

Characterization of Protease Inhibitors from the Seeds of *Adenantha Pavonina*

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Abstract

Enzyme inhibitors such as protease inhibitors are widely distributed in nature inhibit the catalytic activity of proteolytic enzymes. They involved in variety of proteolytic process of biological/physiological significance. The protease inhibitors, APPI-1, APPI-2 and APPI-3 have been isolated and purified from the seeds of *Adenantha pavonina* employing salt fractionation, Sephadex G-10 and Sephadex G-50 gel-permeation chromatography and RP-HPLC. The APPI-1, APPI-2 and APPI-3 was purified to 31.24, 36.02 and 19.21 fold with a recovery of 41.10%, 51% and 43.28%, respectively. Further, APPI-1, APPI-2 and APPI-3 showed a specific inhibitor activity of 16.15, 20.43 and 11.06, respectively. The purified APPI-1, APPI-2 and APPI-3 showed a molecular weight of 7 - 8 kDa, 11 - 12 kDa and 13 - 14 kDa (approximately) as determined by gel filtration chromatography. All the three purified inhibitors are both pH and temperature stable. The APPI-1, APPI-2 and APPI-3 showed antimicrobial activity on *E. coli* and streptococcus species.

Key words: *Adenantha pavonina*, Protease inhibitors, Isolation, Purification, Characterization, Antibacterial properties.

INTRODUCTION

Seeds are one of the storage organs of plants contains variety of proteins which includes carbohydrate binding proteins, ribosome activating proteins, inhibitors of various enzymes, chitin degrading enzymes etc. Among these proteins, enzyme inhibitors such as inhibitors of proteolytic enzymes which are produced in response to pest or pathogen attack attracted a great attention of researchers due to their participation in variety of biological functions. In plants, these inhibitors of proteolytic enzymes acts as storage proteins, involved in the regulation of endogenous proteases thereby preventing unwanted proteolytic degradation, act as defense proteins preventing the attack of insect pests or pathogens thereby prevents crop loss in agriculture. Pharmacologically. The inhibitors of proteolytic enzymes can also be used in the treatment of various proteolytic enzymes involved pathogenic process of human diseases such as emphysema, arthritis, pancreatitis, thrombosis, high blood pressure, muscular distropy, cancer and even AIDS [1 – 6].

Adenanthera pavonina is a perennial and non-climbing species of leguminous tree. Its uses include food and drink, traditional medicine and timber. It is commonly called Red Lucky Seed and it is cultivated for forage. It is also grown as medicinal plant, an ornamental garden plant or urban tree. The young leaves and seeds can be cooked and eaten. This tree is used for making soap and a red dye can be obtained from the wood. Since the seeds are used as medicine and no research work on protease inhibitor from this plant is carried out, the present work is undertaken. In the present study, partial purification and characterization of protease inhibitor is described.

MATERIALS AND METHODS

Seeds of *Adenanthera pavonina* were collected from areas of Puthige Panchayath of Kasaragod District of Kerala state. Bovine Serum Albumin, trypsin, BAPNA, Casein, Sephadex G-10 and Sephadex G-50 were obtained from Sigma chemical company, USA. All other chemicals used were of technical grade.

METHODS:

Isolation and purification

The seeds of *Adenanthera pavonina* were soaked for overnight and dehulled. The acetone powder was prepared blended in a homogenizer with chilled acetone for 5 mins as described by chandrashekharaiyah [7]. It was filtered and the cake obtained was dried at 37° C which was powdered and used for the extraction of protease inhibitor. The protease inhibitor extract was prepared in sodium phosphate buffer, pH 7.0 by stirring over a magnetic stirrer for 1.5 hr at 4° C. The extract was then

centrifuged at 10,000 rpm for 15 mins at 4° C. Quantitative analysis of proteins, and protease inhibitor activity was performed. Solid powdered ammonium sulphate was added while stirring over magnetic stirrer for 30 min to obtain 0 - 80% saturation. After the addition of ammonium sulphate salt, the solution was centrifuged at 10,000 rpm for 30min after allowed to stand for 3hr at 4°C and the pellet obtained was dissolved in small amount of buffer used for extraction. The dissolved pellet was fractionated on sephadex G-10 as described by Chandrashekharaiyah et al [8]. Activation of Sephadex G-10 was done and then equilibrated with 0.05 M sodium phosphate buffer, pH 7.0, packed into a glass column (1.0 cm X 110.0 cm) under gravity. The packed column was equilibrated same buffer. The ammonium sulphate pellet was loaded on to the gel and the proteins were eluted with a fraction volume of 2.0 ml. Quantitative analysis of proteins and protease inhibitor activity was performed. The sephadex G-10 fractions containing protease inhibitor activity were fractionated on Sephadex G-50 chromatography as described previously. The fractions containing protease inhibitor activity were pooled, concentrated and applied on to the sephadex G-50 gel. The chromatography was performed as described to Sephadex G-10 gel and the fractions were collected with a fraction volume of 2.0 ml. Quantitative analysis of proteins and protease inhibitor activity was performed was performed for all the fractions obtained. The fractions contained protease inhibitor activity were pooled, concentrated, dialyzed and subjected to purification by HPLC. RP-HPLC is carried out on Reversed-phase octadecylsilica (C18) column using binary solvent system with binary gradient capability and a UV detector. Buffer A is 0.1% (v/v) TFA in water and Buffer B is 100% acetonitrile containing 0.1% (v/v) TFA. Column Equilibration and Blank Run was carried out using Buffer A with a flow rate of 1 mL/min at 220 and 280 nm respectively. Once the stable line is obtained, the sephadex G-50 precipitated sample was injected and eluted the sample with a linear gradient from 0 to 100% buffer B for 30 min.

Protein estimation: Protein was estimated from the protease inhibitor extract and fraction of sephadex G-10 and G-50 according to the method of Lowry et al., [9].

Trypsin and Chymotrypsin activity

The trypsin and chymotrypsin activity was determined using casein as the substrate according to the method of Kakade et al., [10] as described by Chandrashekharaiyah [7]. Absorbance of the filtrate was measured at 280 nm (275 nm in case of chymotrypsin) using UV - visible spectrophotometer. One trypsin (chymotrypsin) unit is arbitrarily defined as an increase in absorbance by 0.01 at 280 nm (275 nm in case of chymotrypsin) under conditions of assay.

Trypsin and Chymotrypsin inhibitor activity

The trypsin and chymotrypsin inhibitor activity was determined using casein as the substrate according to the method of Kakade et al. [11] as described by Chandrashekharaiyah [7]. Enzyme solution was pre incubated with known aliquots of the inhibitor extract in a total volume of 1 ml at 37°C for 10 min in 0.01 M sodium phosphate buffer, pH 7.6, containing 0.15 M NaCl. The residual enzyme activity was determined as described above. Inhibitory units: Trypsin or chymotrypsin inhibitory unit is defined as the number of trypsin units or chymotrypsin units inhibited under the assay conditions. The temperature stability of purified trypsin inhibitors were studied by pre-incubating, the purified trypsin inhibitors at different temperatures (0 - 90 °C) for 30 min. The incubated samples were rapidly cooled and assayed at room temperature. Trypsin inhibitor assay was performed as described earlier.

Antimicrobial activity

Antibacterial activity by well diffusion method:

Antibacterial activity of the purified inhibitors on different bacterial strains (*E.coli*, *Pseudomonas aerogenosa*, *streptococcus sps* and *Klebsella*) was determined using agar well diffusion assay methods as described by Chandrashekharaiyah [7]. Approximately 20ml of molten and cooled media (nutrient agar) was poured in sterilized Petri dishes the bacterial test organisms were grown in nutrient broth for 24hrs. A hundred ml nutrient broth culture of each bacterial organism was used to prepare bacterial lawns. Agar wells of 5mm diameter were prepared with the help of a sterilized cork borer and labeled. Purified inhibitor was loaded with tetracycline. The plates were incubated at 37⁰ C for 24 hours. The plates were examined for evidence of zones of inhibition.

RESULTS AND DISCUSSION

The protease inhibitors from the seeds of *A.pavonina* were purified by employing salt fractionation, sephadex G-10 and sephadex G-50 chromatography and RP-HPLC. The protein fractionated from the protease inhibitor extract was loaded onto a Sephadex G-10 column. The proteins were eluted using 0.05 M sodium phosphate buffer, P^H 7.0 with a fraction volume of 2 ml. Four peak fractions of proteins were obtained, fraction-I, fraction-II and fraction -III. The Sephadex G-10 fraction-II (Fig. 1) containing protease inhibitor activity was pooled, concentrated and applied on to a Sephadex G-50 column. The proteins were eluted with a fraction volume of 2.0 ml at a flow rate of 12 ml/h. The protease inhibitor activity were eluted in a three fractions, fraction-I, II and III (Fig.2). All the three fractions containing protease inhibitor activity were further purified by RP-HPLC (Fig.3a, b &c). Three *A.pavonina* protease

inhibitors (APPI-1, APPI-2 and APPI-3) were purified. APPI-1, APPI-2 and APPI-3 was purified to 31.24, 36.02 and 19.21 fold with a recovery of 41.10%, 51% and 43.28%, respectively. Further, APPI-1, APPI-2 and APPI-3 showed a specific inhibitor activity of 16.15, 20.43 and 11.06, respectively.

The purified APPI-1, APPI-2 and APPI-3 showed a molecular weight of 7 - 8 kDa, 11 - 12 kDa and 13 - 14 kDa (approximately) as determined by gel filtration chromatography. A protease inhibitor with a molecular weight of 8 kDa was isolated and purified from the seeds of *Vigna mungo* [12]. Scarafoni et al. [13] purified a protease inhibitor consisted of a single polypeptide chain having a molecular weight of 6 kDa from *Lupinus albus* seeds. Similarly, protease inhibitor with a low molecular mass of 6.2 kDa was isolated from the seeds of *Opuntia sterptacantha* [14]. Diana Molina et al. [15] isolated, purified and characterized a protease inhibitor with a single polypeptide chain having a molecular weight of 12 kDa from the seeds of *Lupinus bogotensis*. The molecular weights of purified APPI-1, APPI-2 and APPI-3 are comparable with protease inhibitors isolated from the seeds of *Vigna mungo*, *Lupinus albus*, *Opuntia sterptacantha* and *Lupinus bogotensis* [7].

The effect of pH on the activities of APPI-1, APPI-2 and APPI-3 indicated that they were found to be stable in the pH range 3 - 9. Similarly, studies on the effect of temperature on the purified inhibitors showed that found to be stable in temperatures ranging from 4-65 °C. The pH and temperature stable protease inhibitors were isolated and characterized from the seeds of *Lupinus albus*, *mung bean*, *Opuntia sterptacantha* [13, 14, 16]. Anti-microbial activity of APPI-1, APPI-2 and APPI-3 was tested on *E.coli*, *Pseudomonas aerogenosa*, *streptococcus sps* and *Klebsella* using the disc method as described earlier. An inhibition zones was observed for *E.coli*, and *streptococcus*.

CONCLUSION

Three protease inhibitors from the seeds of *A.pavonina* have been purified using *salt* fractionation, gel-filtration chromatography and RP-HPLC. Purified inhibitors are pH and temperature stable and showed antimicrobial properties.

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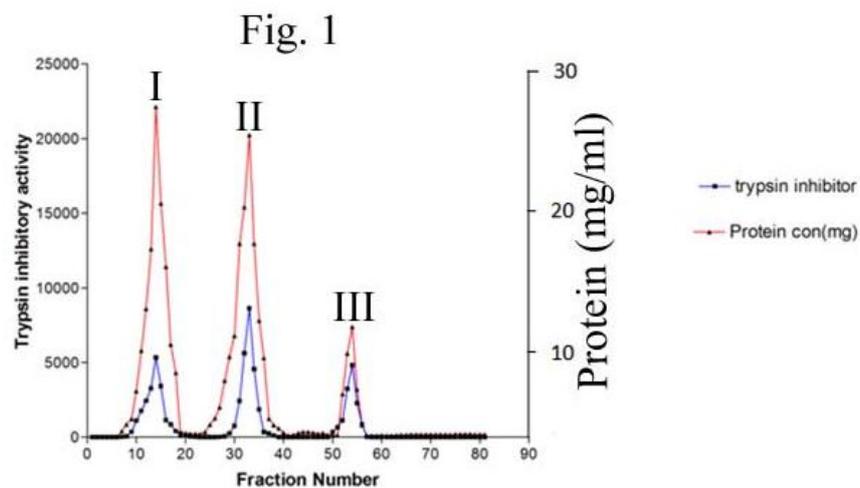


Fig. 1. Sephadex G-10 Chromatography of of Protease inhibitor activity from the seeds of *Adenantha pavonina*

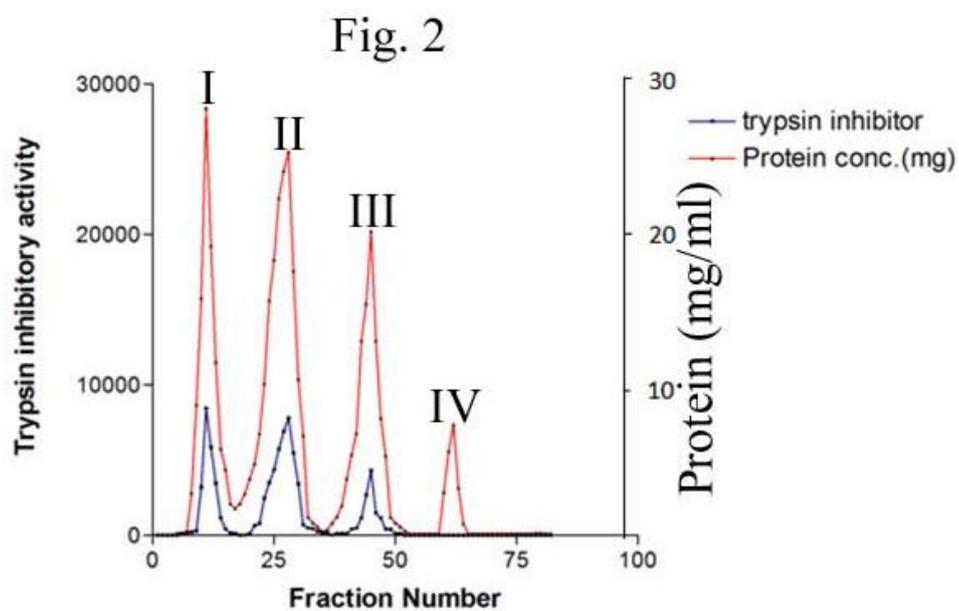


Fig. 2. Sephadex G-50 Chromatography of Protease inhibitor activity of fraction –II of Sephadex G-10 chromatography from the seeds of *Adenantha pavonina*

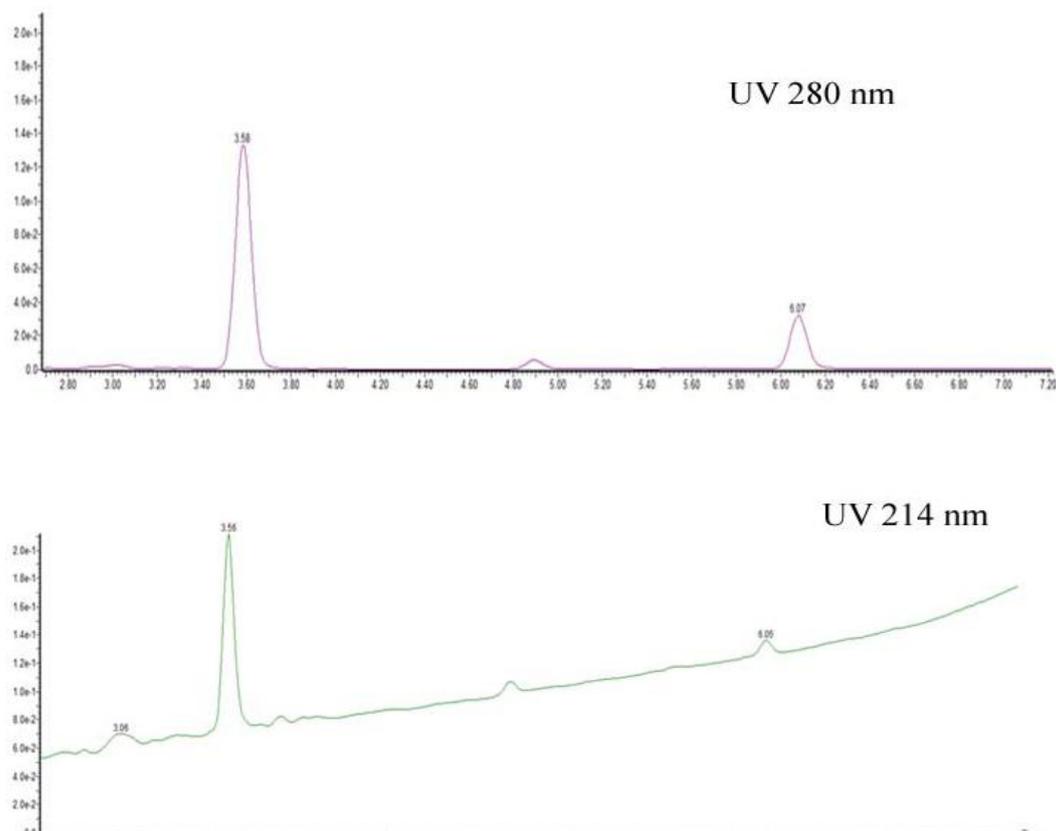


Fig.3a: RP-HPLC profile of Fraction –I of Sephadex G-50 trypsin inhibitor activity

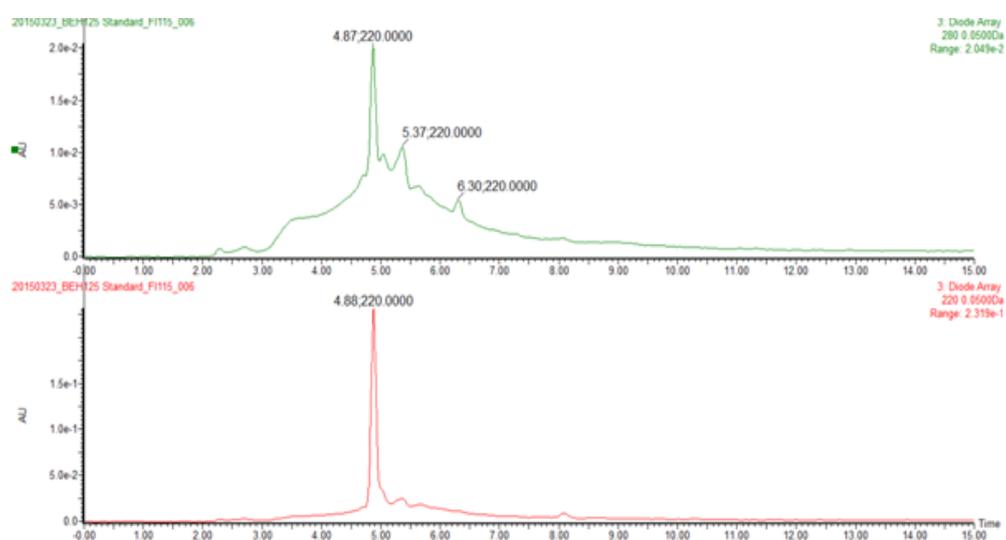


Fig.3b: RP-HPLC profile of Fraction –II of Sephadex G-50 trypsin inhibitor activity

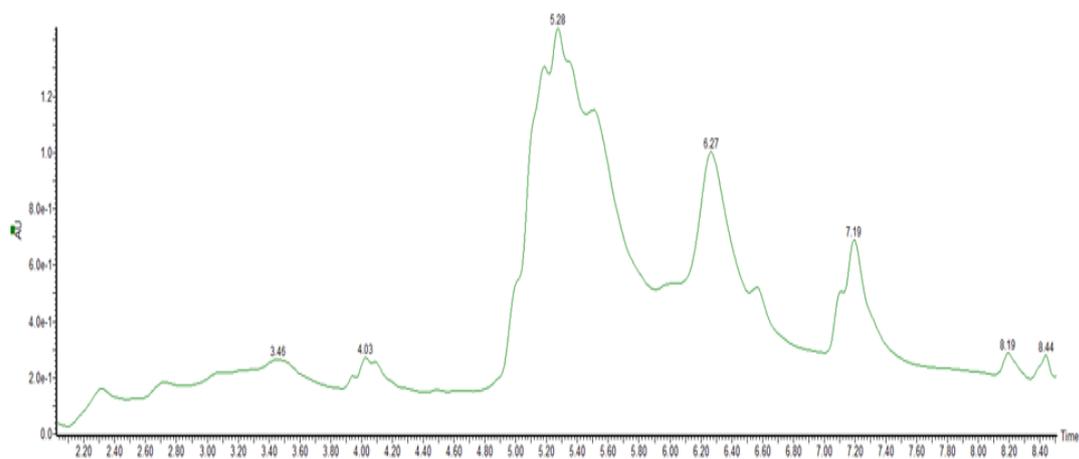


Fig.3c: RP-HPLC profile of Fraction –III of Sephadex G-50 trypsin inhibitor activity

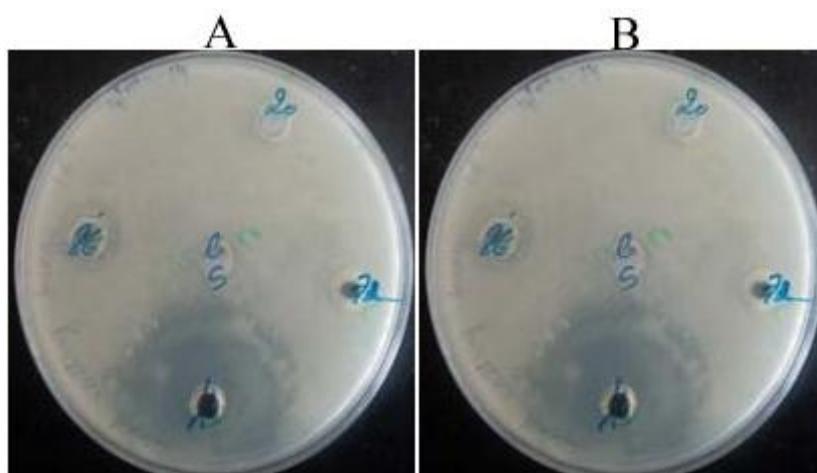


Fig.4. Antibacterial activity of Sephadex G-50 Trypsin inhibitor

A-Inhibition of Sephadex G-50 fraction against *Streptococcus*

B-Inhibition of Sephadex G-50 fraction against *E.coli*

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