Plant Physiology and Biochemistry 83 (2014) 77–87

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Structural and functional characterization of proteinase inhibitors from seeds of *Cajanus cajan* (cv. ICP 7118)

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ARTICLE INFO

Article history:
Received 28 February 2014
Accepted 11 July 2014
Available online 21 July 2014

Keywords:
Achaea janata
Activity staining
BBI
Castor
Isoinhibitors
MALDI-TOF/TOF
Two-dimensional (2-D) gel electrophoresis

ABSTRACT

Proteinase inhibitors (C11PI) from mature dry seeds of *Cajanus cajan* (cv. ICP 7118) were purified by chromatography which resulted in 87-fold purification and 7.9% yield. SDS-PAGE, matrix assisted laser desorption ionization time-of-flight (MALDI-TOF/TOF) mass spectrum and two-dimensional (2-D) gel electrophoresis together resolved that C11PI possessed molecular mass of 8385.682 Da and existed as isoinhibitors. However, several of these isoinhibitors exhibited self association tendency to form small oligomers. All the isoinhibitors resolved in Native-PAGE and 2-D gel electrophoresis showed inhibitory activity against bovine pancreatic trypsin and chymotrypsin as well as *Achaea janata* midgut trypsin-like proteases (AjPs), a devastating pest of castor plant. Partial sequences of isoinhibitor (pI 6.0) obtained from MALDI-TOF/TOF analysis and N-terminal sequencing showed 100% homology to Bowman-Birk Inhibitors (BBIs) of leguminous plants. C11PI showed non-competitive inhibition against trypsin and chymotrypsin. A marginal loss (<15%) in C11PI activity against trypsin at 80 °C and basic pH (12.0) was associated with concurrent changes in its far-UV CD spectra. Further, *in vitro* assays demonstrated that C11PI possessed significant inhibitory potential (IC_{50} of 78 ng) against AjPs. On the other hand, *in vivo* leaf coating assays demonstrated that C11PI caused significant mortality rate with concomitant reduction in body weight of both larvae and pupae, prolonged the duration of transition from larva to pupa along with formation of abnormal larval-pupal and pupal-adult intermediates. Being smaller peptides, it is possible to express C11PI in castor to protect them against its devastating pest *A. janata*.

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1. Introduction

The castor oil plant, *Ricinus communis*, is a plant species of the Euphorbiaceae family. Castor seed is the source of castor oil which has a wide variety of uses. The seeds contain between 40–60% of oil that is rich in triglycerides, mainly ricinolein. Castor seed oil has special chemical and physical properties. Its bio-degradable and eco-friendly nature makes it a vital industrial raw material for more than 700 industrial products, including high quality lubricants, paints, coatings, plastics, soaps, medications for skin infections and cosmetics (Ogunniyi, 2006). The recent application of castor oil is its use as biofuel for the production of biodiesel with reduced sulfur emission. Further, traditional ayurvedic medicine considered castor oil as the king of medicinals for curing arthritic diseases (Kalaiselvi et al., 2003). It has many therapeutical uses including anti-inflammatory and free radical scavenging activity (Ilavarasan et al., 2006; Saini et al., 2010), anti-diabetic effect (Rao et al., 2010) and hepato-protective activity (Visen et al., 1992).

Among the pests that damage the castor field, *Achaea janata* (castor semilooper) is a major feeder which causes about 30–70% loss in its production. Several recent studies indicated that among pest management methods used for crop protection, development of insect resistance by incorporating genes that express proteins with insecticidal activity is a novel approach (Dunse et al., 2010;

Abbreviations: AjPs, *Achaea janata* midgut trypsin-like proteases; AjPIs, *Achaea janata* midgut trypsin-like proteinase inhibitors; BBIs, Bowman-Birk inhibitors; IEF, isoelectric focusing; MALDI-TOF/TOF MS, matrix assisted laser desorption ionization time-of-flight mass spectrometry; PIs, proteinase inhibitors.

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http://dx.doi.org/10.1016/j.plaphy.2014.07.009
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Smigocki et al., 2013). It is therefore important to select appropriate candidate genes such as proteinase inhibitors (PIs), lectins and amylase inhibitors for expression in transgenic plants to strengthen the plant defense against the target pest in a sustainable manner (Foissac et al., 2000). Among them, PIs which are present in storage organs like seeds and tubers are promising candidates that confer resistance against insect pests (Benjakul et al., 2000). In plants, serine PIs are well documented class of inhibitors and are ubiquitous in nature (Haq et al., 2003). However, most lepidopteran pests like *A. janata*, *Helicoverpa armigera* and *Spodoptera litura* principally depend on serine proteases for digestion of proteins consumed through food (Chikate et al., 2013; Srinivasan et al., 2006; Telang et al., 2003; Budatha et al., 2008). When ingested by larvae, PIs inhibit digestive proteases leading to starvation of the insect for essential amino acids (Giri et al., 2004). The insects respond to this situation by overproducing gut proteases to compensate for the inhibited activity of digestive proteases. But, synthesis of additional proteases further deplete the pool of essential amino acids and result in developmental abnormalities and growth retardation (Oliva et al., 2010). Further, during the process of this co-evolution between plants and insects, adaptation of insects against plant PIs is the main limitation for the PI based defense strategy. Insect herbivores have developed multiple adaptive mechanisms viz. (i) over expression of PI-sensitive enzymes (De Leo et al., 1998); (ii) proteolytic cleavage of PIs by insect proteases (Yang et al., 2009); (iii) expression of proteases that are insensitive to inhibition by PIs (Brito et al., 2001). However, to combat this multifaceted adaptive mechanism by the insects, the identification of potential PIs which could target insect's digestive proteases from non-host plants is essential and would definitely make the insect pests adaptation more difficult (Lopes et al., 2004).

Thus, during this current scenario of host-pest co-evolution, pigeonpea (*Cajanus cajan*), a crop plant of the order Fabales, family Leguminosae has been reported as the potential source of (PIs) against wide varieties of lepidopteran larvae (Chougule et al., 2003; Lomate and Hivrale, 2011; Padul et al., 2012; Parde et al., 2012; Prasad et al., 2010a, 2010b, 2009). Earlier reports suggested that different varieties of *C. cajan* possessed PIs resistant to proteases of *A. janata* (Prasad et al., 2010a, 2009). The cultivars ICP 14770 and ICP 7118 of *C. cajan* were found to be the best non-host resource of potential PIs effective against gut proteases of *A. janata* (Prasad et al., 2010a, 2009; Swathi et al., 2012). The PIs from ICP 14770 were found to belong to BBI family (Prasad et al., 2010b). However, some of the earlier reports indicated the existence of Kunitz inhibitors in *C. cajan* (Haq and Khan, 2003). Therefore, in the present study, we purified the PIs from ICP 7118 and, characterized structurally and functionally to reconfirm that the PIs which were effective in controlling *A. janata* belonged to BBI family.

2. Methods

2.1. Seed material and chemicals

Seeds of *C. cajan* (cv. ICP 7118 or C11) were obtained from International Crops Research Institute for Semi-Arid Tropics (ICRISAT). *A. janata* insects were obtained from Directorate of Oil seeds Research (DOR), Hyderabad, India. Bovine serum albumin (BSA), bovine pancreatic trypsin and α-chymotrypsin were procured from Sisco Research Laboratory (Mumbai, India). DEAE-cellulose, cyanogen bromide-activated-Sepharose 4B, Sephadex G-50, *N*-α-benzoyl-DL-arginine-*p*-nitroanilide (BAPNA), *N*-glutaryl-L-phenylalanine-*p*-nitroanilide (GLUPHEPA), BBI, sorbitol, tricine, gelatin and Coomassie Brilliant Blue (CBB) R-250 were purchased from Sigma (St. Louis, MO). Immobiline dry strips (pH 4–7 linear, 11 cm), IPG buffer (4–7 linear), dithiothreitol (DTT) and iodoacetamide (IDA) were procured from GE Healthcare Bio-Sciences AB (Uppsala, Sweden). Bicinchoninic acid (BCA) protein estimation kit was purchased from Thermo Scientific (USA). Amicon filters (3 kDa cut-off) were purchased from Millipore Corporation, USA. All other chemicals and reagents used were of analytical grade.

2.2. Crude protein preparation and purification of C11PI

The seed powder prepared from mature dry seeds was extracted in to 50 mM Tris–HCl, pH 8.0 containing 1% polyvinylpyrrolidone in 1:6 (w/v) ratio under mild continuous stirring for overnight at 4 °C as described in Prasad et al. (2009). The clear supernatant obtained after centrifuging twice at 10,000 rpm for 20 min (4 °C) was subjected to 0–25%, 25–75% and 75–100% $(NH_4)_2SO_4$ fractionation for 1 h (4 °C). The corresponding precipitates were dialyzed (3.0 kDa cut-off) against 50 mM Tris–HCl pH 8.0 and estimated for protein (Smith et al., 1985) as well as trypsin inhibitor (TI) activity. The 25–75% $(NH_4)_2SO_4$ fraction with maximum TI activity was purified by passing sequentially through DEAE-cellulose column, cyanogen bromide-activated-trypsin Sepharose 4B column and Sephadex G-50 column using AKTA prime plus (GE healthcare) fast protein liquid chromatography (FPLC) system. The eluted fractions (1.0 ml) from different chromatography columns were analyzed for total protein (A_{280}) and TI activity. The protein fractions showing significant TI activity were pooled, dialyzed, concentrated using Freeze dryer (Labconco)/Amicon filters (3.0 kDa cut-off) and stored as "C11PI" at −20 °C for further use.

2.3. Proteinase inhibitor assay and determination of inhibitor constant (K_i)

The inhibitory activity of C11PI was evaluated against pancreatic trypsin/chymotrypsin/*A. janata* midgut trypsin-like proteases (AjPs), respectively, as described in Prasad et al. (2010b). After addition of respective proteases, the assay mixture was incubated for 15 min at 37 °C. The residual protease activity was determined after 45 min incubation at 37 °C with 1 mM BAPNA (Erlanger et al., 1961) or GLUPHEPA (Mueller and Weder, 1989) and the reaction was terminated with 30% acetic acid (v/v). One TI/chymotrypsin inhibitor (CI)/*A. janata* midgut trypsin-like proteinase inhibitor (AjPI) unit was defined as the amount of C11PI required to inhibit 50% of the BAPNA or GLUPHEPA hydrolysis by trypsin/AjPs and chymotrypsin, respectively. The K_i values of C11PI against trypsin and chymotrypsin were determined by pre-incubating the respective enzymes with increasing concentrations of C11PI [20, 50 and 100 nM for trypsin (or) 500, 2000 and 5000 nM for chymotrypsin] for 15 min followed by 45 min incubation at 37 °C with different concentrations of BAPNA or GLUPHEPA (0.125, 0.165, 0.250, 0.375, 0.500, 0.625 and 0.750 mM), respectively. The K_i values were estimated from the Lineweaver–Burk plots using Sigma Plot 11.0, Enzyme Kinetics Module 1.3 (Systat Software Inc., San Jose, California, USA).

2.4. Electrophoresis

Tricine-SDS-PAGE was performed using 4% stacking gel and 15% separating gel as described by Schagger and Jagow (1987) under reducing and non-reducing conditions. C11PI was reduced with 50 mM DTT at 56 °C for 1 h followed by alkylation with 2-fold molar excess of IDA (100 mM) for 45 min in dark at room temperature (25 °C). The protein molecular mass standards (Puregene, Genetix, India) ranging from 4.6 to 180 kDa were used. Also, commercially available purified soybean trypsin chymotrypsin inhibitor (Bowman-Birk Inhibitor, BBI) with molecular mass 8.0 kDa

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