STRUCTURAL AND FUNCTIONAL ASPECTS OF PROTEINASES AND INHIBITORS: CLONING, STRUCTURAL MODIFICATION, INFLAMMATION, HOMEOSTASIS, CELL BIOLOGY AND INSECT GROWING

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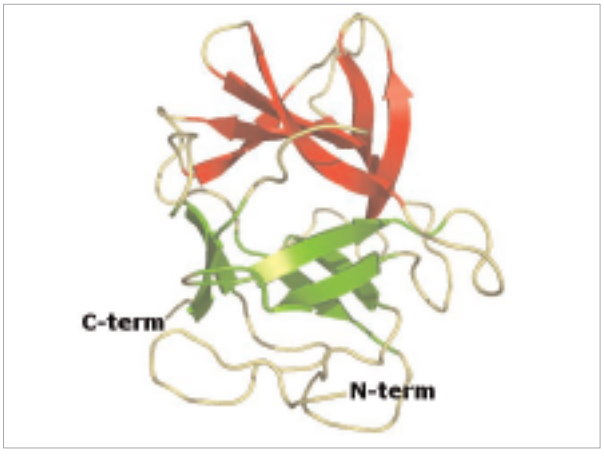
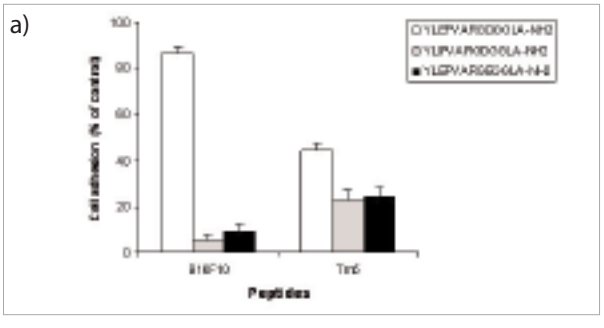
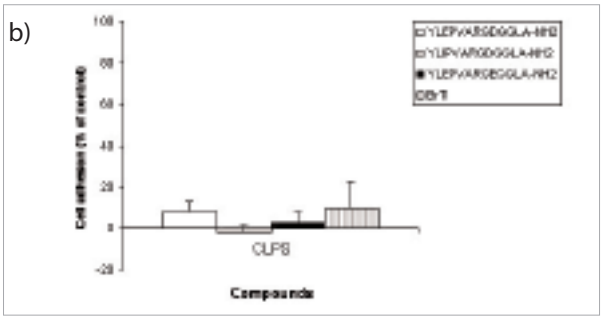


The overall structure of the recombinant Bauhinia bauhinioides *cruzipain inhibitor (rBbCI) . rBbCI has a , β-trefoil fold, formed by six two-stranded hairpins. Three of these hairpins form a barrel structure (in green) and the other three are in a triangular array that caps the barrel (in red).*





(a) Effect of synthetic peptides on cell adhesion of B16F10 (high-metastatic B16 murine mouse melanoma cell line) and of Tm5 (murine melanoma cell lines derived from a nontumorigenic lineage of pigmented murine melanocytes, melan-a). (b) The cell line was preincubated with synthetic peptides or BrTI (70 µM) for 15 minutes, and then subsequently added onto fibronectin coated plates (10 µg/ml) and incubated for 1 hour

Proteinases control a large number of key physiological processes such as cell-cycle progression, cell proliferation, cell death, DNA replication, tissue remodeling, homeostasis (coagulation), wound healing and the immune response. Also, these enzymes have been correlated with the invasion process of many parasites, demonstrating interactions with the host immune system.

Multiple molecular forms of inhibitor proteins have been characterized from microorganisms, animals and plants. These proteins are believed to participate in various physiological functions such as the regulation of proteolytic cascades and safe storage of proteins, as well as defense molecules against plant pest and pathogens. Concerning proteinase inhibitors, many members of the plant Kunitz family, purified from leguminous plants, showed an ability to inactivate some enzymes involved in blood clotting and fibrinolysis. Such inhibitors also exhibited anti-inflammatory effects, and decreased bradykinin release.

Brazilian forests, Pantanal and Cerrado regions are considered as great sources for natural products, among which are proteins capable of inhibiting proteinases. Such proteins can be used as models to study physiopathological mechanisms. Regarding clotting enzymes' inhibitors, the structural features that determine their specificity may be identified for target enzymes. For this purpose, the aim of this project includes: (a) the comparative study of serine proteinase inhibitors isolated from Leguminosae seeds; (b) primary and secondary structures determination, and modeling of inhibitors in order to identify specific amino acid residues important for biological action; (c) design of peptide substrates based on the inhibitor's reactive structure; (d) design of synthetic inhibitors structurally related to native proteins for blood clotting enzyme assays; (e) cloning and molecular modification for blood clotting enzyme assays will also be used on proteins isolated from Brazilian snakes, leeches and caterpillars; (f) characterization of the interaction between isolated and designed inhibitors and enzymes will be investigated in different biological processes such as inflammation, receptor and cell membrane association with endothelial cells, tumors, bacteria, yeast and the development of insect growth.

SUMMARY OF RESULTS TO DATE AND PERSPECTIVES

We reported that the inhibitors BbKI and BbCI, obtained from seeds of *Bauhinia bauhinioides*, are 18 kDa proteins similar to other plant Kunitz-type inhibitors, but differ by the absence of disulfide bridges, and in their inhibition specificity. Following the heterologous expression and production of BbCI and BbKI recombinants in *E. coli*, both proteins showed potent inhibitory activities towards their respective proteinases, similar to the wild-type proteins. BbCI inhibits the human serine proteinase neutrophil elastase and pancreatic porcine elastase, and the cysteine proteinases cathepsin L and cruzipain from *Trypanosoma cruzi*. In spite of BbKI's structural identity to BbCI (84%), it differs from the latter by inhibiting plasma kallikrein, bovine trypsin and human plasmin. We are currently evaluating the inhibitory capacity of these proteinase inhibitors on cell viability of different tumor cell lines, primary human fibroblasts and on the proliferation capacity of human mesenchymal stem cells.

In parallel our collaborators also studied the interaction of human high molecular weight kininogen, a cystatin, with either endothelial or tumor cells. Human kininogens are intravascular proteins of blood plasma and play a role in cell and vascular biology. High molecular weight kininogen (HK) presents antithrombotic, antiadhesive and profibrinolytic activities. HK binds to endothelial cells where it can be cleaved by plasma and tissue kallikreins and release kinins. Heparan and chondroitin sulfate proteoglycans are described as kininogen receptors on the cell surface. This study analyzes the influence of proteoglycans on HK interaction with the cell surface. HK assembly, on endothelial cells (RAEC), was totally blocked by a peptide equivalent to a sequence from HK domain 5. Confocal microscopy experiments showed that HK co-localizes with heparan sulfate proteoglycans (HSPG) and cathepsin B on the cell surface. Our data show that HK is endocytosed by endothelial (RAEC) and tumorigenic cells (CHO-K1). In contrast, CHO-745 cells, which are almost completely devoid of glycosaminoglycan synthesis, do not take up HK. The endocytosed HK was detected in acidic endosomal vesicles. The process of HK internalization was blocked by low temperature, chloroquine, methyl-beta-cyclodextrin, FCCP and 2-deoxy-D-glucose indicating an active process of endocytosis dependent on membrane lipid raft domain. Cellular HK uptake occurs concomitantly with kinin release at the cell surface; besides serine protease, we also detected the involvement of neutral cysteine protease in kinin release. The present data report the HK endocytosis process by HSPG, as a novel additional mechanism for controlling kinin generation at the cell surface, suggesting that endocytosis of HK/HKa molecules is a control process of both angiogenic and antiangiogenic stimuli respectively mediated by these molecules.

MAIN PUBLICATIONS

Oliva MLV, Sampaio MU. Bauhinia Kunitz-tipe inhibitors: structural characteristics and biological properties. *Biological Chemistry*. 2008, *in press*.

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