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Synthesis, cloning and expression in *Escherichia coli* of a gene coding for the Met8→Leu CMTI I – a representative of the squash inhibitors of serine proteinases

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Abstract A chemically synthesized gene coding for a *Cucurbita maxima* trypsin inhibitor modified at position P'3 (Met8→Leu CMTI I), i.e. at the third position downstream of the reactive site bond (Arg5-Ile), was cloned into a derivative of the plasmid pAED4 that utilizes a T7 expression system. The gene was expressed in *Escherichia coli* as a fusion protein that accumulates in inclusion bodies. After reduction and CNBr cleavage of the fusion protein followed by oxidative refolding and reverse-phase HPLC, about 5 mg of pure protein was obtained per l of cell culture. Association constants of recombinant Leu-8-CMTI I with bovine β-trypsin and human cathepsin G are the same, within experimental error, as for CMTI I isolated from a natural source.

Key words: Proteinase inhibitor; Gene cloning; Fused gene; Fused protein; Disulfide bridge; *Escherichia coli*

1. Introduction

Proteinases play a central role in such basic physiological processes as posttranslational processing, fertilization, prohormone processing, complement activation, blood coagulation, and fibrinolysis [1]. Proteinases have also been implicated in certain diseases including trauma, tumor metastasis, emphysema, and arthritis [2]. An essential mechanism of their regulation involves inactivation of proteinases by forming complexes with protein inhibitors. The structure and mechanism of action of inhibitors have been extensively studied [3,4].

A large group of serine proteinase inhibitors has been identified in squash seeds [5]. The compact and very rigid structure of these small proteins consists of 27–32 amino acid residues, stabilized by three disulfide bridges. Because of their small size and rigidity, the squash inhibitors provide an excellent model for studying serine protease-protein inhibitor interaction.

For mechanistic studies extensive comparative binding experiments using a large variety of single or multiple mutants are the most informative. Many such analogues of squash inhibitors have been synthesized by the solid-phase method and their association constants with trypsin, elastase, and chymotrypsin have been measured [5]. Nevertheless, chemical synthesis of polypeptide chains of about 30 amino acid residues is time consuming and expensive. The already high cost of synthesis would become exorbitant in the case of ^{15}N- and/or ^{13}C-enriched proteins required for multidimensional NMR experiments; these are particularly desirable in conformational studies of inhibitor-enzyme complexes. The best way to solve the problem is to apply gene cloning and site-directed mutagenesis techniques.

In this paper we describe an efficient expression system for a squash inhibitor in *E. coli*. We realized that there is little likelihood of achieving significant production of such a small protein by direct cytoplasmic expression. The final yield of small gene proteins expressed in *E. coli* is usually very low, mostly due to metabolic instability of the expression products. A successful approach to avoid this problem is to clone the protein gene fused with a sequence coding for a long, hydrophobic polypeptide chain leading the recombinant protein to form inclusion bodies.

The plasmid used by us, a derivative of pAED4 [6], has been used previously to produce recombinant proteins [7,8] and peptides [9] in *E. coli*. The plasmid utilizes a T7 expression system from the pET3a plasmid [10] and includes an F1 replication origin to allow for single-stranded mutagenesis. The gene for CMTI I is preceded by the sequence containing a part of the leader region and a part of *trpE* gene of the *trp* operon of *E. coli*. This sequence codes for *LE1413* polypeptide [11] – an effective leader of fusion proteins into inclusion bodies [12]. All methionines and cysteines in the *LE1413* sequence have been replaced by alanines to facilitate purification procedure [13].

In the system used by us the desired biological material is obtained from the fusion protein by CNBr cleavage at Met residue. The sequence of *Cucurbita maxima* trypsin inhibitor I (CMTI I) – the best known representative of the squash inhibitor family – contains a single Met residue at position 8. We have substituted it by leucine (see Fig. 1) as a residue which is similar to methionine.

2. Materials and methods

All chemicals used were of the highest purity grade. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase and a dideoxy sequencing kit were from Amersham. Plasmid pAED4 was a generous gift from Professor P.S.Kim. A sample of native CMTI I was isolated from figleaf gourd (*C. ficifolia*) as described [14]. Bovine β-trypsin was obtained according to the method of Liepnicks and Light [15]. Human cathepsin G was a kind gift from Dr. W. Watorek.

2.1. Plasmid construction

The nucleotide sequences coding for Leu-8-CMTI I was programmed according to its respective amino acid sequence (Fig. 1) with the codon preferences established for *E. coli* [16]. Oligonucleotides used for synthesis were purchased from the Center for Molecular and Macromolecular Research of the Polish Academy of Sciences, Lodz. All oligonu-

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cleotides, except terminal ones, were 5′-phosphorylated with T4 polynucleotide kinase, annealed and ligated in a single step using standard procedures [17]. A ligation product of the expected size was isolated from polyacrylamide gels and cloned into *Hind*III/*Bam*HI sites of the derivative of pAED4 plasmid [6] in frame with the sequence coding for *LE1413* polypeptide. The sequence of the gene was confirmed by the dideoxy sequencing method.

2.2 Protein expression and purification

Expression and cyanogen bromide cleavage of the protein were carried out, with small modifications, as described [13]. *E. coli* strain BL21(DE3)-pLys(S) cells were transformed with pAED4 plasmid bearing the CMTI I gene. The transformed cells were grown from overnight cultures at 37°C in Luria groth containing 100 μg/ml ampicillin and 30 μg/ml chloramphenicol. The cells were induced at OD_{590}= 1.0 with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and harvested after 2.5 h. The cells were lysed by freezing, followed by sonication in 50 mM Tris, pH 8.7, 15% glycerol, 100 mM $MgCl_2$, 10 mM $MnCl_2$, 10 μg/ml DNAse I and centrifuged. The pellet was resuspended in 50 mM Tris, pH 8.7, 1% Nonidet (NP40), 1% deoxycholic acid, 1 mM EDTA, 200 mM NaCl, sonicated and centrifuged.

The pellet was dissolved in 6 M GuHCl, 50 mM Tris, pH 8.7, 10 mM oxidized glutathione, and incubated for 15 min at room temperature. Next, the samples were diluted 10-fold with water and centrifuged. The pellet was suspended in 10 ml of 70% formic acid and 200 mg of CNBr was added. After 2.5 h the reaction mixture was spun under vacuum for 4 h, 10-fold diluted with water, and freeze-dried. The lyophilizate was dissolved in 6 M GuHCl, 0.1 M dithiotreitol, 0.1 M Tris, pH 8.7, and dialyzed against 5% acetic acid. The supernatant was mixed with an equal volume of Tris (0.3 M), KCl (0.6 M), EDTA (3 mM), oxidized glutathione (10 mM), pH 9.0, giving final pH 8.5, and incubated overnight. The native form of inhibitor was purified on a semi-preparative Vydac C18 column in acetonitrile/water gradient in the presence of trifluoroacetic acid (0.1%).

3. Results

The final yield of the Leu-8-CMTI I was estimated spectrophotomerically from its Tyr27 absorption to be about 5 mg/l of culture. The molecular mass of the resulting material was found by laser desorption mass spectrometry to be 3257 ± 3 which is in excellent agreement with the calculated value, 3257. The HPLC elution profile of the mixture of CMTI I and Leu-8-CMTI I indicates that the latter elutes at a slightly higher concentration of acetonitrile.

In order to check the effect of Met8→Leu substitution on the energy of association with proteinases, we determined association constants with two different enzymes: bovine β-trypsin and human cathepsin G. In both cases the association constants were the same (within experimental error) as for the wild-type inhibitor (Table 1).

4. Discussion

An attempt to produce in an *E. coli* system a trypsin inhibitor very close to CMTI I was made previously by Chen at al. [18]

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                1                                   10
     Trp Met Arg Val Cys Pro Arg Ile Leu Leu Glu Cys Lys Lys Asp Ser Asp
GCTT TGG ATG CGT GTT TGC CCG CGT ATC CTG CTG GAA TGC AAA AAA GAC TCT GAC
   A ACC TAC GCA CAA ACG GGC GCA TAG GAC GAC CTT ACG TTT TTT CTG AGA CTG
ind  III

                20                                  29
ys Leu Ala Glu Cys Val Cys Leu Glu His Gly Tyr Cys Gly ter ter
GC CTG GCT GAA TGC GTT TGC CTG GAA CAC GGT TAC TGC GGT TAA TAG 3'
CG GAC CGA CTT ACG CAA ACG GAC CTT GTG CCA ATG ACG CCA ATT ATC CTAG
                                                          BamH I
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Fig. 1. Gene and amino acid sequences of Leu-8-CMTI I.

Table 1
Comparison of association and hydrolysis constants for CMTI I and Leu-8-CMTI I

Inhibitor	Association constant (M^{-1})		Hydrolysis constant
	β-trypsin	cathepsin G	
CMTI	3.3×10^{11}	1.7×10^{8}	1.84
Leu-8-CMTI I	1.2×10^{11}	1.2×10^{8}	4.74

Association constants were determined in 0.1 M Tris, 20 mM $CaCl_2$, 0.01% Triton X100, pH 8.3, at 22°C as described by Otlewski and Zbyryt [22]. In the case of cathepsin G the buffer additionally contained 500 mM NaCl. Hydrolysis constants of the reactive site peptide bond Arg5-Ile were determined in 100 mM Mes, pH 6.0, 20 mM $CaCl_2$, 2 mol% of bovine β-trypsin were added to a 0.1 μM solution of the inhibitor and the progress of hydrolysis was monitored using a C18 μBondapak reversed-phase column as described [22].

using the plasmid pWR590-1. The results were not satisfactory. The total yield of purified, active inhibitor was only about 200 μg per 1 of cell culture. As our results have shown, using a plasmid that utilizes a T7 expression system, a much higher yield can be obtained (5 mg/l), high enough, in fact, to keep at a reasonable level the cost of production of ^{15}N- and/or ^{13}C-labeled protein.

Met8→Leu substitution does not have any noticeable effect on the activity of CMTI I inhibitor (see Table 1) despite the fact that methionine at position 8 is strongly conserved in the squash inhibitors. Of 24 unique amino acid sequences of squash inhibitors 22 contain Met and two sequences contain Lys 5. Our data are in agreement with the X-ray structure of CMTI I-bovine trypsin complex showing that Met8 is not in contact with the proteinase [19].

We find, however, a significant effect of the substitution on the hydrolysis constant value. The K_{hyd} is increased 2.6-fold upon substitution. Unlike the association constant, this parameter does not describe an enzyme-inhibitor interaction, but is an exclusive property of an inhibitor. An increased value of K_{hyd} suggests that either the intact form of Leu-8-CMTI I has increased free energy compared to the wild-type inhibitor, or that the cleaved form of Leu-8-CMTI I is stabilized compared to the cleaved form of the wild type CMTI I. At present the first possibility cannot be excluded. The conservative substitution Ile6→Leu in homologous EETI II inhibitor resulted in a significant increase of the binding loop disorder [20]. On the other hand, the side chain of Met8 does not contact any other part of the inhibitor, either in the crystal [19] or in the solution structure [21]. It thus seems more likely that upon reactive site hydrolysis Leu8 undergoes an interaction or interactions which are absent or weaker in the Met8 CMTI I.

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