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Expression in Escherichia coli of chemically synthesized gene for a novel opiate peptide α-neo-endorphin

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ABSTRACT

Chemically synthesized α-neo-endorphin gene was fused to the *Escherichia coli* β-galactosidase gene on the plasmid pKO13. The resulting recombinant DNA was used to transform *E. coli* cells. Radioimmunoassay for α-neo-endorphin in CNBr-treated bacterial cells showed that α-neo-endorphin was synthesized at approximately 5 x 10^5 molecules per single *E. coli* cell. One of the transformants, WA802/pαNE2, was used for α-neo-endorphin purification. From 10.9 g of wet cells, we isolated 4 mg of chemically pure and biologically active α-neo-endorphin.

INTRODUCTION

A novel endogenous opiate peptide, α-neo-endorphin, was isolated from porcine hypothalamus by K. Kangawa et al (1). This peptide is the first form that was found as a big Leu-enkephalin. Recently, the complete amino acid sequence of this opiate peptide was determined to be H-Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys-OH (2). The structure of α-neo-endorphin is unique because Leu-enkephalin sequence (H-Tyr-Gly-Gly-Phe-Leu-OH) is located at the amino acid terminus of it and followed by a characteristic basic amino acid pair of Arg^6-Lys^7, which is usually observed in prohormone sequence (3). The presence of such a basic amino acid pair in the molecule suggests that α-neo-endorphin may be as a precursor of Leu-enkephalin. The opiate activity in the guinea pig ileum assay of α-neo-endorphin was about 21 times more potent than Leu-enkephalin (2). This potent opiate activity suggests that α-neo-endorphin possesses its own physiological role *in vivo*.

To study physiological functions of this new opiate peptide, it is essential to get sufficient quantities of pure material. The recent advences in chemical synthesis of DNA and recombinant DNA technology made it possible to produce a large amount of proteins in bacterial cells (4, 5, 6). In this communication, we describe the design and construction of

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a recombinant plasmid which carries the synthetic α-neo-endorphin gene fused to E. coli β-galactosidase gene. We also describe the efficient expression of a hybrid β-galactosidase/α-neo-endorphin gene in E. coli and successful purification of mature and biologically active α-neo-endorphin.

MATERIALS AND METHODS

Bacterial strains and media. E. coli K-12 strains, WA802 (metB1, lac3 or lacY, galK2, galT22, λ^-, supE44, hsd3) and W3110, were kindly provided by Dr. B. Bachmann of E. coli Genetic Stock Center and Dr. H. Ogawa, respectively.

L-broth (pH 7.3) contained polypepton 10 g, yeast extract 5 g, and NaCl 5 g per liter of water. EMB-lactose medium contained polypepton 8 g, yeast extract 1 g, NaCl 5 g, K_2HPO_4 2.5 g, eosin 0.6 g, methylene blue 0.1 g, lactose 10 g, and agar 15 g per liter of water.

Enzyme and reagents. T4 ligase and restriction endonucleases, EcoRI, BamHI and HindIII, were obtained from Takara Shuzo. T4 polynucleotide kinase and E. coli alkaline phosphatase were purchased from P-L Biochemicals and Worthington, respectively. Chemically synthesized α-neo-endorphin was obtained from Peptide Institute, Protein Research Foundation.

The following reaction buffers were used; kinase buffer, 50 mM Tris-HCl, pH 8.0/10 mM $MgCl_2$/10 mM dithiothreitol (DTT)/2 mM spermidine/ 0.1 M KCl; ligation buffer, 20 mM Tris-HCl, pH 7.6/10 mM $MgCl_2$/10 mM DTT; EcoRI buffer, 100 mM Tris-HCl, pH 7.4/10 mM $MgCl_2$/150 mM NaCl/2 mM 2-mercaptoethanol; radioimmunoassay (RIA) standard buffer, 50 mM phosphate buffer (pH 7.4) containing 0.25% bovine serum albumin (BSA), 80 mM NaCl and 25 mM ethylenediamine tetra-acetic acid (EDTA).

Chemical synthesis of the α-neo-endorphin gene. Eight oligodeoxyribonucleotides containing α-neo-endorphin gene, labeled F1 through F8 (Fig. 1), were synthesized by the modified triester method (7). The purity and nucleotide sequence of each fragment was confirmed by two-dimentional homochromatography after labeling of the oligomers with [γ-^{32}P] ATP in the presence of T4 polynucleotide kinase (8).

Assembly of α-neo-endorphin gene. 5'-Hydroxyl end of the oligodeoxyribonucleotides, F2, F3, F4, F6, F7 and F8, were individually phosphorylated by T4 polynucleotide kinase. Two hundreds microcurries of [γ-^{32}P] ATP ($\approx$7400 Ci/m mol, Amersham) was incubated with the oligonucleotide (10 μg) and 10 units of T4 polynucleotide kinase in a 60 μl of

kinase buffer at 37°C for 20 min. Then, ATP (10 nmol) and T4 kinase (10 units) were added and the reaction was continued for an additional hour. The kinase was inactivated by heating at 90°C for 5 min.

Phosphorylated fragments, F2, F3, F4, F6, F7 and F8 (2.5 μg each), were combined with 5'-OH fragments, F1 and F5 (2.5 μg each), and the mixture was dialyzed against 1 liter of water overnight at 4°C. To anneal these fragments, the dialyzed mixture was heated at 95°C for 2 min in the ligation buffer without DTT and then slowly cooled to 25°C. Then, ATP and DTT were added to the mixture at a concentration of 0.4 mM and 10 mM, respectively. After the reaction mixture (80 μl) was cooled to 11°C, T4 ligase (25 units) was added and the mixture was incubated at the same temperature for 24 hr. The reaction was terminated by heating for 5 min at 65°C. DNA fragments were precipitated with ethanol and purified by electrophoresis on a 15% polyacrylamide gel. The DNA estimated to be 44 base pairs long was extracted from the sliced gel.

Construction of recombinant plasmids. pBR322 (0.5 μg) was digested with EcoRI (4 units). The digested pBR322 DNA was ligated to 1 μg of EcoRI digested λplac5 DNA with T4 ligase (2 units) in 40 μl of ligation buffer. The ligated DNA was used to transform *E. coli* W3110. The transformants were selected for tetracyclin resistance on the EMB-lactose plate containing the antibiotic (10 μg/ml). A plasmid pKO3 was shown to carry the λplac5 fragment in the desired orientation.

To eliminate the EcoRI site proximal to the *lac* operator on the pKO3 plasmid, the plasmid pKO3 (10 μg) was partially digested with EcoRI (1.6 units) and the large linear DNA fragment was purified on a 0.7% agarose gel. The EcoRI cohesive termini of the fragment were filled with T4 DNA polymerase (1 unit). The DNA was precipitated with ethanol and resuspended in 40 μl of ligation buffer. The T4 DNA ligase (2 units) was then added and the mixture was incubated at 14°C for 20 hr. The ligated DNA was used to transform *E. coli* W3110 and Tc^r transformants were selected on nutrient agar-antibiotic medium. Restriction enzyme analysis revealed that a pKO13 plasmid obtained from one of the Tc^r colonies retained one EcoRI site located between the *lac* and Tc promoters.

To construct the *lac*-α-neo-endorphin plasmid, the synthetic α-neo-endorphin DNA (25 ng) was ligated to the larger EcoRI-BamHI fragment of pKO13 (1 μg) with T4 ligase (2 units) in 30 μl of ligation buffer. The ligation mixture was used to transform *E. coli* WA802.

Radioimmunoassay for α-neo-endorphin. A sample solution was diluted

with RIA standard buffer to 100 μl in an acryl test tube and added to a tube containing 100 μl of ^{125}I-labeled α-neo-endorphin (18000 cpm), 100 μl of diluted rabbit anti-α-neo-endorphin antiserum (9) and 200 μl of RIA standard buffer. The tube was incubated at 4°C for 40 hr. Bound and free ligands were than separated as follows. After 100 μl of 1% solution of γ-globulin (bovine) in RIA standard buffer was added to each tube, 1 ml of 25% polyethyleneglycol #6000 was introduced. The tubes were centrifuged at 3000 rpm at 4°C for 15 min. The supernatant was removed by aspiration and radioactivity in the pellets was counted in a gamma-scintillation counter.

Purification of α-neo-endorphin. A transformant *E. coli* strain, WA802/pαNE2, was grown to late logarithmic phase in a 5 liter of L-broth containing 40 mg of ampicillin per liter. Isopropyl-β-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and the cells were further grown for 2 hr at 37°C. The wet cell paste (10.9 g) obtained from the culture was tested with 80 ml of 70% formic acid containing 1.5 g of cyanogen bromide at room termperature in dark for 24 hr.

The reaction mixture was diluted with water to 5.6 liters and applied on a SP-Sephadex C-25 column (free form). The column was washed with 1 liter of 0.1 M acetic acid, 500 ml of water and 2 liters of 2 M pyridine, and then adsorbed materials were eluted with 1 M ammonia. The final eluate was lyophilized and the residue was submitted to gel filtration through a column of Sephadex G-25 after being dissolved in 15 ml of 1 M acetic acid. Each fraction from the Sephadex G-25 chromatography was assayed for α-neo-endorphin by RIA. The immunoreactive fractions thus obtained were collected and further chromatographed through a CM-cellulose CM-52 column. The immunoreactive fractions from the CM-cellulose chromatography were purified by high performance liquid chromatography (HPLC) using a μ-Bondapak C-18 column (reverse phase, Waters Associate) which was pre-calibrated with various standard peptides.

Peptide analysis. Peptide (4 μg) was hydrolyzed with 6 N HCl containing 0.1% phenol and 0.02% 2-mercaptoethanol for 20 hr at 110°C and then analyzed on an amino acid analyzer (Hitachi #835). Sequence analysis of the peptide was performed by dansyl-Edman method (10). The dansylated amino acids, after hydrolysis, were identified on a polyamide sheet.

RESULTS AND DISCUSSION

Chemical synthesis and assembly of α-neo-endorphin gene. The α-neo-endorphin gene was designed according to the idea that was used for the somatostatin gene (3). Figure 1 shows the amino acid sequence of α-neo-endorphin and the nucleotide sequence designed for the peptide. This gene was fused to *E. coli* β-galactosidase gene on plasmid pKO13. Transformation of *E. coli* with the chimeric plasmid DNA will lead to the synthesis of hybrid polypeptide, including the sequence of amino acids corresponding to α-neo-endorphin. Because α-neo-endorphin has no methionine residue in the amino acid sequence, the α-neo-endorphin can be obtained by cyanogen bromide cleavage of the precursor. The α-neo-endorphin gene was divided into eight oligodeoxyribonucleotide fragments, labeled F1 through F8, and they were synthesized by the improved phosphotriester method (7). The eight oligonucleotide fragments were assembled by ligation with T4 ligase as described in Materials and Methods. During the above procedure, F1 and F5 fragments were not phosphorylated to eliminate unfavorable polymerization of these fragments through their cohesive ends (EcoRI and BamHI). The desired α-neo-endorphin gene was isolated from preparative polyacrylamide gel electrophoresis.

Construction of plasmid for expression of the α-neo-endorphin gene. Figure 2 illustrates outlines of construction of the expression plasmid carrying the α-neo-endorphin gene. Plasmid pBR322 was digested with EcoRI endonuclease and then ligated by T4 ligase in the presence of the EcoRI-cleaved fragment of λ*plac5*, which contained *lac* promoter region and the majority of the β-galactosidase structural gene (11). The ligated plasmid DNA was used to transform *E. coli* W3110. The *lac*-EcoRI fragment from λ*plac5* can be inserted into pBR322 plasmid in two orientations; thus, only half of the clones obtained after transformation should have the desired orientation. The orientation of *lac* fragment on the plasmids obtained from six tetracycline-resistant and β-galactosidase constitutive clones was checked by restriction analysis (11). Three of the six clones tested were shown to carry the fragment in the desired orientation, that is, *lac* transcription going into the Tc^r gene of the plasmid. To eliminate the EcoRI site proximal to the *lac* operator, one of the plasmids, pKO3, was further modified as shown in Fig. 2 and plasmid pKO13 was obtained. This plasmid was used for transformation of *E. coli* WA802 and cloning the synthetic α-neo-endorphin gene.

Plasmid pKO13 was digested with EcoRI and BamHI endonucleases and

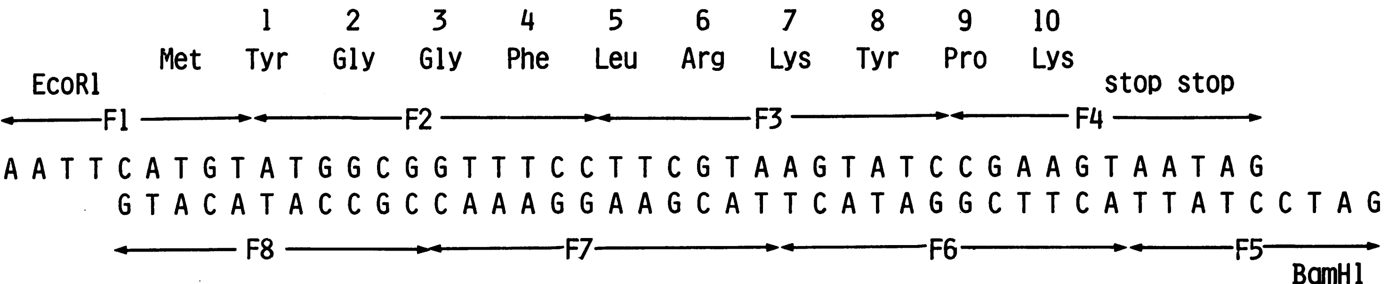

Figure 1. Design of a gene for α-neo-endorphin. The gene for α-neo-endorphin was designed from the amino acid sequence of the porcine peptide (2). The 5' ends of the gene have single stranded cohesive termini for the EcoRI and BamHI restriction endonucleases to insert correctly the gene into plasmid pKO13 (Fig. 2). Eight oligodeoxyribonucleotide fragments, labeled F1 through F8, were synthesized by the modified triester method (7).

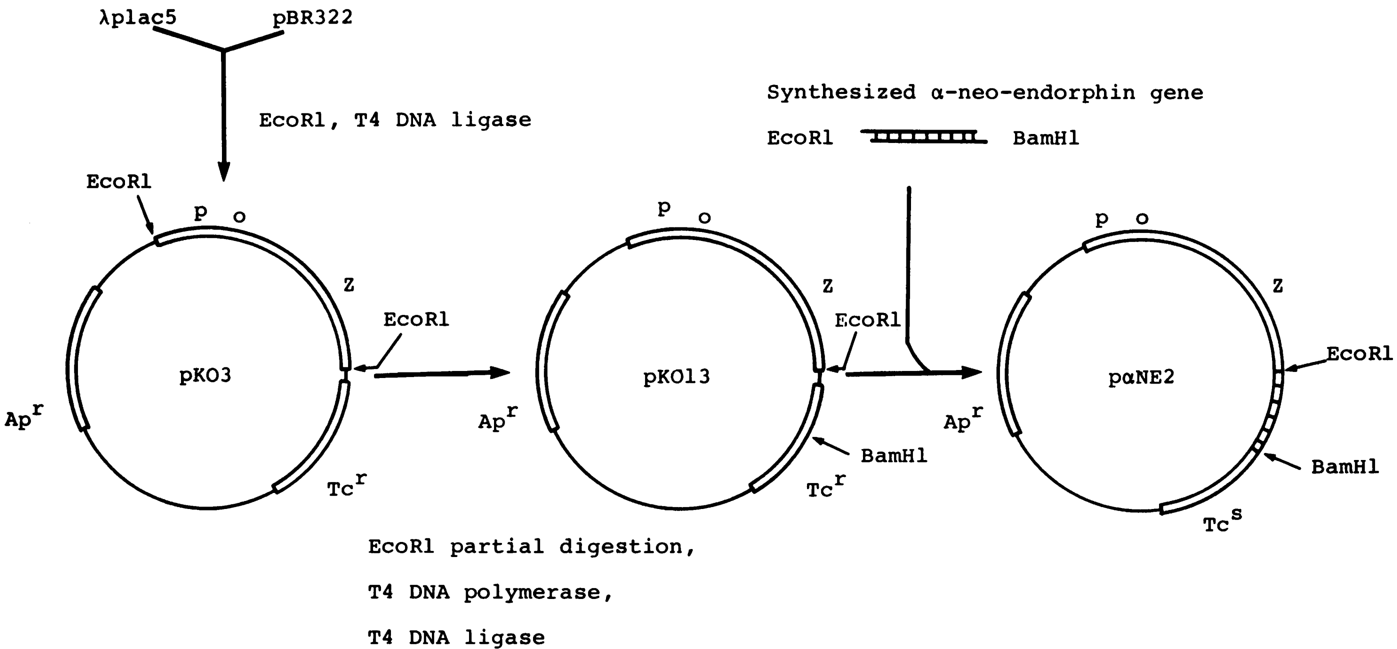

Figure 2. Construction of recombinant plasmids. See Materials and Methods.

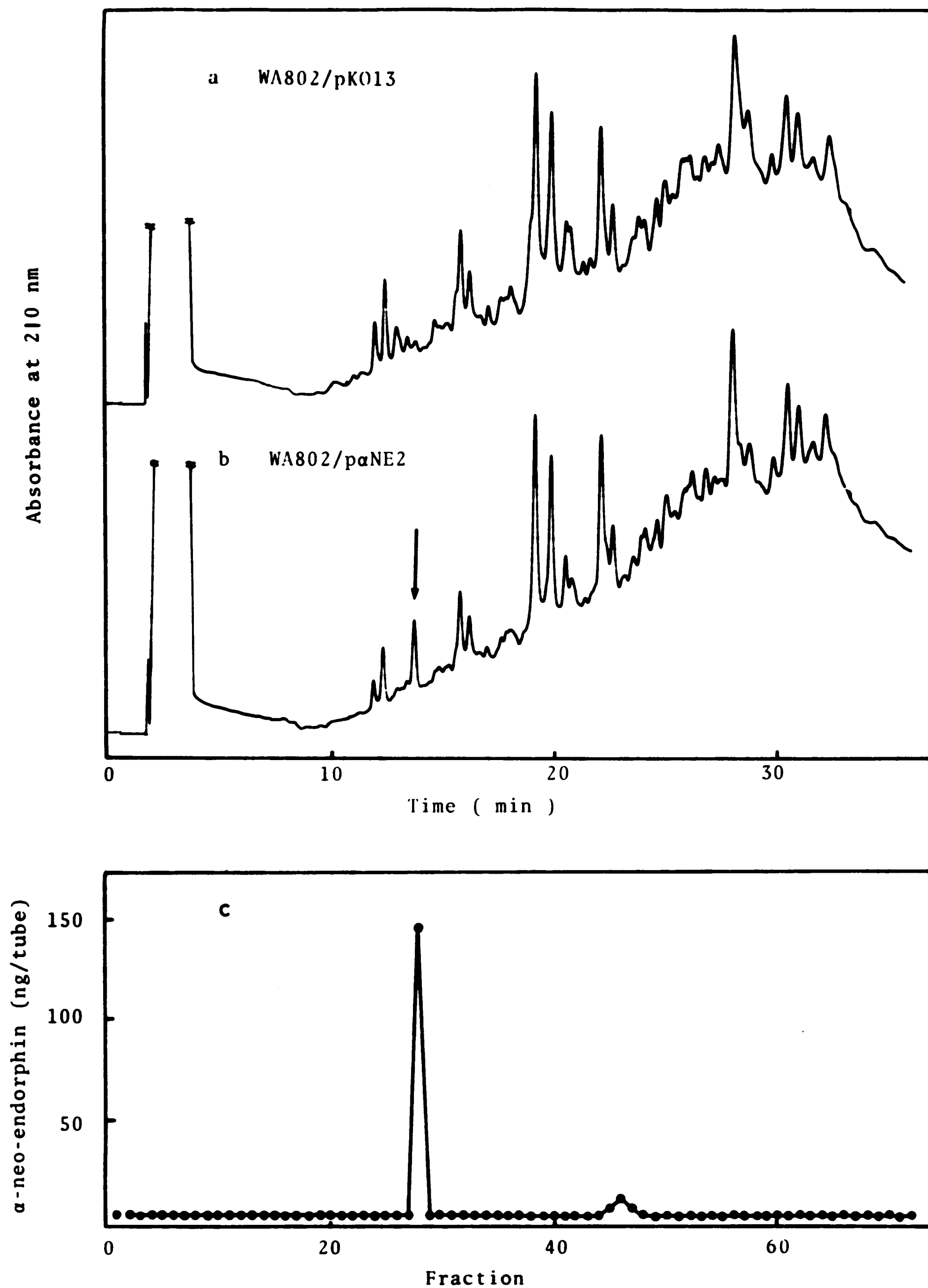

Figure 3. Analysis of α-neo-endorphin expression by HPLC and RIA. *E. coli* strains, WA802/pKO13 and WA802/pαNE2, were grown at 37°C to approximately 4 x 10^8 cells/ml in 2 ml of L-broth

containing ampicillin (40 μg/ml). IPTG was then added at a concentration of 1 mM and growth was continued for 2 hours. A portion (1 ml) was centrifuged and the pellet was suspended in 500 μl of 70% of formic acid containing CNBr (5 mg/ml). After standing for 24 hours at room temperature, the suspension was lyophilized and the residue was dissolved in 1 M acetic acid (200 μl). A portion (150 μl) was loaded on a column of SEP-pak C18 (Waters Associate). The column was washed with 1 M acetic acid (2 ml) and water (2 ml). Adsorbed materials were eluted from the column with 2.5 ml of 10 mM $HCOONH_4/CH_3CN$ (50:50 vol/vol) and evaporated. The residues were dissolved in 1 M acetic acid (200 μl). A portion (100 μl) of the solution was subjected to HPLC on a column (0.4 x 25 cm) of μ-Bondapak C-18 (Waters Associate). The elution buffer was 50 mM KH_2PO_4 (pH 2.0) with an acetonitrile gradient of 10 to 50%. Fractions of 1 ml were collected. Solid line, A_{210}; the position of the authentic α-neo-endorphin was indicated by an arrow in Fig. 3a and 3b.
Amounts of α-neo-endorphin per fraction in Fig. 3b were measured by RIA in Fig. 3c.

the large fragment was purified by agarose gel electrophoresis. The purified fragment was ligated with T4 ligase in the presence of the synthetic α-neo-endorphin DNA. This ligated DNA was used to transform *E. coli* WA802. One hundred and ten ampicillin-resistant transformants were screened for tetracycline sensitivity and 12 (WA802/pαNE1 to WA802/pαNE12) of them were examined for the presence of the α-neo-endorphin DNA of the proper size fragment, that is, these plasmids derived from pKO13 carried the fused genes of β-galactosidase and synthetic α-neo-endorphin. One of the plasmids, pαNE2, was digested with EcoRI and BamHI and the small fragment obtained from the plasmid was subjected to nucleotide sequence analysis by Maxam and Gilbert method (12). The result indicated that plasmid pαNE2 contained a complete nucleotide sequence of the synthetic α-neo-endorphin gene attached to the EcoRI and BamHI site of plasmid pKO13 (data not shown).

Expression of synthetic α-neo-endorphin gene. *E. coli* strains, WA802/pKO13 and WA802/pαNE2, were examined for expression of α-neo-endorphin gene. Both strains were cultivated in 2 ml of L-broth at 37°C and the β-galactosidase was induced by IPTG. Cells from 1 ml of the culture were treated with 70% formic acid containing cyanogen bromide. Without further purification steps, the CNBr-cleaved peptides were analyzed by HPLC on a reverse-phase C18 column. It should be noted that the HPLC elution profile obtained from the cells containing plasmid pαNE2 demonstrated a clearly detectable peak corresponding to α-neo-endorphin, which was absent in the case of WA802/pKO13 cells (Fig. 3a and 3b).

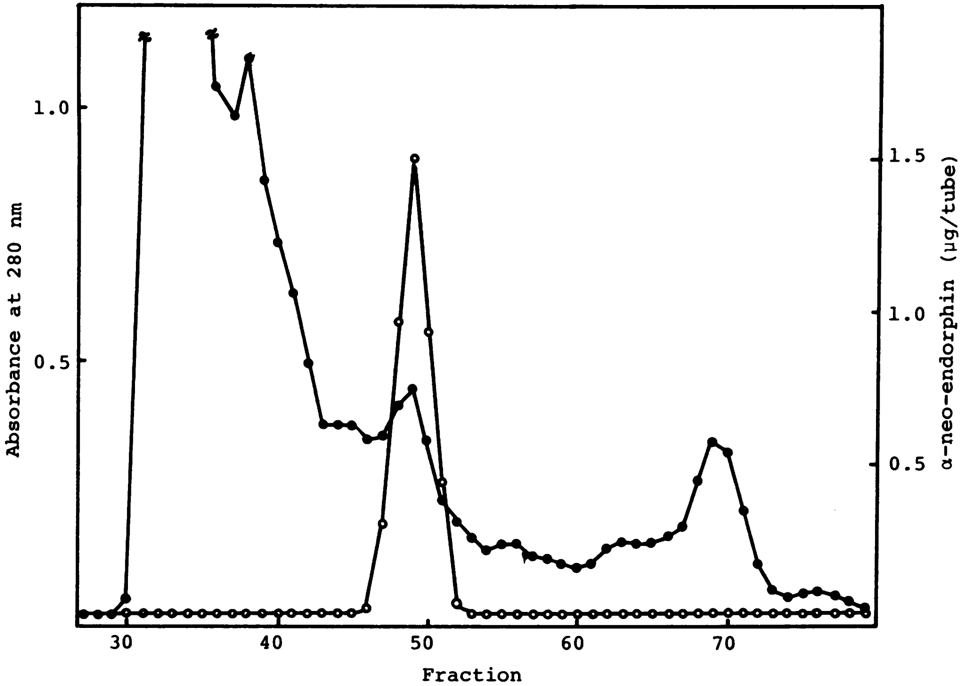

Figure 4. Gel filtration on Sephadex G-25 of the 1 M ammonia eluate from SP-Sephadex C-25. Basic peptides (214 mg) recovered from the CNBr-treated E. coli WA802/pαNE2 cells by SP-Sephadex C-25 treatment was lyophilized and dissolved in 15 ml of 1 M acetic acid. The solution was applied to a column (3 x 151 cm) of Sephadex G-25 equilibrated with 1 M acetic acid. Fractions of 15 ml were collected. •——•, A_{280}; o——o, amounts of α-neo-endorphin per tube measured by RIA.

RIA for α-neo-endorphin on each fraction of the HPLC clearly showed that the peak indicated by the arrow in Fig. 3b was that of α-neo-endorphin. This was also verified by the fact that chemically synthesized α-neo-endorphin was eluted at the same position as that peak (data not shown). The results of HPLC and RIA suggested that the hybrid β-galactosidase/α-neo-endorphin gene was expressed quite efficiently. No α-neo-endorphin immunoreactivity was observed in CNBr-treated WA802/pKO13 cells. The minor peak on the chromatogram (Fig. 3c) seems to be incomplete cleavage products of the β-galactosidase and α-neo-endorphin fused protein with cyanogen bromide.

Stability of plasmid pαNE2 was tested as follows. E. coli strain WA802/pαNE2 was grown in L-broth without ampicillin at 37°C for 24 hr and

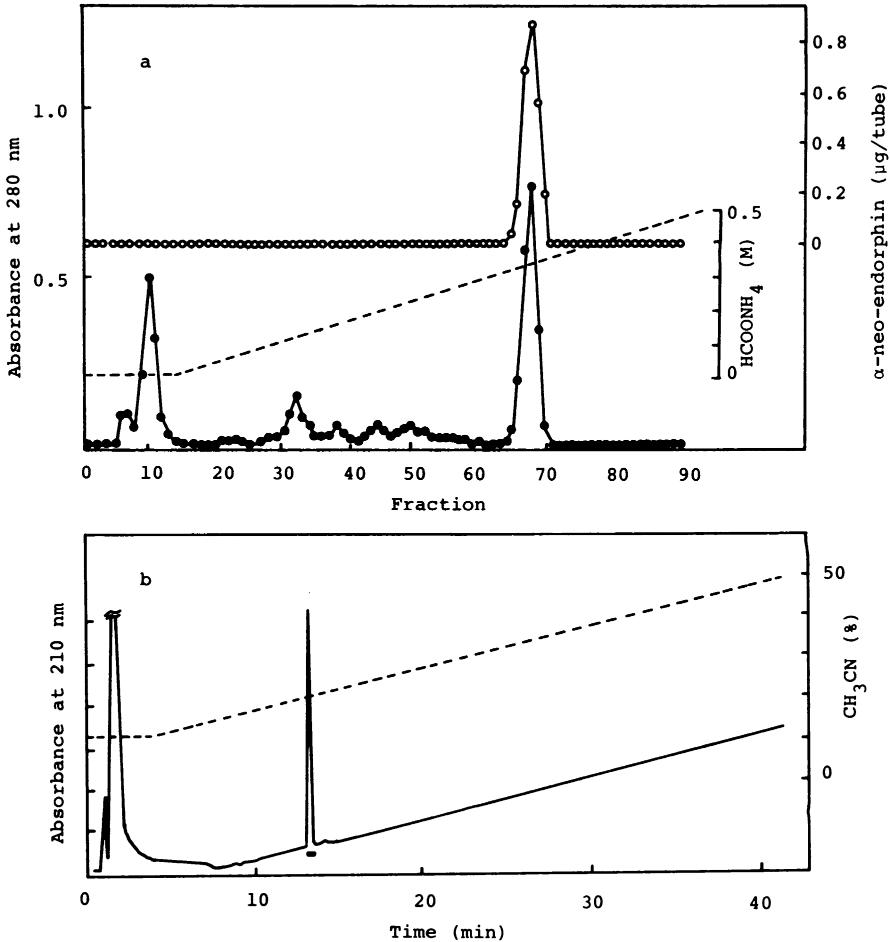

Figure 5. Purification by CM-cellulose chromatography and reverse phase HPLC. Fig. 5a, Fractions 47 to 51 in Fig. 4 were pooled, lyophilized and dissolved in 8 ml of 10 mM $HCOONH_4$. The sample was applied to a 0.9 x 48 cm column of CM-cellulose CM-52 and eluted with 800 ml linear gradient of 10 to 500 mM $HCOONH_4$. Fractions of 5 ml were collected. •——•, A_{280}; o——o, amounts of α-neo-endorphin per tube measured by RIA; -----, expected concentration of $HCOONH_4$.
Fig. 5b, Reverse phase HPLC analysis of the samples purified by CM-cellulose. An aliquot (20 μl) of the immunoreactive fractions (66 to 69 in Fig. 5a) was diluted with 180 μl of 1 M acetic acid. A portion (40 μl) was analyzed by HPLC on a column (0.4 x 25 cm) of reverse phase C18. The elution buffer was 50 mM KH_2PO_4 (pH 2.0) with an acetonitrile gradient of 10 to 50%. Solid line, A_{210}; Black bar, fractions α-neo-endorphin immunoreactivity;-----, expected concentration of CH_3CN.

the cells were plated on nutrient agar 1.5% (BBL). The colonies formed on the plates after 24 hr incubation at 37°C were replicated on nutrient agar plates with or without ampicillin (40 μg/ml) and the plates were incubated at 37°C for 24 hr. Ninety-five percent of the colonies tested showed ampicillin resistance.

Purification and characrerization of α-neo-endorphin from E. coli. Alfa-neo-endorphin was isolated from a 5 liter culture of WA802/pαNE2, as described in Materials and Methods. The following procedure was based on an earlier expreience with the purification of α-neo-endorphin from porcine hypothalamus (1) and on the known properties of the α-neo-endorphin (2). Cyanogen bromide-treated cells were applied on a SP-Sephadex C-25 column (free form) and the 1 M ammonia eluate was sumbitted to gel filtration through a Sephadex G-25 (Fig. 4). Each fraction from the Sephadex G-25 column was assayed for α-neo-endorphin immunoreactivity. The fractions showing the immunoreactivity were pooled and further chromatographed on a CM-cellulose column (Fig. 5a). As shown in Fig. 5a, efficient purification was obtained at this step, where a sigle immunoreactive material was well separated from the other meterials. This was verified by followings. An aliquot of the pooled fractions (66 to 69 in Fig. 5a) was analyzed by HPLC on a reverse-phase C18 column (Fig. 5b). A single main peak was observed at the position exactly identical with that of authentic α-neo-endorphin, although a negligiblly minor peak was found behind the main peak. Immunoreactivity of α-neo-endorphin was located only in the main peak.

The immunoreactive portion (Fig. 5b) was subjected to amino acid analysis. The composition was found to be Pro 1.0, Gly 2.1, Leu 1.0, Tyr 2.0, Phe 1.0, Lys 2.1, Arg 1.0. Amino acid sequence was determined by dansyl-Edman method (10) to be Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys. Thus, the purified peptide from E. coli was verified to be exactly identical with native α-neo-endorphin (2). As summarized in Table 1, our purification method of α-neo-endorphin from E. coli WA802/pαNE2 was simple and efficient. By three-step purification (SP-Sephadex chromatography, Sephadex G-25 gel filtration and CM-cellulose chromatography), we could isolated 4 mg of chemically pure α-neo-endorphin from 10.9 g of wet cells. The isolation yield of α-neo-endorphin from initial content (6.6 mg) was 59%. From these data, it is calculated that a single cell could produce approximately 5×10^5 molecules of the decapeptide. This yield is about 20 times higher than that reported for somatostatin (4) and

Table 1. Purification of α-neo-endorphin from E. coli WA802/pαNE2

	Total protein (mg)*	α-neo-endorphin content (mg)†	Yield %
1. CNBr treatment	1271	6.6	100
2. SP-Sephadex chromatography	214	6.5	98
3. Sephadex G-25 chromatography	11	4.9	74
4. CM-cellulose chromatography	4	3.9	59

10.9 g of wet cells were obtained from a 5 liter culture of E. coli strain WA802/pαNE2. 1271 mg of total protein extracted from the cells which were treated with cyanogen bromide was used for purification of α-neo-endorphin.

* calculated from amino acid analysis data.
† calculated from radioimmunoassay.

2 times higher than that of insulin (5).

Finally, we tested the opiate activity of α-neo-endorphin thus purified by using the guinea pig ileum assay (13). The IC_{50} (concentration of half maximal inhibition) of α-neo-endorphin purified from the E. coli was 5.2 nM whereas that of authentic sample was 5.6 nM. The opiate activity of the test material was also found to be completely blocked by the opiate antagonist naloxone.

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