

ANTIBACTERIAL ACTIVITY OF A TRYPSIN-CHYMOTRYPSIN-ELASTASE INHIBITOR ISOLATED FROM *LAVATERA CASHMERIANA* CAMB. SEEDS

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ABSTRACT

Protease inhibitors were extracted from the seeds of *Lavatera cashmeriana* Camb by ammonium sulphate precipitation and purified by chromatography on DEAE-cellulose and Sephadex G-100 column. The bound protein eluted into four major peaks which we named as LC-pi I, II, III and IV, all showing strong anti protease activity against trypsin, chymotrypsin and elastase. LC-pi I was screened for antibacterial activity against *Klebsiella pneumoniae*, *Escherichia coli* and *Pseudomonas aeruginosa*. The protease inhibitor showed strong antibacterial activity against *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* but was less active against *Escherichia coli*.

Key words: *Lavatera cashmeriana* Camb, antibacterial activity, LC-pi I, nutrient agar.

INTRODUCTION

The protease inhibitors (PIs), the natural antagonists of proteases, are small proteins which are quite common in nature. They have been isolated and characterized from a large number of organisms, including plants, animals and microorganisms (Fritz, 2000; Mosolov *et al.*, 2001; Valueva and Mosolov, 2004; Christeller and Wang 2005; Mosolov and Valueva, 2005; Macedo *et al.*, 2007; Lingaraju and Gowda, 2008). In plants they can be counted among the defensive mechanisms displayed against phytophagous insects and microorganisms (Huma and Khalid, 2007). The defensive capacities of plant PIs rely on inhibition of proteases present in insect guts or secreted by microorganisms, causing a reduction in the availability of amino acids necessary for their growth and development (De Leo *et al.*, 2002). Most PIs interact with their target proteases by contact with the active site of the protease, resulting in the formation of a stable protease-inhibitor complex that is incapable of enzymatic activity (Norton, 1991). Recently, the rapid emergence of microbial pathogens that are resistant to currently available antibiotics has triggered considerable interest in the isolation and investigation of the mode of action of antimicrobial proteins (Alasbahi and Melzig, 2008). Protease inhibitors from plants potentially inhibit the growth of a variety of pathogenic bacterial and fungal strains and are therefore excellent candidates for the development of novel antimicrobial agents.

MATERIALS AND METHODS

Mature seeds of *Lavatera cashmeriana* Camb. were procured from Department of Botany, University of Kashmir, J&K, India. Trypsin, chymotrypsin, elastase, N-

α -benzoyl-DL-arginine-p-nitroanilide (BAPNA), acetyl-L- tyrosine ethyl ester (ATEE), N-succinyl-L-(ala)₃ -p-nitroanilide, DEAE- cellulose and Sephadex G-100 were purchased from Sigma Aldrich Company, USA. Other chemicals were of analytical grade

Isolation of protease inhibitors: Twenty five grams dried seeds of *Lavatera cashmeriana* Camb. were soaked in 0.15N NaCl for 16 hours. The swollen seeds were homogenized with 300ml of ice cold saline Tris buffer (20mM, pH 8.0) containing 1mM sodium metabisulfite in a Remi auto mix blender at 4°C for 10 min. The homogenate was filtered through 4 layers of cheese cloth. The filtrate was centrifuged at 12,000g on a Remi R-24 cooling centrifuge for 20 min. From the supernatant, the proteins were precipitated by ammonium sulphate at 90% saturation. Crude extract was further purified by ion exchange chromatography on DEAE-cellulose column using 20mM Tris buffer, pH 8. The protein was eluted using linear sodium chloride gradient from 0.05 to 0.6M. The fractions eluted from ion-exchange column were chromatographed separately on Sephadex G-100 column in 0.1M Tris buffer, pH 8 and 0.4M NaCl.

Protein estimation: Protein concentration was determined by the method of Lowry *et al* (1951) using BSA as the standard protein.

Measurement of antitryptic activity: The antitryptic activity of inhibitors was estimated by measuring the % inhibition activity of trypsin, using synthetic substrate benzoyl-DL-arginine-p-nitroanilide (Lin *et al.*, 1991).

Measurement of antichymotryptic activity: Inhibition of the activity of chymotrypsin was determined on acetyl-L- tyrosine ethyl ester using the method of Birk *et al.*, (1985).

Measurement of antielastase activity: The activity

evaluation for the elastase inhibition of the protein was determined according to a modified continuous assay method of Bieth *et al.*, (1974) using N-succinyl-L-(ala)₃ – p-nitroanalide as substrate.

Measurement of antibacterial Activity: The antibacterial activity of purified protease inhibitor was determined by agar well diffusion method as described by Perez *et al.*, (1990) against *Klebsiella pneumoniae*, *E.coli* and *Pseudomonas aeruginosa*. Nine gms of nutrient agar was dissolved in 250ml of double distilled water and shaken smoothly. Mixture was boiled on hot plate taking keen notice that it should not char at bottom. After preparation, the media was dispensed into the boiling tubes (20ml in each). Tubes were plugged with cotton and autoclaved for 25 mins at 15lb pressure. Tubes were kept into refrigerator for further use after cooling them.

The agar well diffusion method was performed on LC-pi I. The bacterial colony was picked from culture slants by an inoculating loop and was passed into the agar pour. Neck of tube was flamed and contents were poured into the petri plate. The petri plate was rotated gently so that medium becomes evenly distributed. The inoculated plates were left to dry for at least 5-10 minutes, after which a well was formed on plates using sterile borer. The wells were loaded by sample (5%w/v) and antibiotic ceftriaxone (1%w/v) as standard. Volume accommodated in one well was 100µl. Loaded plates were kept as such for some time under laminar hood. The plates were then incubated at 37°C for 24 hrs in an incubator. Diameter of zone of inhibition around each well was measured.

RESULTS AND DISCUSSION

Protease inhibitors were extracted from the seeds of *Lavatera cashmeriana* Camb by ammonium sulphate precipitation. Purification of crude extract was achieved by ion exchange chromatography and gel filtration. Ion exchange chromatography was performed on DEAE-Cellulose column, equilibrated with 20mM Tris buffer, pH 8.0. The bound protein was eluted using a linear sodium chloride gradient from 0.05 to 0.6 M of 20mM Tris buffer, pH 8.0 in 5ml fractions at a flow rate of 30ml/hr. The bound protein eluted into many small peaks and four major peaks from the column. The fractions under minor peaks didn't show any anti protease activity whereas fractions under peak I, II, III and IV showed anti protease activities against trypsin, chymotrypsin and elastase. The fractions under each peak were pooled and concentrated. The individual peaks were labeled as LC-pi I, LC-pi II, LC-pi III & LC-pi IV and further chromatographed onto Sephadex G-100 column. All the four peaks resolved into single protein peak.

The antimicrobial activity of purified LC-pi I was tested against three bacterial strains viz *Klebsiella pneumoniae*, *E.coli* and *Pseudomonas aeruginosa* by agar

well diffusion method. Ceftriaxone was used as the standard for comparison. After incubation at 37°C for 24h the diameters of the inhibition zones were measured (Table 1). As shown in Fig. 1, LC-pi I strongly inhibited the growth of *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* but was less active against *E. coli*.

Protease inhibitors in plant tubers and seeds are generally thought to serve as storage proteins and to act in the defense against insects and microorganisms (Huma and Khalid, 2007). They are also known to possess potent antibiotic activity against bacteria, fungi, and even certain viruses (Alasbahi and Melzig, 2008; Kim *et al.*, 2009; Satheesh and Murugan, 2011). For instance, a trypsin inhibitor from wheat kernel also elicits a potent antifungal effect (Chilosi *et al.*, 2000). Another Kunitz type protease inhibitor from *Prosopis juliflora* with activity against papain, trypsin and chymotrypsin exerts insecticide effects against *C. maculatus* larvae by blocking the affected enzymes in the insect's digestive system (Macedo *et al.*, 2004). Protease inhibitors also have recently attracted attention because of their potent anti-carcinogenic effect in various in vivo and in vitro systems (Jedinak and 2005; Gills *et al.*, 2007).

Several nontoxic protease inhibitors, mostly of bacterial or plant origin (e.g., from barley seeds, cabbage leaves and Streptomyces), have been purified and are now commercially available for use in preventing protease-induced peri-anal dermatitis (Ruseler-van Embden *et al.*, 2004). A 5.6KDa trypsin-cymotrypsin protease inhibitor isolated from the tubers of potato has been found to possess a strong antimicrobial activity against many pathogenic microbial strains (Kim *et al.*, 2005). Satheesh and Murugan (2011) have reported the isolation of a protease inhibitor from the leaves of *Coccinia grandis* (L.) Voigt. The PI has been found to exhibit marked growth inhibitory effects on colon cell lines and pathogenic microbial strains, including *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Proteus vulgaris*, *Escherichia coli*, *Bacillus subtilis* and pathogenic fungus *Candida albicans*, *Mucor indicus*, *Penicillium notatum*, *Aspergillus flavus* and *Cryptococcus neoformans* (Satheesh and Murugan, 2011). In the present study, we purified the protease inhibitors from *Lavatera cashmeriana* Camb seeds and examined the effect on growth of *Klebsiella pneumoniae*, *E.coli* and *Pseudomonas aeruginosa*. The study showed that LC-pi I exhibited strong antimicrobial effects on *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, which are gram negative bacteria and cause many human infections like urinary tract infections, pneumonia and septicemia. However, the antimicrobial activity against *E.coli* was not very significant. Therefore, LC-pi I, a potent protease inhibitor may have the potential to serve a lead compound for the development of novel antimicrobial drug.

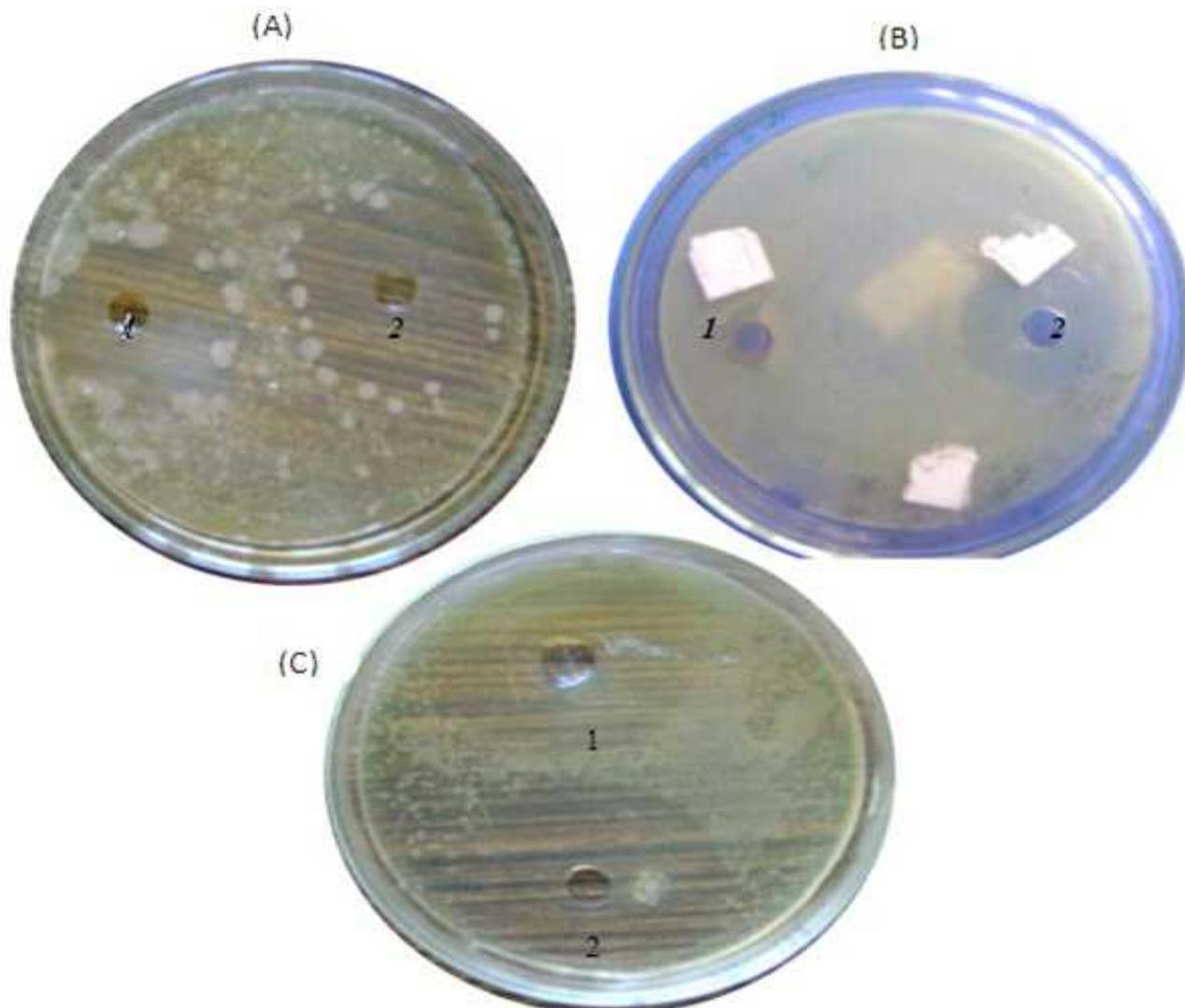


Fig. 1: Antibacterial assay of LC-pi I with three bacterial strains (A) *Klebsiella pneumoniae*, (B) *E.coli*, (C) *Pseudomonas aeruginosa*. Ceftriaxone was used as standard. 1 represents LC-pi I and 2 represents standard

Table 1: Antibacterial activity of LC-pi I

S. No	Strains of microorganisms	Diameter of zone of inhibition(mm)	
		LC-pi I	Standard
1	<i>Klebsiella pneumoniae</i>	10	16
2	<i>E.coli</i>	3	8
3	<i>Pseudomonas aeruginosa</i>	12	16

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