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Arginine substitution by alanine at the P1 position increases the selectivity of CmPI-II, a non-classical Kazal inhibitor

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ABSTRACT

CmPI-II is a Kazal-type tight-binding inhibitor isolated from the Caribbean snail *Cenchritis muricatus*. This inhibitor has an unusual specificity in the Kazal family, as it can inhibit subtilisin A (SUBTA), elastases and trypsin. An alanine in CmPI-II P1 site could avoid trypsin inhibition while improving/maintaining SUBTA and elastases inhibition. Thus, an alanine mutant of this position (rCmPI-II R12A) was obtained by site-directed mutagenesis. The gene *cmpiR12A* was expressed in *P. pastoris* KM71H yeast. The recombinant protein (rCmPI-II R12A) was purified by the combination of two ionic exchange chromatography (1:cationic, 2 anionic) followed by and size exclusion chromatography. The N-terminal sequence obtained as well as the experimental molecular weight allowed verifying the identity of the recombinant protein, while the correct folding was confirmed by CD experiments. rCmPI-II R12A shows a slightly increase in potency against SUBTA and elastases. The alanine substitution at P1 site on CmPI-II abolishes the trypsin inhibition, confirming the relevance of an arginine residue at P1 site in CmPI-II for trypsin inhibition and leading to a molecule with more potentialities in biomedicine.

1. Introduction

Protease inhibitors are attractive tools for the study of structure-function relationship with their cognate enzymes and for biotechnological and biomedical applications [1,2]. The Kazal-type inhibitor family (MEROPS: I1) has more than a hundred known or predicted homologues [3]. These inhibitors show a canonical conformation loop with a convex shape, that is able to interact with the concave active site of the target proteases [4,5].The reactive-site of inhibitor, located in this region, is energetically the most important. The residues of this site are extremely variable in the family but some regularities have been described such as: the presence of an arginine or lysin residue at P1 position indicates inhibition of trypsin while an alanine, valine or leucine residue as P1 site is related with elastase-like and subtilisin-like inhibition [6,7]. Furthermore, other residues in Pn and Pn' sides at canonical-loop could contribute energetically to interaction of Kazal-type inhibitors with serine proteases [4,8]. The variability of amino acid sequence in this interaction loop, as well as the conformation and flexibility given by differences in the position of disulfide bridge CysI-CysV have influence on specificity and strength of interaction with target proteases [9,10].

During the last 20 years, our group have isolated some protease inhibitors from Cuban marine invertebrates with exceptional structural and functional characteristics [11–15]. In 2007, was reported the isolation and functional characterization of a protease inhibitor from the littorinid Caribbean snail *Cenchritis muricatus* (CmPI-II) (UNIPROT ID: IPK2_CENMR) [13].CmPI-II is a single domain protein belonging to the Kazal serine proteases inhibitors family [16]. Taking into account the CysI-CysV bridge position in the protein sequence, Hemmi et al., 2002 classified Kazal inhibitors in classical and non-classical (group 1 and group 2). However, based on this feature on CmPI-II, the group 3 of non-classical inhibitors was proposed in 2007 [16]. In addition, the reactive site Arg^{12}-Glu^{13} (P1–P1′) of CmPI-II was assigned based on multiple sequence alignment with other Kazal-type domains [16].

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Recently, CmPI-II three-dimensional (3D) structure determined by NMR, allowed confirming the reactive site position as well as the classification of non-classical Kazal inhibitors in three groups [17]. This molecule is a tight-binding inhibitor of trypsin (*Ki*=1.1 nM), which is in correspondence with the basic residue at P1 site. However, it shows a broad specificity, inhibiting also human neutrophil elastase (HNE) (*Ki* = 2.6 nM), subtilisin A (SUBTA) (*Ki* = 30 nM) and porcine pancreatic elastase (PPE) (*Ki* = 145 nM) [16].

Only a few Kazal-type inhibitors of SUBTA with basic residues at P1 site are known [18]. SUBTA inhibition by CmPI-II has been proposed to be related with a more open subtilisin S1 cleft that allows the accommodation of the bulk site chain of the arginine at P1 site [16]. Hence, an alanine in CmPI-II P1 site could avoid trypsin inhibition while improving/maintaining SUBTA and elastases inhibition.

In addition, the ability of CmPI-II to inhibit elastases and subtilisins is very interesting, due to the implication of these enzymes in inflammatory [19,20] and respiratory diseases [21,22] as well as infections [23,24]. However, the inhibition of trypsin is a drawback in the biomedical application of this inhibitor, so obtaining more active and selective CmPI-II mutants could increase the potentialities of this molecule.

Furthermore, recombinant CmPI-II (rCmPI-II) was obtained with molecular and kinetic features similar to those described for the natural protein [25], demonstrating that this molecule could be used as template to obtain CmPI-II mutants.

In this work, we inform the obtention of a mutant at P1 position of CmPI-II (rCmPI-II R12A). rCmPI-II R12A was expressed in *Pichia pastoris* system and purified by the combinations of three different chromatographies. Finally, the recombinant protein obtained in this work, results in a more selective inhibitor than the wild-type variant.

2. Materials and methods

2.1. Materials

Strains and vectors: *Escherichia coli* DH5α (F'/endA1 hsdR17 (rk-mk -) supE44 thi-1 recA1 gyrA (Nalr) relA1Δ(laclZY-argF) U169 deoR (φ80dlacΔ(lacZ) M15)). and *P. pastoris* KM71H *(arg4, his4, aox1:ARG4, HIS4)* strains were used for plasmids amplification and protein expression, respectively. pPICZαA vector and *P. pastoris* KM71H were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). pCM101 vector [25] was provided by the Center for Protein Studies, Havana University.

Primers: 5′ and 3′AOX1 sequencing primers and primers for the site-directed mutagenesis were purchased from Invitrogen Life Technologies.

DNA-modifying enzymes: Taq DNA polymerase was obtained from Fermentas (Thermo Fisher Scientific, MA, USA), *Not*I and DNA T4 ligase were purchase from Promega Corporation (Madison, USA). SacI and *Xho*I enzymes were purchase from Invitrogen Life Technologies. *QIAEX II Agarose Gel Extraction* kit, zeocine and hygromicine B were supplied by *QIAGEN GmbH* (Hildern, Germany).

Chromatography columns: STREAMLINE *Direct* HST, Hitrap Q-Sepharose FF and *Superdex* 75 HR 10/30 resins were obtained from GE Healthcare (Upsala, Sweden).

Proteases, substrates and inhibitors: Bovine pancreatic trypsin (EC 3.4.21.4), chymotrypsin A (EC 3.4.21.1), porcine pancreatic elastase (EPP) (EC 3.4.21.36) and p-nitrofenil-4′-benzoato de guanidine (NPGB) were from Sigma-Aldrich Co.(http://www.sigmaaldrich.com/), *Bacillus licheniformis* subtilisin A (SUBTA) (EC 3.4.21.62) supplied by Calbiochem *Novabiochem* Corp (La Jolla, EE.UU.). N-Benzoyl-arginil-p-nitroanilide (BAPA), Z-glycil-glycil-leucil-p-nitroanilide (Z-G-G-L-pNA), Succinil-alanil-alanil-prolyl-phenyl-p-nitroanilide (Suc-A-A-P-F-pNA) and Succinil-alanil-alanil-alanil-p-nitroanilide (Suc-A-A-A-pNA) are from BACHEM (Bubendorf, Switzerland). rCmPI-II [25] was provided by the Center for Protein Studies, Havana University.

Media: composition of YPD medium, salt solution and trace elements solution was used according to literature [25]. Minimal medium: salt solution, 1 ml/L trace elements solution, 0.4 μg/L biotin, 0.5% v/v methanol and pH adjusted to 5.5 with NH_4OH 30% m/v.

2.2. Methods

Cloning and obtaining of rCmPI-II R12A mutant: Site-directed mutagenesis was performed as described in literature [26]. A first PCR was carried out using the pCM101 vector as template [25] and the specific primers cmpi1fw (5′-CCAATTGACAAGCTTTTGATTTTAACGAC -3′) and cmpi3rv (5′- AACTGGATACCACTC**AGC**AGTACAAGC-3′), that hybrid in the 3'→5′ region of the codon to be modified (bold letters). A second PCR was performed using the same vector, the cmpi10rv (5′-GGGGGATCCGCACAAACG-3′) and a specific forward primer cmpi2FW (5′-GCTTGTACT**GCT**GAGTGGTATCCAGTT-3′), which hybrid the 5'→3' region of codon to be mutated (bold letters). The cmpi2-mutated gene was obtained by a third flapping PCR which included the previously obtained PCR products and the flanking external primers, cmpi1fw and cmpi10rv. PCR conditions were 1x (5 min at 96 °C; (40s at 96 °C; 40s at 55 °C; 1.20 min at 72 °C) 40x; 5 min at 72 °C; 16 h at 4 °C. The resultant fusion mutated gene-containing bands were digested with *Xho*I/*Not*I and subcloned into previously digested-pPICZαA expression vector. The new vector was transformed on *P. pastoris* KM71H strain by electroporation, after linearization with *Sac I*. The cloning strategy is showed in Fig. 1. Transformant clones were selected with increasing zeocine concentrations (50–3000 μg/mL) and screened for the mutated *cmpi2* gene by colony PCR [27] followed by sequencing. Cm5 was the name assigned to the expression strain.

Protein expression and purification: rCmPI-II R12A was expressed using a shake-flask procedure. 1.9L of MGY medium (three 2L-flasks with 650 mL each one) was inoculated with 25 mL of Cm5 strain, grown in YPD medium for 24 h at 180 rpm and 28 °C. The inoculated cultures were incubated at 28 °C and 180 rpm until OD_{600nm} reached 8UA. Then, the cells were collected by centrifugation and resuspended into 195 mL MM medium in a 1L-flask and maintained with 0.5% (v/v) methanol every 24 h during three days in an orbital shaker at 180 rpm and 28 °C. The culture supernatant was collected by centrifugation (10,000×*g* for 10 min at 4 °C) and inhibitory activity against trypsin and SUBTA was checked.

The recombinant protein was purified following the methodology described for the wild-type protein [25]. Briefly, a cation exchange chromatography in STREAMLINE *Direct* HST column (1.5 × 3.5 cm) was used as first step, followed by an anionic exchange chromatography in column Hitrap Q-Sepharose FF (1 mL). Finally, a size exclusion chromatography in *Superdex 75* HR 10/30 column, pre-equilibrated with phosphate saline buffer solution (137 mmol/L NaCl; 2.7 mmol/L KCl; 1.0 mmol/L Na_2HPO_4; 1.8 mmol/L KH_2PO_4, pH 7.4).

The purity of rCmPI-II R12A as well as the purification parameters evaluation were estimated as described for the wild-type protein [25]. In addition, an SDS PAGE was performed.

Molecular characterization: Molecular masses were determined by MALDI-TOF mass spectrometry using a Bruker Biflex spectrometer (Bruker Daltonics Inc., Billerica, MA, USA) and α-cyano-4-hydroxycinnamic acid as matrix. ProtParam tool [28] available at http://www.expasy.org was used to calculate the theoretical molecular mass of the mutant. N-terminal of rCmPI-II R12A was carried out using Edman degradation technique in PPSQ-23 Sequencer (Shimadzu, Tokyo, Japan).

The far-UV CD spectra were obtained in the ranges of 190–260 nm, in quartz cuvettes with a path length of 0.1 cm. The spectra were collected by continuous scanning at 0.1 nm intervals, a scanning speed of 100 nm/min, a response time of 1s, a bandwidth of 1 nm. Fifteen accumulations were averaged in each experiment, and the background spectra were subtracted. The protein concentrations were 38 μmol/L for rCmPI-II and 43 μmol/L for rCmPI-II R12A (both in PBS diluted 1/10 with purified water). The correction of the baseline was done in all experiments using a purified water as a control. The mean molar ellipticity per residue $[\theta]_\lambda$

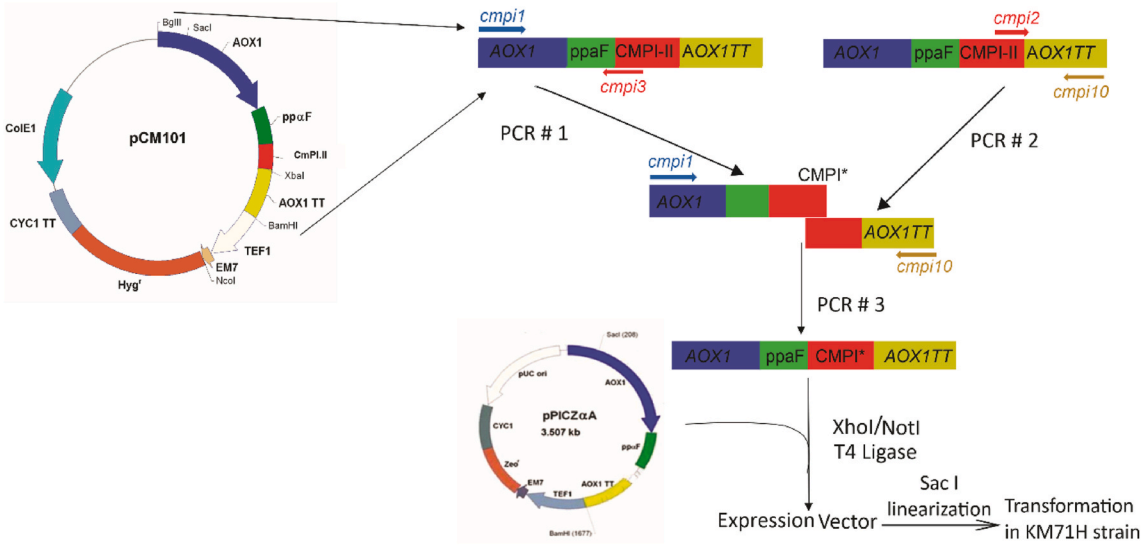


Fig. 1. Site-direct mutagenesis and cloning strategies of rCmPI-II R12A heterologous expression in KM71H *P. pastoris* strain. Maps of pCM101 and pPicZαA vectors are represented, as well as the PCR strategy and the restriction enzymes used for cloning and linearization.

was calculated according to the literature [29]. The analysis of the deconvolution of the experimental CD spectra using the CDSSTR algorithm and the data base 4 reference protein on the Dichroweb Internet server (http://dichroweb.cryst.bbk.ac.uk) allowed to obtain the protein secondary structure [30,31].

In addition, the melting temperature (Tm) was determined at 222 nm increasing temperature (25–95 °C, 1 °C/min) using Jasco PTC-510 Peltier temperature controller and Jasco MCB-100 water circulation mini-bath. Three replicates were evaluated for each protein. Normalized CD data (taking into account the maximum and minimum value of the data set) were plotted in function of the temperature. Melting temperature (Tm) was determined fitting experimental data to Boltzmann equation implemented on GraphPad Prism 8.01 software.

Protein concentration was calculated by spectrophotometric method and the extinction coefficient ($\xi^{1\%,\ 280\ nm} = 16.4$) determined using the ProtParam tool [28] available at http://www.expasy.org/.

Measurement of IC_{50} values: Inhibitory activity of rCmPI-II and rCmPI-II R12A was assayed as previously described [25]. In this case the inhibitors were evaluated against bovine pancreatic trypsin, SUBTA, porcine pancreatic elastase and chymotrypsin using the synthetic substrates Bz-Arg-pNA (1.0 mmol/L, 1.0 K_M) [32], Suc-Ala-Ala-Pro-Phe-pNA (0.2 mmol/L, 1.0 K_M) [33], Suc-Ala-Ala-Ala-pNA (1.1 mmol/L, 1.0 K_M [34] and Suc-Ala-Ala-Pro-Phe-pNA (1 mmol/L, 1.0 K_M [35], respectively.

In addition, IC_{50} parameter was calculated, in parallel, for both inhibitors versus the aforementioned enzymes following the procedure recommended for tight-binding inhibitors [36] and the tested parameters for the natural and wild-type protein [25]. Fixed protease concentrations were tested with different initial rCmPI-II or rCmPI-II R12A concentrations (Table 1).

Table 1
Experimental conditions for IC_{50} values determination.

Condition	bovine pancreatic trypsin	subtilisin A	porcine pancreatic elastase	chymotrypsin A
[E] mol/L	$8.6{\cdot}10^{-8}$	$4.0{\cdot}10^{-9}$	$2.0{\cdot}10^{-9}$	$2.3{\cdot}10^{-7}$
$[S_0]$ mmol/L	1.0 mmol/L (1.0 K_M)	0.2 mmol/L (1.0 K_M)	1.1 mmol/L (1.0 K_M)	1 mmol/L (1.0 K_M)
$[I_0]$ mmol/L	$(3.8–95){\cdot}10^{-9}$ (rCmPI-II) $I_0/E_0 = 200$ (rCmPI-II R12A)	$(0.9–151){\cdot}10^{-9}$ (rCmPI-II) $(0.05–105){\cdot}10^{-9}$ (rCmPI-II R12A)	$(1.1–12){\cdot}10^{-5}$ (rCmPI-II) $(2.7–106){\cdot}10^{-8}$ (rCmPI-II R12A)	$I_0/E_0 = 200$ (rCmPI-II) $I_0/E_0 = 200$ (rCmPI-II)

3. Results

In order to improve CmPI-II selectivity the arginine residue at the P1 position of CmPI-II was substituted by an alanine using site-directed mutagenesis. This new variant (rCmPI-II R12A) was obtained and compared with the wild-type inhibitor.

3.1. rCmPI-II R12A expression and purification

The CmPI II mutant was obtained by site-directed mutagenesis (Fig. 1). Two purified 374-pb (PCR1) and 561-pb (PCR2) bands were flapped by a third PCR using the cmpi1fw and cmpi10rv external primers. The presence of an 825-pb-band of the mutant was checked by restriction assays. A transformant KM71H clone (Cm5) was isolated at the highest antibiotic concentration and used for shake-flask fermentations. The presence of the mutated *cmpi2* gene was corroborated by colony PCR followed by sequencing.

As shown in Fig. 2, there is an increase in the protein concentration (measured as of OD $_{280nm}$) and a reduction in the SUBTA but not trypsin residual activity in a time-dependent manner during the 72 h of induction. These results suggest the expression of rCmPI-II R12A as soluble protein in the Cm5 supernatant.

The purification of the protein, from *P. pastoris* culture supernatant was achieved by the combination of two ion exchange chromatography (cationic and anionic), and gel filtration (Fig. 3).

As result of the cationic exchange chromatography, was recovered an 87% of rCmPI-II R12A, with an increase in 37-fold of the purification grade, compared to initial quantity of inhibitor (Table 2). The fractions with activity against SUBTA eluted from STREAM line Direct HST chromatography, were pooled and loaded in the Hitrap Q-Sepharose FF column. The recombinant protein was purified until homogeneity using a molecular exclusion chromatography in Superdex 75 HR column. As result of the complete procedure was obtained a purification grade increase of 182-fold with yield of 12% (Table 2). This represents 5.1 mg of pure rCmPI-II R12A per liter of culture.

The high purity of the recombinant inhibitor was confirmed by the presence of an acute and symmetric peak from C4-HPLC column, as well as the unique band with the expected molecular weight (around 5.3 kDa) observed on SDS-PAGE gel (Fig. 3 D and E).

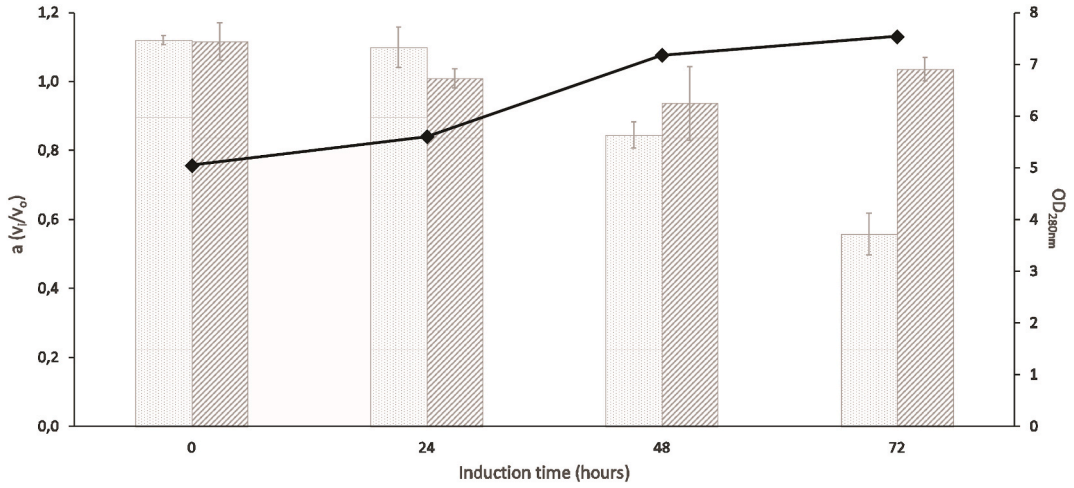


Fig. 2. Heterologous expression of CmPI-II R12A in *P. pastoris* culture supernatant. Variation of OD_{280nm} (◆), residual SUBTA activity (dotted grey bars) and residual trypsin activity (listed bars) in the culture supernatant, during a 72h induction period with methanol. The figure shows the average and standard deviation of three measurements.

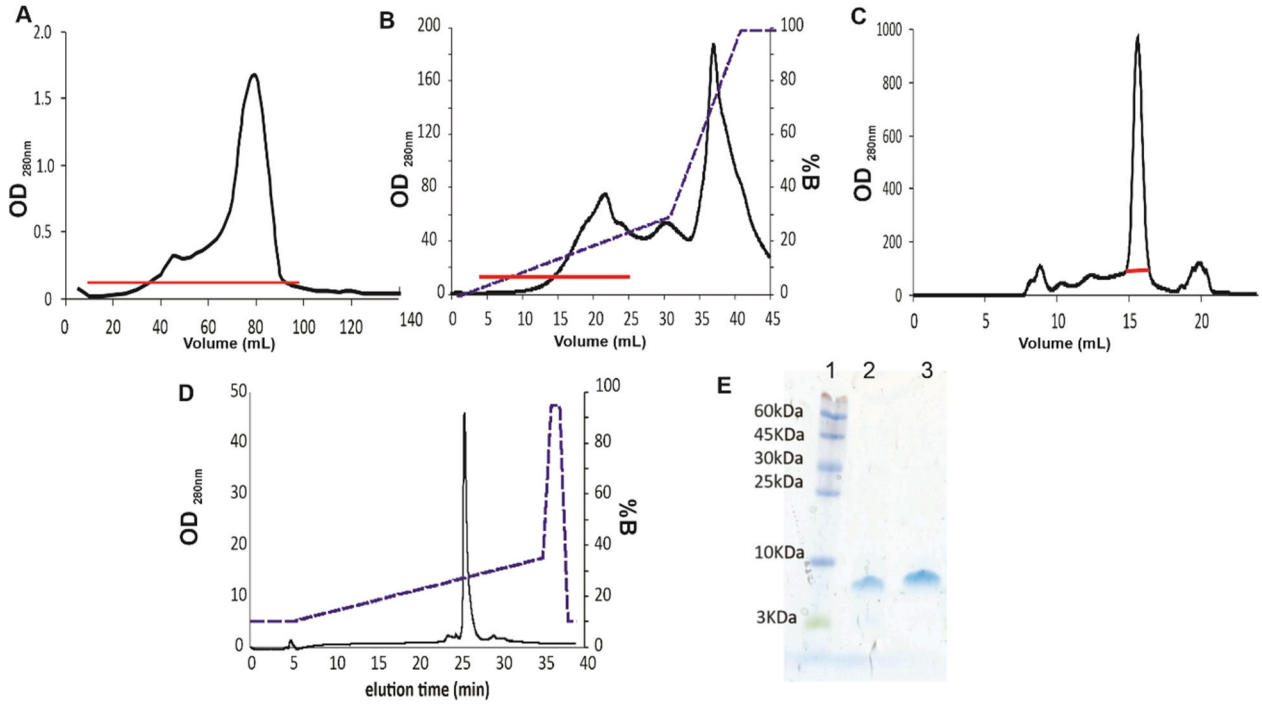


Fig. 3. Purification procedure of rCmPI-II R12A. A: Typical chromatograms for the cationic exchange chromatography in STREAMLINE *Direct* HST matrix, B Anionic exchange chromatography in Hitrap Q-Sepharose FF, C: Gel filtration in *Superdex 75* HR 10/30. D: RP-HPLC in matrix C4. E: SDS-PAGE 15% of purified simples line 1: Molecular Weight Marker, line 2: rCmP-II R12A, line 3: rCmP-II. Proteins were visualized by Coomassie Brilliant Blue R-250 staining. Experimental conditions of each chromatography are described in the methods section. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 2
Summary of the purification of rCmPI-II R12A.

Purification step	Protein[a] (mg)	Inhibitory Activity [b] (U)	Specific Inhibitory Activity (U/mg)	Purification Grade (fold)	Yield (%)
Culture supernatant	1470	60,659	41	1.0	100
CEC Streamline HST	34	44,767	1526	37	87
AEC Hitrap Q-Sepharose	7.2	25,210	3482	84	41
SEC Superdex 75 HR 10/30	0.9	7188	7491	182	12

[a] Total amount of protein from 195 mL of culture.
[b] Inhibitory activity (U): amount of inhibitor needed to inhibit 1 U of SUBTA enzymatic activity.

3.2. Molecular and functional characterization of recombinant inhibitors

The N-terminal sequences showed the presence of the twelve first residues (AED*X*VGRKA*X*TA) expected for the recombinant mutant protein, where *X* represents no phenylthiodantoin-amino acids detected. This is in agreement with the presence of Cys^4 and Cys^{10} in the natural and recombinant CmPI-II [13,25]. In addition, the presence of an alanine at the P1 position was confirmed. The experimental molecular mass (5394 Da) matches with the theoretical one calculated for rCmPI-II R12A amino acid sequence. On the other hands, in agreement with the features of natural CmPI-II [13] and evidence for rCmPI-II [25], *P. pastoris* system does not glycosylate potential N-linked glycosylation sites ($N^{30}FSA^{33}$ and $N^{41}ITI^{45}$) found in the primary structure of the rCmPI-II R12A.

The content of secondary structure elements in R12A CmPI-II mutant and wild type rCmPI-II were evaluated in parallel by CD far-UV. The CD spectra in the far-UV range showed no major conformational changes occurred as a result of the mutation (Fig. 4 A and B).

In addition, for both proteins, Tm values were close to 75 °C, showing the relative high stability of the inhibitors. However, the melting temperature for the mutant was 85.9 ± 3.4 °C while the Tm obtained for wild-type inhibitor was 75.2 ± 0.9 °C (Fig. 4C).

3.3. Kinetic characterization

The specificity studies of rCmPI-II R12A against bovine pancreatic trypsin, SUBTA, porcine pancreatic elastase and chymotrypsin were evaluated in parallel with wild-type rCmPI-II. The IC_{50} values were calculated by adjusting the residual activity (obtained from the experimental data) and inhibitor concentrations to the corresponding equation [36] (Fig. 5).

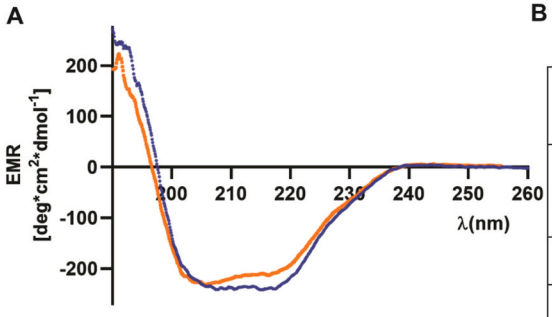
As result of alanine substitution at P1 site on CmPI-II, activity against trypsin was abolished, even using an inhibitor/enzyme molar ratio equal to 200 ([rCmPI-II R12A] = $1.7{\cdot}10^{-5}$ mol/L in the assay) there was no inhibition. However, the mutant variant increases EPP and SUBTA inhibition respect to wild-type molecule. The IC_{50} values of rCmPI-II R12A against EPP and SUBTA are 560 and 3-fold decreased, respectively. rCmPI-II nor rCmPI-II R12A showed inhibition of chymotrypsin in the assayed conditions ($[I_0]/[E_0] \geq 200$). IC_{50} values showed by rCmPI-II in this work are in the same order to Ki values obtained for natural CmPI-II [13] and IC_{50} values obtained, in a previous work, for rCmPI-II [25].

4. Discussion

For the serine proteases canonical inhibitors is well stablished that the amino acid residue at the P1 position, in the reactive site, is the main determinant of specificity [4]. For CmPI-II, residues Arg^{12}-Glu^{13} (P1–P1′) were assigned as the reactive site of CmPI-II, based on multiple alignments including the amino acid from Kazal-type domains [16,17]. This inhibitor has an unusual specificity in the Kazal family because; with a basic residue at the P1 site, it can strongly inhibit not only trypsin, but also elastases and SUBTA [16,25].

Considering that a basic residue at the P1 position is usually critical for trypsin inhibition in canonical serine protesas inhibitors [18] introducing and alanine in the P1 position of CmPI-II probably will disrupt trypsin inhibition. Thus, an alanine mutant of this position could be a more selective inhibitor, a molecule inactive against trypsin while maintaining/improving SUBTA and elastases inhibition.

Therefore, using the platform of rCmPI-II expression in *P. pastoris* previously established [25] the R12A variant of CmPI-II was obtained by site-directed mutagenesis and cloned into *P. pastoris* KM71H. The use of a purification procedure with three steps, as described for rCmPI-II and ^{15}N rCmPI-II [25] let to obtain pure rCmPI-II R12A from *P. pastoris* culture supernatant. The selected expression/purification procedure allowed obtaining 5.1 mg of pure rCmPI-II R12A per liter of culture. This result is twice lower to the obtained for rCmPI-II [25] probably due to the use of a shake-flask fermentation system instead of a benchtop fermenter for the protein expression. Nevertheless, this value is in the range reported for *P. pastoris* expression system [37] and the quantity of



B

Molecule	α (%)	β (%)	Random (%)
natural CmPI-II (Gonzalez et al., 2007)	24	17	59
rCmPI-II	15	29	56
rCmPI-II R12A	12	32	56

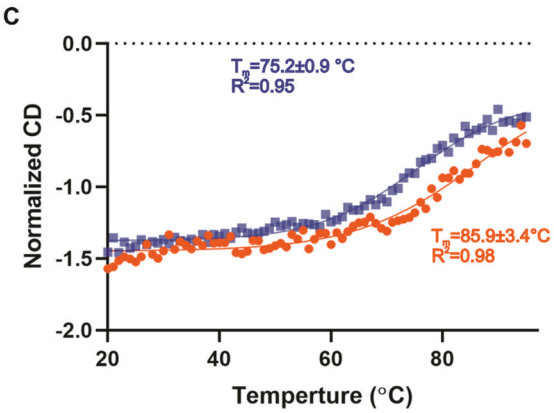


Fig. 4. Conformational characterization of rCmPI-II R12A A) Far-UV CD spectra of rCmPI-II R12A (orange, 43 μmol/L) and rCmPI-II (black, 38 μmol/L). B) Estimations of the secondary structure content of rCmPI-II R12A compared to wild-type inhibitor rCmPI-II. Secondary structure estimation was developed on Dichroweb server, with the reference set data CDSSTR. C) Melting curves obtained for rCmPI-II (blue) and rCmPI-II R12A (orange). The curves were recorded at 220 nm during increase of the temperature from 20 to 95 °C (1 °C/min). Tm value was calculated fitting to Boltzmann equation the experimental data using GraphPad Prism 8 software. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

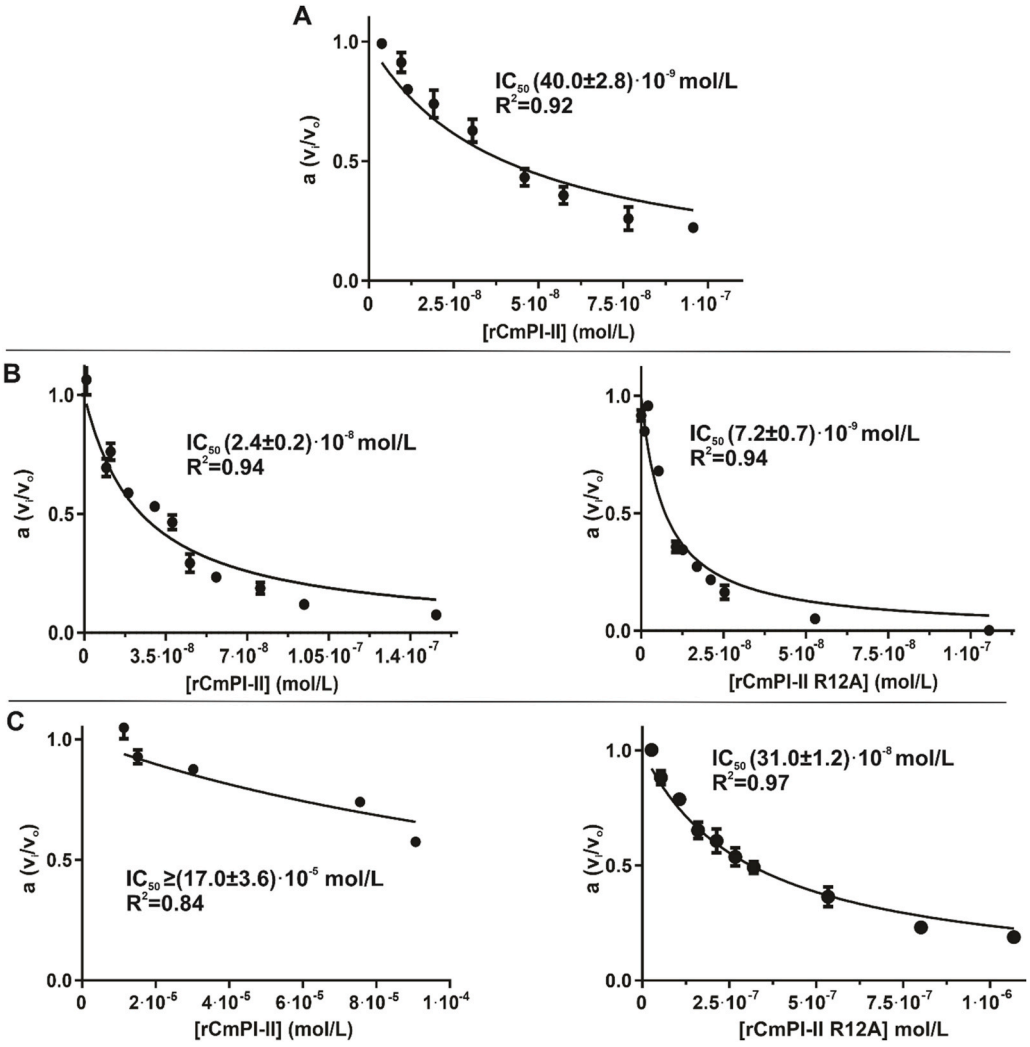


Fig. 5. Functional characterization of rCmPI-II and rCmPI-II R12A. The graphic shows the fit of experimental data (*a* vs [Io]) to IC_{50} equation implemented on GraphPad Prism 8.0.1 software as well as the IC_{50} values determined for trypsin (A), SUBTA (B) and EPP (C).

protein obtained allowed us to accomplish functional and molecular characterization.

The N-terminal sequence and experimental molecular mass obtained showed the expected differences between wild-type and mutant protein. The rCmPI-II and mutant CD spectra showed two negative shoulders around 200 and 225 nm, and a positive maximum in the 185–195 regions. This signal is observed in almost all folded proteins. The spectra features are similar to the reported for a Kazal-type inhibitor [38] corroborating the correct folding of the expressed recombinant proteins. Quantitative results show a similar global folding of rCmPI-II and rCmPI-IIR12A.

In addition, both inhibitors showed values of Tm higher than 75 °C, indicating a very high thermal stability. This result is in concordance with the existence in Kazal inhibitors and particularly in CmPI-II structure of three disulphide bonds and a hydrophobic core formed by residues located in the β sheet and the α helix [17,18,39]. However, rCmPI-II R12A was more stable than rCmPI-II. The change of the Arg in P1 position by an Ala means the loss of a positive charge in the surface possibly leading an increase in the mutant stability. In studies of engineered proteins with charge introduction, charge reversal and/or charge redistribution showed the importance of surface charge-charge interactions for protein stability [40].

Finally, the concave shape of the inhibition curves (Fig. 5) obtained for rCmPI-II R12A is similar to wild-type protein, indicating a reversible inhibition.

The concept that the inhibitors with basic residue at P1 position tend to inhibit trypsin holds true for Kazal domains as the OMCHI3 [41], as Arg and Lys residues are the specificity determinants of this enzyme. This fact is demonstrated in this work, because the P1 substitution by an alanine on CmPI-II abolishes the trypsin inhibition and turnover a more specific inhibitor.

For SUBTA inhibition IC_{50} values indicated that the substitution of Arg12 in CmPI-II for an alanine led to an increase of potency in three times which indicates that the P1 has a relative significance for SUBTA inhibition and that it might be others residues involved in the rCmPI-II interaction with this protease. In general, for Kazal inhibitors, hydrophobic residue in P1 position increase SUBTA inhibition [18,42].

In addition, wild-type and mutant IC_{50} values against EPP were $17{\cdot}10^{-5}$ mol/L y $3.1{\cdot}10^{-7}$ mol/L^{-1}, respectively. These values suggest that rCmPI-II R12A is a better inhibitor for EPP than rCmPI-II. This is coherent with the characteristics of EPP S1 pocket and the presence of a Val216 and Thr 226 residues at top of the pocket exclude bulk residues in P1 position [9]. Similar results were obtained previously for the substitution of P1 Lys of BPTI by alanine, transforming this molecule in a tight binding inhibitor of elastase [43]. In addition, the Kazal-type inhibitor OMTKY3 showed a similar behavior, where P1 variants L18K and L18R are weak elastase inhibitors whether the variant L18A is a tight-binding inhibitor [42].

In the case of chymotrypsin, rCmPI-II nor rCmPI-IIr R12A are tight-binding inhibitors for this protease. Chymotrypsin is a specific enzyme for bulk hydrophobic residues justifying absence of inhibition observed for both inhibitors [44].

Furthermore, bioinformatics analysis of CmPI-II binding loop with SUBTA and ENH along with alanine scanning showed that the most important residues for this interactions are P1 (Arg12), P2 (Thr11) and P2' (Trp14) [16,17]. The mutant rCmPI-II/W14A, reported previously [17], is active against trypsin but it does not show activity against SUBTA even using up to 200-fold molar excess of inhibitor, confirming relevance of a tryptophan residue at P2' position for SUBTA inhibition as was predicted by bioinformatics. Therefore, obtaining more mutants of P1, P2 and P2′ positions to reveal the insights into the structure-function relationship of rCmPI-II and its target proteases is in order and will be carried out in the future.

5. Conclusions

In this work, rCmPI-II R12A was obtained correctly folded and was able to inhibit target proteases as expected according to the mutation introduced in the protein. The alanine substitution at the P1 position in rCmPI-II abolish trypsin inhibition and retains SUBTA inhibition potency. Hence, the molecule obtained in this work is a more selective inhibitor and leads the way to obtain more potent and specific inhibitors with potential biomedical applications using the CmPI-II scaffold.

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Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

Not applicable.

Code availability

Not applicable.

Declaration of competing interest

All authors declare that they have no conflict of interest.

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