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Cloning a synthetic gene for human stefin B and its expression in *E. coli*

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A gene coding for human stefin B was synthesized by the solid-phase phosphite method and cloned in the pUC8 cloning vector. The insert with the verified DNA sequence was subcloned into two expression vectors and expressed in *E. coli* as a fusion protein with β-galactosidase and as a native protein. The CNBr cleaved fusion protein and the native recombinant stefin B were inhibitory to papain and reacted with antibodies against human stefin B.

Stefin B; Cysteine proteinase inhibitor; Synthetic gene; Fusion protein; Gene expression; (*E. coli*, Human)

1. INTRODUCTION

Stefin B is a protein inhibitor of the cysteine proteinases. It belongs to the stefin family of the cystatin superfamily [1]. It has been isolated from human spleen and liver and its amino acid sequence has been fully determined [2,3]. Stefin B is distributed widely among the tissues and is mostly localized intracellularly as other stefins but it has also been found extracellularly [4]. Its role is generally accepted as a protector against the proteinases leaking from lysosomes. In order to obtain more of the protein for study of its interactions with cysteine proteinases and for further elucidation of its physiological functions we decided to clone the gene for stefin B and to produce the protein using transformed bacteria.

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Abbreviations: DMSO, dimethylsulfoxide; IPTG, isopropyl-β-D-thiogalactopyranoside; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; TFA, trifluoroacetic acid; PAGE, polyacrylamide gel electrophoresis

2. MATERIALS AND METHODS

2.1. *Materials*

The enzymes used for the various experiments described below were purchased from Pharmacia (Uppsala) except the Sequenase kit for DNA sequencing which was from the United States Biochemical Corporation. Protocols for standard procedures were those as published [5], unless otherwise specified.

2.2. *Design of the synthetic gene*

Design of the synthetic gene was done with help of the program package 'KLON' (unpublished), written especially for this purpose. We inserted restriction sites to ease the future mutagenesis, obeyed the optimal codon usage of *E. coli* [6] and tried to avoid stable secondary structures. Conceptual fragmentation of the gene into the oligonucleotides was done with the program 'REGEN' as described [7].

2.3. *Chemical synthesis, purification and phosphorylation of the oligonucleotides*

Oligonucleotides were synthesized with an Applied Biosystems 381A synthesizer using methyl phosphoramidites. Crude oligonucleotides were purified by reversed-phase HPLC on an Zorbax ODS column (4.6 mm × 25 cm) before the detritylation (chromatographic conditions: solvent A, 5% acetonitrile, 0.1 M trimethylammonium acetate in H_2O; solvent B, 80% acetonitrile; flow rate, 1 ml/min; gradient, 0–50% B in 30 min). Final purification was achieved by gel electrophoresis on 15% polyacrylamide gel. The oligonucleotides were visualized by UV-shadowing, excised from the gel and eluted with water. Phosphorylation on the 5′-terminus was done with T_4 polynucleotide kinase.

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2.4. *Ligation of the synthetic gene into the pUC8 vector*

The phosphorylated oligonucleotides (20 pmol each, except in nos 1 and 16 at 40 pmol) were mixed in a total volume of 35 μl in the 67 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, heated at 100°C for 2 min and cooled to room temperature for an hour. 20 pmol of the *Bam*HI and *Sal*I digested and dephosphorylated vector pUC8 (purchased from Pharmacia) was added, ATP to the final concentration of 1 mM and DTT to 10 mM. Ligation was carried out after the addition of the 1 U of T_4 DNA ligase at 14°C for 16 h.

2.5. *Identification of the recombinant clone*

Competent cells (*E. coli* DH5α-purchased from BRL) prepared by the DMSO method [8] were transformed by the ligation mixture. Recombinant colonies were identified by the X-gal test. Restriction analysis of the mini preps cut with *Pst*I/*Kpn*I identified the plasmids with the correct insert – pUC8-SB. Plasmid was sequenced by the Sequenase kit as suggested by the producer.

2.6. *Construction of expression vectors*

The gene for stefin B was excised from the pUC8-SB plasmid by digestion with *Bam*HI and *Sal*I, eluted from the 5% polyacrylamide gel and ligated into the pKP1500 expression vector (provided kindly by T. Miki and T. Imoto, Kyushu University, Japan, [9]) and into the pUR288 (provided by H.G. Gassen [10]), treated previously with the same enzymes.

2.7. *Expression and purification of recombinant stefin B*

Cells, harboring pKP1500-SB plasmid were harvested 3 h after the induction with IPTG and lysed as described [11]. Stefin B was isolated by means of affinity chromatography on carboxymethyl-papain Sepharose followed by the gel filtration on Superose 12 column [3]. For the amino acid sequence determination the protein was isolated by HPLC using gradient of the acetonitrile/0.1% TFA. Cleavage with CNBr was done in 16 h in 80% formic acid using weight ratio 1:10 (CNBr/protein) and the protein concentration 10 mg/ml. The sample was lyophilized and twice evaporated with H_2O. The inhibitory activity was determined against papain [12].

2.8. *N-terminal amino acid sequence analysis*

Sequential Edman degradation was performed by the Applied Biosystems (Foster City, CA) 470A protein sequencer by the analysis of the phenylthiohydantoin derivatives of amino acids on-line with 120A model HPLC of Applied Biosystems.

2.9. *Immunological detection*

Protein mixture was separated on the SDS-PAGE using the Phast system from Pharmacia and electrophoretically transferred onto the nitrocellulose membrane and treated with anti-human stefin B antibodies as described [13].

3. RESULTS AND DISCUSSION

Since human stefin B is only 98 amino acids long, cloning via the synthetic gene appeared to be a reasonable approach. Moreover the protein only has two methionines positioned as the first two amino acids and they would as such facilitate cloning as a fusion protein, producing by cleavage with CNBr almost intact protein. The inhibitory activity of the protein lacking the first two methionines should not be severely perturbed as was observed with the homologous rat cystatin β [14].

The gene was assembled from 17 oligonucleotides as designated in fig.1. Annealing of the oligonucleotides and ligation were done in one reaction mixture. We obtained approximately one half of the recombinant clones from which the correct plasmids were identified by restriction analysis. After confirming the DNA sequence of the insert, we subcloned it into the pUR288 vector in frame at the C-terminus of the β-galactosidase. The SDS-PAGE of the cell extract was blotted on a nitrocellulose membrane and the major protein band, corresponding to the M_r of the fusion protein, was identified with an anti-human stefin B antibody (fig.2). The fusion protein was not inhibitory active to papain. However, after cleavage of the whole protein extract with CNBr, the inhibitory activity was generated, confirming that the protein without the N-terminal methionines is also active.

As the next step the insert was transferred into the pKP1500 expression vector to allow the intracellular production of stefin B in the yield of approx. 4 mg/l of bacterial culture after induction with 0.5 mM IPTG. The identity of the recombinant stefin B was confirmed by protein sequencing. Sequential Edman degradation yielded the methionine amino-terminal (255 pmol) and the 14 subsequent residues as follows: Met (288 pmol), not determined (Cys in the natural stefin B), Gly (392 pmol), Ala (162 pmol), Pro (118 pmol), Ser (113 pmol), Ala (146 pmol), Thr (84 pmol), Gln (182 pmol), Pro (58 pmol), Ala (87 pmol), Thr (72 pmol), Ala (165 pmol) and Glu (72 pmol). The sequence was the same as predicted from the nucleotide sequence. The engineered protein differed from the natural only by not being acetylated on the N-terminal methionine. Besides the full-length protein, another protein species was found lacking the first two methionines, which we attribute to a proteolytic cleavage during isolation on immobilized papain, similar as was already reported [14]. Both long and short forms exhibited the same inhibitory activity. Preliminary kinetic studies show that the natural and both recombi-

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         10         20         30         40         50         60         70         80
      MetMetCysGlyAlaProSerAlaThrGlnProAlaThrAlaGluThrGlnHisIleAlaAspGlnValArgSer
  (----------------F1-----------------)(---------------F2----------------)(------
  GATCCATGATGTGTGGTGCTCCGTCTGCTACTCAGCCGGCTACTGCAGAAACTCAGCATATCGCTGACCAGGTTCGTTCT
      GTACTACACACCACGAGGCAGACGATGAGTCGGCCGATGACGTCTTTGAGTCGTATAGCGACTGGTCCAAGCAAGA
      (-------F9a-------)(------F9b--------)(-----------------F10-------------)(-

         90         100        110        120        130        140        150        160
  GlnLeuGluGluLysTyrAsnLysLysPheProValPheLysAlaValSerPheLysSerGlnValValAlaGlyThrAsn
  ---------F3----------------)(--------------------F4--------------------)(---------
  CAGCTGGAAGAGAAATACAACAAGAAATTCCCGGTTTTCAAAGCTGTAAGCTTCAAATCTCAGGTTGTTGCTGGTACCAAC
  GTCGACCTTCTCTTTATGTTGTTCTTTAAGGGCCAAAAGTTTCGACATTCGAAGTTTAGAGTCCAACAACGACCATGGTTG
  ---------------F11--------------)(-----------------F12---------------------)(----

       170        180        190        200        210        220        230        240
  TyrPheIleLysValHisValGlyAspGluAspPheValHisLeuArgValPheGlnSerLeuProHisGluAsnLysPro
  ---------F5-----------------)(----------------F6-----------------)(-----------
  TACTTCATCAAAGTTCACGTTGGCGATGAAGACTTTGTTCACCTGCGCGTTTTCCAGTCTCTGCCGCACGAGAACAAACCG
  ATGAAGTAGTTTCAAGTGCAACCGCTACTTCTGAAACAAGTGGACGCGCAAAAGGTCAGAGACGGCGTGCTCTTGTTTGGC
  -------------F13----------------)(----------------F14-----------------)(------

      250        260        270        280        290        300
  LeuThrLeuSerAsnTyrGlnThrAsnLysAlaLysHisAspGluLeuThrTyrPhe x  x
  --F7--------------)(---------------------F8-----------------)
  CTGACTCTGTCCAACTACCAGACCAACAAAGCTAAGCACGACGAGCTGACCTACTTCTAGTAG
  GACTGAGACAGGTTGATGGTCTGGTTGTTTCGATTCGTGCTGCTCGACTGGATGAAGATCATCAGCT
  ------F15--------------)(---------------------F16----------------)
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Fig.1. Amino acid sequence of human stefin B and construction of the gene from 17 oligonucleotides.

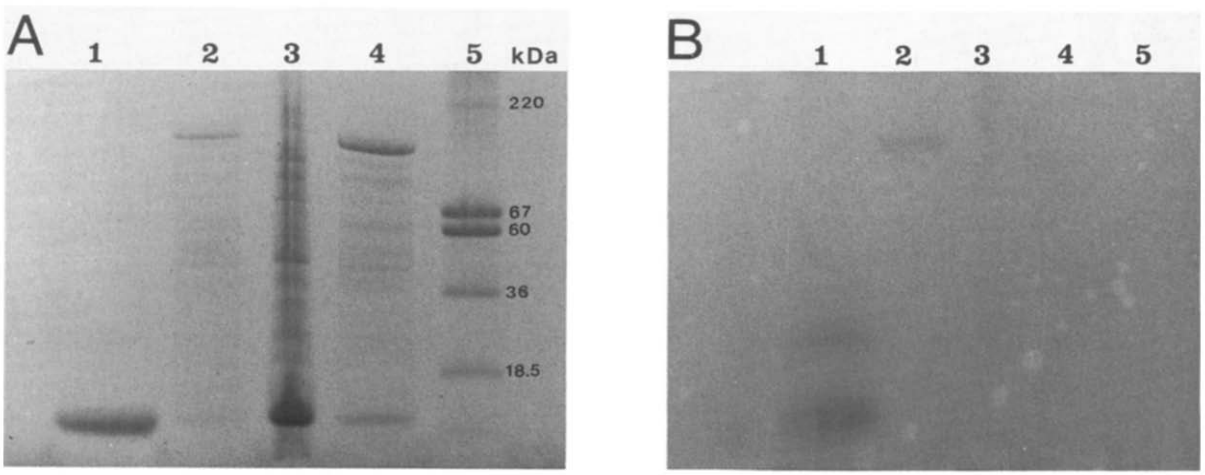

Fig.2. (A) SDS-PAGE under reducing conditions on the Phast system. Lanes: 1, human stefin B; 2 and 3, extract of *E. coli* DH5α/pUR288-SB after 3 h and before induction with IPTG; 4, extract of *E. coli* DH5α-pUR288 3 h after induction; 5, molecular mass standards. (B) Western blot of the gel from A with polyclonal anti-human stefin B antibodies.

nant forms of stefin B have comparable K_i values.

Thus, we have demonstrated the feasibility of expressing active stefin B in *E. coli* via cloning by total gene synthesis. This system we are currently utilizing for site-directed mutagenesis to produce mutants that will be used to study the nature of the dimerization process in native stefin B [2,3] and the mechanism of action with its target enzymes.

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