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Heterologous expression of *Cenchritis muricatus* protease inhibitor II (CmPI-II) in *Pichia pastoris* system: Purification, isotopic labeling and preliminary characterization

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ABSTRACT

Cenchritis muricatus protease inhibitor II (CmPI-II) is a tight-binding serine protease inhibitor of the Kazal family with an atypical broad specificity, being active against several proteases such as bovine pancreatic trypsin, human neutrophil elastase and subtilisin A. CmPI-II 3D structures are necessary for understanding the molecular basis of its activity. In the present work, we describe an efficient and straightforward recombinant expression strategy, as well as a cost-effective procedure for isotope labeling for NMR structure determination purposes. The vector pCM101 containing the CmPI-II gene, under the control of *Pichia pastoris* AOX1 promoter was constructed. Methylotrophic *Pichia pastoris* strain KM71H was then transformed with the plasmid and the recombinant protein (rCmPI-II) was expressed in benchtop fermenter in unlabeled or ^{15}N-labeled forms using ammonium chloride (^{15}N, 99%) as the sole nitrogen source. Protein purification was accomplished by sequential cation exchange chromatography in STREAMLINE DirectHST, anion exchange chromatography on Hitrap Q-Sepharose FF and gel filtration on Superdex 75 10/30, yielding high quantities of pure rCmPI-II and ^{15}N rCmPI-II. Recombinant proteins displayed similar functional features as compared to the natural inhibitor and NMR spectra indicated folded and homogeneously labeled samples, suitable for further studies of structure and protease-inhibitor interactions.

1. Introduction

Proteases and their inhibitors have been the subject of intense research for studying the protein-protein interactions, and also for the development of new drugs. In 2007, a serine protease inhibitor (CmPI-II) (UNIPROT: IPK2_CENMR) was isolated and characterized from the marine mollusk *Cenchritis muricatus* [1]. CmPI-II is a Kazal-type inhibitor with 50 residues, three disulfide bridges and a molecular weight of 5480 Da. Kazal-like inhibitors are single- or multidomain-proteins with six cysteine residues engaged in disulfide bonds arranged in a conserved pattern. The number of residues between CysI and CysII (**m** value), and between CysIV and CysV (**n** value) defines the distinction between 'classical' and 'non-classical' Kazal inhibitors [2]. CmPI-II was classified as a 'non-classical' Kazal type inhibitor, but differs in its **m** and **n** values from the two previous 'non-classical' groups reported. CmPI-II has a greater number of amino acid residues between CysI and CysV and the disulfide bridge is shifted toward the C-terminus compared to the

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Abbreviations

3D	three-dimensional
^{15}N rCmPI-II	recombinant CmPI-II labeled with ^{15}N
AEC	anionic exchange chromatography
AOX1/2	alcohol oxidase 1/2
Bz-Arg-pNA	Benzoyl-arginyl-p-nitro-anilide-HCl
CEC	cationic exchange chromatography
CmPI-II	*Cenchritis muricatus* proteases inhibitor II
rCmPI-II	recombinant CmPI-II
[E_o]	initial enzyme concentration
HSQC	Heteronuclear Simple Quantum Coherence
[I_o]	initial inhibitor concentration
K_i	Inhibition constant
MALDI-TOF	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight
MEC	Molecular Exclusion Chromatography
NMR	Nuclear Magnetic Resonance spectroscopy
NOESY	Nuclear Overhouser Effect Spectroscopy
NPGB	p-nitrophenyl-p′-guanidinobenzoate
$OD_{280\ nm}$	Optical Density at 280 nm
RP-HPLC	Reversed-Phase High-Performance Liquid
rShPI-1A	recombinant *Stichodactyla helianthus* Protease Inhibitor 1
Suc-Ala-Ala-Pro-Phe-pNA	Succinyl-alanyl-alanyl-prolyl-phenyl-p-nitro-anilide-HCl
TFA	trifluoroacetic acid
TOCSY	Total Correlation Spectroscopy

'non-classical' domains previously described. According to this, it was proposed that this protein defines a new group (group 3) of non-classical inhibitors from the Kazal family (Fig. S1) [3]. CmPI-II is a tight-binding inhibitor active against trypsin ($K_i = 1.1$ nM), human neutrophil elastase (HNE) ($K_i = 2.6$ nM), subtilisin A ($K_i = 30.8$ nM) and porcine pancreatic elastase ($K_i = 145$ nM). This inhibitor has a basic residue in P1 position (arginine) [3], which has been widely related with the trypsin inhibition, but not with subtilisin A and HNE inhibition [4–8].

The capability of CmPI-II to inhibit HNE and subtilisin A is very attractive, due to the role these enzymes play in respiratory and inflammatory pathologies [9–11], as well as in many infectious diseases [12–14], respectively. Nevertheless, the structural determinants for the inhibition of these enzymes by CmPI-II are still unknown. Only several models to explain its HNE inhibition were constructed [3]. Thus, the tridimensional (3D) structure of this protein should be solved, alone and in complex with the target enzyme.

Different approaches can be used for the structural characterization of this protein. Among them, Nuclear Magnetic Resonance (NMR) spectroscopy is a powerful method for obtaining structural information of macromolecules and its molecular complexes [15]. This method, as X-ray crystallography, is a biophysical one, which can provide atomic-resolution of structures of biological molecules such as proteins, nucleic acids and their complexes [16]. In addition, the use of NMR experiments allows determining epitope interaction between two proteins and also to determine the dissociation constant (in the micro to millimolar range). This approach also allows establishing the molecular orientation during protein interaction and its effect in the conformation and mobility of the involved regions [17,18]. Despite the advance in the methodologies for protein structural studies, significant mass (milligrams) of pure proteins is still needed.

The methylotrophic yeast *Pichia pastoris* is a well-established host for the expression of recombinant proteins. In addition, this microorganism is capable of expressing proteins with their correct folding and several posttranslational modifications. Taking into account this feature, *P. pastoris* has proved to be useful for the expression of proteins with several disulfide bridges, such as protease inhibitors [19–22]. On the other hand, *P. pastoris* is able of metabolizing methanol as sole the carbon source. The first step in the methanol metabolism is its oxidation to formaldehyde using molecular oxygen by the enzyme alcohol oxidase. Two genes in *P. pastoris* encode for alcohol oxidase, the AOX*1* and AOX*2* [23,24]. However, the majority of alcohol oxidase activity in the cell is attributable to the product of the AOX*1* gene. Therefore the protein expression under *AOX*1 promoter allows for an efficient control of the fermentation due to its tight expression control, which is only expressed when the cells are growing in methanol as the sole carbon source [24–26].

This host has also been used to obtain isotopically labeled protein for NMR studies [27–29]. In this case, the protein should be enriched in active nuclei, for example ^{15}N and/or ^{13}C. Although *E. coli* is usually employed to express heavy isotope-labeled recombinant protein, the product of interest is often expressed as inclusion bodies and has to be refolded. For that reason, in the last years *P. pastoris* has become the second most popular expression system for this purpose.

In this study, we describe the cloning and heterologous expression of rCmPI-II and ^{15}N-labeled rCmPI-II in *P. pastoris* system for future NMR studies. Recombinant proteins were successfully purified, correctly folded displaying the kinetic behavior of natural ones. Finally, the samples obtained yielded good quality preliminary NMR spectra, proving feasibility of structure determination and interaction studies by NMR.

2. Materials and methods

2.1. Materials

2.1.1. Strain

Pichia pastoris KM71H strain *(arg4, his4, aox1::ARG4, HIS4)* was used as expression host of rCmPI-II. *Vectors:* pBM333 contained the CmPI-II synthetic gene, codon-optimized for *P. pastoris,* which was previously obtained in our lab (D. Gil, unpublished results). The synthetic gene was designed taking into account the amino acid sequence of CmPI-II and was acquired from GeneArt (Regensburg, Germany) (Fig. S2); vector pBM301 [19] (Fig. 1).

2.1.2. Enzymes, substrates and other reagents

The restriction enzymes *Xba*I, *Sal*I, *Sac*I and T4 ligase were purchased from Roche Applied Sciences. Isotope-enriched (99%) ^{15}N-NH_4Cl was obtained from Cambridge Isotope Lab (Andover, MA, USA). Bovine pancreatic trypsin (EC 3.4.21.4) (Sigma-Aldrich, St. Louis, MO, USA)), subtilisin A (EC 3.4.21.62), Bz-Arg-pNA and NPGB were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Suc-Ala-Ala-Pro-Phe-pNA was purchased from Calbiochem-Novabiochem (San Diego, CA,USA). Q-Sepharose-FF and STREAMLINE *Direct* HST matrixes were purchased from GE Healthcare (Uppsala, Sweden). Other reagents used were of analytical purity.