

Research Article

***Streptomyces lavendulae* Protease Inhibitor: Purification, Gene Overexpression, and 3-Dimensional Structure**

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Received 12 April 2015; Accepted 5 July 2015

Academic Editor: Ioannis G. Roussis

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Protease inhibitors trypsin (STII, *Streptomyces* trypsin inhibitor 1) has been identified, purified by ammonium sulfate precipitation and Sephadex G-100 gel filtration. SDS-PAGE of protease inhibitor showed molecular weight of approximately 10 KDa. PCR product (~1615 bp) of *stil* gene was cloned in expression vector pACYC177/ET3d and transformed in *Escherichia coli* JM109. *Protease inhibitors* trypsin was purified and used as antiviral against Coxsackievirus B3 (CVB3). CVB3 is one of the major causative agents of chronic, subacute, acute, and fulminant myocarditis as well as pancreatitis and aseptic meningitis. It has been reported that more than 50% of human myocarditis is associated with CVB3 infection.

1. Introduction

Protease inhibitors (PIs) are a class of antiviral drugs that are widely used to treat HIV/AIDS and hepatitis caused by hepatitis C virus. Blocking proteolytic cleavage of protein precursors is necessary for the production of infectious viral particles [1, 2]. For the last 10 years, attention on protease inhibitor as the drugs target has increased slightly due to their role in 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 have been done to find natural protease inhibitor from many kinds of sources, such as virus, bacteria, and fungi and also other organisms, such as sponges and tunicate [3]. Among sea organisms, sponge was the most potential producer of bioactive agents including enzyme inhibitor components [4]. Coxsackievirus B3 (CVB3) belongs to the family Picornaviridae and the genus *enterovirus* can be transmitted via fecal and oral-route. CVB3 is one of the major causative agents of chronic, subacute, acute, and fulminant myocarditis as well as pancreatitis and aseptic meningitis. It has been reported that more than 50% of human myocarditis is associated with CVB3

infection. It has been estimated that about 10%–20% of people (approximately 20,000–40,000 patients per year in the USA) with symptoms of acute myocarditis induced by CVB3 infection will develop chronic disease, and chronic myocarditis progresses to dilated cardiomyopathy at a frequency of 3.5–8.5 cases/100,000 persons (9000–20,000 new cases per year in the USA) [5]; they often require heart transplantation [6]. It has been also found that CVB3 infects stem cells in the neonatal central nervous system [6] and also can induce type 1 diabetes mellitus [7]. So it often does great harm to patients' hearts, pancreases, and central nervous systems, especially in neonates, young children, and immunocompromised adult patients [8]. However, no antiviral agents against CVB3 are available for clinical use [9].

2. Materials and Methods

2.1. Microorganism and Growth Conditions. The bacterium isolated and used in this study was *Streptomyces lavendulae* DAMH105 [10] which was isolated from sea water. The *E. coli* strains used in this study were described in Table 1. *Escherichia coli* strains were grown at 37°C in LB broth with vigorous agitation. They were used for the detection of

TABLE 1: Bacterial strains and plasmids used in this study.

Strain/plasmid	Description	Source
Strains: <i>E. coli</i> JM109	F' <i>traD36 proA</i> ⁺ B ⁺ <i>lacI</i> ^q Δ(<i>lacZ</i>)M15/Δ(<i>lac</i> - <i>proAB</i>) <i>glnV44 e14</i> ⁻ <i>gyrA96 recA1 relA1 endA1</i> <i>thi hsdR17</i>	New England Biolabs
<i>E. coli</i> DA100	JM109 (pDA1)	Competent cells
Plasmids: pACYC177/ET3d/ yNMT	Promoter for expression: T7 (<i>phi10</i>) Replicon: p15A	ATCC
pDA1	pACYC177/ET3d/yNMT + 1626 bp <i>stil</i> gene	This work

insertional inactivation of the *stil* gene in expression vector. The chemical reagents listed were purchased from Sigma.

2.2. Molecular Biology Techniques

2.2.1. Gene Isolation. DNA extraction, polymerase chain reaction (PCR), and DNA sequencing genomic DNA were Employed with a modification of the method of [11]. The PCR amplification was 15 carried out in a 50 μL mixture, the forward and reverse primers were as follows:

Fwd *stil*ScaI ggcagctacttgcgcaacaccgcgcgctg,

Rev *stil*PstI ggctgcagaagataaacgcgctcgcg.

PCR was performed by following standard procedures using BIOTAQ DNA polymerase (Bioline). The Expand High Fidelity PCR System (Boehringer) was used to generate PCR products for cloning purposes. Thirty cycles were performed with the following conditions: 30 s at 90°C, 1 min at 50°C, and 1 min at 72°C. Where longer PCR products were sought, the Expand Long Template PCR System from Boehringer was used according to the manufacturer's instructions. Primers for PCR were synthesized using an Applied Biosystems' PCR-MATE DNA synthesizer.

2.2.2. Plasmid Extract. Plasmid DNA was isolated from *E. coli* strains using the alkaline lysis method [12] or the QIA-GEN column purification kit (any one). *E. coli* strains were transformed by electroporation with a Gene-Pulser apparatus (Bio-Rad) as described by [12–14], respectively. Restriction digestion by *ScaI*, *PstI*, and Klenow treatment and DNA ligations were done according to standard procedures [11]. Restriction enzymes, the Klenow fragment of the *E. coli* DNA polymerase I, and T4 DNA ligase were purchased from New England Biolabs. Restriction-endonuclease-digested DNA was eluted from agarose gels using a GeneClean II kit from Bio101.

2.2.3. Construction of Expression Plasmid pACYC177/ET3d/yNMT. The expression vector pACYC177/ET3d/yNMT has promoter T7 (*phi10*), was constructed by insertion of *Stil* fragment using restriction site *ScaI*, *PstI* vector. The vector has kanamycin antibiotic resistance, so it is grown

in LB medium with kanamycin. Fragment was removed by restriction enzymes 237 bp, and the fragment inserted was 1626 bp. *Escherichia coli* is the most widely used bacterium as expression host for overexpression trials of genes (expression construct in Figure 4). In this recent study we have shown that it is possible to engineer or select the *E. coli* strains transformed with protease inhibitor gene which have great medical applications [15].

2.3. Enzyme Preparation. Crystallized salt-free trypsin was used for the estimation of inhibitor effects. 0.2 mL filtrate from shake culture and 0.8 mL trypsin solution, containing about 5 μg trypsin, were incubated for 30 min at 30°C. Blanks were incubated with 0.8 mL trypsin solution and 0.2 mL distilled water. The remaining free trypsin, which was not inhibited, was estimated as caseinolytic activity at pH 9.5 according to the method of [16] because a correlation between inhibition and concentration of inhibitor is linear only to a certain limit. Culture filtrates diluted to give about 50% inhibition of the trypsin were used. Inhibitor activity was calculated as the amount of trypsin (μg) that was inhibited to 50% by 1 μL of culture filtrate. Protein concentration was determined by the method of [17], with bovine serum albumin as standard.

2.4. Partial Purification of the Protease

2.4.1. Ammonium Sulphate Precipitation. The culture of *E. coli* JM109 strain bearing plasmid pACYC177 was grown in LB broth supplemented with 50 μg/mL kanamycin at 30°C supernatant containing the extracellular enzyme that was first subjected to ammonium sulphate precipitation. Ammonium sulphate fractions of 0–30%, 30–50%, 50–75%, and 75–100% (v/v) were collected by centrifugation at 10,000 g and the pellet obtained in each fraction was suspended in a minimal volume of 100 mM Tris-HCl, pH 8.0.

2.4.2. Sephadex G-100 Gel Filtration. The 50–75% (v/v) ammonium sulphate fraction was subjected to gel filtration on a Sephadex G-100 column (3 cm × 100 cm) equilibrated with 25 mM Tris-HCl, pH 8.0. Enzyme fractions of 5 mL were collected at a flow rate of 25 mL/h with the same buffer. Protein content (Abs 280 nm) and protease activity were measured. Fractions showing protease inhibitor activities were pooled. Fraction (20–30) contained STII (this protease inhibitor trypsin which was expressed by *stil* gene), which was >95% pure as judged by Coomassie blue stained SDS-PAGE. Protein concentration was determined by the method of Lowery.

2.4.3. Gel Electrophoresis and Molecular Weight of STII. 5 mL from fraction IV of purification was withdrawn and heated at 95°C for 2 min; each 20 μL was loaded in a lane. The solution was pipetted into the assembled vertical slab gel unit in the casting mode leaving 2 mL from the front. A layer of distilled water was pipetted on the top of solution. The gel was allowed to polymerize at room temperature and the water was poured from the upper surface. The stacking gel solution was prepared and added onto the top of separating gel where

the comb was inserted. The gel polymerization was allowed. The assembled vertical slab containing the polymerized gels was put into the electrophoresis chamber filled with the tank buffer. The protein samples were loaded into the wells after removing the comb by using Hamilton syringe in loading; a protein marker was also loaded (200 volts and tank buffer 1x). For electrophoresis, the gel was stained in 50 mL of staining solution for 45 min with shaking at room temperature and then destained in destaining solution, and, finally, gel image was taken. Sample was taken from bacterial cells of *Streptomyces* sp. Directly. One milliliter from overnight culture incubated in shaking incubator at 37°C and 120 rpm cells was collected by centrifugation at 10,000 rpm for 15 min. Bacterial pellets were resuspended well in 60 μ L distilled water and then 20 μ L sample buffer and 10 μ L protein tracking dye were added. Polyacrylamide gels were prepared with unheated samples as described before [18]. Centrifugation for 15 min at 10,000 rpm was done and placed on ice until loading. *Stereo structure diagram of STII* is studied by Chimera program 10.1 2014.

2.5. Protease Inhibitor Purified Preparation. The drug (*protease inhibitor purified*) was sterilized by filtration through Millipore 0.45 μ m. Then a series of dilutions of the drug (20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL) were prepared in test medium and kept at 4°C until used.

2.6. Cell Culture. Vero cell lines were cultivated in Eagle's minimum essential medium (EMEM) (Lonza, Belgium) supplemented with 10% heat inactivated fetal bovine serum (FBS), 100 units/mL penicillin, and 100 μ g/mL streptomycin. The medium with 2% FBS was used for the cytotoxicity and antiviral assays (all reagents were purchased from Lonza, Belgium).

2.7. Cytotoxicity Assay. The cell viability was evaluated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) method according to [19]. Briefly, Vero cells were grown in 96-well plates at concentration of 5×10^3 cells/well. The grown cells were incubated for 24 hours at 37°C under 5% CO₂ humidified atmosphere. The culture medium was removed and the various concentrations of the drug (20, 10, 5, 2.5, 1.25, 0.625, and 0.3125 mg/mL) were added onto Vero cells. After incubation period of 48 h, the drug was removed from wells and replaced by 100 μ L of MTT solution (5 mg/mL) for 4 h at 37°C. After removal of MTT, 50 μ L dimethyl sulfoxide (DMSO) was added to wells for 30 min in CO₂ incubator at 37°C. Optical density (OD) was measured by using a spectrophotometer reader at 540 nm. Data were obtained from triplicate wells. The percentage of cytotoxicity was calculated as $[(A - B)/A] \times 100$, where *A* was the optical density of untreated cells and *B* was the optical density of treated cells.

2.8. Study of Antiviral Activity of Protease Inhibitor Purified with Mode of Action of Drug on CVB3 by TCID₅₀/1mL Determination. Vero cells were seeded in 96-well plates in concentration of 5×10^3 cells/well and after 24 h incubation at

37°C. The medium was removed from the wells and different doses of virus suspension (10^{-3} – 10^{-8}) with or without drug were added to cells in three different ways as follows.

2.8.1. Virucidal Assay. Virus suspensions were incubated with an equal volume of medium including the drug (*protease inhibitor purified*) for 1 h at 37°C. Then 100 μ L of mixed suspension was added to vero cells. After 1 h incubation, the mixed suspension was removed and the cells were rinsed twice with PBS; then 200 μ L of test medium and 2% FBS were added to wells.

2.8.2. Compound Treatment before Virus Infection (Pretreatment). The drug was incubated with the cells for 24 h at 37°C in 5% CO₂ atmosphere. The drug (*protease inhibitor purified*) was discarded and the cells were challenged with virus suspensions. After 1 h incubation, unabsorbed virus was removed and the cells were rinsed twice with PBS; then 200 μ L of test medium and 2% FBS were added to wells.

2.8.3. Compound Treatment after Virus Infection (Posttreatment). Confluent cell monolayers were challenged with virus suspensions for 1 h; then the unabsorbed virus was discarded and replaced with media containing the drug.

All plates were incubated at 37°C under CO₂ incubator for 5 days; then the CPE was checked under light microscope. The virus titer was estimated from cytopathogenicity and expressed as 50% tissue culture infectious doses (TCID₅₀/1 mL) by Karber method [20]. The inhibition percentage was estimated as described by Bastos et al. [21] using the following formula: (IP) = $(1 - T/C) \times 100$, where *T* is the drug-treated viral titers and *C* is the control viral titers without drug.

3. Results

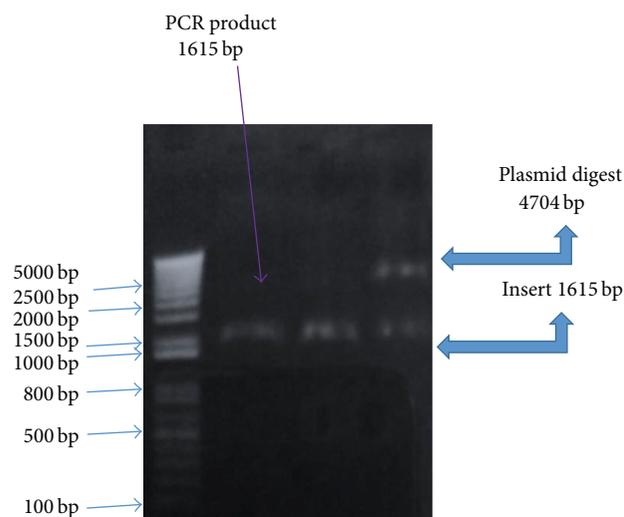
An extracellular alkaline protease inhibitor was partially purified from the culture filtrate of *Streptomyces* using ammonium sulphate precipitation (40, 45%). The purification steps were summarized in Table 1. *Streptomyces lavendulae* produces protease inhibitor applied with different cultural conditions and optimized in a synthetic medium. To improve enzyme gene expression, protease inhibitor gene was isolated from *Streptomyces lavendulae* and transformed using expression vector pACYC177/ET3d/yNMT, cloning of expression vector in *E. coli* JM109. Purification in this case showed specific activity protease inhibitor up to 363.2 μ /mg; see Table 2.

The data showed that about 94.7% of the yield was with 10.94-fold purification. The partially purified inhibitor was further purified using Sephadex G-100 column chromatography. A single peak of the inhibitor activity was noticed in the elution pattern. The inhibitor was purified 77.24-fold with 91.8% enzyme recovery and specific activity of 112 U/mg of protein.

Total DNA was isolated from these strains, and the structural nisin gene was amplified by PCR, resulting in a 1564 bp DNA fragment. The presence of protease inhibitor genes was determined by direct sequencing of the PCR

TABLE 2: Purification procedures of protease inhibitor from *Streptomyces*.

Purification step	Total protein (mg) \pm SD	Total activity (units) \pm SD	Specific activity (U/mg protein) \pm SD	Enzyme recovery %	Purification fold
Crude enzyme	126 \pm 15	543 \pm 10	4.31 \pm 0.1	100	1
Amm. sulfate fraction (40–80%)	10.5 \pm 14	300.1 \pm 17	28.58 \pm 2	55.26	6.631
Sephadex G100 fraction (25–37)	1.3 \pm 12	287.8 \pm 19	221.38 \pm 14	53.001	51.36

FIGURE 1: Digest construct with gene insertion and PCR product *stil* gene.

product. Sequence ladder was obtained, indicating that a single protease inhibitor gene species was present in the strains as illustrated in Figures 1 and 2.

The gene encoding the protein was localized to a 1615 bp *SacI*, *PstI* DNA fragment. Analysis of this sequence indicated an open reading frame which agreed with tryptic peptide amino acid sequence data. A restriction map for the gene region indicating the sequencing strategy is shown in Figure 2. The complete nucleotide and amino acid sequences are shown in Figure 1. Construct formation depends on restriction endonuclease map (Figure 3).

STII was comparable in SDS-PAGE at 10 kDa STII found in culture supernatants of *Streptomyces* sp. Since the STII produced by *E. coli* appeared to have the same size as the mature products from *Streptomyces* (Figure 5), it seemed likely that similar posttranslational processing events were occurring. These results indicate that the *Streptomyces* translational elements can be utilized by *E. coli* and that *Streptomyces*-derived secretion information functions in *E. coli*. The protein and its gene exhibit a high degree of homology and, as expected, the gene encodes presumptive signal peptides required for secretion of the protein. The proteins are protein protease inhibitors related to the *Streptomyces subtilisin* inhibitor isolated from *Streptomyces albobiseolus* [22, 23].

3.1. Cytotoxicity Results. The 50% cytotoxic concentration of the drug was 5.7 mg/mL. Therefore, 5 mg/mL of the drug was used as safe dose for antiviral experiment.

3.2. Antiviral Results. To assess whether the test drug has antiviral activity against CVB3 three approaches were taken into consideration. The first one is the inhibition of CVB3 by binding the drug to viral capsid and preventing virus entry into host cells. The second one is the inhibition of virus pathogenicity by blocking viral receptors on the surface of host cells. The third one is the inhibition of virus by affecting one or more steps of virus replication inside the host cells. For this purpose, the drug was added during, before, and after virus infection. Our results demonstrated that the drug showed the maximum inhibitory effect when the drug was incubated with cell before virus infection with reduction of virus titer by 2.75 \log_{10} TCID₅₀/1 mL (inhibiting the viral replication by 54%); see Table 3.

4. Discussions

Protease inhibitors are one of the most abundant classes of proteins in the world. They are found in numerous plants, animals, and microorganisms [24]. Serine proteases comprise a large group of peptidases characterized by presenting a catalytic serine residue. In fact, three residues (serine, histidine, and aspartic acid) are essential in catalytic process. They work together to cleave the peptide bond of the substrate Hedstrom [25]. Trypsin, chymotrypsin, and elastase are the largest and best studied serine proteases and they are involved in the protein digestion of the diet in mammals, including human. They have the same three-dimensional structures and active site architecture but differ in the substrate specificity: while trypsin cleaves peptide bonds on the C-terminal side of a positively charged residue (Lys or Arg), except when it is followed by proline, chymotrypsin prefers large hydrophobic residues (Phe, Trp, and Tyr) and elastase acts on small neutral amino acid residues (Ala, Gly, and Val) [25].

4.1. Purification of a Protease Inhibitor. Trypsin and chymotrypsin from bovine pancreas are serine proteases more used in *in vitro* assays for determination of the presence of inhibitory activity by the crude extracts of several origins, by accompaniment of inhibitory activity during all the isolation process, and for characterization of inhibitor purified, formation of inhibitor-serine protease complex, and studies about stability of the inhibitory activity [26–29].

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atgacaacagctgacgaatacaaaagcagaaggttaacaaatattttgctgctaaagaattt
M T T A D E Y K A E G N K Y F A A K E F
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E K A I E A F T K A I E A S P E P N H V
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L Y S N R S G S Y A S L K D F N N A L K
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D A Q E C V K I N P N W A K G Y N R I A
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V A K L E R L K T N P Q L G N P D M F S
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D P R L L T G F C C F M G I D M D L P N
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M G F T A P N E S Q S N A S E P K L E P
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K S V P E S K P E P K A E Q K E E E S T
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S F A R L G N I Y L K K D E L P E A V K
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N F E K S L T E H R T P D V L N K L R S
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T Q R E I K T R E L N A Y I D P E K A E
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G G K S I H E I D E L M N K A T Y Q R F
caagccattgaaggtgaaactcctgaacaaactatggaaagattt
Q A I E G E T P E Q T M E R V
    
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FIGURE 2: Nucleotide and amino acid sequence of *Streptomyces* secreted proteins, STII: the residues have been confirmed by amino acid sequence analysis of tryptic fragments of the mature protein. As can be seen, ~95% of the sequence of the mature protein was confirmed.

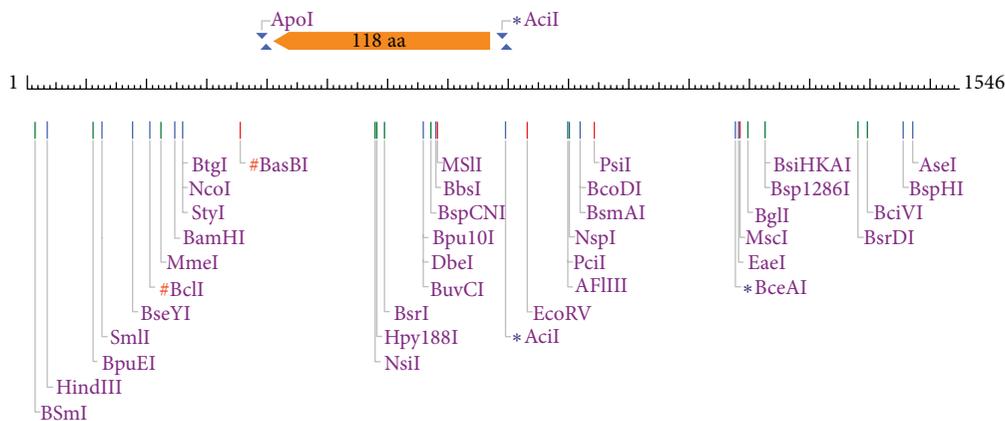


FIGURE 3: Restriction endonuclease map of the *S. lavendulae* genomic DNA region surrounding the *stil* gene. The open reading frame is boxed. Arrows underneath represent the restriction fragments sequenced.

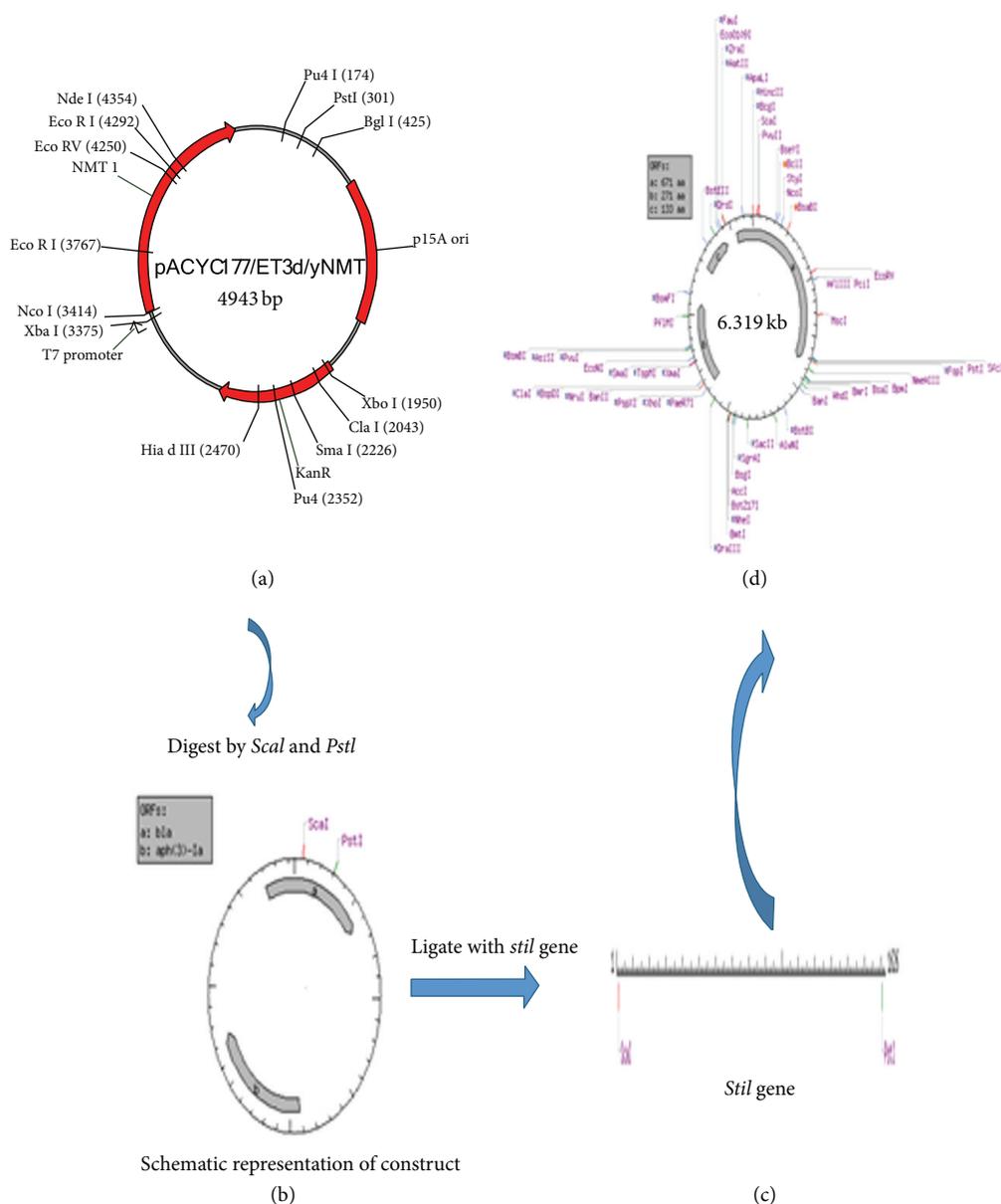


FIGURE 4: Steps of Construction of *stil* gene/pACYC177/ET3d/yNMT. (A) pACYC177/ET3d/yNMT expression vector map. (B) 237 bp *ScaI*–*PstI* fragment containing the N-terminal half of the ampicillin-resistance gene was excised out of the pACYC177/ET3d/yNMT vector. (C) *stil* gene purified was digested using *ScaI*–*PstI*. (D) 1615 bp *ScaI*–*PstI* fragment was ligated to the pACYC177/ET3d/yNMT expression vector *ScaI*–*PstI* backbone fragment to create 6319 bp construct/*stil*.

The data showed that about 94.7% of the yield was with 10.94-fold purification. The partially purified inhibitor was further purified using Sephadex G-100 column chromatography. A single peak of the inhibitor activity was noticed in the elution pattern. The inhibitor was purified 77.24-fold with 91.8% enzyme recovery and specific activity of 112 U/mg of protein. This significantly agrees with that obtained for *Coccinia grandis* PI [30] and is higher than that obtained for *Archidendron allipticum* PI [31].

4.2. Gene Isolation of *stil*. Cloning the *stil* Gene of *S. lavendulae*. The 10 kDa protein defined by SDS-PAGE analysis was

purified from *S. lavendulae* conditioned medium after 30 h in culture by a combination of ammonium sulfate precipitation and preparative SDS-PAGE as described in experimental procedures. The reduced denatured protein was digested with trypsin and the fragments isolated. Sequence analysis of the resulting peptides provided sufficient information accounting for >80% of the protein.

4.3. Protease Inhibitor Molecular Weight. Protease inhibitor from *S. griseoincarnatus* [32, 33]. Protein protease inhibitors have been classified based on their size, structure, and disulfide 3 bond arrangement [34]. The SSI-like class of inhibitors

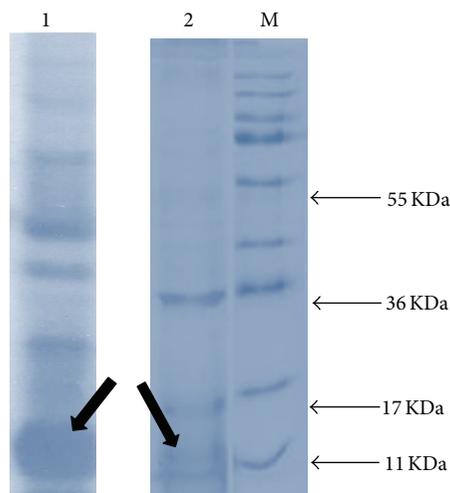


FIGURE 5: SDS-PAGE (15% gel), lane 1: protein profile from 2 mL culture supernatant of *E. coli* JM109 with expression vector *stiI*; lane 2: protein profile from 2 mL culture supernatant of *Streptomyces* sp.; lane 3: protein marker.

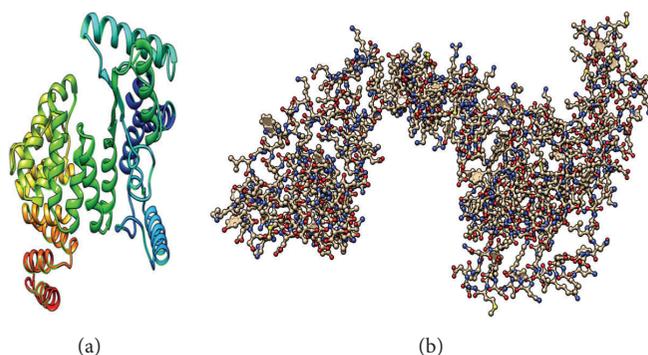


FIGURE 6: Stereo diagram of STII docking by Chimera program using *stiI* gene sequence. (a) STII in interactive form (Ribbons). (b) Secondary structure of STII with all atoms in interactive form.

has two non-overlapping disulfide bonds and monomeric molecular masses of ~10 kDa; see Figure 5. The genes for *stiI* were transformed into *E. coli* under control of the promoter. Following heat induction, these fractions were subjected to SDS-PAGE, STII overproduced and purified analyzed by SDS-PAGE (15% gel).

4.4. Stereo Structure Diagram of STII. In general terms, all the PIs bind to their specific protease preventing access of the substrate to the active site. For some PIs, the docking occurs directly in the protease active site, while for others the binding takes place in a neighborhood of the catalytic centre, leading to its steric hindrance [35]. The majority of the known PIs, specially the serine protease inhibitors, interact with the enzyme catalytic sites in a “canonical” manner, similar to the enzyme-substrate interaction, via an exposed reactive site loop of conserved conformation [36] (Figure 6). The canonical conformation of the reactive loop characterizes a mechanism of competitive inhibition and is also found

in the trypsin inhibitors from *Erythrina caffra* seeds and *Psophocarpus tetragonolobus* chymotrypsin inhibitor [37, 38]. Trypsin inhibitors, such as those from *Swartzia pickelii*, can present also glutamine residue in the reactive site [39]. Specific inhibitor for chymotrypsin, in general, possesses leucine residue in its reactive site [40] and in the *Bauhinia rufa* elastase inhibitor one valine was identified in the reactive site [41].

Mechanistically, the drug inhibited the virus replication by blocking the virus receptor preventing the virus entry into host cells. The drug showed less effect on virus replication when added to cells after virus infection with reduction in virus titer by 1 log₁₀ TCID₅₀/1 mL (inhibiting the viral replication by 16%). Mechanistically, the drug inhibited the virus replication by affecting one or more steps in viral life cycle after entry of virus into host cells. However, no antiviral activity was shown when the drug was preincubated with virus prior to infection (the results are summarized in Table 3).

TABLE 3: The antiviral activity of the drug against CB3 with the mode of action on Vero cell line.

Virus titer without drug ^a	Virucidal		Treatment before infection			Treatment after infection		
	Virus titer with drug ^b	Reduction of virus titer ^R	Virus titer without drug ^a	Virus titer with drug ^c	Reduction of virus titer ^R	Virus titer without drug ^a	Virus titer with drug ^d	Reduction of virus titer ^R
10 ⁶	10 ⁶	0	10 ⁶	10 ^{3.25}	10 ^{2.75}	10 ^{6.25}	10 ⁵	10 ¹

5. Conclusion

Finally, the *Streptomyces* secretion signals would function in a heterologous bacterial system, the *stil* gene, including their putative ribosome binding sites and transcription initiation start sites, and they were subcloned downstream of the promoter in the *E. coli* expression vector. Protease inhibitor trypsin obtained can be used as drug and has high antiviral activity against CVB3 infection, particularly when the virus is added to cells prior to infection, and further experiments are required to assess whether the drug has antiviral activity against virus *in vivo*.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors would like to express their special thanks to Sigma and Lab Technology Company and Virus Lab in the National Research Centre.

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