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Action of *Bauhinia bauhinioides* synthetic peptides on serine proteinases

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Abstract

The kallikrein inhibitor found in *Bauhinia bauhinioides* seeds (BbKI) differs from classical Kunitz plant inhibitors in the lack of disulfide bridges in its structure [Biochim. Biophys. Acta 1477 (2000) 64–74]. In this study, we examined whether structural properties may be involved in inhibitory specificity and, if so, whether those properties might be useful tools in designing compounds that interfere with enzyme activity. Peptides structurally related to the BbKI (RPGLPVRFESPLRINIIKE-NH_2) reactive site were synthesized by solid-phase method and assayed for serine proteinase activity. The peptides RPGLPVRFESPLRINIIKE-NH_2, RPGLPVRFESPL-NH_2, and GLPVRFES-NH_2 were efficient tissue kallikrein inhibitors, with I_{50} values of 0.54 μM, 0.87 μM, and 0.5 mM, respectively. The lasting inhibitory effect was observed in incubation periods of up to 120 min. None of the studied peptides interfere with the activity of thrombin, factor Xa or trypsin, although the native protein BbKI is a potent trypsin inhibitor.

Keywords: *Bauhinia bauhinioides*; Kallikrein; Kunitz inhibitor; Plant inhibitor; Peptides; Primary structure; Serine proteinase; Tissue kallikrein

Serine proteinases such as elastase, kallikrein, plasmin, and thrombin play important roles in various processes. They are associated with, among other events, inflammation, coagulation, tissue remodeling, tumor invasion, and fibrinolysis [1–3]. Kallikreins, the essential enzymes of the kallikrein-kinin physiological system, are involved in the regulation of blood pressure, inflammation, and other processes. Kallikreins, found in glandular cells, neutrophils, and biological fluids, are divided into two main groups: tissue or glandular kallikrein and plasma kallikrein [4,5]. Kinins, produced by the action of kallikreins on kininogens, are vasoactive components that mediate various responses to injuries. Two different receptors (B1 and B2) mediate the mechanism of kinin action [3,5].

Endogenously, the proteolytic activity of these enzymes is regulated by a superfamily of inhibitors known as serpins, which share structural similarity and a common mode of action [6,7]. Since serpins with identical amino acid residues at the P1 position of the reactive site inhibit different proteinases, other residues must be involved in specificity determination. The amino acid sequence of the reactive site varies among serpins and determines the specificity of the inhibitors. Investigation of this subject revealed a distinct contribution of different residues in the inhibition of target proteinases [8,9].

Among the different families of plant inhibitors [10], the Kunitz type comprises a large number of proteins that are characterized in terms of structure and mechanism of action [11–13]. Kunitz type inhibitors have been characterized by their ability to inhibit proteinases that are involved in physiological events such as coagulation, inflammation, and cancer [14]. Studies have clearly demonstrated that both the sequence and the amino acid localized at P1 position are important to be recognized by the target enzyme [15,16]. Due to the involvement of proteinases in several pathological events, characterizing the structure and function of new inhibitors can be important tools for designing new compounds to be used as therapeutic agents.

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A new type of Kunitz inhibitor, *Bauhinia bauhinioides* kallikrein inhibitor (BbKI), which is purified from *B. bauhinioides* seeds, has a structure devoid of disulfide bridges. The similarity of BbKI to the bradykinin moiety in kininogen may explain its stronger binding to plasma kallikrein [17]. In previous studies, we have also shown that, due to the high specificity of BbKI structural features for trypsin or trypsin-like enzyme activities, these features can be useful as substrates for investigating enzyme specificity [13,15]. They may be especially useful for investigations involving the large family of insect enzymes. Moreover, a synthetic peptide containing the BbKI reactive site has been demonstrated to inhibit the proteolytic activity of human plasma kallikrein [13]. To further characterize the roles of the reactive site region residues in complex formation and in specificity determination, we synthesized peptides based on the BbKI structure and analyzed their inhibitory properties on enzymes involved in blood coagulation, inflammation, and fibrinolysis.

Materials and methods

Enzymes, substrates, and peptides. Human plasma kallikrein (HuPK) (EC 3.4.21.34) was purified by a previously described procedure [18]. Porcine pancreatic kallikrein (PoPK) (EC 3.4.21.35), trypsin (EC 3.4.21.4), human thrombin (EC 3.4.21.5), and plasmin (EC 3.4.21.7) were purchased from Sigma Chemical Company. Human neutrophil elastase (EC 3.4.21.37) and porcine pancreatic elastase (PPE) (EC 3.4.21.36) were purchased from Calbiochem. The H-D-Pro-Phe-Arg-AMC, Suc-Ala-Ala-Pro-Val-AMC, Boc-Val-Pro-Arg-AMC, Ac-Phe-Arg-AMC, Suc-Ala-Ala-Pro-Val-pNan, and H-D-Val-Leu-Lys-pNan were purchased from Calbiochem (San Diego, USA) and the $N\alpha$-benzoyl-D-L-arginine-*p*-nitroanilide (BAPA) was purchased from Merck.

Peptides. Peptides containing the amino acid sequence of the BbKI site were synthesized and solubilized in HEPES/DMSO 4:1 (v/v) [12].

Inhibition assays and kinetics. The assays were performed in a 96-well plate in a final volume of 250–270 μl as described by Oliva et al. [12,13]. The peptides were incubated at 37 °C with one of the following serine proteinases: porcine pancreatic elastase (18 nM at pH 8.0) in 0.1 M Tris–HCl buffer containing 0.5 M NaCl; trypsin (0.2 μM at pH 8.0) in 50 mM Tris–HCl buffer containing 0.02% $CaCl_2$; porcine pancreatic kallikrein (18 nM at pH 9.0) in 50 mM Tris–HCl buffer containing 0.1% albumin; human plasma kallikrein (14 nM at pH 8.0) in 0.1 M Tris–HCl buffer containing 1 M NaCl; thrombin (2.0 uNHI at pH 8.0) in 50 mM Tris–HCl buffer; or plasmin (55 nM at pH 7.4) in 0.1 M Tris–HCl buffer containing 0.2 M NaCl. In all assays, K_m substrate concentration was applied at least twice, with the exception of during investigation of inhibitory mechanisms, in which case the experiment was performed with increasing substrate concentration.

Investigation of the reactivity of the peptides with target proteinases. For the kinetic assays, enzymes and inhibitors in concentrations sufficient to inhibit more than 50% of enzyme activity were incubated at 37 °C in the appropriate buffer. At various times (0–120 min), aliquots of the reaction mixture were removed, the appropriate substrate for each enzyme was added, and the reaction was followed for 10–20 min. Radioimmunoassay PoPK (48 nM) in 50 mM Tris–HCl buffer, pH 9.0, without or with the peptides, was incubated with kininogen (0.5–0.6 μM) in a final volume of 20 μl at 37 °C for 2 h. The kinin was extracted according to the technique described by Araujo et al. [19].

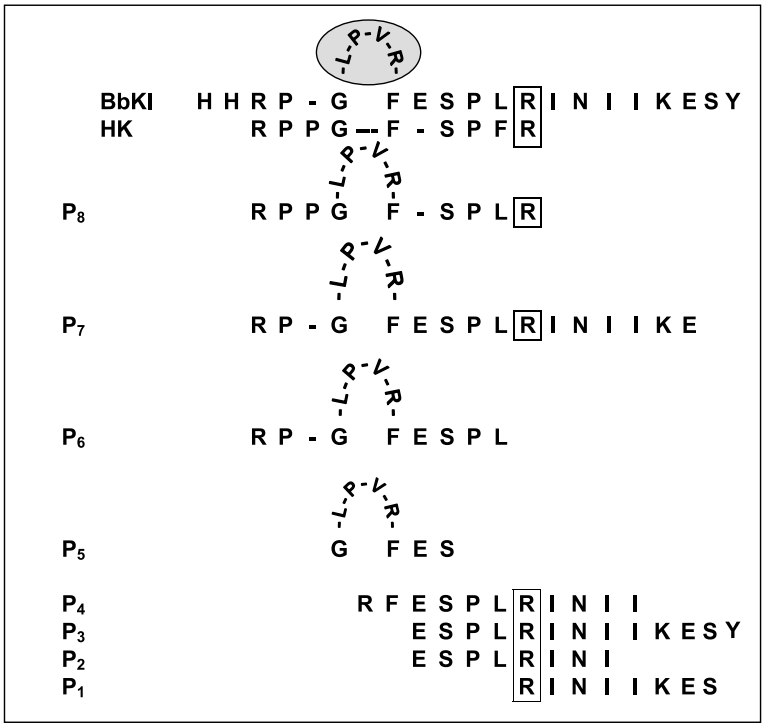

Fig. 1. (BbKI) *B. bauhinioides* kallikrein inhibitor reactive site homology to the kinin moiety in human kininogen (HK). P_1–P_8 Synthetic peptides derived from BbKI reactive site.The Arg at P1 position of BbKI reactive site are in the box.

Results and discussion

Previously published results have shown that fluorogenic substrate derived from the BbKI reactive site and hydrolyzed by bovine trypsin or trypsin-like enzymes from insect sources displays resistance to hydrolysis by other serine proteinases [12,13,15]. This led us to investigate the reactivity of BbKI-derived synthetic peptides to target and non-target proteinases (Fig. 1A).

The peptides ESPLRINI-NH_2 (P_2) and RFESPLRINII-NH_2 (P_4), containing the amino acid Arg at the P1 position of the reactive site of trypsin inhibition, had no effect on the activity of any enzyme analyzed. Those comprising P1 and P2 (RINIIKES-NH_2 [P_1] and ESPLRINIIKESY-NH_2 [P_3]) interfere with HuPK and PoPK activities.

The largest peptide, RPGLPVRFESPLRINIIKE-NH_2 (P_7), which presents the sequence **L-P-V-R-** (loop shown in Fig. 1) and the amino acid at the P1 position for trypsin inhibition, has a deleterious effect on plasma and tissue kallikrein activity. However, it has no effect on the activities of trypsin or of any other serine proteinase.

Our studies involving peptide–enzyme interaction indicate that RINIIKES-NH_2 binds to the enzyme as a substrate, evidenced by the abolition of peptide interference with enzyme activity during various phases of pre-incubation. The results show that, once hydrolyzed, the peptide no longer competes with other commercial H-D-Pro-Phe-Arg-MCA substrates.

Analyzing the contribution of the N-terminal side of Arg at the P1 position, the results showed a significant effect of RPGLPVRFESPL-NH_2 (P_6) and GLPVRFES-NH_2 (P_5) on H-D-Pro-Phe-Arg-MCA hydrolysis by PoPK.

The peptides RPGLPVRFESPLRINIIKE-NH_2 (P_7), RPGLPVRFESPL-NH_2 (P_6), and GLPVRFES-NH_2 (P_5) strongly inhibit PoPK (Figs. 2A–C) and display tight interaction with the native protein. The resistance of these peptides to hydrolysis by tissue kallikrein was

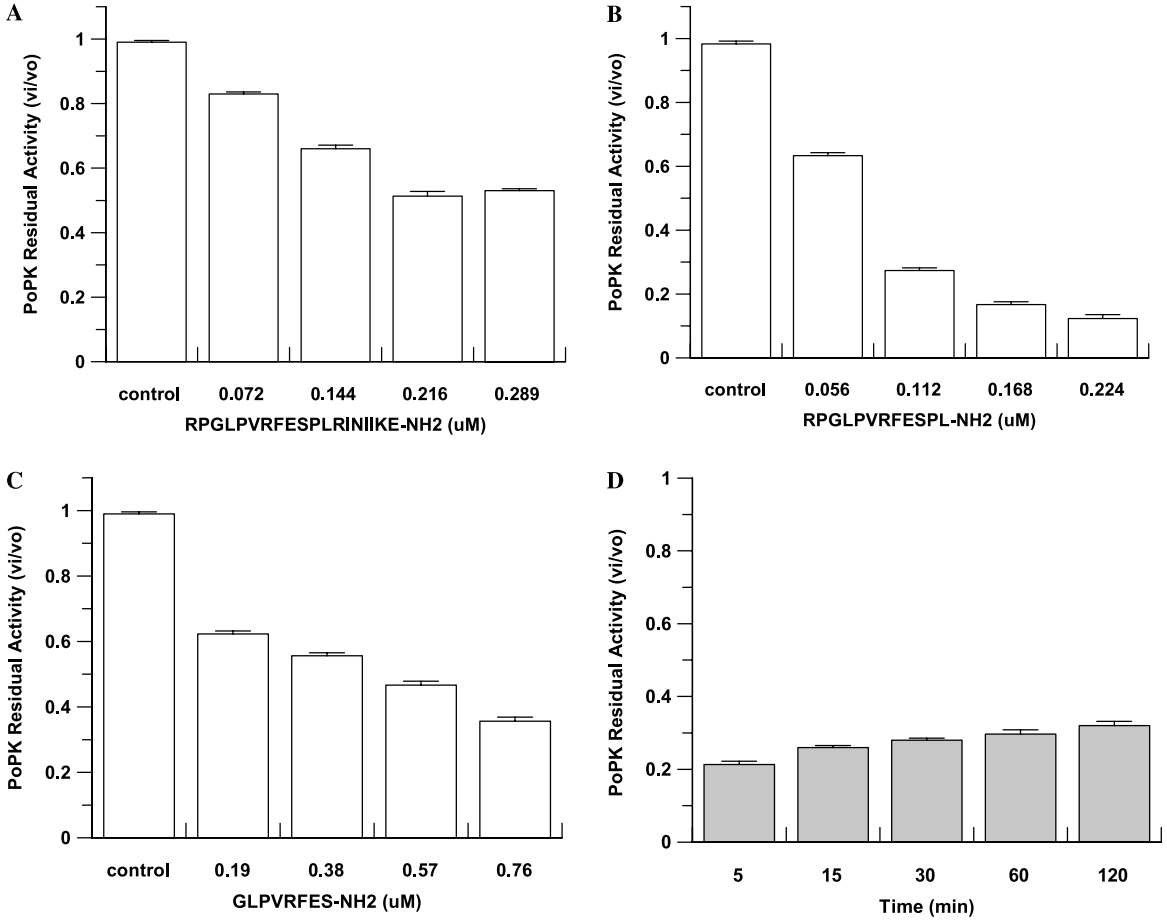

Fig. 2. Inhibitory effect of peptides containing BbKI-related sequences on PoPK activity. PoPK (18 nM) was incubated (20 min, 37 °C in 50 mM Tris–HCl buffer, 0.1% albumin, pH 9.0) with increasing amounts of (A) RPGLPVRFESPLRINIIKE-NH_2, (B) RPGLPVRFESPL-NH_2, and (C) GLPVRFES-NH_2. The residual PoPK activity was assayed on H-D-Pro-Phe-Arg-MCA (0.2 mM) substrate. (D) Effect of RPGLPVRFESPL-NH_2 on PoPK activity. PoPK (18 nM) was incubated with peptides (0.4 mM) for 5–120 min, 37 °C in 50 mM Tris–HCl buffer, and 0.1% albumin, pH 9.0. Enzyme activity was detected as previously described.

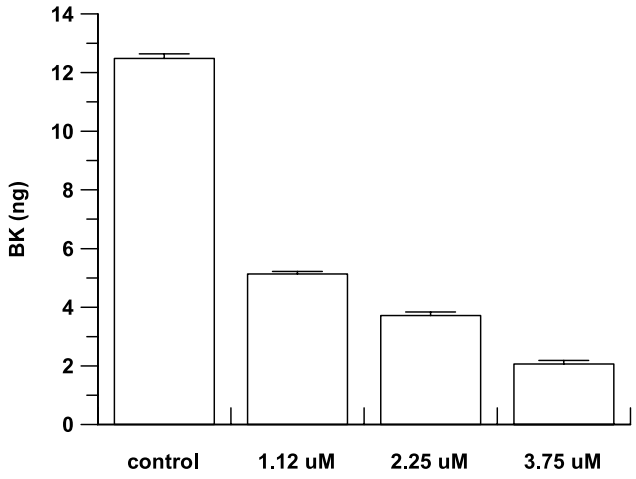

Fig. 3. Effect of RPGLPVRFESPLRINIIKE-NH_2 on kallikrein kinin release activity. Increasing amounts of RPGLPVRFESPLRINIIKE-NH_2 were pre-incubated for 20 min, 37 °C in 50 mM Tris–HCl buffer, 0.1% albumin, pH 9.0, with PoPK (48 nM). BK release was detected as described in Methods.

confirmed by a longer incubation period (up to 120 min) as shown in Fig. 2D for the peptide RPGLPVRFESPL-NH_2 (P_6). The resistance to hydrolysis by PoPK was confirmed by reverse-phase chromatography, in which the enzyme and peptides were incubated for 30 min and the reaction mixture was subjected to RP-HPLC analysis. No differences were observed between enzyme–peptide and peptide elution profiles (data not shown).

The I_{50} values for PoPK were determined at 0.54 μM, 0.87 μM, and 0.5 mM, respectively. A common feature of peptides that exhibit an inhibitory effect on tissue kallikrein is the presence of the sequence **LPVR-E** that is inserted into the bradykinin motif in the native protein (Fig. 1). The enzyme activity recovered by increasing substrate concentration characterizes these peptides as competitive inhibitors. The action of the peptides on PoPK activity was also observed by radioimmunoassay and is shown for RPGLPVRFESPLRINIIKE-NH_2 in Fig. 3.

The data show that other regions in the BbKI structure are contact sites for the maintenance of the complex. Furthermore, the reactive site sequence should be capable of interacting with HuPK or PoPK and thereby forming a stable complex.

None of the peptides studied blocked elastase, thrombin, factor Xa or trypsin, although the native protein BbKI is a potent trypsin inhibitor.

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