Volume 238, number 1, 116–118 FEB 06342 September 1988

Total synthesis of the cystatin α gene and its expression in *E. coli*

Nobuhiko Katunuma, Masayuki Yamato[+], Eiki Kominami[°] and Yoshimasa Ike[*]

Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770 and **Central Research Institute, Mitsui Toatsu Chemical, Inc., Biotechnology Laboratory, Mobara, Chiba 297, Japan*

Received 8 August 1988

A gene encoding cystatin α has been chemically synthesized, cloned and expressed in *E. coli*. The gene of 318 base pairs was assembled by enzymatic ligation of 19 oligonucleotides and cloned into a pBR322-derived expression plasmid down stream of the tac promoter. The expression product of the synthetic gene has been purified by Sephadex G-50 column chromatography and shown to have the same properties as those of the authentic protein isolated from rat epidermis.

Cystatin α; Cysteine proteinase inhibitor; Synthetic gene; *(E. coli)*

1. INTRODUCTION

Cystatin α is one of the specific endogenous inhibitors of cysteine proteinases and a member of the evolutionary superfamily [1]. Cystatin α, with an M_r of about 11000, lacks cysteinyl residues and shows limited distribution in epidermis, squamous epithelia and neutrophils [2,3].

A new method for the large scale production of cystatin α using recombinant DNA technology has been developed to study the relationship between structure and inhibitory activity using X-ray crystallography, and also to develop new drugs of therapeutic use for specified diseases based on abnormal increase of cysteine proteinases such as muscular dystrophy [4], distal myopathy [5] as well as for the suppression of proliferation of some RNA viruses [6]. This paper deals with the total synthesis of the cystatin α gene, its expression in *E. coli* and purification of the product.

Correspondence address: N. Katunuma, Division of Enzyme Chemistry, Institute for Enzyme Research, The University of Tokushima, Tokushima 770, Japan

[+] *Present address:* Department of Food Microbiology, School of Medicine, The University of Tokushima, Tokushima 770, Japan

[°] *Present address:* Juntendo University School of Medicine, Bunkyo-ku, Tokyo 113, Japan

2. MATERIALS AND METHODS

2.1. *Design of the gene*

Based on the amino acid sequence of cystatin α [7], 19 overlapping oligonucleotide fragments were designed according to the codon usage of *E. coli* [8] as shown in fig.1. The recognition sites of *Eco*RI and *Bam*HI were placed at the 5′- and 3′-ends of the gene, respectively. As the N-terminal amino acid of cystatin α is methionine, an additional codon for it was unnecessary for the initiation of translation. Tandem translation stop codon was incorporated at the C-terminal.

2.2. *Chemical synthesis of the oligomers*

The 19 oligomers were synthesized by the phosphoramidite method [9] using an automatic DNA synthesizer (Microsyn 1450A). The product was removed from the solid support and purified by polyacrylamide gel electrophoresis (PAGE). The product band was detected by UV light, cut out, eluted and precipitated from ethanol.

2.3. *Assembly of the gene fragments and its cloning in* E. coli

The assembly was performed as shown in fig.2. The fragments 1, 2, 11, 12 and 13 were mixed and phosphorylated. After annealing, the mixture was ligated to make segment A. Segment A was then purified by PAGE and DE-52 cellulose column chromatography. Other segments B, C and D were prepared in the same manner. Segments A and B (C and D) were ligated to segment AB (CD), which was purified by PAGE. Segments AB and CD were ligated to construct the total gene of cystatin α. The resulting gene was purified by extraction with phenol/chloroform followed by ethanol precipitation. The chemically and biochemically synthesized cystatin α gene was then ligated to pBR322 previously digested with *Eco*RI and *Bam*HI. The plasmid was introduced into *E. coli* MC1061 and the resulting ampicillin-resistant transformants were screened

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/88/$3.50

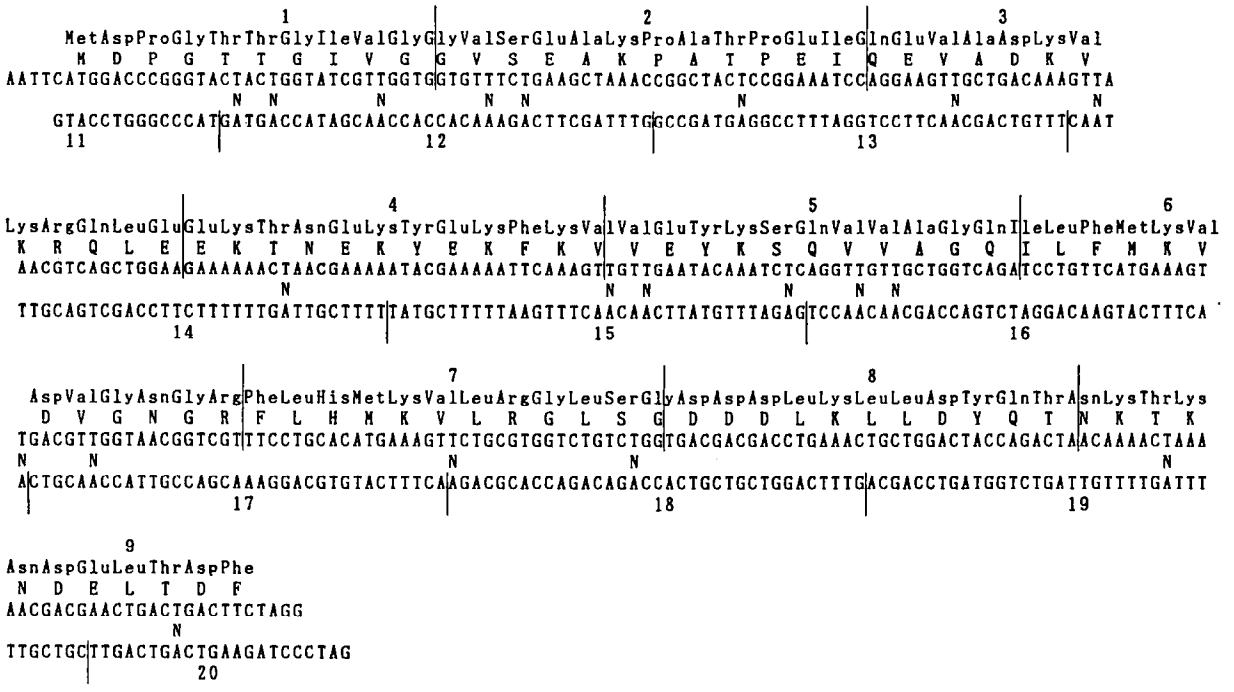

Fig.1. Design of synthetic gene fragments based on the amino acid sequence of cystatin α. The recognition sites of *Eco*RI and *Bam*HI were placed at the 5′- and 3′-ends of the gene, respectively. Tandem translation stop codon was inserted at the 3′-end.

by hybridization to radiolabeled oligomer fragment 1 and fragment 20. Restriction enzyme analysis revealed that five of eight hybridization positive transformants contained the desired *Eco*RI-*Bam*HI fragment corresponding to the synthetic gene of cystatin α. The nucleotide sequence of the fragment was confirmed by the method of Maxam and Gilbert.

2.4. *Expression of cystatin α gene in* E. coli

The *Eco*RI-*Hin*dIII fragment of pTPI-010 containing the *Eco*RI-*Bam*HI fragment was ligated to the *E. coli* tac expression plasmid (pKK223-3) [10] at the *Eco*RI and *Hin*dIII sites (fig.3). The resulting plasmid was introduced into *E. coli* MC1061. Transformants were screened by the same method as mentioned above. One of the positive transformants, E204, was employed for the purification of cystatin α.

2.5. *Culture of a recombinant strain of* E. coli

The recombinant strain E204 was grown at 37°C for 16 h in L-broth (1% tryptone, 0.5% yeast extract (Difco), 1% NaCl and 30 μg/ml ampicillin). The cells were harvested by centrifugation at 10000 × *g* for 30 min.

2.6. *Purification of cystatin α from* E. coli

Harvested cells were frozen and thawed twice and suspended in 5 vols of 25 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA. The suspension was vortex-mixed for 30 s and centrifuged at 10000 × *g* for 10 min at 4°C. The supernatant was then applied to a column of Sephadex G-50 equilibrated with 25 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and eluted with the same buffer. Cystatin α fractions were pooled,

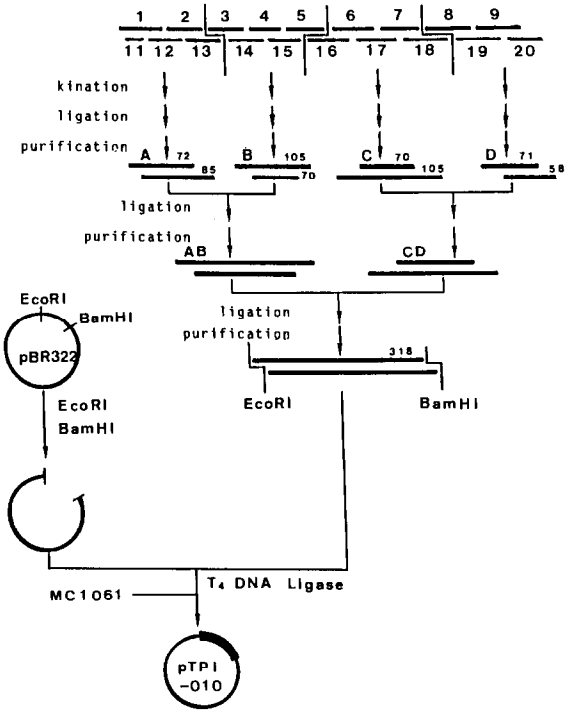

Fig.2. Construction of a plasmid containing chemically synthesized cystatin α gene.

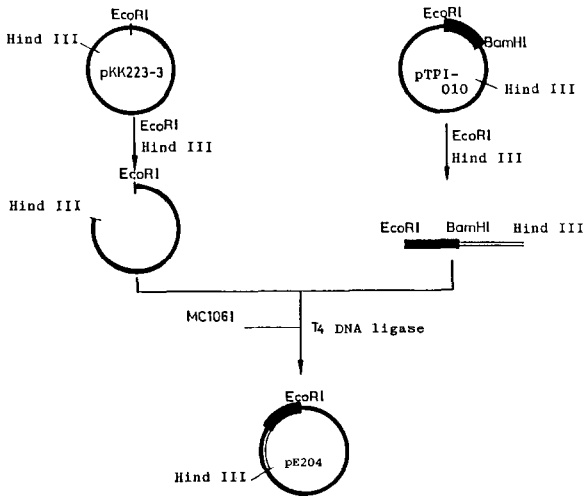

Fig.3. Construction of a plasmid for the expression of cystatin α gene under tac promoter control.

concentrated by ultrafiltration using a YM-10 membrane and dialyzed overnight against 10 mM Tris-HCl buffer, pH 8.0. The dialysate was further purified on a column of DE-52 with the gradient elution of 0–0.2 M NaCl in 10 mM Tris-HCl buffer, pH 8.0. Cystatin α fractions were pooled. Amino acid analysis of the purified cystatin α was performed as described previously [3].

3. RESULTS AND DISCUSSION

3.1. *Purification of cystatin* α

Cystatin α can be solubilized easily from recombinant *E. coli* by freezing and thawing, and by following extraction with buffer. Sephadex G-50 column chromatography proved to be very useful in the next process. By further purification with DE-52, it gave a single band on SDS-PAGE (not shown). The best overall yield was 20 mg of protein from 1 g of wet cells. Though only E204 strain is referred to in this paper, better recombinant strains producing higher amounts of the inhibitor are now constructed by improving the expression system of the plasmid. Strain improvement for the secretion of cystatin α remains to be studied.

3.2. *Properties of recombinant type cystatin* α

The purified recombinant cystatin α inhibited papain and cathepsins B, H and L to the same extent as the naturally-occurring counterpart. The cystatin α showed the same mobility on the electrophorsis in the presence and absence of SDS and also reacted with the antiserum against the natural one (not shown). The amino acid composition of the recombinant cystatin α is shown in table 1. It is in good agreement with that of rat cystatin α [7]. These results demonstrate that both cystatins are identical to each other. Our studies have made possible the supply of cystatin α in quantities large enough to use in studying its therapeutic effectiveness and biological functions as well.

Acknowledgement: We would like to thank Dr Y. Saito (Abe Trading Co., Ltd) for preparation of the oligomers.

REFERENCES

[1] Barrett, A.J., Fritz, H., Grubb, A., Isemura, S., Jarvinen, M., Katunuma, N., Machleidt, W., Muller-Esterl, W., Sasaki, M. and Turk, V. (1986) Biochem. J. 236, 312.
[2] Kominami, E., Bando, Y., Wakamatsu, N. and Katunuma, N. (1984) J. Biochem. 96, 1437–1442.
[3] Katunuma, N. and Kominami, E. (1985) Curr. Top. Cell. Regul. 27, 345–360.
[4] Kominami, E., Ii, K. and Katunuma, N. (1987) Am. J. Pathol. 127, 461–466.
[5] Ii, K., Hizawa, K., Nonaka, I., Sugita, H., Kominami, E. and Katunuma, N. (1986) Am. J. Pathol. 122, 193–198.
[6] Korant, B.D. et al. (1986) J. Cell. Biochem. 32, 91–95.
[7] Takio, K., Kominami, E., Bando, Y., Katunuma, N. and Titani, K. (1984) Biochem. Biophys. Res. Commun. 121, 149–154.
[8] Grantham, R., Gantier, C. and Gouy, M. (1981) Nucleic Acids Res. 9, 43–96.
[9] Froehler, B.C. and Matteucci, M.D. (1986) Tetrahedron Lett. 27, 469–472.
[10] De Boer, H.A., Comstock, L.J. and Vasser, M. (1983) Proc. Natl. Acad. Sci. USA 80, 21–25.