Leu-enkephalin purification from *E. coli* cells carrying the plasmid with fused synthetic leu-enkephalin gene

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ABSTRACT

Chemically synthesized leu-enkephalin gene was fused to a large Eco RI-Bam HI fragment of pBR322 along with a Eco RI fragment of Ch4A phage DNA carrying the promoter and most of the *E.coli* β -galactosidase gene. The resulting recombinant DNA was used to transform *E. coli* cells. Transformants were screened for Tc-sensitivity, Am-resistance, and β -galactosidase constitutional synthesis. Restriction endonuclease analysis combined with DNA sequencing of the plasmid DNAs revealed a complete nucleotide leu-enkephalin sequence and Eco RI lac-operon fragment in two possible orientations. Radioimmunoassay for leu-enkephalin activity in BrCN-treated bacterial extracts showed that *in vivo* leu-enkephalin is synthesized only in strains carrying plasmids with the proper lac-fragment orientation. About $5 \cdot 10^4$ molecules of the former are synthesized per single *E. coli* cell. One of the clones was used for leu-enkephalin purification. Using 100 g of cells it is possible to obtain about 2 mg of practically pure leu-enkephalin.

INTRODUCTION

Recent progress in methods for the chemical-enzymatic synthesis of defined polynucleotides combined with the use of recombinant DNA have provided the means for the large-scale production of various proteins by bacterial cells via cloning and expression of synthetic genes with a help of DNA vehicles. This has been aptly demonstrated in the synthesis of the somatostatin [1] and insulin [2] genes. Large-scale *in vivo* production of neuropeptides might be of certain practical value. The present report is concerned with the design and construction of a recombinant plasmid, carrying the synthetic leu-enkephalin gene, which codes for a neuro-pentapeptide [3], using the aforementioned approach. Some new publications on the cloning and expression of synthetic genes have appeared [4,5] since preliminary reports [6,7] of this investigation had been published.

MATERIALS AND METHODS

Materials. Sephadex G-50 (superfine) was purchased from Pharmacia Fine Chem.;

Tris (hydroxymethyl)-aminomethan - from Merck; ethidium bromide - from Sigma; dithiothreitol - from Calbiochem; 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal), sodium dodecyl sulphate (SDS), cyanogen bromide and EDTA - from Serva; 2-mercapthoethanol, acrylamide, N,N'-methylene-bisacrylamide, N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, Coomassie brilliant blue R-250, and agarose - from Bio-Rad; γ - ^{32}P ATP and ^{125}I - from Amersham; nitrocellulose filters HAWP - from Millipore Corp.; Whatman 3MM paper - from Whatman Ltd.; Leu-enkephalin was kindly provided by Dr. V.T.Ivanov from our Institute.

Methods. T_4 DNA ligase was purified by a new procedure worked out in our laboratory [8]. Polynucleotide kinase was isolated according to [9]. Eco RI, Bam HI, Sal I, and Hind III restriction endonucleases were obtained and used as described previously [10]. *E. coli* alkaline phosphatase was diethylpyrocarbonate treated to remove trace DNAase and RNAase contamination [11].

Plasmid DNAs were isolated according to the method of Zasloff et al. [12]. Restriction endonuclease generated DNA fragments were analyzed by electrophoresis on polyacrylamide [13] or agarose [14] gels.

T_4 DNA ligase reactions were carried out in 20 mM Tris-HCl, pH 7.6, 10 mM MgCl_2 , 10 mM DTT, 0.5 mM ATP at 4°C for 24 h. The reaction mixture contained in total volume of 20-100 μl : 0.9 nmoles of Eco RI treated Ch4A DNA [15], 0.8 nmoles of Eco RI-Bam HI fragment of pBR322, and 15 nmoles of the synthetic leu-enkephalin gene. The reaction mixture was heated at 90°C for 2 min, cooled and used to transform *E. coli* 802 or HB 101 strains as described by Cohen et al. [16]. The transformants were plated on 1.2% LB agar plates containing 20 μg of ampicillin per ml and 40 μg of X-gal. The plates were incubated at 37°C for 24 h. The efficiency of transformation was about 10^5 colonies per 1 μg of recombinant DNA. The transformants were selected for ampicillin resistance (Ap^r), tetracycline sensitivity (Tc^s), and constitutive β -galactosidase synthesis as identified by the blue colour of the colonies [17]. Leu-enkephalin was labeled with ^{125}I by a chloramine T procedure [18].

Radioimmunoassay for leu-enkephalin was carried out in a reaction mixture containing 0.05 M Tris-HCl pH 7.0, 0.1% NaN_3 , 0.5 units of leu-enkephalin antisera and 10^5 cpm of ^{125}I -enkephalin. Non-labeled leu-enkephalin competing analysis was employed to determine its specific activity in BrCN-treated bacterial extracts. Calibration control was performed by addition of various amounts (5-30 pmoles) of synthetic non-labeled leu-enkephalin to a standard radioimmunoassay. Competing analysis was performed the same way except that appropriate dilutions of BrCN-treated bacterial extracts in aliquots were

added. After 4 h at 4°C bound [125 I]-enkephalin was separated from free peptide by retaining the antibody-enkephalin complex on Millipore HAWP filters. Radioactivity was counted in an Ultrogamma counter (LKB).

Restriction endonuclease analysis of recombinant plasmid DNAs isolated from the selected clones. The synthetic leu-enkephalin gene was synthesized by Drs. Efimov, V.A., and Chakhmakhcheva, O.G. [19] and kindly provided for the cloning experiments. The design of the leu-enkephalin gene is shown in Fig. 1. In addition to 5 amino acid codons of the gene it contained a methionine preceding codon and a nonsense "ochre" terminating codon. To facilitate the insertion of the gene into plasmid DNA, the 5'-ends of the former contained single-stranded cohesive termini for Eco RI and Bam HI restriction endonuclease specific sites. Such a design with use of the "TAA" stop codon brings to a loss of Bam HI restriction site after construction of the recombinant molecule due to a G-C pair substitution for A-T at a Bam HI cohesive terminus of the gene preventing restriction endonuclease cleavage. The recombinant plasmid construction is outlined in Fig. 2. In contrast to a parental plasmid pBR322, the resulting plasmids lack a Bam HI restriction site and carry one additional Eco RI site. The approach used in plasmid construction and transformant screening suggest two possible opposite orientations of the lac-operon fragment respectively to the gene. Expression of leu-enkephalin gene can proceed only with a proper orientation of this fragment when the COOH-terminus of the β -galactosidase gene is fused to the former. The asymmetric location of a Hind III restriction site within the lac-operon fragment can indicate which plasmids have transcription proceeding into the leu-enkephalin gene. Orientation of the lac-operon fragment was determined with the aid of Hind III-SalI simultaneous cleavage of the recombinant plasmid DNAs and subsequent agarose gel electrophoresis of the two resulting fragments.

In the case of the proper orientation two restriction fragments of 3.88 and 2.72 megadaltons were observed. Otherwise two fragments of 5.96 and 0.64

Fig. 1. Nucleotide sequence of the synthesized DNA fragment containing leu-enkephalin gene.

Fig. 2. Design and construction of a recombinant plasmid carrying synthetic leu-enkephalin gene (E) fused to *E. coli* lac-operon fragment bearing lac-promoter (P) and β-galactosidase gene (β-Gal) on a vehicle plasmid pBR322. Arrows indicate start points and direction of transcription of the genes coding for ampicillin and tetracycline resistance (Ap^r, Tc^r) and β-galactosidase.

megadaltons could be detected. In 4 of 8 analyzed clones the desired lac-operon orientation was observed (plasmids pEK-1, pEK-4, pEK-6, and pEK-9), in 3 clones it was opposite (plasmids pEK-2, pEK-10, and pEK-11). Plasmid DNAs of pEK-1 and pEK-4 were partially sequenced in order to confirm a complete leu-enkephalin gene nucleotide sequence.

DNA-sequencing. Plasmid DNAs were digested with Eco RI, labeled with the aid of γ-³²P ATP and polynucleotide kinase, and then cleaved by SalI restriction endonuclease. The smaller DNA fragment supposed to contain the leu-enkephalin gene was electrophoretically purified and sequenced by Maxam and Gilbert technique [20] with several modifications [21]. Both the plasmids were found to contain a complete nucleotide sequence of the leu-enkephalin gene, attached to the altered Bam HI site of pBR322.

RESULTS AND DISCUSSION

The synthetic leu-enkephalin gene expression in *E. coli* cells. A number of selected clones was assayed for leu-enkephalin gene expression as described

Table 1. Radioimmunoassay for leu-enkephalin activity in *E. coli* strains carrying recombinant plasmids with fused synthetic leu-enkephalin gene*

Experiment No	Competing substance	Bound [125 I]-enkephalin** (cpm)	Experiment No	Competing substance	Bound [125 I]-enkephalin** (cpm)
1	-	4810 $\pm$ 190	8	40 μ l pEK-6	1030 $\pm$ 130
2	10 pmoles of enkephalin	1530 $\pm$ 50	9	40 μ l pEK-6	1220 $\pm$ 40
3	40 pmoles of enkephalin	680 $\pm$ 40	10	40 μ l pEK-2	4880 $\pm$ 30
4	10 μ l pEK-1	2060 $\pm$ 180	11	40 μ l pEK-11	4390 $\pm$ 160
5	40 μ l pEK-1	1220 $\pm$ 70	12	40 μ l pEK-10	4660 $\pm$ 120
6	10 μ l pEK-1 + IPTG	1320 $\pm$ 180	13	40 μ l pEK-10 + IPTG	4780 $\pm$ 240
7	40 μ l pEK-4	1740 $\pm$ 110	14	40 μ l pBR322	4920 $\pm$ 70

* 10 μ l of cell extract correspond to about 10^8 cells. Each figure represents a triplicate assay average.

** Excluding a non-specific [125 I]-enkephalin retention on Millipore filters observed without antisera (1040 $\pm$ 150 cpm). IPTG - isopropylthiogalactoside.

treated bacterial extracts of the four clones with proper lac-operon fragment orientation. There was no effect when extracts of the three other clones with opposite lac-fragment orientation were examined. Appropriate dilution curves (Fig. 4) were plotted in order to estimate leu-enkephalin content in bacterial extracts of the clones in question. Considering the curves in the Fig. 4, it can be found that 50% label dilution is observed if 7 pmoles of non-labeled leu-enkephalin (curve 1) or 5 μ l of non-induced *E. coli* HB101/1 BrCN-treated extracts (curve 2) are added to the radioimmunoassay. Thus 1 μ l of the extract contains about 1.4 pmoles of leu-enkephalin. 1 μ l of bacterial extract cor-

Fig. 4. Radioimmunoassay for leu-enkephalin activity in BrCN-treated bacterial extracts of *E. coli* strains carrying the recombinant plasmids. According to a standard procedure cells were grown in 5 ml of LB (Luria broth) to a cell density of 10^9 cells per ml and directly used for the bacterial extract preparation or exposed to the IPTG induction. After the induction with 1 mM IPTG samples were shaken at 37°C for additional 2 h. Then cells were pelleted by centrifugation and resuspended in 1 ml of 70% HCOOH containing 5 mg of BrCN. After 24 h at 22°C in the dark extracts were diluted with 9 ml of water and evaporated. Dry residues were suspended in 0.4 ml of 50 mM Tris-HCl pH 7.0, 0.1% Na₂S₂O₅, and the suspension was clarified by centrifugation after pH adjustment with IM NaOH. Supernatants were saved and assayed for enkephalin content as described in Materials and Methods.

- (1) ●—● - non-labeled enkephalin, pmoles
- (2) x—x - HB 101/1 *E. coli* extract, μ l
- (3) o—o - HB 101/1 *E. coli* extract, IPTG induction, μ l
- (4) +—+ - HB 101/1 *E. coli* extract, μ l

responds to $1.2 \cdot 10^7$ of initial cells as can be calculated from the extract preparation procedure described in the legend to the Fig. 4. It was evaluated that a single cell could produce about $5 \cdot 10^4$ molecules of the pentapeptide. Lac-operon induction by ImM isopropyl- β -thiogalactoside enhanced the extent of expression in the clones with the proper lac-operon fragment orientation 3-4 times (curve 3). Thus, it was confirmed that the constructed plasmid could direct *in vivo* leu-enkephalin synthesis in *E. coli* cells.

Leu-enkephalin purification from *E. coli* pEK-6 cells. As it was estimated about $1\text{--}2 \cdot 10^5$ leu-enkephalin molecules are produced by a single cell in the presence of an inducer. This is 10 times higher than observed with somatostatin expression [1] and similar to A and B insulin chain synthesis [2]. Thus, 100 g of packed cells contain at least 5 mg of fused leu-enkephalin. We therefore undertook purification on the former.

Growth of recombinant plasmid-carrying strains. *E. coli* pEK-6 was grown in 3 bottles of 10 l LB medium each, containing 10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, 5 g of NaCl per 1 l of 0.01 M Tris-HCl pH 7.4 at 37°C. When the culture reached a cell density of 10^9 cells per ml, lactose was added to 1 mg/ml as an inducer and aeration was continued for two additional hours. Then cells were harvested by centrifugation at 8000 rpm (Beckman J-21 B) and stored at -40°C.

Cell disruption and isolation of the cell wall and membrane fraction. 67 g of wet cells were resuspended in 230 ml of 0.05 M Tris-HCl pH 8.0 containing 10 mM MgCl_2 , 50 mM NaCl and 5 mM 2-mercapthoethanol. 250 mg of lysozyme in 20 ml of the same buffer were added and the suspension was incubated at 37°C for 15 min, cooled and sonicated (22 KHz). The temperature was not allowed to rise above 10°C. Crude extract was centrifuged for 30 min at 18000 rpm (J-21B). Then supernatant and pellet were analyzed for β -galactosidase and leu-enkephalin specific activities. β -galactosidase was found in both fractions; leu-enkephalin was detected only in the pellet.

Guanidine chloride fractionation. The pellet was resuspended in 60 ml of 6 M guanidine chloride containing 1% 2-mercaptoethanol. The suspension was stirred for 30 min at 4°C and centrifuged for 1 h at 30000 rpm (Spinco, Ti, 35). Supernatant was dialyzed for 16 h against 30 l of H_2O . Precipitate formed during the dialysis was centrifuged for 15 min at 15000 rpm.

BrCN cleavage of the protein fraction. The resulting pellet was resuspended in 150 ml of 75% HCOOH and incubated with 2 g of BrCN added for 24 h at room temperature in the dark. Then it was diluted with 9 volumes of distilled water, and the mixture evaporated. The dried material was suspended in 60 ml

of 50% CH_3COOH and clarified by centrifugation. Clear supernatant was concentrated by evaporation to 4 ml.

Sephadex G-50 column chromatography. A column (2·100 cm) of superfine Sephadex G-50 was prepared and equilibrated with 50% CH_3COOH . Leu-enkephalin fraction was applied to the column at 4 ml/h. The chromatography profile of leu-enkephalin activity elution and A_{280} are plotted in Fig. 5. Leu-enkephalin activity eluted as a single sharp peak. Fractions 36-40 were pooled, concentrated by evaporation to 0.5 ml, and used for further purification.

Leu-enkephalin descending paper chromatography. The pooled enkephalin fraction from Sephadex G-50 column was applied to a Whatman 3 MM sheet (20 cm wide) and dried. Then descending paper chromatography of enkephalin was carried out in a system containing pyridine-butanol- CH_3COOH - H_2O (60:40:12:48). The paper strip was air-dried, thin strips from both sides were cut off and stained with ninhydrin. The major part of ninhydrin staining material was in a single band. The non-stained part of the band was cut off, eluted with 10 ml of 10% CH_3COOH and evaporated to 0.2 ml. The fraction was then chromatographed on a Sephadex G-10 column (0.8·28 cm). Rechromatography on a thin-layer cellulose plate revealed that ninhydrin staining material moves as a compact spot with the same R_f as the control leu-enkephalin. It gave a specific reaction with anti-leu-enkephalin antibodies also. Data on leu-enkephalin purification are summarized in Table 2.

Samples of the standard and isolated enkephalin were hydrolyzed in 5.7 M

Fig. 5. Sephadex G-50 column chromatography profile of BrCN-treated "debris" fraction. See details in the text.

Table 2. Leu-enkephalin purification from *E. coli* pEK-6 cells

Purification step	Total protein, g	Enkephalin content, mg	Yield, %
I. Crude extract	9,6	3,96	100
II. "Debris"	2,4	3,39	85,6
III. Guanidine-HCl eluate from fraction II	0,33	2,40	60,6
IV. Sephadex G-50 chromatography	-	0,88	22,2
V. Whatman 3 MM Descending paper chromatography	-	0,52	13,1

HCl at 105°C under vacuum for 24 h and analyzed for amino acid composition [23] using analyzer model D-500, Durrum. The results are shown in Table 3. It can be seen that amino acid composition of the purified pentapeptide is completely identical to the standard leu-enkephalin. Slight reduction in tyrosine content was due to a partial degradation of tyrosine molecules under conditions specified.

This work was carried out in accordance with current USSR guidelines on

Table 3. Amino acid composition analysis of the synthetic and purified leu-enkephalin

Amino acids	Synthetic Leu-enkephalin (mol %)	Purified leu-enkephalin (mol %)
Gly	43,1	43,4
Leu	22,8	20,7
Tyr	15,3	16,4
Phe	18,7	18,6

Samples of the standard leu-enkephalin and of that purified from pEK-6 *E. coli* cells were hydrolyzed in 5.7 M HCl at 105°C under vacuum for 24 h. Amino acid analysis was carried out as described [23]. Amino acid contamination in both samples was less than 10% compared to the figures given in the table.

recombinant DNA research with P3 physical containment facilities employed.

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