High-level Production of Human β -Endorphin in *Escherichia coli*Kenji NAGAHARI, Tadashi HISHIDA, Tamotsu NIKI,
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A human opioid peptide hormone, β -endorphin, was expressed in *Escherichia coli* as the C-terminal portion of a fused protein. The fused protein consisting of the N-terminal 62% of *E. coli* anthranilate synthetase (323 amino acids), a peptide corresponding to the linker DNA (7 amino acids), and the precursor form of β -endorphin (47 amino acids), was highly expressed under the control of a tryptophan promoter. The level of expression of β -endorphin was estimated to be 17 mg/liter broth by radioimmunoassay, and 51 mg/liter broth by SDS polyacrylamide gel electrophoresis followed by a densitometric analysis. β -Endorphin was cleaved from the fused protein and purified by HPLC, and is presumed to be identical with human β -endorphin because of its amino acid composition and amino acid sequence.

β -Endorphin (β -EP) is a peptide hormone consisting of 31 amino acid residues. Human β -EP, isolated from the pituitary, has significant analgesic activity and also various pharmacological activities including induction of grooming behavior, catalepsy, and hypothermia.¹⁾ In addition to these effects, Gilman *et al.* reported effects of β -EP on a lymphocyte function.²⁾ β -EP is synthesized from the opioid precursor called pro-opiomelanocortin (POMC). From the study of bovine cDNA for POMC, it was shown that seven active peptides, including the β -EP, adrenocorticotrophic hormone (ACTH), and α -, β -, and γ -melanocyte stimulating hormones (α -, β -, and γ -MSH's) are coded sequentially in one mRNA.³⁾ A human gene for POMC was cloned⁴⁾ and shown to have a structural relationship with that of bovine.

Using recombinant DNA techniques, many pharmacologically important proteins have been produced in *Escherichia coli* but relatively small peptides cannot be produced directly. They are made in a form of a fused protein with a protein of the host cell.⁵⁾ Small peptides can not be produced effectively because of their sensitivity to the proteolytic activity of

the host cell.

Recently, Shine *et al.* reported the expression of mouse β -EP which was fused to an *E. coli* β -galactosidase and the successful *in vitro* cleavage of β -EP from the fused protein.⁶⁾ We report here the high expression of human β -EP in the form of a fused protein (TrpE β EP) with *E. coli* anthranilate synthetase (TrpE) and the purification of β -EP from the fused protein.

MATERIALS AND METHODS

Enzymes and reagents. Restriction enzymes were obtained from Takara Shuzo Co., Ltd. except when otherwise noted. T4 DNA polymerase was purchased from Bethesda Research Laboratories, Inc., ClaI restriction enzyme and RNA polymerase were obtained from Boehringer Mannheim GmbH. Trypsin was purchased from Worthington Diagnostic Systems, Inc., and the radioimmunoassay kit for β -EP was from New England Nuclear.

Plasmids; bacteria, and media. RSF2124-*trp* plasmid⁷⁾ containing the whole *E. coli* tryptophan operon was used as a source of DNA for the *trp* promoter and a *trpE* (anthranilate synthetase) gene. pYT3-24 plasmid, a derivative of a plasmid containing human ACTH- β -LPH precursor gene,⁴⁾ was constructed by Teranishi (unpub-

lished) and was used for the β -EP gene. *E. coli* RR1 was used as the host cell of the constructed plasmid. M13mp7 phage and its host, *E. coli* JM103 were obtained from Bethesda Research Laboratories, Inc. M9 glucose minimal medium with 0.2% casamino acid was used to grow cells for the expression of β -EP and for the isolation of plasmid DNA. L-plate containing (per liter) 10 g of Bactotryptone, 5 g of Bactoyeast extract, and 10 g of NaCl was used for the selection of the transformants.

Gel electrophoresis. Agarose and acrylamide gel electrophoresis for DNA were done in TBE buffer (90 mM Tris base, 2.5 mM EDTA, 90 mM H_2BO_3). SDS polyacrylamide gel electrophoresis (SDS PAGE) and SDS PAGE containing 7 M urea were done by the method of Laemmli,⁸⁾ and Swank and Munkres,⁹⁾ respectively. To localize the protein bands which can be detected by radioimmunoassay (RIA) for β -EP, the gel was sliced and each slice was extracted with 0.05 M NH_4HCO_3 containing 0.05% SDS and then tested for RIA.

Purification of the *TrpE* β EP fused protein. The cell pellet from 1 liter of culture was suspended in 25 ml of sample buffer with a high concentration of SDS (100 mM Tris-HCl pH 6.8, 10% glycerol, 0.5 M β -mercaptoethanol, and 4.8% SDS), and boiled in a water bath for 5 min. Two milliliters of the sample was run in preparative 10% SDS PAGE (1 $\times$ 14 $\times$ 14 cm). The part of the gel containing the fused protein was cut out and the protein was eluted and purified. SDS was removed from the protein by Bio-Rad AG11-A8 resin.

Cleavage of β -EP from the fused protein. Blocking the lysine residue of the fused protein by citraconic anhydride and digestion of the fused protein at the arginine residue by trypsin were done by the method of Seidah *et al.*¹⁰⁾ After lyophilization, the sample was used in SDS PAGE containing 7 M urea or HPLC to purify β -EP.

Peptide purification and its analysis. Reverse-phase HPLC on a column of RP-300 (4.6 $\times$ 250 mm; Brownlee labs) was used for the purification of the peptide. The amino acid composition was analyzed by a Durrum Analyzer. The N-terminal amino acid was identified by the dansyl method. The amino acid sequence was analyzed by stepwise Edman degradation, using the LKB 4020 sequencer.

RESULTS

Construction of the expression vector

The structural gene for *E. coli trpE* which codes for anthranilate synthetase is 1560 base pairs (bp) long and has two *Bgl*II sites at positions 965 and 1026 (A of the ATG for f-Met of *TrpE* being 1).¹¹⁾ RSF2124-*trp* plas-

mid⁷⁾ consisting of *E. coli* plasmid RSF2124, the whole *E. coli* tryptophan operon, and λP_L and *N* genes of λ phage yields a 2.3 Kb fragment upon digestion with *Bgl*II. This fragment was proved to have the λP_L promoter, *N* gene of λ phage, the *E. coli trp* promoter (P_T), and part of the *trpE* gene (Fig. 1). To construct an expression vector which has an *Eco*RI cloning site inside the *trpE* gene, the 2.3 Kb *Bgl*II fragment described above was inserted into the *Bam*HI sites of M13mp7 replicative form. M13mp7 hybrid plasmid with the 2.3 Kb fragment was then digested with *Eco*RI to yield the 2.3 Kb fragment with *Eco*RI cohesive ends at both termini. This is possible because two *Eco*RI sites are at the outer sides with regard to two *Bam*HI sites in the multi-cloning site of M13mp7.¹²⁾ Purified 2.3 Kb *Eco*RI fragment was then inserted into the *Eco*RI site of pBR322. A pBR322 derivative (pTrp100) with the *Eco*RI fragment in the desired orientation was selected and isolated (Fig. 1). To remove one of the two *Eco*RI sites that was upstream from the *trp* promoter, pTrp100 was partially digested with *Eco*RI and the DNA fragment with a unit length was purified by agarose gel. The *Eco*RI site of the fragment was then filled in by T4 DNA polymerase and blunt end ligated by T4 DNA ligase. *E. coli* RR1 was transformed by the ligated DNA sample and plasmid pTrp110 was isolated. As shown in Fig. 1, pTrp110 has one *Eco*RI site.

Isolation of an *Eco*RI fragment containing a gene for human β -EP

A 160 bp *Hae*III fragment carrying a human β -EP gene derived from plasmid pYT3-24 was purified by acrylamide gel electrophoresis, then inserted into the *Hinc*II sites of M13mp7. A 190 bp *Eco*RI fragment containing the β -EP gene was isolated from a constructed M13mp7 hybrid plasmid and then inserted into the *Eco*RI site of pTrp110.

After transformation by the ligated DNA sample and analysis by miniscreening, several transformants were found to have a plasmid carrying the 190 bp *Eco*RI fragment in the correct orientation (Fig. 1). This plasmid was

FIG. 1. Construction of an Expression Vector for the TrpE β EP Fused Protein.

P_L and P_T represent the promoters of λP_L and the tryptophan operon, respectively. The P_T promoter and the direction of transcription are indicated by the solid thick arrow.

The DNA region coding for TrpE, the precursor portion of POMC and the linker are also indicated by $\square$ and β -EP by $\blacksquare$.

E represents the restriction site for *EcoRI*.

named pTrp110 β EP. In the course of constructing pTrp110 β EP, a deletion in the linker DNA occurred. The DNA sequence at the junction between the *trpE* and β -EP genes is shown in Fig. 2. The reason for this deletion is not known.

Removal of the λP_L promoter from the pTrp110 β EP plasmid

pTrp110 β EP in *E. coli* RR1 was unstable (data not shown), probably because of constitutive transcription from the λP_L promoter on the plasmid. Therefore, we tried to remove

the λP_L promoter from the pTrp110 β EP plasmid.

pTrp110 β EP has two *HpaI* sites, one in the *trp* promoter and the other in the *N* gene. In an *in vitro* experiment, RNA polymerase bound to the promoter region protects DNA from nuclease attack. Therefore, *HpaI* digestion was done in the presence of *E. coli* RNA polymerase and then the DNA was digested with *SalI*. A 2.43 Kb *HpaI*, *SalI* fragment purified from the gel and a vector fragment produced by sequential treatment of pBR322 with *EcoRI*, T4 DNA polymerase and *SalI* were

FIG. 2. Deletion of the λP_L Promoter from the pTrp110 β EP Plasmid.

H, E, and S represent the restriction sites for *Hpa*I, *Eco*RI, and *Sal*I, respectively. The DNA sequence and its corresponding amino acid between the *trpE* and the POMC gene are also shown.

ligated by T4 DNA ligase and introduced into *E. coli* RR1. A plasmid pTrp β EP, was isolated from a transformant and used for further study (Fig. 2).

Expression of *TrpEβEP* fused protein

E. coli RR1 (pTrp β EP) was grown overnight in L-broth, and 1 ml of overnight culture was inoculated into 50 ml of M9 minimal medium containing 0.2% casamino acids. When the cell density of the medium reached

FIG. 3. Expression of the *TrpEβEP* Fused Protein in Cells Carrying pTrp β EP.

Cells were cultured with (+IAA) or without (−IAA) a *trp* promoter inducer, indoleacrylic acid, and the cells were subsequently removed at the time after induction indicated above the gel. Proteins were electrophoresed on an SDS PAGE, and stained with Coomassie brilliant blue.

FIG. 4. RIA for β -EP Eluted from the Gel.

Protein marker were also run and the molecular weights are indicated below the gel.

to 30 Klett units, indoleacrylic acid (IAA: $20\mu\text{g}/\text{ml}$) was added to induce the *trp* promoter. Cells in 1 ml of culture were collected by centrifugation, suspended in $200\mu\text{l}$ of sample buffer (100 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, and 0.5 M β -mercaptoethanol), and then boiled for 5 min. Five to $15\mu\text{l}$ of the sample (depending on the cell density) were electrophoresed in 10% SDS polyacrylamide gel electrophoresis. Figure 3 shows that the density of the protein band corresponding to 39 K molecular weight depends on the culture time. Five hours after the addition of IAA, the amount of 39 K protein

was estimated to be 23.5% of the total cellular protein by densitometric analysis (data not shown). With the addition of $100\mu\text{g}/\text{ml}$ of tryptophan in the medium, the 39 K band in the gel disappeared (data not shown). Figure 4 shows that a 39 K protein has β -EP activity in RIA. β -EP activity in the smaller protein molecule would be the result of the degradation of the 39 K protein. The same sample which was electrophoresed in SDS PAGE was also analyzed by RIA. The amount of β -EP calculated from the RIA was 17 mg (equivalent to β -EP)/liter broth.

FIG. 5. HPLC Profile of the Tryptic Peptides Derived from the TrpE β EP Fused Protein.

Purification of the TrpE β EP fused protein and its tryptic digestion

Purification of the TrpE β EP fused protein and removal of SDS were done as described in MATERIALS AND METHODS. TrpE β EP fused protein was citraconated and digested by trypsin. After decitraconylation by adding formic acid, samples were injected into an HPLC column. Peptides were separated by size as shown in Fig. 5. The location of the peak indicated by an arrow corresponded to that of authentic β -EP. The fractionated peptide corresponding to this peak has an activity of β -EP in RIA, and was used for further study.

Peptide analysis

The N-terminal amino acid of the peptide described above was tyrosine like that of human β -EP (data not shown). The amino acid composition of the peptide reflected the theoretical value (Table I). Partial sequence analysis of the peptide indicated that the N-terminal amino acid sequence was X-Gly-Gly-Phe-Met-Thr-X-X-X-X-X-Thr-Pro-Leu-Val-Thr-Leu-Phe (X represents the missing amino acids). This amino acid sequence is practically identical to the N-terminal portion of human

TABLE I. AMINO ACID COMPOSITION OF β -EP FROM THE TrpE β EP FUSED PROTEIN

Amino acid	Theoretical	Observed
Ala	2	2.0
Asx	2	2.3
Glx	3	3.3
Gly	3	3.1
Ile	2	1.1
Leu	2	2.0
Lys	5	4.5
Met	1	0.3
Phe	2	1.8
Pro	1	1.5
Ser	2	2.0
Thr	3	2.7
Tyr	2	1.8
Val	1	1.5

β -EP.

DISCUSSION

We first tried to express β -EP in a precursor form called pro-opiomelanocortin whose C-terminal part is β -EP. We also tried to express β -EP with additional amino acid residues at the N-terminal side by a combination of various promoters and DNA sequences. But in

both cases, very low levels of expression of β -EP were observed, suggesting that these peptides are very difficult to express or the peptides expressed are very sensitive to proteolytic attack in *E. coli*. Smith *et al.* recently reported the high expression (~ 2 mg/liter) of β -urogastron containing a peptide of 67 amino acid residues.¹³⁾

The fused proteins so far reported, consisting of an *E. coli* protein and a short peptide, were expressed using almost all of the *E. coli* original protein,^{5,6)} but only the N-terminal 62% of *E. coli* anthranilate synthetase was used in this study. As long as the fused protein is stable, the smaller the *E. coli* protein, the more molecules can be expected to be produced. We tried to decrease the number of amino acid residues in part of *E. coli* protein (TrpE) included but we could not obtain a plasmid which yields more fused protein molecules than the level reported here, indicating that the N-terminal 62% portion of *E. coli* anthranilate synthetase (TrpE) is necessary for high expression and stability in *E. coli*.

Denatured as well as native TrpE β EP fused protein can be detected by RIA, suggesting that the β -EP portion is outside of the native TrpE β EP fused protein molecule. However, three times the reactivity was obtained by RIA after cleavage of β -EP from the fused protein (data not shown).

Therefore, 51 mg/liter is a more reliable value for the level of expression of β -EP. This value is in good agreement with that obtained from the SDS PAGE electrophoresis of the

fused protein.

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