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# Purification and characterization of a novel heterodimer protease inhibitor from *Streptomyces* spp. VL J2 with potential biopesticidal activity against *H. armigera*

Kiran Marathe, Sainath Kasar, Ambalal Chaudhari, Vijay Maheshwari*

*School of Life Sciences, North Maharashtra University, Jalgaon, India*

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ABSTRACT

A protease inhibitor (PI) was isolated and purified from halo-alkaliphilic *Streptomyces* spp. VL J2. SDS-PAGE of purified PI revealed it to be a heterodimer of two unidentical subunits of 27.5 and 11.08 kDa, which corroborates well with intact molecular mass of 38.5 kDa obtained by GPC and MALDI –TOF. Inhibitory activity was confirmed by activity staining and reverse zymogram studies and the inhibitor was found to retain activity at <50 °C and pH 2–9.5. It showed presence of two isoforms with isoelectric point of 5.5 and 5.7. The inhibition of trypsin and chymotrypsin indicated it to be a serine protease type belonging to serpin family. The stoichiometry of trypsin-inhibitor interaction was 1:2. Modification of amino acid showed presence of arginine and free sulfhydryl group at active site. Kinetic studies revealed the non-competitive type of inhibition of trypsin with low Ki value ($9.4 \times 10^{-9}$ M). Activity against *H. armigera* showed significant decline and delay in larval (51%), pupal weights and periods, respectively, prominent physical abnormalities and reduced nutritional indices as a function of treatment. The results suggest that the purified PI has promising pesticidal activity and could serve as a potential candidate gene for transgenic plant research.



## 1. Introduction

Enzyme inhibitors have evoked interest as useful tools in study of enzyme structures and reaction mechanisms, therapeutic and biocontrol agents in pharmacology and agriculture [1,2]. Of these, protease inhibitors (PI) are the protein repertoire with ability to inhibit a wide variety of enzymes in all life forms [3] and hence, emerged as an alternative tools to control pest and pathogens and therapeutic for various human microbial (hepatitis, herpes, AIDS, aspergillosis), mortal (arthritis, muscular dystrophy, malaria, cancer, obesity) neurodegenerative and cardiovascular diseases [4].

The disease spectrum in plants is continuously changing and depends on the dynamic nature of pests and crop systems. Hence, disease management in crop production system is a crucial functional component and could be achieved by use of (i) chemical control, (ii) insect resistant crop varieties and (iii) integrated cultural practices including biopesticides. To circumvent the drawbacks of chemical insecticides, several alternative strategies such as plant breeding, molecular marker linked selection, built in resistance, gene pyramiding and transgenic plants have been developed to control insect pest [5]. PIs are small size defense related proteins in certain plants mainly belonging to 3 families *Leguminosae, Solanaceae* and *Gramineae* that selectively inhibit proteases of invading pathogens or insect pest thereby, limiting essential amino acids for growth and reproduction and emerging as anti-metabolic proteins for their control [6]. At present there are several protease inhibitors of small molecular size specific for each of mechanistic classes of proteases (serine, cysteine, aspartyl and metallo) identified from plants and animals [7–9] but very few reports from microbial source exist [10,11].

Of the insect pests, polyphagous lepidopteran insects have a broad host range (>180) and voraciously feed on the nitrogen rich plant structure of diverse plant species, causing massive economic losses [12]. The management of lepidopteran insects has become a major challenge both on economic and ecological fronts and warrants a safe ecofriendly alternative. Under these circumstances, several natural compounds of plant, animal and microbial origin have provided adequate protection against them [6]. Among natural sources, actinobacteria contributed various bioactives [13] and demonstrated that they can play a key role in their management [14,15].

* Corresponding author at: School of Life Sciences, North Maharashtra University, Jalgaon, 425001, M.S., India.
*E-mail address:* vlmaheshwari@rediffmail.com (V. Maheshwari).

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The alkaline gut of lepidopteran larvae predominantly has serine proteases (trypsin and chymotrypsin) [16] and diet incorporation assays can be used to study the effects of serine protease inhibitors on them. PI can bind to gut proteases of insect larvae, resulting in low amino acid turnover, thereby affecting growth and development of insect, reducing fecundity and enhancing mortality [17]. The inhibitory activity of several PIs against alkaline larval midgut proteases has been demonstrated [15,17–21] and proved highly effective against lepidopteran insects [22].

Although a plethora of PIs from various microbes have been reported, but the majority of small molecule inhibitors as antinutritional agents are available from *Actinomycetes* such as (i) amastatin and bestatin as amino peptidase inhibitor against red flour beetle [23], (ii) pepastatin A as aspartate protease inhibitor against cowpea bruchid [24] and (iii) leupeptine as serine protease inhibitor against western corn rootworm [25]. In addition, several serine protease inhibitors such as streptomyces subtilisin inhibitor (SSI) family, alkaline protease inhibitor (API-2C), streptomyces trypsin inhibitor 2 (STI2) have been characterized from different *Streptomyces* spp. [26] and comparative studies about their primary structures have been carried out [27]. Recently, we screened actinobacterial isolates from soda lake habitat as a source of PI for antifungal activity against phytopathogens and reported its insecticidal activity against *C. chinensis* as well [28]. The present study focused on purification, characterization of protease inhibitor isolated from the *Streptomyces* spp. and further systematic evaluation against one of the most damaging pre-harvest pests, *Helicoverpa armigera*.

## 2. Materials and methods

### 2.1. Materials

All chemicals, media and reagents were analytical reagent (AR) grade, mainly procured from M/s. HiMedia, Mumbai, India. Lower range protein molecular weight markers and purified enzymes were obtained from Sigma Aldrich (St. Louis, MO, USA).

### 2.2. Insect pest

Early third instar *Helicoverpa armigera* larvae were obtained from R & D division, Nirmal Seeds Pvt. Ltd., Pachora, Jalgaon (MS). The larvae of the insect were initially fed on natural diet of young, fresh, non-Bt cotton leaves before the start of the experiment. The insecticidal assay was carried out under laboratory conditions on an artificial diet as per protocol provided by Central Institute for Cotton Research (CICR), Nagpur (India).

### 2.3. Purification of protease inhibitor

PI was purified from the culture broth of a *Streptomyces* spp. VL J2 carried out by a four step process as described by Bijina et al. [9]. *Streptomyces* spp. VL J2 was grown in an Erlenmeyer flask containing 1L of previously optimized medium [28]. The cells were removed by centrifugation (5000 X g) and the supernatant was used as a source of PI.

### 2.4. Protease inhibitor assay

The Kunitz caseinolytic assay with slight modifications was used for assaying PI activity [29]. The reaction mixture (2 mL) contained trypsin (100 μg/ml) in 0.1 M Tris HCl buffer, (pH 8.4), casein (1%), *actinomycete* culture extract (0.1 mL) as a source of PI and incubated at 37 °C for 20 min. The reaction was terminated by adding 2 mL of TCA (5%), the tubes were kept for 30 min at room temperature and content was filtered through Whatman no. 1 filter paper. The absorbance of tyrosine in the filtrate was taken at 275 nm against a standard tyrosine solution (UV 1601, Shimadzu, Japan). Simultaneously, absorbance of the blank and control without inhibitor and substrate was also recorded.

### 2.5. Characterization of purified PI from Streptomyces spp. VL J2

#### 2.5.1. Molecular weight determination

Molecular weight (MW) of the purified PI was estimated both by SDS-PAGE under reducing condition (with β-mercaptoethanol) and gel filtration chromatography through Sephadex G-50 using known molecular weight markers (Sigma Aldrich, USA) [17]. Intact molecular weight of isolated PI was also determined by MALDI-TOF/TOF on MS Bruker Daltonics ULTRAFLEX III. The sample was mixed with SA (Sinapinic acid) matrix in 1:1 ratio i.e 1 μL of sample +1 μL of matrix. The resulting 2 μL then spotted on MALDI target plate and was allowed to air dry. The MALDI TOF instrument was calibrated with protein standard mix. The matrix-sample co-crystallized spot was then analyzed by acquiring the spectrum in the desired range and using FLEX ANALYSIS software and mass values were assigned to the spectrum.

For molecular weight determination of purified PI by gel filtration on a Sephadex G-50 column (62 × 2.5 cm), the void volume of the column was determined by loading 2.0 mL of 0.2% blue dextran-2000 (MW ~$2 \times 10^6$) and eluting it at a flow rate of 0.5 mL $min^{-1}$ by passing Tris-HCl buffer (pH 8.2). The column was calibrated using the standard protein marker such as bovine serum albumin (66 kDa), ovalbumin (4 kDa), papain (23 kDa) and lysozyme (14.3 kDa). A calibration curve (log MW × $10^4$ versus $V_e/V_o$) was plotted where, $V_e$ = elution volume at which the protein peak was obtained and $V_o$ = void volume was determined. Purified PI (2 mg) was applied separately on the gel filtration column and fractions of 1 mL each were collected and analyzed for protein content. The molecular weight of PI was determined by extrapolating the $V_e/V_o$ value on the calibration curve.

#### 2.5.2. Heterodimeric nature of purified PI

The heterodimeric nature of the purified PI was verified by treating it (1 mg/ mL) with 1% SDS for 30 min in reaction buffer (10 mM Tris HCl, pH 8.2). After incubation, the treated PI and untreated PI (native) were loaded separately on to the Sephadex G-50 column and molecular weight was determined in both conditions. Peak fractions obtained from the gel filtration column, were pooled and loaded onto SDS PAGE and also assayed for PI activity. The molecular weight was determined by comparing the bands on gel with the molecular weight markers.

#### 2.5.3. Isoelectric focusing, activity staining and reverse zymography of purified PI

Protein sample/s were allowed to run on a 7 cm, 3–10 IPG (immobilized pH gradient) strip in Bio-Rad's IEF cell and stained with silver nitrate. Along with the sample, IEF standards were processed for comparison as per Sapana [30].

The inhibitory activity of the purified PI was visualized by activity staining using artificial substrate BApNA as per the method of Felicioli et al. [31] with slight modification. It was further confirmed by reverse zymogram on Gelatin-PAGE performed by adding gelatin (0.1% final concentration) to the polyacrylamide gel prepared according to the method of Felicioli et al. [31].

#### 2.5.4. Stability studies of isolated PI

The pH stability of PI was determined in different pH buffers (KCl-HCl buffer pH 1.8–2, citrate buffer pH 4–6, phosphate buffer pH 7, Tris-HCl buffer pH 8–9, borax/NaOH buffer pH 10, Glycine-NaOH buffer pH 11) for 24 h at 4° C. After incubation, the PI was dialyzed against distilled water to remove the respective buffer and