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Research article

Antibacterial serine protease from *Wrightia tinctoria*: Purification and characterization

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ABSTRACT

A serine protease was purified from the leaves of *Wrightia tinctoria* by sequential flow through method comprising screening, optimization, ammonium sulfate precipitation, gel filtration and ion exchange column chromatography. The yield and purification fold obtained were 11.58% and 9.56 respectively. A single band of serine protease was visualized on SDS-PAGE and 2-D gel electrophoretic analyses were revealed with the molecular mass of 38.5 kDa. Serine protease had an optimum pH of 8.0 and was stable at 45°C with high relative protease activity. The addition of metal ions such as Mg2+ and Mn2+ exhibits a high relative activity. Serine protease had a potent antibacterial activity against both Gram-positive and Gram-negative bacteria. A 10 μg/ml of serine protease was tested against S. aureus, M. luteus, P. aeruginosa and K. pneumoniae which had 21, 20, 18 and 17 mm of zone of inhibition respectively. Serine protease from *W. tinctoria* degrades the peptidoglycan layer of bacteria which was visualized by transmission electron microscopic analysis.

1. Introduction

In the latest two decades, the interest received by plant proteases and its natural products has been on the rise. So the number of industrially employed enzymes of plant origin is still less but growing fast. In this respect, proteases are the most commercially important enzymes due to their multiple uses in the food, pharmaceutical and detergent industries, also for the preparation of leather and wool, among others (Feijoo-Siota and Villa, 2011). Commercial proteolytic enzymes are used in the processes such as brewing, tendernization of meat and dairy processing (Marques et al., 2010). Proteases mainly hydrolyze the peptide bonds. They can act near the ends of polypeptide chains (exopeptidases) or within them (endopeptidases) (Palma et al., 2002). They are differentiated according to their substrate specificity as aminopeptidases, which are cleaves the peptides at the N-terminus, and carboxypeptidases, degrades peptides at the C-terminus. Proteases are classified according to their catalytic mechanism, which acts specifically in the enzyme active site. The MEROPS Database considers seven families of proteases: Aspartic, Cysteine, Glutamic, Metallo, Asparagine, Serine and Threonine respectively. In plants, five classes of endoproteases have been described: serine, cysteine, aspartic, metallo and threonine protease (Ako and Nip, 2007).

Proteases derived from plants find a promising use in food and biotechnological industries due to their broad substrate specificity, high stability in extreme conditions, good solubility, and activity over a wide range of pH and temperature. In plants, proteases are involved in almost all aspects of the life cycle ranging from the assembling of storage proteins during seed germination to the initiation of cell death and senescence programs (Schaller, 2004). Different parts of plants are valuable reservoirs for biomolecules and proteases from plant leaves represent an important segment of them. Members of several plant families such as, Apocynaceae, Euphorbiaceae and Caricaceae Moraceae exude leaves on healing wounding and skin related diseases, which constitutes organic and inorganic compounds and enzymes mainly proteases. These extracellular proteases play a defensive role for plants against herbivores, insects, and pests (Konno et al., 2004; Rajesh et al., 2007). *Wrightia tinctoria* is a small, deciduous medicinal plant widely used by folk medicine practitioners and tribal communities in various districts of Central and Southern states of India, for its

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anti-inflammatory antinociceptive immunomodulatory, anti-hemorrhagic, hepatoprotective, antipsoriatic and antidiarrheal properties (Tetali et al., 2009). Besides, protease from leaves could be a potential source of proteases due to ease of purification methods, low levels of interfering substances during purification resulting in good yield of proteases.

Serine proteases were once thought to be rare in plants; in recent years, however, several proteases have been isolated, characterized and purified from various plant species in which they occur in distinct parts, ranging from the leaves to the s, latex (Liggieri et al., 2009) and the fruits (Chauhan et al., 2013). Serine proteases use the active site Ser as a nucleophile. The catalytic mechanism is very similar to that of cysteine proteases, and some serine proteases are even evolutionarily related to cysteine proteases (Srinivasan et al., 2006).

In the present investigation, screening of leaf extracts of 20 different plant species for protease activity was evaluated by protease plate assay method. The extract obtained from the leaves of *W. tinctoria* showed relatively higher protease activity than the extract of protease obtained from other plants. Therefore, attempts have been made to isolate, optimize, purify and characterize the protease from the extract of *W. tinctoria*. The protein was purified using ammonium sulphate fractionation, Sephadex G-100 and CM-Cellulose cation exchange column chromatography techniques to its homogeneity. The purity of the protein was verified by SDS-PAGE and intact mass spectrum analyses. The antibacterial activity of the purified protease against several human pathogenic bacteria belonging to Gram-positive and Gram-negative groups has also been evaluated. The effect of Sp on the structural destabilization of bacterial membrane has been analyzed by transmission electron microscopy.

2. Materials and methods

2.1. Materials

Acetonitrile, Acrylamide, Ammonium bicarbonate, Ammonium sulphate, Azocasein, Bovine serum albumin, Casein, CM-Cellulose, Coomassie brilliant blue G/R-250, Dialysis sacks, Diisopropyl fluorophosphate, 5 5′-Dithiobis-2-nitrobenzoic acid, Dithiothretiol, L-trans-epoxysuccinylleucylamide (4-guanidino) butane-N- [N- (L-3-transcarboxyirane-2-carbonyl) -L-leucyl]agimatine, Ethylenediaminetetraacetic acid, Ethylene glycol-bis (2-aminoethyl ether)-N,N,N′,N'-tetraacetic acid (EGTA), Folin Ciocalteu's phenol reagent, Gelatin, Glycerol, Hide powder azure, Iodoacetic acid, Leupeptin, N,N-methylene bis-acrylamide, N-ethylmaleimide, *o*-phenanthroline, Phenylmethansulfonyl fluoride, Polyvinyl polypyrrolidone, Soyabean trypsin inhibitor, Sephadex G-100, Sodium tetrathionate, Trichloroacetic acid, Trifluoroacetic acid and β-mercaptoethanol were obtained from Sigma Chemical Co, USA. Mueller-Hinton agar and broth, 2-Propanol, Benzene, Dimethyl sulphoxide, Ethanol, Hexane, Methanol, Toluene and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide were from Himedia, India. All other chemicals were of the high purity commercially available.

2.2. Collection of plant samples and microorganisms

Healthy and fresh leaves of plant species were collected from University of Madras, Guindy campus, Chennai, Tamilnadu, India. They were washed thoroughly and blotted on sterile filter paper. The samples were ground with appropriate buffer and estimated for protein and its protease assay. Their antimicrobial potentials were tested against Gram positive bacteria *Staphylococcus aureus* MTCC 3160, *Micrococcus luteus* MTCC 106 and Gram negative bacteria which includes *Pseudomonas aeruginosa* MTCC 741 and *Klebsiella pneumoniae* MTCC 3040 and they were obtained from the Microbial Type Culture Collection, Institute of Microbial Technology, Chandigarh, India. They were subcultured with every 2 weeks of interval on agar media and kept at 4 °C. Before using them for experiments, the cultures were propagated twice in liquid media overnight.

2.3. Crude extract preparation

Apparently healthy leaves of plant species were washed several times with distilled water, blotted and ground in pre-cooled mortar and pestle with acid washed sand at 4 °C. Cell free homogenates were obtained by using phosphate buffer (50 mM; pH 7.0) containing 0.1% (w/v) insoluble PVPP (Sigma Chemical Co, USA) filtering through four layers of cheese cloth and the filtrate was centrifuged at 12000 × g for 15 min at 4 °C. The supernatant was used as enzyme source.

2.4. Optimization studies of crude protease activity

2.4.1. Crude protease activity in different buffer system

The selected plant which had highest protease activity was *W. tinctoria*. The leaves of *W. tinctoria* were taken i.e 1.0 g in 3.0 ml of different buffer systems Glycine-HCL buffer (50 mM; pH 2.0, 2.5 and 3.0), Citrate phosphate buffer (50 mM; pH 3.0, 4.0 and 4.5), Sodium acetate buffer (50 mM; pH 5.0, 5.5), Sodium phosphate (50 mM; pH 6.0, 6.5 and 7.0), Tris-HCl buffer (50 mM; pH 7.5, 8.0, 8.5) and Glycine-NaOH buffer (50 mM; pH 9.0 and 10.0) and ground in the above mentioned buffers. The extracts were centrifuged at 12000 × g for 20 min at 4 °C and then the supernatant was estimated for protein and tested for protease activity. The activities were recorded and the buffer systems which had highest protease activity were used for further analysis.

2.4.2. Crude protease activity in different millimolar concentration

The selected buffer which showed highest protease activity was Tris-HCl (pH 7.5). About 1.0 g of *W. tinctoria* leaves in 3.0 ml of different millimolar concentrations (10, 20, 30, 40, 50, 100, 150, 200, 300, 400, 500, 600, 700, 800, 900 and 1000 mM) were taken and ground in the above mentioned millimolar concentrations. The extracts were centrifuged at 12000 × g for 20 min at 4 °C and then the supernatant were estimated for protein and protease activity. The buffer systems which showed highest protease activity were used for further analysis.

2.4.3. Crude protease activity in different temperature

The selected mill molar concentration buffer which showed highest protease activity was Tris-HCL (50 mM; pH 7.5). Approximately 1.0 g of *W. tinctoria* leaves in 3.0 ml of Tris-HCL (50 mM; pH 7.5) were taken and subjected to different temperatures (−40, −20, 0, 4, 10, 25, 50 and 75 °C) and ground in the above mentioned temperatures. The extracts were centrifuged at 12000 × g for 20 min at 4 °C and then the supernatant was estimated for protease activity by plate assy. The optimum temperature which showed highest protease activity was used for further analysis.

2.5. Crude protease activity in different ammonium sulphate fractionation

The leaves of *W. tinctoria* were ground in the optimized condition (i,e Tris-HCl; pH, 7.5; 50 mM at 4 °C) homogenized and the crude protease was centrifuged at 12000 × g for 30 min at 4 °C. Ammonium sulphate was added to the supernatant which were subjected from 0 to 20, 20–40, 40–60, 60–80, 80–100% (w/v)