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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 be considered for the development of compounds with biological activity. Inhibitors from the Bowman-Birk and plant Kunitz-type family have been characterized by proteinase specificity, primary structure and reactive site. Our group mostly studies the genus *Bauhinia*, mainly the species *bauhinioides*, *rufa*, *ungulata* and *variegata*. In some species, more than one inhibitor was characterized, exhibiting different properties. Although proteins from this group share high structural similarity, they present differences in proteinase inhibition, explored in studies using 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 development. Also, these enzymes have been correlated with the invasion process of many parasites, which demonstrates important interactions with the host immune system (Renslo and McKerrow 2006 – review).

These inhibitors interact reversibly with proteinases forming stoichiometric complexes and competitively influencing the catalytic activity (Radisky et al. 2004).

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

Multiple molecular forms of protein inhibitors have been characterized from animals, microorganisms and plants (Ryan 2000, Birk 2003).

The interest in enzyme inhibitors obtained from plants began in the 1940s, when Kunitz (1946, 1947) isolated and purified from soybean a protein which inhibited trypsin. Inhibitor proteins have been studied as model systems for elucidating proteinase inhibition

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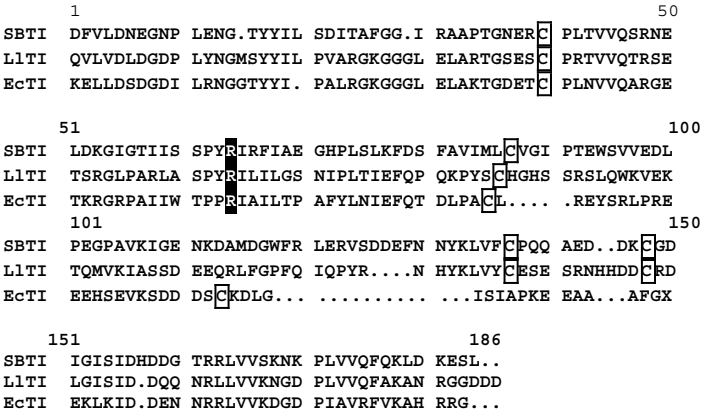

Fig. 1 – Comparative sequences of related Kunitz inhibitors SBTI (soybean trypsin inhibitor); LlTI – *Leucaena leucocephala* (Oliva et al. 2000) and EcTI – *Enterolobium contortisiliquum* (Batista et al. 1996). SBTI identical residues are in gray; the cysteine residues are indicated by black boxes. The 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

Plant Kunitz-type inhibitors are easily found in leguminous seeds. As mentioned previously, the first inhibitor from this family (SBTI) was obtained from *Glycine max* 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 *Bauhinia* seeds inhibit blood clotting enzymes, as well as other serine and cysteine proteinases. The inhibitors BbKI and BbCI, obtained from seeds of *Bauhinia bauhinioides*, a

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 four cysteine residues forming two disulfide bridges in one polypeptide chain, Cys_{38}-Cys_{85} and Cys_{135}-Cys_{144} (SbTI numbering). gBrTI, glycosylated *B. rufa* trypsin inhibitor has a single disulphide bridge (Cys_{41}-Cys_{85}) (Sumikawa et al. 2006); BbKI, *B. bauhinioides* kallikrein inhibitor has a single cysteine residue (Cys_{154}) (Oliva et al. 2001a, b); BbCI, *B. bauhinioides* cruzipain inhibitor (de Oliveira et al. 2001), and BrTI, *B. rufa* trypsin inhibitor are devoid of cysteine residues (Nakahata et al. 2006). EcTI, *Enterolobium contortisiliquum* trypsin inhibitor (Batista et al. 1996) and LlTI, *Leucacena leucocepha* trypsin inhibitor (Souza-Pinto et al. 1996) present four cysteine residues and two polypeptide chains. Inhibitor and proteinase were incubated at 37°C with one of the following proteinases and respective substrates: cathepsin L (18 nM), cruzain (3.2 nM) and cruzipain (18 nM) activated with 100 mM sodium phosphate buffer, pH 6.3 containing 10 mM EDTA, 400 mM NaCl, and 2 mM dithiothreitol; 0.3 mM Z-Phe-Arg-MCA; cathepsin 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 in 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 mM 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, murine 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 nM 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 M 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 NaCl; 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 7.4 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 the 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 Kunitz family.

BbCI and BbKI recombinants were obtained by heterogonous expression and production in *E. coli*, and both proteins similar to the wild-type proteins (Araújo et al. 2005) showed potent inhibitory activities towards their target proteinases. What distinguishes BbCI is the inhibition of two different classes of proteinases, e.g., it inhibits the serine proteinases human neutrophil elastase and pancreatic porcine elastase, and the cysteine proteinases cathepsin L and cruzipain from *Trypanosoma cruzi*. Alanine in the P1 position is essential for these inhibitions. Although BbKI primary structure is highly identical to BbCI (84%), it differs by inhibiting bovine trypsin, human plasmin and plasma kallikrein (Table I). BbKI does not affect the activity of cysteine proteinases,

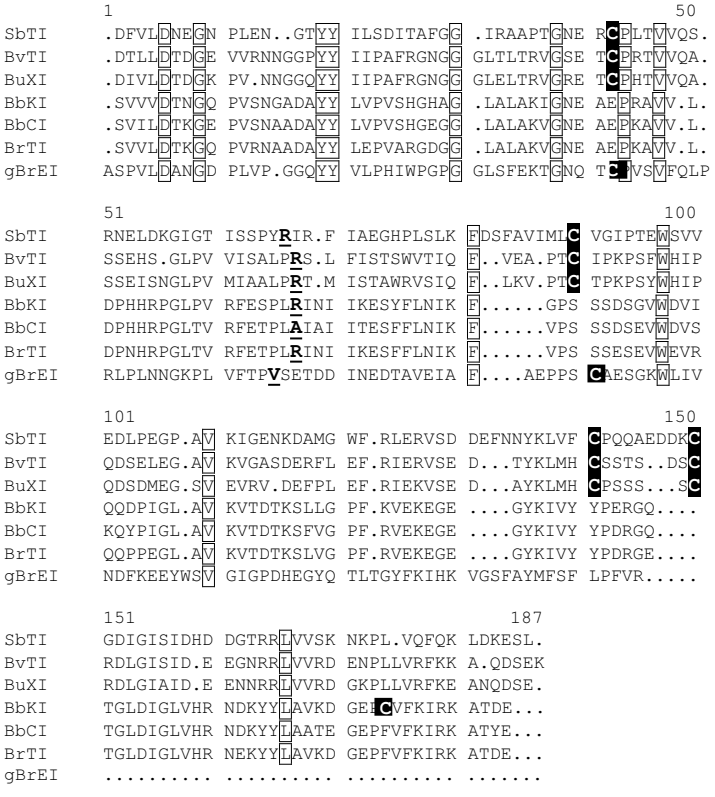

Fig. 2 – Comparison of partial sequences of related Kunitz inhibitors. SBTI, soybean trypsin inhibitor; BvTI, *Bauhinia variegata* Trypsin Inhibitor (Oliva et al. 2003); BuXI, *Bauhinia ungulata* Factor Xa Inhibitor (Oliva et al. 2003); BbKI, *Bauhinia bauhinioides* Kallikrein Inhibitor (Oliva et al. 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.

BrTI, a Kunitz-type proteinase inhibitor purified from *Bauhinia rufa* seeds, contains the RGD sequence 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 attachment was not affected. Moreover, this peptide was functional only when the sequence present in the native pro-

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

Obtidas de sementes leguminosas, várias proteínas inibem proteinases de origem animal, incluindo humanas, e podem ser consideradas para o desenvolvimento de compostos com atividade biológica. Inibidores da família Bowman-Birk e da família Kunitz vegetal tem sido caracterizados em relação a especificidade para proteinase, estrutura primária e sitio reativo. O nosso grupo majoritariamente vem estudando o gênero *Bauhinia*, principalmente as espécies *bauhinioides*, *rufa*, *ungulata* e *variegata*. Em algumas espécies, mais de um inibidor com propriedades diferentes foi caracterizado. Embora tais proteínas apresentem alta similaridade estrutural, diferem quanto à inibição de proteinases, e foram exploradas em estudos utilizando diversos modelos biológicos.

Palavras-chave: Bowman-Birk, quimotripsina, inibidores Kunitz, calicreína plasmática, estrutura primária, tripsina.

REFERENCES

ARAÚJO APU, HANSEN D, VIEIRA DF, DE OLIVEIRA C, SANTANA LA, BELTRAMINI LM, SAMPAIO CA, SAMPAIO MU AND OLIVA MLV. 2005. Kunitz type *Bauhinia bauhinioides* inhibitors devoid of disulfide bridges: isolation of the cDNAs, heterologous expression and structural studies. Biol Chem 386: 561–568.

BATISTA IFC, OLIVA MLV, ARAÚJO MS, SAMPAIO MU, RICHARDSON M, FRITZ H AND SAMPAIO CAM. 1996. Primary structure of a Kunitz-type trypsin inhibitor from *Enterolobium contortisiliquum* seeds. Action on blood clotting contact phase enzymes. Phytochemistry 41: 1017–1022.

BATISTA IF, NONATO MC, BONFADINI MR, BELTRAMINI LM, OLIVA MLV, SAMPAIO MU, SAMPAIO CAM AND GARRATT RC. 2001. Preliminary crystallographic studies of EcTI, a serine proteinase inhibitor from *Enterolobium contortisiliquum* seeds. Acta Crystallogr D Biol Crystallogr 57: 602–604.

BIRK Y. 2003. In: Plant Protease Inhibitors: Significance in Nutrition, Plant Protection, Cancer Prevention and Genetic Engineering. ed. (Berlin Heidelberg: Springer-Verlag) p. 1–126.

BODE W AND HUBER R. 1992. Natural protein proteinase inhibitors and their interaction with proteinases. Eur J Biochem 204: 433–451.

DE OLIVEIRA C, SANTANA LA, CARMONA AK, CEZARI MH, SAMPAIO MU, SAMPAIO CA AND OLIVA ML. 2001. Structure of cruzipain/cruzain inhibitors isolated from *Bauhinia bauhinioides* seeds. Biol Chem 382: 847–852.

DELAISSÉ JM, EECKHOUT Y AND VAES G. 1984. *In vivo* and *in vitro* evidence for the involvement of cysteine proteinases in bone resorption. Biochem Biophys Res Commun 125: 441–447.

DENHARDT DT, GREENBERG AH, EGAN SE, HAMILTON RT AND WRIGHT JA. 1987. Cysteine proteinase cathepsin L expression correlates closely with the metastatic potential of H-ras-transformed murine fibroblasts. Oncogene 2: 55–59.

HANSEN D, MACEDO-RIBEIRO S, VERÍSSIMO P, YOO IM S, SAMPAIO MU AND OLIVA ML. 2007. Crystal structure of a novel cysteinless plant Kunitz-type protease inhibitor. Biochem Biophys Res Commun 360: 735–740.

KRAUCHENCO S ET AL. 2001. Crystallization and preliminary X-ray diffraction analysis of a novel trypsin inhibitor from seeds of *Copaifera langsdorffii*. Acta Crystallogr D Biol Crystallogr 57: 1316–1318.

KRAUCHENCO S, NAGEM RA, DA SILVA JA, MARANGONI S AND POLIKARPOV I. 2004. Three-dimensional structure of an unusual Kunitz (STI) type trypsin inhibitor from *Copaifera langsdorffii*. Biochimie 86: 167–172.

KUNITZ M. 1946. Crystalline soybean trypsin inhibitor. J Gen Physiol 29: 149–154.

KUNITZ M. 1947. Isolation of a crystalline protein compound of trypsin and of soybean trypsin-inhibitor. J Gen Physiol 30: 311–320.

LASKOWSKI MJR AND KATO I. 1980. Protein inhibitors of proteinases. Annu Rev Biochem 49: 593–626.

MACEDO MLR, GARCIA VA, FREIRE MGM AND RICHARDSON M. 2007. Characterization of a Kunitz trypsin inhibitor with a single disulfide bridge from seeds of *Inga laurina* (SW.) Willd. Phytochemistry 68: 1104–1111.

MORRISON JF. 1982. The slow-binding and slow, tight-binding inhibition of enzyme-catalyzed reactions. TIBS 7: 102–105.

NAKAHATA AM ET AL. 2006. Structural and Inhibitory proprieties of plant proteinase inhibitor containing a RGD motif. Int J Biol Macromol 40: 22–29.

NEUHOF C, OLIVA ML, MAYBAUER D, MAYBAUER M, DE OLIVEIRA C, SAMPAIO MU, SAMPAIO CA AND NEUHOF H. 2003. Effect of plant Kunitz inhibitors from *Bauhinia bauhinioides* and *Bauhinia rufa* on pulmonary edema caused by activated neutrophils. Biol Chem 384: 939–944.

OLIVA MLV, SAMPAIO MU AND SAMPAIO CAM. 1987. Serine and SH-proteinase inhibitors from *Enterolobium contortisiliquum* beans. Purification and preliminary characterization. Braz J Med Biol Res 20: 767–770.

OLIVA MLV, ANDRADE S, BATISTA IFC, SAMPAIO MU, JULIANO M, FRITZ H, AUERSWALD EA AND SAMPAIO CAM. 1999a. Human plasma kallikrein and tissue kallikrein binding to a substrate based on the reactive site of a factor Xa inhibitor isolated from *Bauhinia ungulata* seeds. Immunopharmacology 45: 145–149.

OLIVA MLV, MENDES CR, JULIANO MA, CHAGAS JR, ROSA JC, GREENE LJ, SAMPAIO MU AND SAMPAIO CAM. 1999b. Characterization of a tissue kallikrein inhibitor isolated from *Bauhinia bauhinioides* seeds: inhibition of the hydrolysis of kininogen related substrates. Immunopharmacology 45: 163–169.

OLIVA MLV, SOUZA-PINTO JC, BATISTA IFC, ARAUJO MS, SILVEIRA VF, AUERSWALD EA, MENTELE R, ECKERSKORN C, SAMPAIO MU AND SAMPAIO CA. 2000. *Leucaena leucocephala* serine proteinase inhibitor: primary structure and action on blood coagulation, kinin release and rat paw edema. Biochim Biophys Acta 1477: 64–74.

OLIVA MLV, MENDES CR, SANTOMAURO-VAZ EM, JULIANO MA, FRITZ H, SAMPAIO MU AND SAMPAIO CAM. 2001a. *Bauhinia bauhinioides* Plasma Kallikrein Inhibitor: Interaction with Synthetic Peptides and Fluorogenic Peptide Substrates Related to The Reactive Site Sequence. Medical Chemistry 8: 977–984.

OLIVA MLV, SANTOMAURO-VAZ EM, ANDRADE SA, JULIANO MA, POTT VJ, SAMPAIO MU AND SAMPAIO CAM. 2001b. Synthetic Peptides and Fluorogenic Substrates Related to the Reactive Site Sequence of Kunitz Type Inhibitors Isolated from *Bauhinia:* Interaction with Human Plasma Kallikrein. Biol Chem 382: 109–113.

OLIVA MLV, ANDRADE SA, JULIANO MA, JULIANO L, SAMPAIO MU AND SAMPAIO CAM. 2003. Kinetic Characterization of Factor Xa Binding Using a Quenched Fluorescent Substrate Based on the Reactive Site of Factor Xa Inhibitor from *Bauhinia ungulata* Seeds. Curr Med Chem 10: 1085–1093.

RADISKY ES, KWAN G, KAREN LU CJ AND KOSHLAND DE JR. 2004. Binding, proteolytic, and crystallographic analyses of mutations at the protease-inhibitor interface of the subtilisin BPN'/chymotrypsin inhibitor 2 complex. Biochem 43: 13648–13656.

RENSLO AR AND MCKERROW JH. 2006. Drug discovery and development for neglected parasitic diseases. Nat Chem Biol 2: 701–710.

RICHARDSON M. 1991. Seed storage proteins: The enzyme inhibitors. Methods in Plant Biochem 5: 259–305.

RYAN CA. 2000. The systemin signaling pathway: differential activation of plant defensive genes. Biochim Biophys Acta 1477: 112–121.

SOUZA-PINTO JC, OLIVA ML, SAMPAIO CA, DAMAS J, AUERSWALD EA, LIMAOS E, FRITZ H AND SAMPAIO MU. 1996. Effect of a serine proteinase inhibitor from *Leucaena leucocephala* on plasma kallikrein and plasmin. Immunopharmacology 33: 330–332.

SUMIKAWA JT, NAKAHATA AM, FRITZ H, MENTELE R, SAMPAIO MU AND OLIVA MLV. 2006. A Kunitz-type glycosylated elastase inhibitor with one disulphide bridge. Planta Med 5: 393–397.

SUMIKAWA JT, BRITO MARLON V, ARAUJO APU, MACEDO MLR, OLIVA MLV AND MIRANDA A. 2008. Action of *Bauhinia*-derivated compounds on *Callosobruchus maculatus*. In: DEL VALLE SUSAN, ESCHER EMANUEL, LUBELL WILLIAM D (Org), Peptides for Youth. New York, NY: American Peptide Society, p. 611–613.

TURK B. 2006. Targeting proteases: successes, failures and future prospects. Nat Rev Drug Discov 5: 785–799.