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Biochemical properties of a bacterially-expressed Bowman-Birk inhibitor from *Rhynchosia sublobata* (Schumach.) Meikle seeds and its activity against gut proteases of *Achaea janata*

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ABSTRACT

Crude proteinase inhibitors (CPIs) extracted from the seeds of *Rhynchosia sublobata*, a wild relative of pigeon pea showed pronounced inhibitory activity on the larval gut trypsin-like proteases of lepidopteran insect pest – *Achaea janata*. Consequently, a full-length cDNA of Bowman-Birk inhibitor gene (*RsBBI1*) was cloned from the immature seeds of *R. sublobata*. It contained an ORF of 360 bp encoding a 119-amino acid polypeptide (13.3 kDa) chain with an N-terminus signal sequence comprising of 22 amino acids. The amino acid sequence and phylogenetic analysis together revealed that RsBBI1 exhibited a close relation with BBIs from soybean and *Phaseolus* spp. A cDNA sequence corresponding to *RsBBI1* mature protein (89 amino acid stretch) was expressed in *E. coli*. The recombinant rRsBBI1 protein with a molecular mass of 9.97 kDa was purified using trypsin affinity chromatography. The purified rRsBBI1 exhibited non-competitive mode of inhibition of both bovine trypsin (*Ki* of 358 ± 11 nM) and chymotrypsin (*Ki* of 446 ± 9 nM). Its inhibitory activity against these proteases was stable at high temperatures (>95 °C) and a wide pH range but sensitive to reduction with dithiothreitol (DTT), indicating the importance of disulphide bridges in exhibiting its activity. Also, rRsBBI1 showed significant inhibitory activity (IC_{50} = 70 ng) on *A. janata* larval gut trypsin-like proteases (AjGPs). Conversely, it showed <1% inhibitory activity (IC_{50} = 8 μg) on *H. armigera* larval gut trypsin-like proteases (HaGPs) than it has against AjGPs. Besides, *in vivo* feeding experiments clearly indicated the deleterious effects of rRsBBI1 on larval growth and development in *A. janata* which suggests it can be further exploited for such properties.

1. Introduction

Insect larvae feed on the vegetative and reproductive organs of plants and digest them with the aid of serine, cysteine, aspartic or metalloproteinases present in their gut environment (Terra and Ferreira, 1994). Upon infestation by larvae, plants display a myriad of defense responses, including the production of bioactive secondary metabolites and proteinaceous molecules such as α-amylase inhibitors, lectins, polyphenol oxidases and proteinase/protease inhibitors (PIs) (Furstenberg-Hagg et al., 2013). Serine PIs, which are active against many serine proteases found in the gastrointestinal tract of insects, are identified generally across the plant kingdom. They are further classified into eight different families viz. Kunitz inhibitors, Bowman-Birk inhibitors (BBIs), Potato inhibitor-I and Potato inhibitor-II, Mustard trypsin inhibitors, Squash inhibitors, Serpins and Cereal trypsin/α-amylase inhibitors (Mosolov and Valueva, 2005).

PIs are highly stable globular proteins constitutively expressed in storage organs such as seeds and tubers. They are also induced in

Abbreviations: AjGPs, *Achaea janata* larval gut trypsin-like proteases; AjGPIs, *A. janata* larval gut trypsin-like protease inhibitors; BBI, Bowman-Birk inhibitor; CI, Chymotrypsin inhibitor; CPIs, crude protease inhibitors; HaGPs, *Helicoverpa armigera* larval gut trypsin-like proteases; HaGPIs, *H. armigera* larval gut trypsin-like protease inhibitors; PIs, Protease inhibitors; RsBBI1, *Rhynchosia sublobata* BBI1 protein; rRsBBI1, Recombinant RsBBI1 protein; TI, Trypsin inhibitor.

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vegetative organs of plants during biotic and abiotic stresses and participate in regulating endogenous proteolysis, seed development, and programmed cell death. In addition, they serve as seed reserves for sulfur-containing amino acids, cysteine (Jamal et al., 2013; Mosolov and Valueva, 2005). PIs act against insect pest by binding to their digestive proteases and block their proteolytic activity. This mechanism lowers the pool of essential amino acids in the insects' gut required for their growth and thereby causing mortality (Jongsma and Bolter, 1997). The PIs from wild relatives and non-host plants are more effective than the PIs from host plants in the management of the insect pests as the digestive enzymes present in their guts have not adapted to such PIs (Harsulkar et al., 1999; Jongsma et al., 1996). Several transgenic plants expressing PIs from the host or non-host plants are produced to counteract the insect pests (Duan et al., 1996; Hilder et al., 1987; Johnson et al., 1989; Macedo et al., 2015).

From an economic perspective, *Helicoverpa armigera* and *Achaea janata* are relatively important among the lepidopteran insect pests. *H. armigera,* being polyphagous has caused significant loss to many crops (Lammers and Macleod, 2007). Further, the management of *H. armigera* continued to be a major challenge as it has developed resistance to a variety of pesticides. Conversely, *A. janata* feeds on an oil-rich *Ricinus communis* and causes severe loss to this cash crop owing to its foliar feeding behavior (Sujatha et al., 2010).

The wild relatives of leguminous crops are known to harbor a valuable gene pool for biotic, abiotic and disease resistance traits (Mallikarjuna et al., 2011). Serine PIs such as BBIs are mostly identified in leguminous plants and they contain two reactive sites for trypsin and chymotrypsin inhibition. Besides, the larval gut environment of lepidopteran insects possessed chiefly trypsin-like and chymotrypsin-like proteases. Therefore, in the present study, a BBI (*RsBBI1*) gene was cloned and sequenced from the immature seeds of *R. sublobata*, a wild relative of pigeon pea. The recombinant RsBBI1 (rRsBBI1) expressed in *E. coli* was examined for its biochemical properties and inhibitory potential against AjGPs and HaGPs. Based on the *in vitro* studies, *in vivo* feeding bioassays were performed to reveal the importance of rRsBBI1 in inducing growth retardation and mortality of *A. janata* larvae.

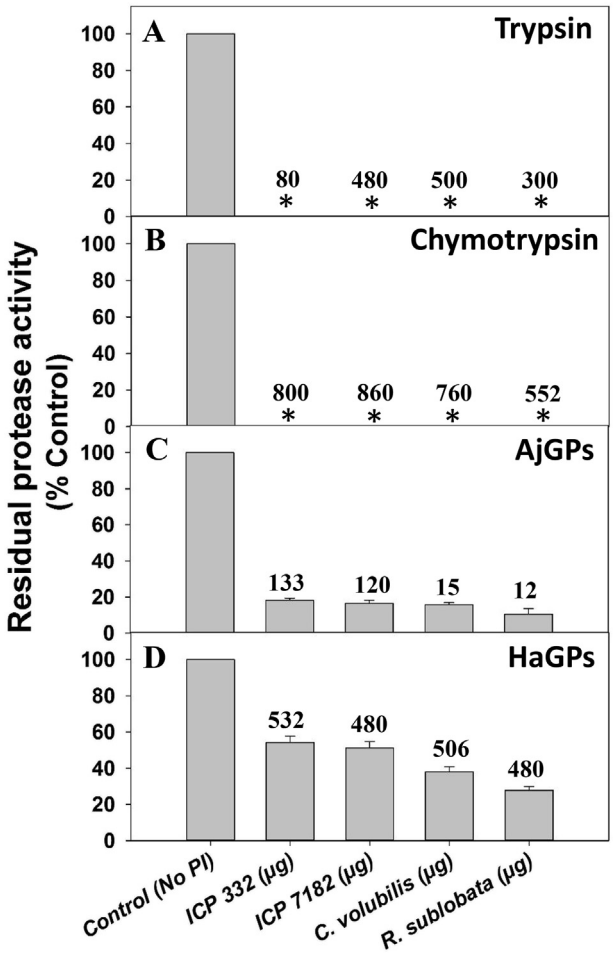

Fig. 1. Protease inhibition by seed CPI from cultivars and wild relatives of *C. cajan*. Residual protease activity of **(A)** bovine trypsin, **(B)** bovine chymotrypsin, **(C)** AjGPs and **(D)** HaGPs on incubation with CPI from seeds of *C. cajan* cultivars (ICP 332, ICP 7182) and wild relatives [*C. volubilis* (ICP 15774), *R. sublobata* (ICP 15868)]. Control bars represent the 100% activity of the different proteases in the absence of CPI. An asterisk (*) indicates the complete loss of protease activity in the presence of CPI. The values indicated above the bars are the corresponding CPI concentrations required to obtain maximum inhibition of respective proteases.

2. Results

2.1. Effect of seed crude PI on gut trypsin-like proteases

Seed crude PI (CPI) extracts of cultivars (ICP 332 and ICP 7182) and wild relatives (*C. volubilis* and *R. sublobata*) of pigeon pea were compared for their inhibitory potential against AjGPs and HaGPs in a wide range of concentration, using trypsin and chymotrypsin as reference controls (data not shown). The amount of CPI required from cultivars and wild relatives to cause maximum inhibition in activity of different proteases varied considerably (Fig. 1A–D). For example, among the bovine proteases tested, an amount of 80 µg of CPI from *C. cajan* cultivar ICP 332 was required to cause 100% inhibition in the activity of trypsin (Fig. 1A). Conversely, an amount of 552 µg of CPI was required from *C. cajan* wild relative *R. sublobata* to cause 100% inhibition in the activity of chymotrypsin (Fig. 1B). Among the larval gut trypsin-like proteases tested, the CPI from both cultivars and wild relatives could not inhibit the activity of AjGPs and HaGPs completely. However, the CPI from *R. sublobata* caused 85 ± 3% inhibition in the activity of AjGPs and 62 ± 5% inhibition in the activity of HaGPs at 12 µg and 480 µg, respectively (Fig. 1C and D). These results indicate *R. sublobata* CPI is 40-fold less active against HaGPs than AjGPs.

2.2. Cloning and sequencing of RsBBI1

In an attempt to clone BBI from *R. sublobabta* (wild relative), a 250 bp RT-PCR product was amplified from the cDNA generated using oligo dT primer (Fig. 2A). Consequently, a full-length transcript sequence of *RsBBI1* was obtained after 5′ and 3′ RACE experiments (Fig. 2B). Primary 5′ and 3′ RACE products obtained were ~250 bp and ~450 bp and the secondary PCR with the nested primers yielded 207 bp and 361 bp, respectively. Development of smaller products by the expected number of bases than the primary RACE products preliminarily confirmed the desired gene amplification. Contig sequence, generated out of the nucleotide sequences of RACE products, yielded complete sequence (505 bp) information of *RsBBI1* transcript comprising of an open reading frame of 360 bp encoding 119 amino acids, 21 bp of 5′ UTR and 109 bp of 3′ UTR ending with a polyA tail (Fig. 2C). BLASTn of cDNA sequence obtained showed greater identity with several other proteinase inhibitors belonging to BBI family (Supplementary Fig. 1). The complete *RsBBI1* CDS sequence was submitted to NCBI GenBank (accession # KT119632.2).

In silico analysis of RsBBI1 protein sequence of 119 amino acids