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Bowman–Birk proteinase inhibitor from *Clitoria fairchildiana* seeds: Isolation, biochemical properties and insecticidal potential

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ABSTRACT

Herein described is the biochemical characterisation, including *in vitro* and *in vivo* assays, for a proteinase inhibitor purified from *Clitoria fairchildiana* seeds (CFPI). Purification was performed by hydrophobic interaction and gel filtration chromatography. Kinetic studies of the purified inhibitor showed a competitive-type inhibitory activity against bovine trypsin and chymotrypsin, with an inhibition stoichiometry of 1:1 for both enzymes. The inhibition constants against trypsin and chymotrypsin were 3.3×10^{-10} and 1.5×10^{-10} M, respectively, displaying a tight binding property. SDS–PAGE showed that CFPI has a single polypeptide chain with an apparent molecular mass of 15 kDa under non-reducing conditions. However, MALDI-TOF analysis demonstrated a molecular mass of 7.973 kDa, suggesting that CFPI is dimeric in solution. The N-terminal sequence of CFPI showed homology with members of the Bowman–Birk inhibitor family. CFPI remained stable to progressive heating for 30 min to each temperature range of 37 up to 100 °C and CD analysis exhibited no changes in spectra at 207 nm after heating at 90 °C and subsequent cooling. Moreover, CFPI was active over a wide pH range (2–10). In contrast, reduction with DTT resulted in a loss of inhibitory activity against trypsin and chymotrypsin. CFPI also exhibited significant inhibitory activity against larval midgut trypsin enzymes from *Anagasta kuehniella* (76%), *Diatraea saccharalis* (59%) and *Heliothis virescens* (49%). Its insecticidal properties were further analysed by bioassays and confirmed by negative impact on *A. kuehniella* development.

1. Introduction

Proteinase inhibitors (PIs) are proteins widely distributed amongst plants and animals and which are able to inhibit proteolytic enzymes from diverse origins. Plants are a rich source of PIs and express these proteins in reproductive and storage organs as well as in vegetative tissues. Plants can produce PIs constitutively during normal development or in an inductive way in response to herbivory which corroborates with its defence function against pathogenic microorganisms and pests (Falco and Silva-Filho, 2003; Lopes et al., 2004; Oliveira et al., 2013).

PIs are classified according to the type of enzymes that they inhibit, and are known as serine, cysteine, aspartic, or metallo-proteinase inhibitors. Amongst these, plant serine PIs are the most common and best studied (Prasad et al., 2010c). They are grouped into families according to primary structure homology, position of reactive sites and number or location of disulphide bonds, highlighting the Kunitz and Bowman–Birk inhibitor (BBI) families, which are abundant in various Leguminosae seeds (Laskowski and Kato, 1980). PIs that constitute the Kunitz family are usually 18–24 kDa proteins with two disulphide linkages and a single reactive site mostly specific for trypsins. BBIs are smaller (6–9 kDa), with a polypeptide chain bridged by seven conserved disulphide bonds, and they possess two reactive sites. In relation to the reactive sites of legume BBIs, there are two domains located on the

Abbreviations: BBI, Bowman–Birk inhibitor; CD, circular dichroism; DTT, dithiothreitol; RP-HPLC, reversed-phase high performance liquid chromatography; Ki, inhibition constant; MALDI-TOF, matrix assisted laser desorption ionisation time of flight mass spectrometry; PI, proteinase inhibitor; TFA, trifluoroacetic acid; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride.

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opposite sides of the protein. These inhibitory domains can interact simultaneously and independently with one molecule of proteinase, forming a 1:1:1 stoichiometric complex of proteinase: inhibitor: proteinase (Barbosa et al., 2007; McBride et al., 1998). Characteristic amino acid residues in the inhibitory domain determine their specificity, e.g. for trypsin or chymotrypsin, which the most important residue being located at the P_1 position, using nomenclature developed by Schechter and Berger (1967).

Diverse physiological functions have been described for BBIs, including as a reserve of sulphur amino acids, regulation of endogenous proteinase levels and protection against insect pests and invading microorganisms (Lioi et al., 2010; Prasad et al., 2010a; Ragg et al., 2006). In addition, anti-carcinogenic or radioprotective activity and immune stimulating properties of BBIs have been reported (Fang et al., 2011; Kumar et al., 2004; Sampaio et al., 1996). *In vivo* and *in vitro* studies have indicated that BBIs are active against proteinases of larval guts, leading to inactive complex formation (Pereira et al., 2007; Prasad et al., 2010a; Rahbé et al., 2003). The impairment in digestion and absorption of amino acids causes a delay in larval growth and development, besides affecting insect survival. For this reason, BBIs have been a promising tool for pest control, mainly in plant engineering, wherein increased expression of these inhibitors could enhance resistance against phytophagous insects in economically important crops (Falco and Silva-Filho, 2003; Pompermayer et al., 2001). Furthermore, small cyclic peptides based on the reactive site of BBIs retain the structure and activity of the loop region from the parent protein, allowing production of highly potent and easily synthesised PIs (McBride et al., 2002).

Insects from the order Lepidoptera abundantly express serine proteases in their digestive physiology and thus these constitute good candidates for bioassays (Terra and Ferreira, 1994). *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae), the Mediterranean flour moth, is a polyphagous pest that feeds on a wide variety of stored products such as grains, fruits, nuts, tobacco and candies, causing economic losses worldwide (Machado et al., 2013). Control of these insects generally demand use of chemical insecticides that are toxic to humans and harmful to the environment. In contrast, plant PIs are considered a natural tool in the control of pests that may be used in integrated pest management (Ramos et al., 2008). Although the PIs have been extensively studied in terms of their potential to combat insect pests, most of the reported studies were conducted using Kunitz-type inhibitors. Therefore, the study of BBI effectiveness against phytophagous insects of different feeding habits could contribute to advances in pest control development, mainly to insects resistant to an inhibitor belonging to another family.

Clitoria fairchildiana R.A. Howard, popularly known in Brazil as Sombreiro, belongs to the subfamily Papilionoideae of the Fabaceae. It is a tree native to the Amazon region that is broadly distributed in the southwest and north Brazilian regions due its use in reforestation programs. The present paper reports the purification and biochemical characterisation of a BBI from *C. fairchildiana* seeds (CFPI). Feeding trials showed negative effects on growth and development of *A. kuehniella* larvae when fed on a diet containing CFPI.

2. Results and discussion

2.1. Purification of the C. fairchildiana proteinase inhibitor (CFPI)

Crude protein extract obtained from the seed powder showed strong inhibitory activity against trypsin and was applied onto a size exclusion Sephadex G-75 column. The crude extract was divided into four peaks, which the fraction represented by UV-absorbing (280 nm) peak G-2 having trypsin inhibitory (TI) activity (Supplementary 1). Peak G-2 was thus used in a subsequent purification step on a phenyl-sepharose hydrophobic interaction column, wherein although five UV-absorbing (220 nm) peaks were obtained only the P-3 peak showed TI activity. For purification of some contaminants of high molecular mass observed in SDS–PAGE (Fig. 1), a gel filtration chromatographic step using a Superdex 75 column was next conducted (Supplementary 1). The chromatogram shows S-1 and S-2 peaks, but only the major peak (S-2) showed TI activity and was pooled and named as CFPI (*C. fairchildiana* proteinase inhibitor). The purification procedures demonstrated that CFPI had a yield of 21.2% with a 17-fold purification (Table 1).

2.2. Matrix assisted laser desorption ionisation time-of-flight (MALDI-TOF) analysis of CFPI

MALDI-TOF analysis of CFPI showed a major peak measuring 7973.14 Da (Supplementary 2) and two similar peaks were observed, measuring 7886.06 and 7757.74 Da. These minor peaks can be isoforms resulting from partial proteolysis of the amino or C-terminus, in agreement with what was verified in BBI from *Lupinus albus* (Scarafoni et al., 2008). BBIs are products of multigene families that justify the existence of multiple isoforms by protein processing at both the amino and carboxylic ends (Domoney et al., 1995). Plant–insect coevolution has resulted in the production of numerous isoinhibitors to combat new insect proteinases (Lopes et al., 2004). Several BBIs isolated from leguminous plants like *Lens culinaris* (Ragg et al., 2006), *Pisum sativum* (Domoney et al., 1995) and *Vigna radiata* (Wilson and Chen, 1983), possess isoinhibitors.

In contrast with MALDI-TOF results, CFPI showed a relative molecular mass of 15 kDa determined by SDS–PAGE under non-reducing conditions, exhibiting a dimeric pattern of self-association in solution typical of BBIs (Kumar et al., 2004; Prasad et al., 2010c). The analysis of CFPI under reducing conditions (0.1 M DTT) displayed a polypeptide chain with a molecular mass of 12 kDa (Fig. 1). Paiva et al. (2006) also verified a different migration of BBI from *Cratylia mollis* in SDS–PAGE under reducing conditions; they attributed this effect to disulphide bonds in the inhibitor that resulted in different stabilities. Some reports verified that ingested

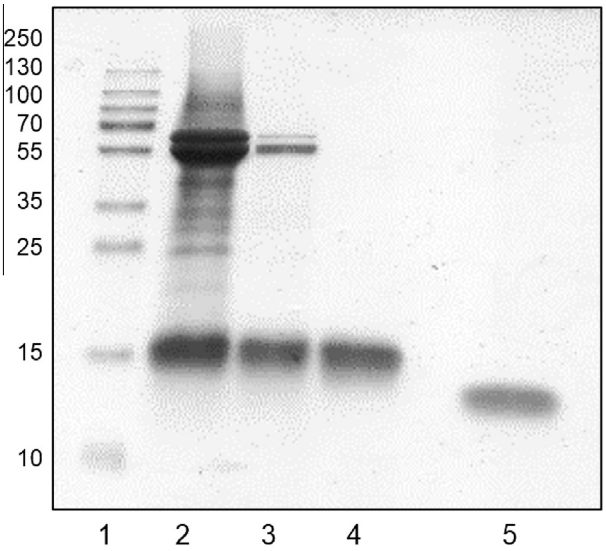

Fig. 1. SDS–PAGE showing the fractions obtained during purification: Lane (1) molar mass markers, (2) crude extract, (3) fraction CFPI-phenyl-sepharose, (4) CFPI, (5) CFPI reduced.

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