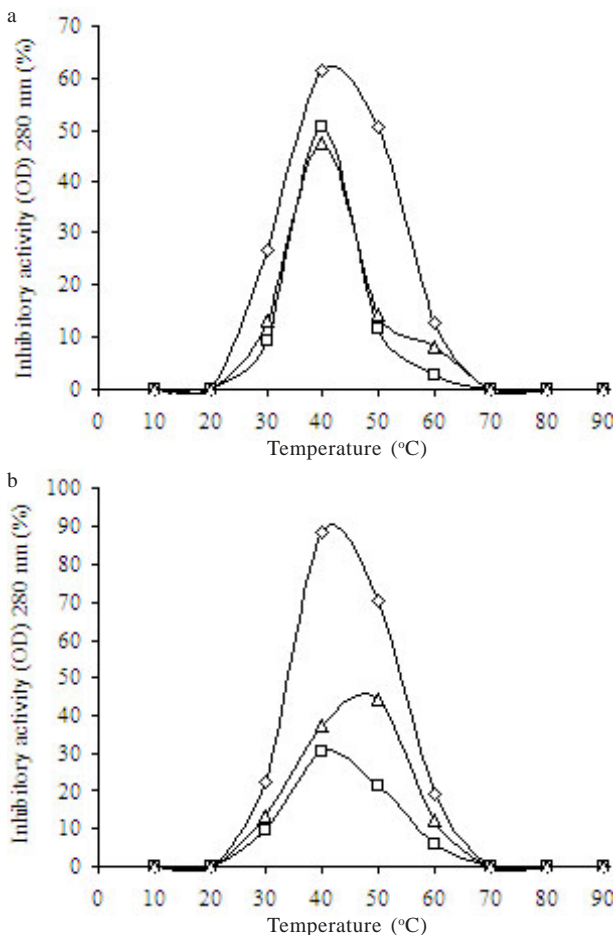


Figure 2. Inhibitory activity of SAB S-12, SAB S-21, and SAB S-17 against various temperature in (a) thermolysin substrate, (b) subtilisin substrate. ◇: isolate SAB S-12, □: isolate SAB S-21, △: isolate SAB S-17.

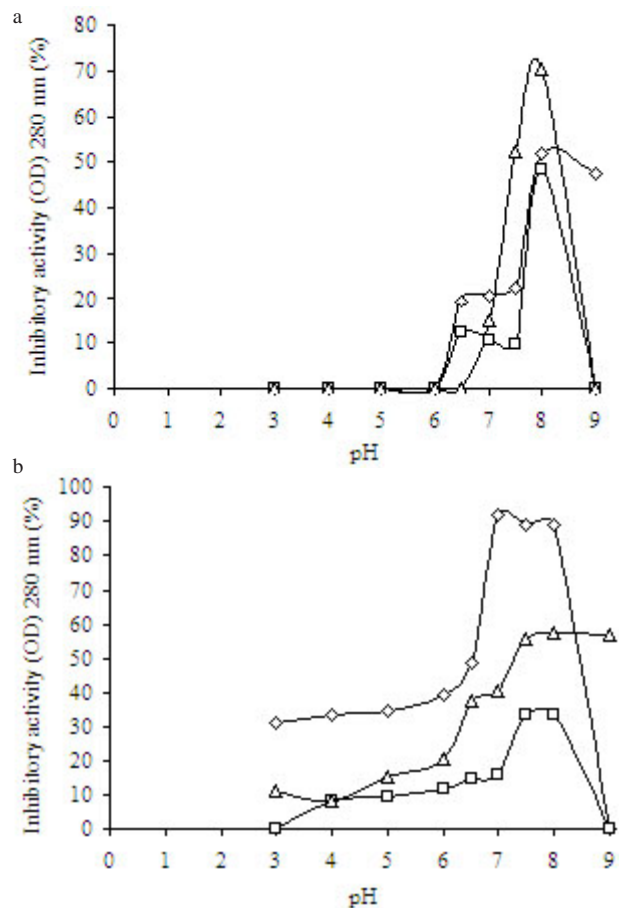


Figure 3. Inhibitory activity of SAB S-12, SAB S-21, and SAB S-17 against various pH levels in (a) thermolysin substrate, (b) subtilisin substrate. ◇: isolate SAB S-12, □: isolate SAB S-21, △: isolate SAB S-17.

Table 1. Homologous search analysis of the 16S rRNA gene sequence of three bacterial isolates producing protease inhibitor isolated from sponge *Jaspis* sp.

Isolate code	Homology	Identity (%)	Access No.
SAB S-12	<i>Providencia</i> sp.	92	AY870456.1
SAB S-17	<i>Paracoccus</i> sp.	86	GU338411.1
SAB S-21	<i>Bacillus</i> sp.	100	FN556567.1

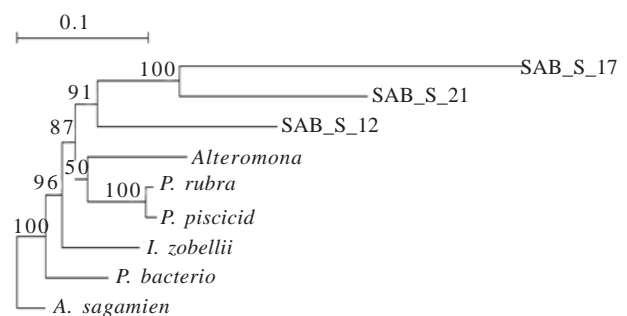


Figure 4. Phylogenetic tree showing position of isolates of SAB S-12, SAB S-21, and SAB S-17 among protease inhibitors-producing bacteria.

DISCUSSION

Three bacterial isolates of SAB S-12, SAB S-17, and SAB S-21 were isolated from marine sponge *Jaspis* sp. showed protease inhibitors activity. Protease inhibitor was produced by SAB S-12 could inhibit protease larger than SAB S-21 and SAB S-17. The preliminary protease inhibitor activity was determined from the disappearance of proteolytic zone of the isolates on the screening medium. Based on screening test results, SAB S-12 showed no inhibitory zone, while SAB S-21 and SAB S-17 showed proteolytic zone, which were 0.1 and 0.1 cm, respectively. The three strains mentioned above also inhibit the serine protease (subtilisin), metalloprotease (thermolysin), and crude extract from pathogenic bacteria (EPEC K.1.1, *S. aureus*, and *P. aeruginosa*). Isolate SAB S-12 had the highest activity in inhibiting subtilisin (91.57%) and crude extract from the pathogenic bacteria (98.84%). Meanwhile, isolate SAB S-21 has the metalloprotease (thermolysin) inhibitory activity at roughly 70.370%.

The similar study was conducted by Nurhayati *et al.* (2006) reported that a bacterium designed as isolate 6A3 (identified as *Chromohalobacter* sp.) isolated from sponge *X. testudinaria* from Kepulauan Seribu, produced protease inhibitor activity (93.5% activity) against protease produce by *P. aeruginosa*). However, they did not classify a protease inhibitor molecule based on their study. Their finding was really different with our results revealing that isolate SAB S-12 has the largest capability to produce serine protease inhibitor molecule or compound. It indicated that this molecule/compound produced by isolate SAB S-12 inhibited protease production of pathogenic bacteria (EPEC K.1.1). In the assay of its activity, casein from skim milk was used as the substrate for the growing of pathogenic bacteria. Normally, pathogenic bacteria produce protease enzyme which digests casein in the skim milk agar medium as the kinds of protein, therefore the pathogenic bacteria would made proteolytic zone around its colony.

Each bacterial isolate of SAB S-12, SAB S-21, and SAB S-17 had maximum protease inhibitor production in each optimum time of cultivation. It indicated that the optical density of them would be higher along with the time of cultivation. The optical density described the density of amount of bacteria in the solution. Thus, the protease inhibitor production would growing along with the growing of optical density until it faced its optimum time of cultivation and then the optical density would decrease and so did its production of protease inhibitor enzyme. The protease inhibitory activity of each bacterial isolates showed different percentage for different substrate. It was indicates that each isolates had specification to different kind of protease (Figure 2).

Isolate SAB S-12 had higher inhibition capability to serine protease (subtilisin) while, SAB S-21 had higher inhibition capability to metalloprotease (thermolysin). pH and temperature also influenced the inhibitory activity produced by each bacterial isolates. Those components

were also could enhanced the protease inhibitory activity of SAB S-12, SAB-21, and SAB-17, respectively (Figure 2). Hames and Hooper (2000) reported that temperature influences the reaction mechanism of protease inhibitor (protein) in 2 ways. The first way is the temperature will increase the mechanism of reaction until it reaches its optimum temperature, meanwhile in further higher temperature, changes in enzyme conformation will be happen. Therefore the enzyme can not enter the active site of inhibitor enzyme. After that, the activity of enzyme inhibitor will decrease. The second way is increasing of molecule thermal energy which develop structure of enzyme inhibitor protein will be affected in destroy of non-covalent interaction so that enzyme inhibitor will denature and its activity will decrease.

Based on 16S rRNA sequence, SAB S-12 homolog with *Providencia*. This bacterium is a Gram negative, motile bacterium of the family Enterobacteriaceae. This genus can be found commonly in soil, water, and sewage (Tumbarello 2004). This result is different from Imada (1985b) who successfully isolated marine bacteria producing protease inhibitor, *Alteromonas* sp. This isolate, produced protease inhibitor of marinostatin. It is also different from Kobayashi *et al.* (2003) who found *Pseudomonas sagamiensis* as the marine bacteria producing protease inhibitor. Based on the phylogenetic tree, it could be known that SAB S-12 (homolog with *Providencia* sp.), SAB 17 (homolog with *Paracoccus* sp.), and SAB S-21 (homolog with *Bacillus* sp.) were in the same groups (Figure 4 & Table 1). They were relatively closed each other than to bacterial reference strains. The others bacteria, such as *Alteromonas*, *P. piscicida*, *P. rubra*, *I. zobellii*, and *A. sagamiensis* were used as the comparator bacteria. They were definitively classified as marine bacteria producing protease inhibitor (Kobayashi *et al.* 2003).

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