PROTEINASE INHIBITORS FROM *BAUHINIA* SPECIES AND DESIGN OF SUBSTRATES AND INHIBITORS FOR PROTEOLYTIC ENZYMES INVOLVED IN BLOOD CLOTTING (A REVIEW)

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Abstract

Acting selectively, proteinase inhibitors isolated from genus Bauhinia distinct species may be a model for enzyme-substrate interactions studies. Bauhinia inhibitors are potent plant Kunitz type proteinase inhibitors that directly inhibit blood coagulation enzymes. BuXI isolated from Bauhinia ungulata is distinguishable from others inhibitors because of the slow tight-binding inhibition of factor Xa while the highly homologous (70%) inhibitor from Bauhinia variegata (BvTI) does not affect factor Xa. The primary sequence data indicate that ungulata and variegata species inhibitors are classical plant Kunitz serine proteinase inhibitors, with a single polypeptide chain and their reactive site situated in a loop closed by disulfide bridge. BbKI, the inhibitor isolated from B. bauhinioides is characterized as a distinct member of the Kunitz family due the absence of disulfide bridges. The most effective plasma kallikrein inhibitor, BbKI bears only one cysteine residue at the C-terminal position (residue 154) and the homology of the sequence neighboring its reactive site to bradykinin moiety in kininogen structure may explain the stronger interaction to plasma kallikrein.

Key Word Index—blood clotting enzymes; factor Xa; kallikrein; Kunitz inhibitor; primary sequence; proteinase inhibitor.

Abbreviations: SBTI, soybean trypsin inhibitor; BvTI, Bauhinia variegata trypsin inhibitor; BuXI, Bauhinia ungulata factor Xa inhibitor; BbKI, Bauhinia bauhinioides kallikrein inhibitor, LOPAP prothrombin activator proteinase isolated from Lonomia obliqua.

Introduction

There are no doubts that plant proteinase inhibitors are important response to natural predators as insects and nematodes (Jongsma & Bolter, 1997; Gatehouse et al., 1998; Franco et al., 2002).

Many if not all plant proteinase inhibitors appear to bind to the target enzyme forming an immediate and tight complex (Richardson 1991). It has been shown that the inhibitory activity and specificity of the inhibitors towards the target enzyme are influenced as much as substrate recognition, by residues in the primary specificity site, as well as in more distal regions like the surface loop.

A comparison of the known sequences of the members of this protein family showed that the primary structure is highly homologous. The structures revealed several features that are conserved in most Kunitz type inhibitors: Mr 20,0 kDa, four cysteine residues and the sequence neighboring the single reactive site, in general, Arg-Ser or Arg-Lys, with high degree of homology and situated in a loop closed by one disulfide bridge (Wenzel & Tscheche, 1995; Richardson, 1991).

Our laboratory has shown that serine proteinase isolated from different species of Bauhinia seeds selectively inhibit blood clotting enzymes, although also inhibiting other serine proteinases.

The leguminous genus Bauhinia spread in the tropics and subtropics is trivially named cow's foot. In South America it is polpular as an alternative medicine in diabetes treatment. In this work, we review the interaction of those inhibitors as well as the structural features, which lead to a specific interaction, and the inhibitory specificity of blood clotting enzymes is also shown.

Purification and Structural Characterization

Bauhinia seeds contain relatively large quantities of serine proteinase inhibitors extracted with 0.15M NaCl and purified by ion-exchange column chromatography on DEAE-Sephadex, gel filtration, Mono Q chromatography, or alternatively, by affinity chromatography on trypsin-Sepharose (Oliva et al., 1999a,b; Bueno et al., 1999; Oliva et al., 2000; Oliva et al., 2001a; Oliveira et al., 2001). Following these procedures, the overall yield is 30% and the inhibitors appear as a single polypeptide chain of molecular mass 20 kDa. For structure determination, the homogeneity of the preparation was assessed by reversed-phase analysis (HPLC system).

From the primary structure determination, BuXI and BvTI (from ungulata and variegata species, respectively) were identified as members of Kunitz serine proteinase inhibitors family with disulfide bridges in conservative position, and the reactive sites limited by the bridge formed by Cys^{39} and Cys^{85}, in the first loop (Figure 1) (Andrade et al., *in press;* Oliva et al., submitted*)*.

BbKI, from B. bauhinioides shows similarity to Kunitz inhibitors but lacks intra chains disulfide bridges. In BbKI the only cysteine residue is at the C-terminal position (residue 154) (Figure 2).

Inhibitory properties

The inhibitory activity on trypsin, chymotrypsin and on the coagulation enzymes plasma kallikrein, factor Xa, thrombin and also fibrinolytic plasmin was investigated.

The dissociation constants were measured by determination of the enzyme residual activity and *Ki* values were determined using the equation described by Morrison for slow-tight binding model (Knight, 1986). The studied inhibitors show high affinity for bovine trypsin, chymotrypsin but the specificity on clotting enzymes shows major differences (Table 1). All studied Bauhinia inhibitors inhibit plasma kallikrein but BbKI is the most effective (Ki = 0.35 nM) (Oliva et al., 2001a,b). Factor Xa is inhibited by BuXI only but none of inhibitors act on thrombin (Andrade et al., *in press*; Oliva et al., submitted). All studied proteins are stable over a relatively broad range of pH values (2-12) and the inhibitory activity was maintained at temperatures up to 60°C.

Blood Clotting

The ability of Bauhinia inhibitors to affect blood clotting assays as thrombin time (TT), prothrombin time (PT), and activated partial thromboplastin time (APTT) was followed with normal human citrated plasma. The increase in APTT indicates that the contact phase of blood clotting is blocked but not TT. Prolongation of TT is not achieved by the exogenous addition of BuXI, leading to the conclusion that BuXI does not interact with factor X proenzyme form, or the interaction is too slow to interfere with this assay system (Oliva et al., submitted). BbKI was shown to inhibit plasma kallikrein leading to a significant prolongation of APTT.

Structural and Specificity Features

Besides the common properties presented by Bauhinia inhibitors, the differences on the inhibitory specificity led us to investigate the relationship between primary structure and selectivity towards proteolytic enzymes, mostly, related to blood clotting.

BuXI structure shows the presence of Met^{67}, Thr^{64} and Met^{59} in the vicinity of the reactive site of the scissile amino acid residues (Figure 3) and which are absent in other inhibitors. Studies developed in our laboratory showed the involvement of both Met residues in BuXI inhibition. Following oxidation with hydrogen peroxide (H_2O_2), BuXI no longer inhibits factor Xa but still inhibits trypsin, thus indicating that those Met residues participate in the interaction of the inhibitor with the enzyme (Oliva et al., submitted).

Concerning BbKI, the structural similarity to bradykinin moiety in kininogen (Figure 4) seems to explain the stronger binding to human plasma kallikrein (Oliva et al., 1999).

Action of Bauhinia inhibitors on other trypsin-like enzymes.

Bauhinia inhibitors were investigated with respect to their ability to inhibit trypsin-like enzymes from digestive extracts of insects. The action of BuXI and BvTI on LOPAP, a prothrombin activator proteinase isolated from Lonomia obliqua venom that resembles factor Xa in prothrombin activation, was also studied (Reis et al., 2001).

Both BuXI and BvTI are effective on the inhibition of Abracris flavolineata and Musca domestica midgut trypsin-like enzymes. BuXI inhibits more efficiently Spodoptera frugiperda midgut activity, while BvTI is more effective on Tenebrio molitor midgut proteinase. However, only BuXI, but not BvTI inhibits LOPAP with Ki 15 nM (Table 2; Andrade et al., in press). BbKI strongly inhibits the enzyme isolated from the digestive system of the cockroach Periplaneta americana (K_i 1.1 nM) but not Diatraea saccharalis trypsin-like activity (Table 2) (Andrade et al., in press).

Compounds Derived from Bauhinia Inhibitors. Structure as Tool to Investigate Biological Processes.

We have also shown that the structural features of the studied Bauhinia inhibitors may drive the design of substrates and also peptide inhibitors.

As the inactivation of factor Xa distinguishes BuXI from other Bauhinia inhibitors, synthetic peptides based on its reactive site sequence were used as substrates for further characterization of factor Xa binding.

The substrate Abz-VMIAALPRTMFIQ-EDDnp (leading peptide) was hydrolyzed by factor Xa with a high catalytic efficiency (k_{cat}/K_m 4.3 x 10^7 $M^{-1}sec^{-1}$), 10^4-fold higher than that of Boc-Ile-Glu-Gly-Arg-AMC widely used as factor Xa substrate, and both Met residues in the substrate influence the binding to factor Xa. The change of threonine (P_1') for serine decreases the catalytic efficiency by four orders of magnitude. Factor Xa did not act on the substrate containing the reactive site sequence of BvTI, that inhibits trypsin but not factor Xa (Andrade et al, in press; Oliva et al., submitted). Like factor Xa, LOPAP cleaves Abz-VMIAALPRTMFIQ-EDDnp (k_{cat}/K_m=3.5x10^4 $M^{-1}sec^{-1}$), but not BvTI derived substrate. Two methionine residues are also involved in LOPAP substrate interaction, in as much as in factor Xa interaction, since the change of both Met residues abolished both factor Xa and LOPAP catalyzed hydrolysis (Andrade et al., in press).

A synthetic peptide containing BbKI reactive site was demonstrated to inhibit the proteolytic activity of human plasma kallikrein (Oliva et al., 2001b). The quenched fluorogenic substrates containing BbKI reactive site sequence are poorly cleaved by kallikrein, factor Xa, factor XIIa, thrombin and plasmin but they are hydrolyzed by the enzyme isolated from Periplaneta americana with catalytic efficiency in the range of 10^8 to 10^9 M^{-1}. sec^{-1} (Oliva et al., 2001b; Andrade et al., in press).

These results show that based on naturally available Bauhinia inhibitors, highly specific substrates may be devised and used to characterize trypsin-like enzyme activities, especially the large family of insect digestive enzymes.

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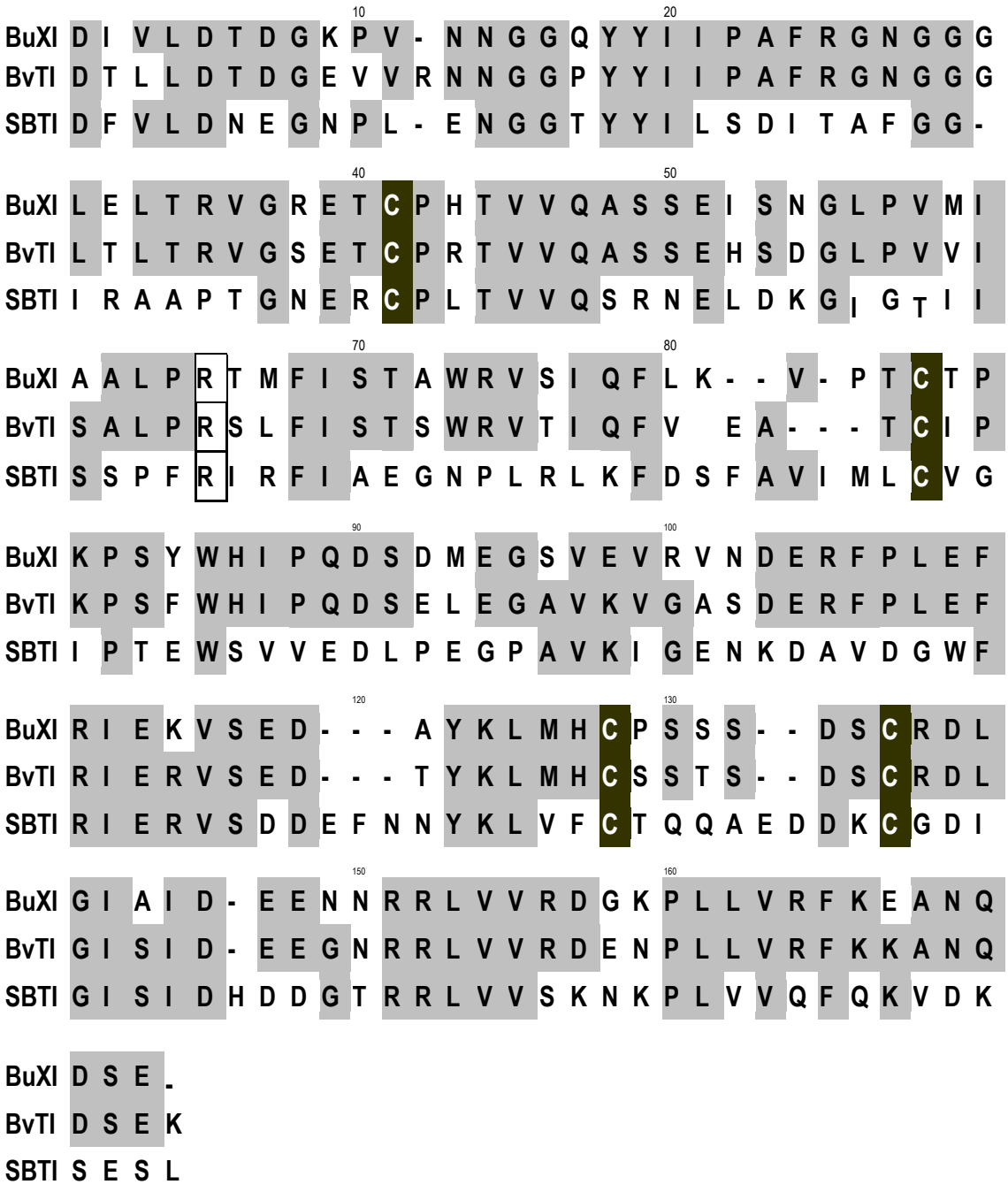
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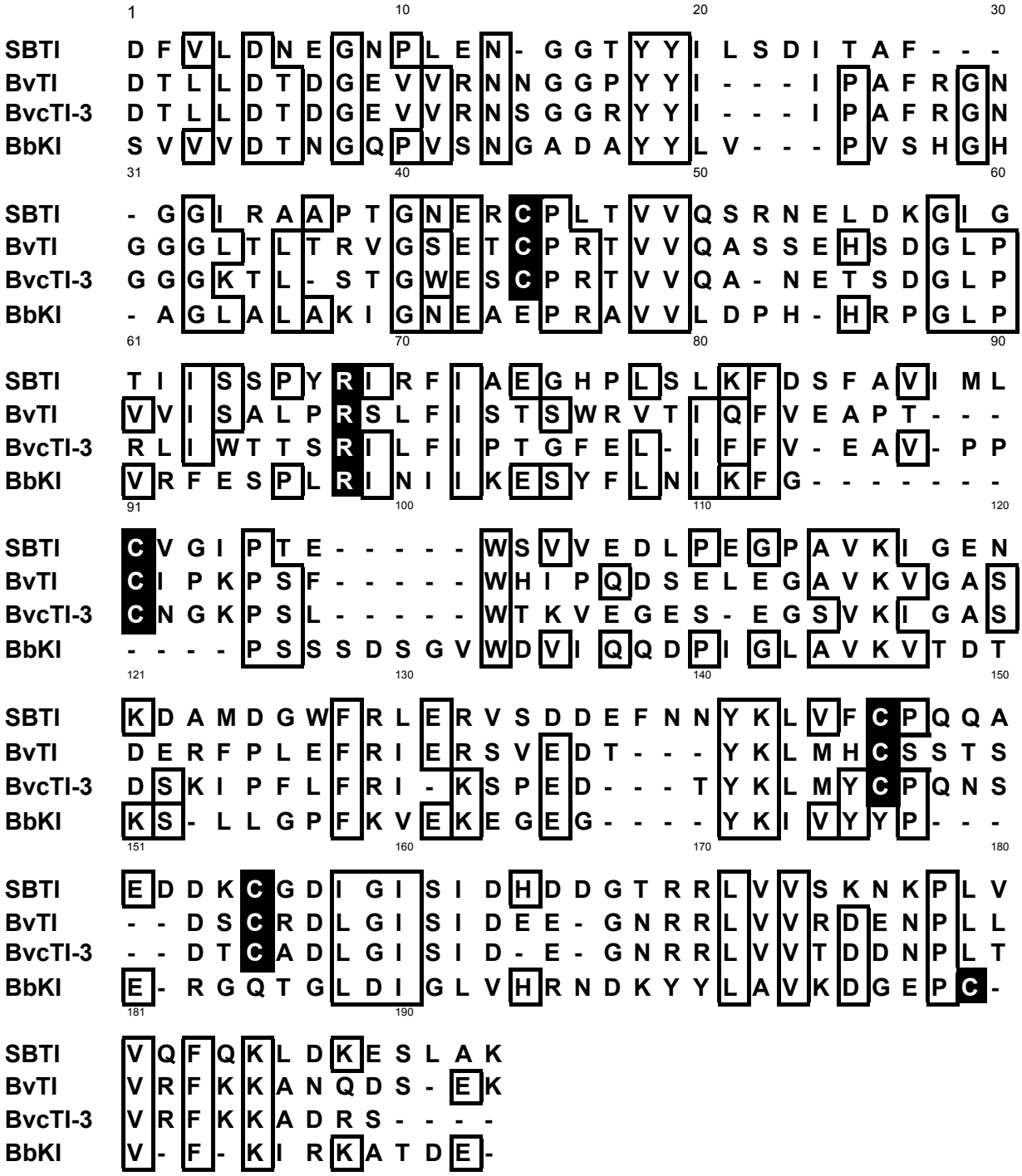

Figure 2. Comparative sequences of related Kunitz inhibitors. 1, SBTI (soybean trypsin inhibitor); 2, BvTI (Bauhinia variegata Trypsin Inhibitor); 3, BvTI-c (Bauhinia variegata, var. *candida* Trypsin inhibitor); 4, BbKI – Bauhinia bauhinioides Kallikrein Inhibitor. Identical residues are in the white boxes and cysteine residues and inhibitors reactive sites are underlayed in black color.

Table 1. Bauhinia Inhibitors Dissociation Constants K_i [M] (Oliva et al., 2001b)

Enzyme	BuXI	BbKI	BvTI
Trypsin	2.0×10^{-8}	0.36×10^{-9}	1.5×10^{-9}
Chymotrypsin	2.7×10^{-9}	3.9×10^{-6}	1.2×10^{-8}
Human factor XIIa	7.4×10^{-8}	1.1×10^{-7}	2.1×10^{-8}
Human plasma kallikrein	6.9×10^{-9}	0.35×10^{-9}	2.3×10^{-8}
Human factor Xa	1.4×10^{-8}	ϕ	ϕ
Plasmin	7.6×10^{-8}	ϕ	2.9×10^{-9}
Thrombin	ϕ	ϕ	ϕ.
Tissue kallikrein	ϕ	ϕ	ϕ

BuXI, Bauhinia ungulata Factor Xa Inhibitor; BbKI, Bauhinia bauhinioides kallikrein inhibitor; BvTI, Bauhinia variegata Trypsin Inhibitor. ϕ no inhibition

The K_i values were determined using specific substrates for each enzyme: tissue kallikrein porcine pancreatic and human urinary kallikrein, H-Pro-Phe-Arg-AMC (40 µM); chymotrypsin, Suc-Phe-pNan (3.0 mM); human plasma kallikrein, Ac-Phe-Arg-pNan (0.8 mM); plasmin, H-D-Val-Leu-Lys-pNan (0.2 mM); thrombin, H-D-Phe-L-Pip-L-Arg-pNan (0.2 mM); factor Xa, Boc-Ile-Glu-Gly-Arg-AMC (0.3 mM).

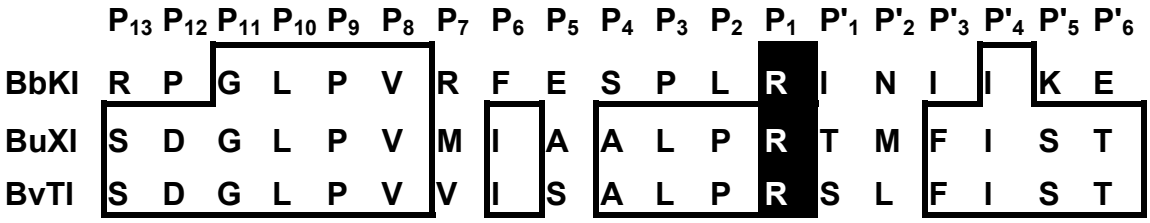

Figure 3. Comparison of partial sequences of related Kunitz inhibitors. BbKI, Bauhinia bauhinioides Kallikrein Inhibitor; BuXI, Bauhinia ungulata Factor Xa Inhibitor; BvTI, Bauhinia variegata Trypsin Inhibitor. Black boxes indicate the reactive site residue for trypsin inhibition. Identical residues are in white boxes (Oliva et al., 2001b).

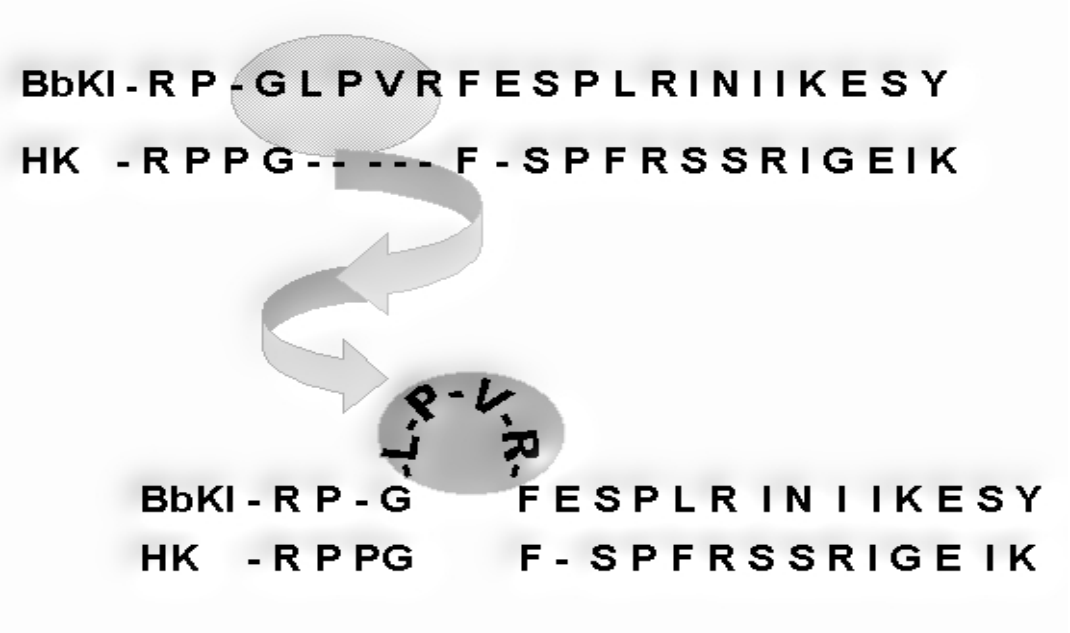

Figure 4. B. bauhinioides kallikrein Inhibitor (BbKI) reactive site homology to the kinin moiety in human kininogen (HK).

Table 2. Dissociation constants (K_i, M) for Bauhinia Inhibitors action on bovine trypsin, bovine chymotrypsin, insect midgut trypsin- like enzymes, and LOPAP (Andrade et al., *in press*).

Enzyme	**BuXI**	**BvTI**	**BbKI**
Bovine trypsin	$2.1x10^{-8}$	$1.5x10^{-9}$	$0.36x10^{-9}$
Bovine chymotrypsin	$2.8x10^{-9}$	$1.2x10^{-8}$	$3.9x10^{-6}$
M. domestica	$6.4x10^{-8}$	$5.3x10^{-8}$	nd
A. flavolineata	$7.5x10^{-8}$	$5.0x10^{-8}$	nd
T. molitor	$1.2x10^{-7}$	$4.8x10^{-8}$	nd
S. frugiperda	$3.0x10^{-9}$	$1.8x10^{-8}$	nd
P. americana	nd	nd	$1.1x10^{-9}$
D. saccharalis	nd	nd	ϕ
LOPAP	$1.5x10^{-8}$	ϕ	nd

BuXI, Bauhinia ungulata factor Xa inhibitor; BvTI, Bauhinia variegata trypsin inhibitor. ϕ -no inhibition; nd not determined. K_i was determined using specific substrates for each enzyme: Bz-Arg-pNan (bovine trypsin and midgut trypsin-like enzymes); Suc-Phe-pNan (chymotrypsin); Abz-YQTFFNPRTFGSQ-EDDnp (LOPAP), Z-Arg-MCA (P. americana enzyme), Suc-Phe-Arg-MCA) (D. saccharalis).