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Action of plant proteinase inhibitors on enzymes of physiopathological importance

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ABSTRACT

Obtained from leguminous seeds, various plant proteins inhibit animal proteinases, including human, and can considered for the development of compounds with biological activity. Inhibitors from the Bowman-Birk a plant Kunitz-type family have been characterized by proteinase specificity, primary structure and reactive site. O group mostly studies the genus *Bauhinia*, mainly the species *bauhinioides*, *rufa*, *ungulata* and *variegata*. In son species, more than one inhibitor was characterized, exhibiting different properties. Although proteins from th group share high structural similarity, they present differences in proteinase inhibition, explored in studies usi diverse biological models.

Key words: Bowman-Birk, chymotrypsin, Kunitz inhibitors, plasma kallikrein, primary structure, trypsin.

INTRODUCTION

Proteolytic enzymes are abundant in living cells and play important roles in intracellular proteolysis. Many studies have shown that proteinases are targets for the investigation of several diseases.

By cleaving proteins, proteinases are involved in the control of a large number of key physiological processes, such as cell-cycle progression, cell death, cell proliferation, DNA replicatin, haemostasis, immune response, tissue remodeling and wound healing (Turk 2006 – review). For instance, in the case of cysteine proteinases, since the imbalance of their enzymatic activities causes serious diseases, such as osteoporosis (Delaissé et al. 1984) and tumor invasion (Denhardt et al. 1987), the search for inhibitors that can moderately control their activity is desired for drug develo Also, these enzymes have been correlated with vasion process of many parasites, which demon important interactions with the host immune (Renslo and McKerrow 2006 – review).

These inhibitors interact reversibly with prot forming stoichiometric complexes and competitiv fluencing the catalytic activity (Radisky et al. 20

Serine proteinases activity is blocked through binding of the enzyme active site and the inhibi sulting in a complex resistant to proteolysis (Las and Kato 1980, Bode and Huber 1992).

Multiple molecular forms of protein inhibito been characterized from animals, microorganis plants (Ryan 2000, Birk 2003).

The interest in enzyme inhibitors obtaine plants began in the 1940s, when Kunitz (1946,

In commemoration of the 75th anniversary of

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      1                                                  50
SBTI  DFVLDNEGNP LENG.TYYIL SDITAFGG.I RAAPTGNERC PLTVVQSRNE
LlTI  QVLVDLDGDP LYNGMSYYIL PVARGKGGGL ELARTGSESC PRTVVQTRSE
EcTI  KELLDSDGDI LRNGGTYYI. PALRGKGGGL ELAKTGDETC PLNVVQARGE

     51                                                 100
SBTI  LDKGIGTIIS SPYRIRFIAE GHPLSLKFDS FAVIMLCVGI PTEWSVVEDL
LlTI  TSRGLPARLA SPYRILILGS NIPLTIEFQP QKPYSCHGHS SRSLQWKVEK
EcTI  TKRGRPAIIW TPPRIAILTP AFYLNIEFQT DLPACL.... .REYSRLPRE
     101                                                150
SBTI  PEGPAVKIGE NKDAMDGWFR LERVSDDEFN NYKLVFCPQQ AED..DKCGD
LlTI  TQMVKIASSD EEQRLFGPFQ IQPYR....N HYKLVYCESE SRNHHDDCRD
EcTI  EEHSEVKSDD DSCKDLG... .......... ...ISIAPKE EAA...AFGX

    151                                 186
SBTI  IGISIDHDDG TRRLVVSKNK PLVVQFQKLD KESL..
LlTI  LGISID.DQQ NRLLVVKNGD PLVVQFAKAN RGGDDD
EcTI  EKLKID.DEN NRRLVVKDGD PIAVRFVKAH RRG...
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Fig. 1 – Comparative sequences of related Kunitz inhibitors SBTI (soybean trypsin inhibitor); LlTI – *Leucaena leucocephala* (Oliva et al. 2000) and EcTI – *Enterolobium contortisiliquum* (Batista et al. 1996). SBTI identical residues are in gray; the cysteine residues are indicated by black boxes. The P_1 residues of the reactive sites are in the black boxes.

mechanisms, as well as for studying the protein-protein associations. Being considered anti-nutritional factors, those inhibitors are believed to participate in various physiological functions, such as the regulation of proteolytic cascades and the safe storage of proteins, as well as to act as defense molecules against plant pest and pathogens (Birk 2003, Sumikawa et al. 2008).

The best known groups of inhibitors obtained from seeds include serine proteinase inhibitors (EC. 3.4.21) of chymotrypsin (EC. 3.4.21.3), trypsin (EC. 3.4.21.4) and subtilisin (EC. 3.4.21.62). Numerous examples of inhibitors are also known for aspartyl proteinases (EC. 3.4.23), cysteine proteinases (EC. 3.4.22) and metalloproteinases (EC. 3.4.12).

Kunitz-type proteinase inhibitors are abundant in seeds from *Leguminosae* subfamilies, i.e. *Mimosoideae*, *Caesalpinoideae* and *Papilionoideae*. This type of inhibitor normally occurs as a single polypeptide chain; however, some inhibitors have also been shown to be dimeric proteins (Richardson 1991, Krauchenco et al. 2001, 2004).

This review deals with our recent data on the structure and function of plant Kunitz-type inhibitor interactions in biochemical processes involved in some diseases.

PLANT KUNITZ-TYPE INHIBITORS

seeds and, over the past three decades, a large number of other inhibitors have been purified and their primary structures determined. This lead **s** to the conclusion that these inhibitors are not restricted only to the leguminous group, but are also found in other plants (Richardson 1991, Birk 2003).

Information on the structure of plant Kunitz-type inhibitors is helpful to understand the mechanisms underlying their specificity for coagulation factors, inflammation and tumors, and to allow us to investigate which region of the protein is responsible for its biological activity.

The primary sequences of inhibitors may be highly similar within the same family. Several structural features are conserved in most Kunitz-type inhibitors: molecular mass of approximately 20 kDa, four cysteine residues and the sequence neighboring the single reactive site, which in general is Arg-Ser or Arg-Lys situated in a loop closed off by one disulfide bridge, and involved in trypsin inhibition (Richardson 1991, Birk 2003).

Souza-Pinto et al. (1996) purified a Kunitz trypsin inhibitor (LlTI) from *Leucaena leucocephala* (Fig. 1). Biochemical studies showed that LlTI blocks enzymes involved in blood clotting and fibrinolysis (Table I), has anti-inflammatory effects and decreases bradykinin release.

Inhibitors isolated from different species of *Bauhi-*

TABLE I
Inhibition effect (K_{iapp},nM) of plant inhibitors on proteinases.

Enzymes \ Inhibitors	BuXI *2Cys−Cys*	BvTI *2Cys−Cys*	BbKI *1Cys*	BbCI *NoCys*	BrTI *NoCys*	gBrEI *1Cys−Cys*	EcTI *2Cys−Cys*	LlTI *2Cys−Cys*
	1 chain						2 chains	
Cathepsin L				0.22				
Cruzipain				1.3				
Cruzain				0.3				
Cathepsin G				160				
Bovine trypsin	28	2.1	2.0		2.9		0.9	2.5
Bovine chymotrypsin	2.7	12	2600				1.11	14
Bovine pancreatic elastase				40		60		
Human neutrophil elastase				5.3			55	
Human plasma kallikrein	6.9	23	2.4		14		6.1	6.3
Human factor XIIa	74	110						
Human factor Xa	14							
Human plasmin	76		33				9.36	0.32
Porcine pancreatic kallikrein			200					
Murine plasma kallikrein		2.2	5.2		13			

BuXI, *B. ungulata* factor Xa inhibitor (Oliva et al. 2003) and BvTI, *B. variegata* trypsin inhibitors (Oliva et al. 2003), with fo cysteine residues forming two disulfide bridges in one polypeptide chain, Cys_{38}-Cys_{85} and Cys_{135}-Cys_{144} (SbTI numbering). gBr glycosylated *B. rufa* trypsin inhibitor has a single disulphide bridge (Cys_{41}-Cys_{85}) (Sumikawa et al. 2006); BbKI, *B. bauhinioid* kallikrein inhibitor has a single cysteine residue (Cys_{154}) (Oliva et al. 2001a, b); BbCI, *B. bauhinioides* cruzipain inhibitor (Oliveira et al. 2001), and BrTI, *B. rufa* trypsin inhibitor are devoid of cysteine residues (Nakahata et al. 2006). EcTI, *Enterolobiu contortisiliquum* trypsin inhibitor (Batista et al. 1996) and LlTI, *Leucacena leucocepha* trypsin inhibitor (Souza-Pinto et al. 199 present four cysteine residues and two polypeptide chains. Inhibitor and proteinase were incubated at 37°C with one of the followi proteinases and respective substrates: cathepsin L (18 nM), cruzain (3.2 nM) and cruzipain (18 nM) activated with 100 mM sodiu phosphate buffer, pH 6.3 containing 10 mM EDTA, 400 mM NaCl, and 2 mM dithiothreitol; 0.3 mM Z-Phe-Arg-MCA; catheps G (0.3μM in 0.1 M Tris/HCl buffer, pH 7.5 containing 0.5 M NaCl; 1.0 mM MeO-Suc-Ala-Ala-Pro-Phe-pNan); trypsin (7.0 nM 0.05 M Tris/HCl, pH 8.0, 0.02% $CaCl_2$; 1.0 mM BAPA), chymotrypsin (76 nM in 0.1 M Tris/HCl, pH 8.0, 0.02% $CaCl_2$; 2.0 m Suc-Phe-pNan), HuPK, human plasma kallikrein (67 nM in 0.05 M Tris/HCl, pH 8.0; 0.5 mM H-D-Pro-Phe-Arg-pNan); rPK, muri plasma kallikrein (6.0 nM in 0.05 M Tris/HCl, pH 8.0; 0.5 mM H-D-Pro-Phe-Arg-pNan); PoPK, porcine pancreatic kallikrein (2.6 n in 0.1 M Tris/HCl, pH 8.0; 0.8 mM Ac-Phe-Arg-pNan), PPE, porcine pancreatic elastase (24 nM in 0.05 M Tris/HCl, pH 8.0, 0.5 NaCl; 0.5 mM MeO-Suc-Ala-Ala-Pro-Val-pNan), HNE, human neutrophil elastase (25 nM in 0.05 M Tris/HCl, pH 7.0, 0.5 M NaC 0.5 mM MeO-Suc-Ala-Ala-Pro-Val-pNan), factor Xa (0.4 nM in Tris/HCl 0.05 M, pH 8.0; 0.25μM Boc-Ile-Glu-Gly-Arg-MCA Factor XIIa (13 nM in 0.05 M Tris/HCl, pH 8.0; 40μM H-D-Pro-Phe-Arg-MCA), and plasmin (3.5 nM in 0.1 M Tris/HCl, pH 7 containing 0.2 M NaCl; 1.0 mM H-D-Val-Leu-Lys-pNan). K_{iapp} values were determined by adjusting the experimental points to t equation for tight binding, using a nonlinear regression with the Grafit 3.01 program (Morrison 1982).

plant known in Brazil by the popular name of "cow paw" due the shape of its leaves, are 18 kDa proteins that present a high primary structure similarity with other plant Kunitz-type inhibitors (Fig. 2), but differ by the absence of disulfide bridges and in their inhibition specificity (Oliva et al. 1999a, b, 2003, de Oliveira et al. 2001). The description of other inhibitors lacking the four conservative cysteine residues (Macedo et al. 2007) reinforces the establishment of a new group of plant

both proteins similar to the wild-type proteins (et al. 2005) showed potent inhibitory activities t their target proteinases. What distinguishes BbC inhibition of two different classes of proteinases, inhibits the serine proteinases human neutrophil e and pancreatic porcine elastase, and the cystein teinases cathepsin L and cruzipain from *Trypan cruzi*. Alanine in the P1 position is essential fo inhibitions. Although BbKI primary structure is

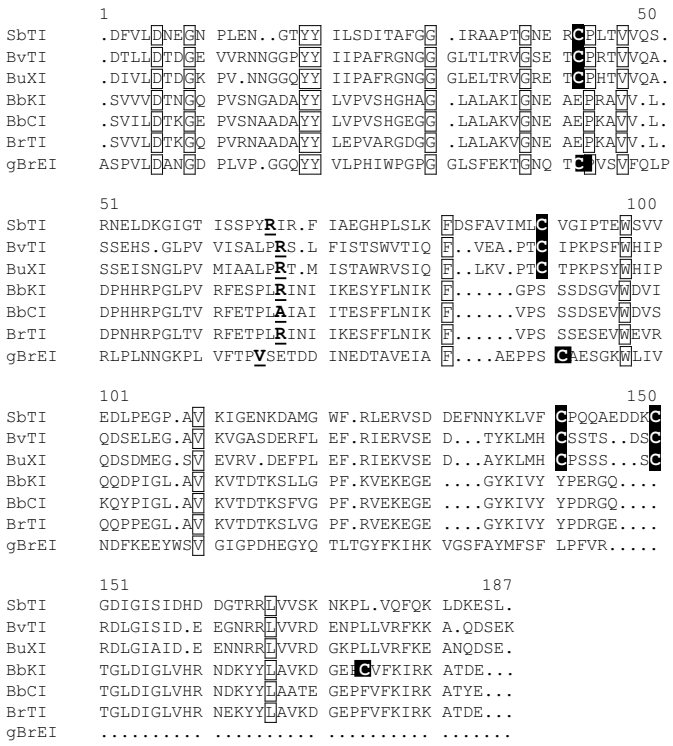

Fig. 2 – Comparison of partial sequences of related Kunitz inhibitors. SBTI, soybean trypsin inhibitor; BvTI, *Bauhinia variegata* Trypsin Inhibitor (Oliva et al. 2003); BuXI, *Bauhinia ungulata* Factor Xa Inhibitor (Oliva et al. 2003); BbKI, *Bauhinia bauhinioides* Kallikrein Inhibitor (Oliva et al. 2001a); BbCI, *Bauhinia bauhinioides* Cruzipain Inhibitor (de Oliveira et al. 2001); BrTI, *Bauhinia rufa* Trypsin Inhibitor (Nakahata et al. 2006) and gBrTI *Bauhinia rufa* elastase Inhibitor, glycosilated form (Sumikawa et al. 2006). Black boxes indicate the reactive site residue for trypsin inhibition. Methionine residues in BuXI are indicated by white boxes.

neither BbCI interfere on blood clotting enzyme activities (de Oliveira et al. 2001, Neuhof et al. 2003).

Hansen and co-workers (2007) reported the three-dimensional structure of the recombinant BbCI at 1.7Å resolution and, in comparison to the structures of BbKI and other plant Kunitz-type inhibitors, it was shown that they share a common β-trefoil fold. Furthermore, the crystallographic structure of BbCI showed that the maintenance of the canonical conformation of the reactive site loop is important for a proper inhibitory function, and that the protein scaffold plays an important role at this site. The absence of disulfide bridges in the structure of BbCI is compensated for by essential interactions that maintain its structural stability and preserve its biological function.

and inhibits human plasma kallikrein and trypsin, but not other related enzymes (Nakahata et al. 2006). A variety of studies have demonstrated that proteinase inhibitors can suppress several stages of carcinogenesis, including tumor initiation, promotion and progression. Although their mechanism of action is not yet clear, in 2006, Nakahata and co-workers reported the inhibitory action of YLEPVARGDGGLA-NH_2, a synthetic peptide containing the RGD sequence derived from the structure of BrTI (Fig. 2). This peptide inhibited the adhesion of B16F10 (a high-metastatic B16 murine melanoma cell line) and Tm5 (a murine melanoma cell line derived from a non-tumorigenic lineage of pigmented murine melanocytes, melan-a) to fibronectin. When Asp9 was changed to Glu (YLEPVARGEGGLA-NH2), cell attach-

tein was preserved, since changing Glu3 to Ile (YLIPVARGDGGLA-NH2) did not interfere with B16F10 cell adhesion and was less effective on the adhesion of Tm5 cells. Neither YLEPVARGDGGLA-NH2 nor YLIPVARGDGGLA-NH2 and YLEPVARGEGGLA-NH2 affected the interaction of RAEC (an endothelial cell line from rabbit aorta) with fibronectin. Differently from other *Bauhinia* inhibitors, BrTI is the only one that exhibits insecticidal activity on *Callosobruchus maculatus* larvae (J.T. Sumikawa et al., unpublished data).

Purified from *Enterolobium contortisiliquum* seeds, EcTI (Fig. 1) appears to be an interesting inhibitor since it shows a strong capacity for inhibiting trypsin ($K_{i(app)}$ 0.88 nM), chymotrypsin ($K_{i(app)}$ 1.11 nM), plasma kallikrein ($K_{i(app)}$ 6.15 nM), plasmin ($K_{i(app}$ 9.36 nM) and human neutrophil elastase ($K_{i(app)}$ 55.00 nM) (Oliva et al. 1987, Batista et al. 1996, 2001) (Table I), but not cysteine proteinases.

The inhibitory capacity of these proteinase inhibitors was investigated on the cell viability of different tumor cell lines, primary human fibroblasts and on the proliferation capacity of human mesenchymal stem cells, in addition to their mechanism of action on blood coagulation, fibrinolysis, inflammation and platelet aggregation.

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RESUMO

dade biológica. Inibidores da família Bowman-Birk
mília Kunitz vegetal tem sido caracterizados em relaç
pecificidade para proteinase, estrutura primária e sitio
O nosso grupo majoritariamente vem estudando o gêne
hinia, principalmente as espécies *bauhinioides*, *rufa*, *u*
e *variegata*. Em algumas espécies, mais de um inibi
propriedades diferentes foi caracterizado. Embora tais
nas apresentem alta similaridade estrutural, diferem q
inibição de proteinases, e foram exploradas em estu
lizando diversos modelos biológicos.

Palavras-chave: Bowman-Birk, quimotripsina, in
Kunitz, calicreína plasmática, estrutura primária, tr

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