REPORT

Novel protease inhibitors active against human neutrophil elastase and plasma kallikrein with therapeutic potentialities: Structure-function relationships

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ABSTRACT

Two new protease inhibitors (PIs), CmPI-II and AdKI were purified and characterized from mollusks *Cenchritis muricatus* and *Aplysia dactylomela*, respectively. They showed different specificities, CmPI-II for human neutrophil elastase (HNE) and AdKI for human plasma kallikrein (HPK). Purification procedures were established, rendering good yields and high purification degree. CmPI-II (UNIPROT: P84755) is a 5480 Da polypeptide of three disulphide bridges belong to the "non-classical" Kazal-type inhibitors. A new group was proposed according to the location of the CysI-CysV disulfide bridge. The presence of a basic residue at the inhibitor active site changed the pre-established requirement of a hydrophobic residue for elastase inhibition. The three-dimensional CmPI-II/HNE complex model contributes to explain the CmPI-II specificity for the enzyme. This is the first PI molecule isolated from C. *muricatus*. On the other hand, AdKI (2.9 kDa polypeptide) is an exception among invertebrate inhibitors in terms of inhibitory strength and selectivity against HPK. A new serine protease, AdSP, was also purified and characterized from the same extract, which could be the target for AdKI. CmPI-II and AdKI are the first inhibitors isolated from the *phyllum Mollusca* against ENH and HPK, respectively.

Keywords: inhibitor, protease, marine invertebrates, mollusk

Introduction

Protease inhibitors (PIs) are relevant for vital processes that have been evidenced by its presence in a wide range of tissues, by hampering uncontrolled proteolysis or guaranteeing partial proteolysis as physiological event [1].

PIs have received interest for multiple applications in biotechnology and biomedicine beyond its most traditional application in protein-protein interaction studied, focused mainly in therapeutics. Recent findings have demonstrated proteolysis control as a pharmacologically valid tool, by using several PIs to treat systemic and infectious diseases. Both, therapeutic efficiency and potentiality of PIs have been exemplified in treating AIDS, inflammatory, immune and respiratory diseases, and cardiovascular and neurodegenerative disorders (as Alzheimer disease) [2].

Among the target proteases for PIs have been found the serine proteases (SP), which are involved in several physiological and pathological processes. One of them, is the human neutrophil elastase (HNE; E.C. 3.4.21.37), belongs to S1 family [3], a target on several diseases. Its inhibitors are efficient tools for studying the HNE physiological functions, also potential therapeutic candidates for pulmonary emphysema, adult respiratory distress, rheumatoid arthritis, and other diseases [4], or even core structures to design more efficient candidates. So far, synthetic PIs developed against HNE have shown high toxicity and collateral effects [5].

Another PIs therapeutic target is the human plasma kallikrein (HPK; E.C. 3.4.21.37), belongs to S1 family [3], intervening in several cardiovascular and inflammatory processes. This enzyme shows antithrombotic, profibrinolytic, proinflamamtory, and vasodilator properties, its PIs causing pro-coagulant, anti-inflammatory and vasoconstrictor effects [6]. Aprotinin, a PI belonging to the Kunitz/BPTI family, is the only PI commercially available for cardiovascular surgery applications but shows a wider specificity low affinity against [2]. Therefore, structure-function studies on the interaction of these target proteases with new inhibitors will provide knowledge on the PIs mechanisms of action and might contribute to an improved design of therapeutic candidates.

Marine invertebrates are natural sources for biomolecules of remarkable biological activity. They are widely distributed throughout Cuban shores as a significant source for bioactive compounds as tight binding PIs, which show protease-inhibitor dissociation constants (Ki) lower than 10-7 mol/L [7-10].

In this work, two new PI molecules, CmPI-II and AdKI, were isolated from the Cuban mollusks *Cenchritis muricatus* and *Aplysia dactylomela*, respectively, being also identified and characterized.

Results

Identification and characterization of PIs from the littorinid C. *muricatus* mollusk

Inhibitory activity of serine proteases was detected in saline extracts of *C. muricatus*, a littorinid gastropod (*Mollusca*) found in Cuban shores. At least three molecular entities were resistant at 60 ºC heating, showing different molecular masses and specificities against

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serine proteases [11, 12]. The major fraction, named CmPI-II, was purified to homogeneity by a simple, reproducible and efficient method, by applying the heated extract supernatant onto a trypsin-Sepharose affinity chromatography matrix. The eluate fraction was further applied onto a Hitrap-Q HP anion exchange chromatography column, yielding a highly pure inhibitor as corroborated by the presence of a single and symmetric peak in a C18 matrix profile obtained from a high resolution system (RP-HPLC). The purified protein had a single N-terminus determined by the automatic Edman degradation method [11, 12].

Specificity studies showed that CmPI-II was able to strongly inhibit SPs belonging to the S1 family, such as HNE (Ki 2.6 ± 0.9 nmol/L), trypsin (Ki 1.1 ± 0.2 nmol/L) and porcine pancreatic elastase (Ki 145.0 ± 4.4 nmol/L), as well as Bacillus licheniformis subtilisin A (Ki 30.8 ± 1.2 nmol/L), belonging to the S8 family (clan SB). However, the inhibitor was unable to affect the activity of other SPs as chymotrypsin, plasma or tissular kallikrein, thrombin, plasmin or papain (cystein proteases) [11, 13] .

CmPI-II (UNIPROT: P84755) is a 50 residues protein of 5480 Da molecular mass and three disulphide bridges, which was established combining the automatic Edman degradation and ESI-MS/MS mass spectrometry [11-13]. The CmPI-II aminoacid sequence was similar to those of other protease inhibitors belonging to the Kazal-type family (MEROPS I1). Multiple sequence alignment for CmPI-II classified as "non-classical" Kazal-type inhibitor allowing establish a new "non-classical" group on the basis of the distinctive positioning of the CysI-CysV disulphide bridge compare with other "non-classical" Kazal-inhibitors from different sources [11, 13].

Additionally, the proposed of three-dimensional (3D) model of CmPI-II revealed properties similar to those of the Kazal-type family: a central α-helix, three β-sheets and a protruding loop at the N-terminus region where the reactive site (P1) is located (Figure 1A) [11, 13].

On the other hand, the CmPI-II specificity became an exception among the Kazal-type inhibitors by inhibiting elastases with a basic residue (Arg12) at P1 site [11-13]. The CmPI/II-HNE complex model (Figure 1B) was proposed by comparison to the crystallographic structure of the turkey ovomucoid third domain (OMTKY3)/HNE complex (PDB code: 1PPF) [14]. The CmPI/II-HNE complex model indicates similar contacts in the primary binding sites P3, P2, P1, P1' and P2' of CmPI-II with the enzyme, and additional contacts within the primary (P6, P1, P1' and P2') and secondary (P11' and P14') binding sites. There were also different contacts at P4 and P5 sites, contributing to explain the strong inhibition of the enzyme (Figures 1C) [11, 13]. The most significant association energy contribution came from the interaction of Arg 12 at P1 residue with Asp226 at the botton of the HNE S1 pocket, as established by the CmPI-II/ENH complex model, suggesting that the CmPI-II Arg12 residue penetrate and fitted at S1 binding site of the enzyme (Figure 1C).

On the other hand, CmPI-II was obtained by recombinant procedures due to its structure and function properties. The synthetic gene was cloned into an expression vector for *Pichia pastoris* system. The recombinant protein was obtained at working bench scale in bioreactors with high yields. The recombinant CmPI-II inhibitor (rCmPI-II) showed structure and function properties similar to those of the natural inhibitor [15]. Besides, protein-protein interaction studies using IF MALDI-TOF MS methodology [16], demonstrated the interaction of rCmPI-IIr with trypsin and subtlisin A (Figure 2). The recombinant availability of the inhibitor supports its application in basic and applied studies at amounts higher than those naturally obtained and without affecting the natural source.

Purification and characterization of a human plasma kallikrein inhibitor and a serine protease from the marine mollusk *A. dactylomela*

An aqueous extract from another gastropod of the Cuban shores, *A. dactylomela*, commonly known as sea hares, showed SP inhibitory activity [17]. An inhibitor active against human plasma kallikrein (HPK), named AdKI, was purified to homogeneity by a different protocol from that described above. The purification was achieved by acetone fractionation (80%) v/v, ion-exchange chromatography on Mono Q column and gel filtration chromatography on Superdex 75 column (FPLC system) [18]. Molecular characterization studies showed that AdKI is a small polypeptide (2.9 kDa), which showed N-terminus with low homology (< 30%) to Kunitz-BPTI family inhibitors.

AdKI is a tight-binding inhibitor against HPK (Ki 2.2 x 10^{-10} M), human plasmin (Ki 1.8 x 10^{-9} M) and pancreatic trypsin (Ki 4.7 x 10^{-9} M), but is inactive against bovine pancreatic chymotrypsin, tissular kallikrein, pancreatic elastase and thrombin, among other SPs. Its strong inhibition on HPK was confirmed on clotting time studies where it was able to increase the activated partial thromboplastin time, without affecting the prothrombin and thrombin times [18].

Additionally, a SP named AdSP was purified from the whole extract of *A. dactylomela* by anion exchange chromatography and gel filtration procedures. This method allowed estimating the molecular mass of the protein as 30.5 kDa. Kinetic studies assaying different small peptide substrates revealed the high catalytic efficiency of the enzyme for the fluorogenic substrate Acetyl-Pro-Phe-Arg-AMC (kcat/KM de 96 969 M-1 s-1), which is a typical substrate of kallikrein-like protease [19]. The enzyme shows an optimum pH of 7.8 at 37 ºC. Its activity is strongly inhibited by benzamidine, soybean trypsin inhibitor (SBTI) and aprotinin, and weakly by PMSF, o-phenanthroline and DTT. The E-64, EDTA and hirudin had no effect on the enzymatic activity. AdSP is a stable SP, because it is able to show 50% of activity after heating at 50 ºC for 1 h. It is also capable of degrading gelatin within a polyacrylamide gel [19]. AdSP could be the target protease for AdKI. Further studies are required to confirm this issue.

Relevance of the study

The scientific novelty of the study can be summarized in: a) A new molecular entity, the CmPI-II protease inhibitor belonging to the Kazal-type family, was detected, purified and characterized in the mollusk *C.*

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muricatus. A new group of "non-classical" Kazal-type inhibitors was proposing taking into account the differences positioning of the CysI–CysV disulphide bridge respect to others inhibitors from the same family. CmPI-II is the first inhibitor that having a basic residue at P1 site, which is able to strongly inhibit the HNE activity; b) CmPI-II (UNIPROT: P84755) was the only inhibitor described so far, active against elastases, subtilisin A and trypsin; c) CmPI-II was the first molecule showing inhibitory activity isolated from *C. muricatus*; d) The recombinant CmPI-II is functionally active and shows the same properties of natural molecule; e) Other two new molecular entities were detected, purified and characterized from the mollusk *A. dactylomela*, the AdKI inhibitor and the serine protease AdSP. The first strongly inhibiting HPK, and the second, could be a target of AdKI; f) AdSP and AdKI were the first molecules showing kallikrein-like proteolytic activity and inhibitory activity, respectively, isolated from *A. dactylomela*.

Theoretical contributions were: a) New values were proposed to identify and classify the "classical" and "non-classical" PIs belonging to the Kazal-type inhibitor family; b) A new group of "non-classical" Kazal-type inhibitors was proposed by the different location of the CysI and CysV disulphide bridge found in CmPI-II; c) The first demonstration of a Kazal-type inhibitor with a basic residue at the P1 site is able to strongly inhibit elastases, declining the pre-established requirement for a hydrophobic residue in that position for inhibition of these enzymes. This property was not only demonstrated by functional studies, but also by model proposed for CmPI-II/HNE complex; d) AdKI is the most selective and strongest PI against HPK discovered in invertebrates. e) The first time was demonstrated the presence of HNE and HPK inhibitors and a SP from *Mollusca* marine organisms.

In addition, this work involved practical contributions by means of the established purification procedures, which represent technologies for production of inhibitors at laboratory scale and also scalable. Based on their structural and kinetic properties, CmPI-II and AdKI are also valuable practical tools for further

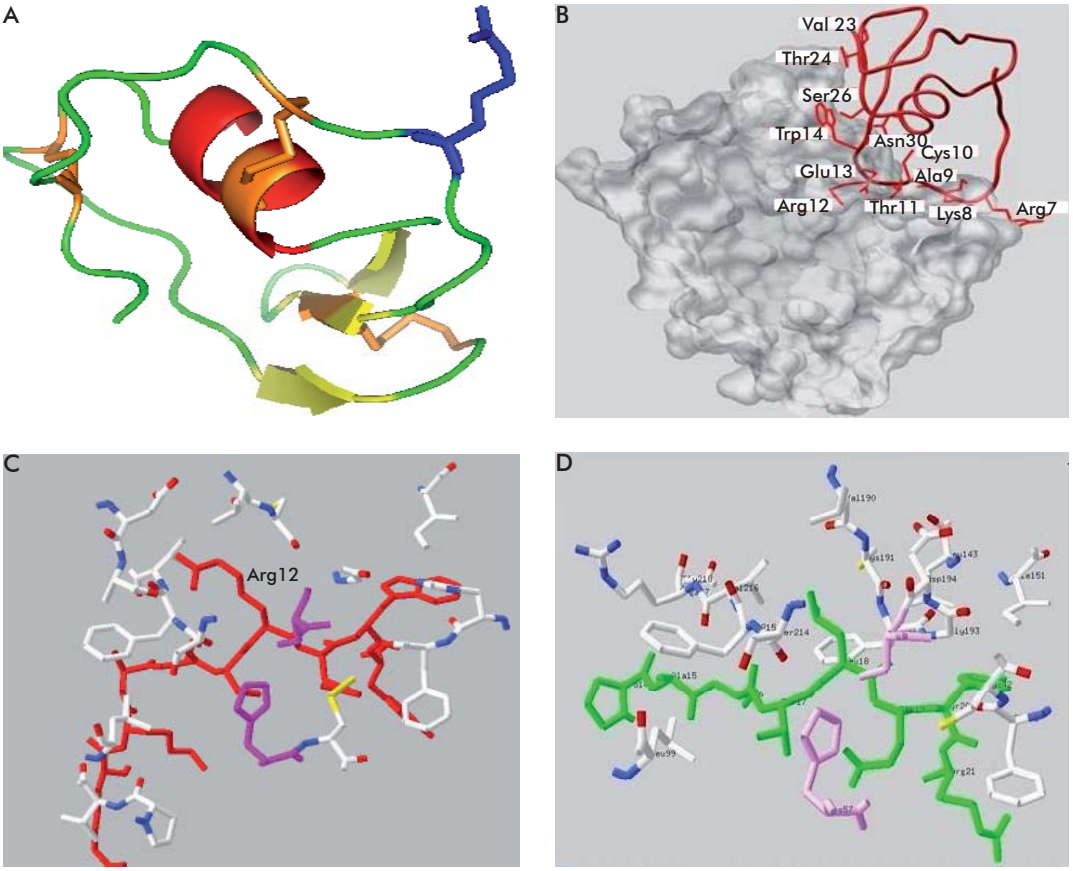

Figure 1. 3D conformational models of CmPI-II and its complex with HNE. A) Richardson´s representation of the 3D conformational model of CmPI-II. Represented are the basic Arg12 residue at theP1 site (blue), α-helix (red), β-sheet (yellow), random sequence (green) and disulphide bridges (orange). B) Proposed model of the 3D structure of CmPI-II/HNE. The surface area of the enzyme is represented in gray and the CmPI-II carbon skeleton in red. Aminoacid residues intervening in the primary binding site P6-P2´ (Arg7, Lys8, Ala9, Cys10, Thr11, Arg12, Glu13, Trp14, respectively) and the secondary binding sites P11´, P12´, P14´, P15´, P18´ (Val23, Thr24, Ser26, Asn30, respectively) are signaled. C) Representation of the region covering the primary binding site (P6-P3') in the proposed 3D conformational model for the complex CmPII/HNE. D) Crystallography structure of the OMTKY3/ENH complex. The residues of the CmPI-II primary binding site and OMTKY3 are depicted in red and green, respectively. Enzyme residues are represented in white (carbon chain), red (oxygen), blue (nitrogen) and sulphur (yellow). Ser195 and His57 are in magenta.

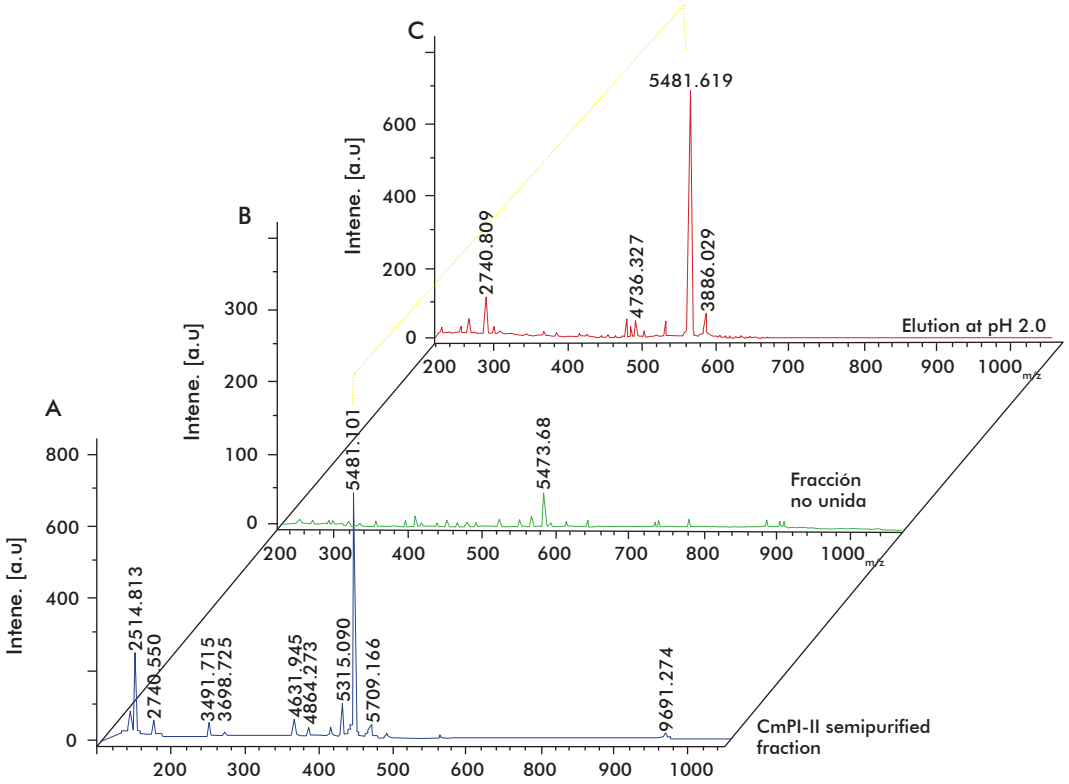

Figure 2. IF MALDI-TOF MS of CmPI-IIr. A) MS spectrum for the semipurified fraction of CmPI-IIr (m/z ion signal of 5481.101 Da). B) The CmPI-IIr interacting with subtilisin A immobilized in a glyoxil-Sepharose matrix (pH 8.5 at room temperature) promotes the disappearance of the signal from the applied fraction. C) The signal re-appears after elution at pH 2.0.

analysis of structure-function relationship studies with their target proteases, especially of HNE and HPC, and models to design new inhibitors against these enzymes of biomedical application.

Conclusions

The existence of proteases and inhibitors in living organisms demonstrates the relevance of these molecules for life perpetuation. In spite of the physiological role of these biomolecules being poorly documented, marine invertebrates are a major source of proteases and inhibitors. Two protein SP inhibitors of different structural and functional properties were identified in the gasteropods *C. muricatus* and *A. dactylomela*, both serving as tools to increase knowledge on the SP structure-function relationship.

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