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Purification and characterization of a trypsin inhibitor from *Putranjiva roxburghii* seeds

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ABSTRACT

A highly stable and potent trypsin inhibitor was purified to homogeneity from the seeds of *Putranjiva roxburghii* belonging to *Euphorbiaceae* family by acid precipitation, cation-exchange and anion-exchange chromatography. SDS–PAGE analysis, under reducing condition, showed that protein consists of a single polypeptide chain with molecular mass of approximately 34 kDa. The purified inhibitor inhibited bovine trypsin in 1:1 molar ratio. Kinetic studies showed that the protein is a competitive inhibitor with an equilibrium dissociation constant of 1.4×10^{-11} M. The inhibitor retained the inhibitory activity over a broad range of pH (pH 2–12), temperature (20–80 °C) and in DTT (up to100 mM). The complete loss of inhibitory activity was observed above 90 °C. CD studies, at increasing temperatures, demonstrated the structural stability of inhibitor at high temperatures. The polypeptide backbone folding was retained up to 80 °C. The CD spectra of inhibitor at room temperature exhibited an α, β pattern. N-terminal amino acid sequence of 10 residues did not show any similarities to known serine proteinase inhibitors, however, two peptides obtained by internal partial sequencing showed significant resemblance to Kunitz-type inhibitors.

1. Introduction

Plant proteinase inhibitors are widely distributed in plant seeds and are most studied class of inhibitors (Richardson, 1991; Mello and Silva-Filho, 2002). The molecular mass of these inhibitors can vary from 4 to 85 kDa, with majority in the range of 8–20 kDa (Hung et al., 2003). In higher plants, several gene families of these protease inhibitors have been characterized. The plant serine proteinase inhibitors, particularly trypsin inhibitors, have been extensively studied (Laskowski and Kato, 1980; Barrett and Salvesen, 1986). They play an important role as plant defense agent against insects and pests attack (Sampaio et al., 1996; Shewry and Lucas, 1997; Walker et al., 1997; Franco et al., 2003) and are known to be involved in many biological functions, such as blood coagulation, platelet aggregation and anti-carcinogenesis (Kennedy, 1998; Oliva et al., 2000). Plant protease inhibitors have been described as endogenous regulators of proteolytic activity (Ryan, 1990; Kato, 2002) and as storage proteins (Xavier-Filho, 1992). These inhibitors have been grouped mainly into Kunitz, Bowman-Birk, Potato I and II and squash, cereal superfamily and thaumatin-like inhibitors (Richardson, 1991). Most serine proteinase inhibitors from seeds have been isolated and characterized from *Leguminosae*, *Cucurbitaceae*, *Solanaceae* and *Graminae* families (Garcia-Olmedo et al., 1987). There are not many reports of purification and characterization of these inhibitors from other plant families. Other families where an inhibitor has been purified and characterized include *Rutaceae* (Shee and Sharma, 2007; Shee et al., 2007a,b) and *Euphorbiaceae* (Sritanyarat et al. 2006) families.

Putranjiva roxburghii belonging to *Euphorbiaceae* family is an ornamental tree of tropical India known as child life tree. Roxburghonic keto acid and some flavonoids, terpenoids and triterpines has been purified and characterized from the leaf and trunk bark of this plant (Garg and Mitra, 1968, 1971a,b; Sengupta et al., 1967). To date, no protein has been characterized from this plant. This paper describes the purification and characterization of a highly stable and potent trypsin inhibitor from the seeds of *P. roxburghii*.

2. Results and discussion

2.1. Purification of *P. roxburghii* trypsin inhibitor (PRTI)

P. roxburghii trypsin inhibitor (PRTI) was purified to homogeneity in three steps by acid precipitation, CM-sepharose cation

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exchange and DEAE-sepharose anion exchange chromatography. In acid precipitation step, low molecular mass proteins were precipitated along with some other proteins. The trypsin inhibitory activity was retained in supernatant. This method has been earlier used for the purification of *Brassica nigra* seed trypsin and subtilisin inhibitor (Genov et al., 1997). In second step on CM-sepharose column, all the low molecular mass proteins were bound to the column while trypsin inhibitory activity was found in flow through. This resulted in complete removal of major low molecular mass protein. In last step, protein with trypsin inhibitory activity was bound to a DEAE-sepharose column. After washing the column extensively, bound proteins were eluted with step gradient of NaCl. The fractions with trypsin inhibitory activity were eluted at 50 and 100 mM NaCl. The purity of the protein in above fractions was analyzed by SDS–PAGE. The fraction eluted at 100 mM NaCl showed single band on SDS–PAGE (Fig. 1a). The protein was further subjected to size exclusion chromatography column on HPLC where it showed a single peak with a retention time of 8.2 min in 50 mM Tris–HCl buffer, pH 8.0 (Fig. 1b). The SDS–PAGE analysis under both reducing (Fig. 1b, inset) and non-reducing (data not shown) conditions showed that PRTI is a single polypeptide chain with a molecular mass of approximately 34 kDa. Interestingly, the molecular mass of PRTI is significantly higher than the typical Kunitz-type inhibitor (20 kDa). Earlier, trypsin inhibitors of 32.5, 33 and 43.5 kDa has been reported from *Crotalaria pallida* seeds (Gomes et al., 2005), *Ipomoea batatas* (sweet potato) root (Hou et al., 2001) and *Avena sativa* L. (Mikola and Kirsi, 1972), respectively.

2.2. N-terminal and partial internal sequencing

In N-terminal sequencing of PRTI, first 10 residues from the N-terminal were obtained. The sequence determined was Arg-Pro-Pro-Gln-Ala-Gly-Tyr-Ile-Gly-Val. The N-terminal sequence of PRTI showed no similarities with any of the known trypsin inhibitors. In partial internal sequencing, sequences of four peptides were obtained. In separate experiments, one peptide (peptide 1) was obtained from LC-MS/MS and three peptides from MALDI-MS/MS studies. The peptide sequenced by LC-MS/MS analysis showed only one match when searched against FASTA database within BioWorks 3.2 program (Thermo Fischer scientific). It showed 100% identity to winged bean chymotrypsin inhibitor-3 (Shibata et al., 1988) (Fig. 2). All the peptides obtained by MALDI-MS/MS analysis showed low score in MASCOT search. Among three peptides only one peptide (peptide 2) with MASCOT score of 23 showed 100, 73 and 73% identity to *Acasia confussa* trypsin inhibitor (Wu and Lin, 1993), *Prosopsis juliflora* trypsin inhibitor (Negreiros et al., 1991) and trypsin isoinhibitors DE5 of *Adenanthera pavonina* L. (Richardson et al., 1986), respectively (Fig. 2). Although the MASCOT score of the peptide 2 is lower than the significant value, it has been included here as no information is available about this

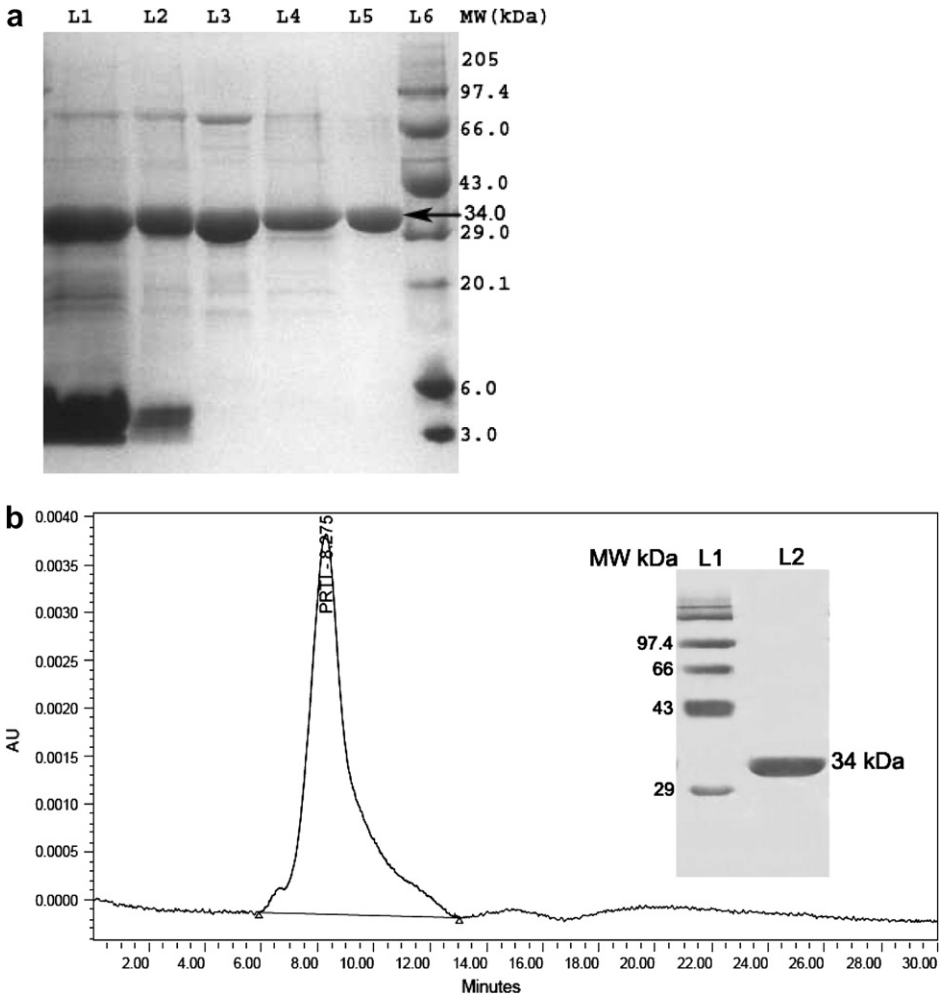

Fig. 1. (a) SDS–PAGE analysis of the protein. L1, crude extract; L2, supernatant after acid precipitation step; L3, CM flow-through; L4, 50 mM NaCl fraction after anion exchange column; L5, purified protein in 100 mM NaCl fraction after anion exchange chromatography; L6, molecular weight markers. (b) Elution profile of purified protein on HPLC gel-filtration column. Insert: SDS–PAGE analysis of HPLC purified PRTI.