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The High Production of Multimeric Angiotensin -converting-enzyme-inhibitor in *E. coli*

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Multimeric angiotensin-converting-enzyme-inhibitor (ACEI) containing a trypsin cleavable linker peptide between ACEI was constructed. We made synthetic DNA coding for the ACEI peptide with asymmetric and complementary cohesive ends of linker nucleotides. A tandemly repeated DNA cassette for the expression of concatameric short peptide multimers was constructed by ligating the basic units. The resultant multimeric peptide expressed as soluble and trypsin treated peptide was shown at the same retention time with chemically synthetic ACEI by HPLC. The present results showed that the technique developed for the production of the ACEI multimers with trypsin cleavable linker peptides can be generally applicable to the production of short peptide.

Key words: Multimeric peptide, High production, Angiotensin-converting-enzyme-inhibitor, *E. coli*

Introduction

The angiotensin-converting enzyme (ACE; peptidyldipeptide hydrolase, EC 3.4.15.1) plays an important physiological role in the regulation of blood pressure. It cleaves angiotensin I to a powerful vasoconstrictor and saltretaining octapeptide, angiotensin II, and inactivates the casodilator and natriluretic nonapeptide, bardykinin (Soffer et al., 1976; Peach et al., 1977). Small molecules with strongly antihypertensive activity are synthesized and used as angiotensin-converting-enzyme-inhibitor (ACEI). Food derived peptide ACEI have recently received attention because of the development of functional foods contributing to homeostasis (Ondetti et al., 1977; Patchett et al., 1980; Ariyoshi et al., 1993). For functional foods or pharmaceutical applications, a large quantity of ACEI needs to be produced economically. Chemical synthesis is not practical because of its high cost and safety issues. Therefore, a biological expression system would be the most cost-effective method for the mass production of the ACEI. In this study, we produced ACEI from the concatameric short peptide multimers that were produced in *E. coli*.

Materials and Methods

Bacterial strains and vectors

E. coli strain XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn10 (Tet^r)]^c) was used as a host for subcloning and *E. coli* BL21 (DE3) (F$^-$ *ompT hsdS*$_B$ ($r_B^- m_B^-$) *gal dcm* (DE3)) was used for gene expression. pGEM4Z (Promega Co.) and pET22b (+) (Novagen Co.) were used as vectors for the multimerization and expression of peptide, respectively.

Oligonucleotides for tandem multimerization of a gene encoding ACEI

For the left adaptor, primer III (5'-CATATGCCC-

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GGTAAACGC-3', *Nde* I site was underlined) and the right adaptor, primer Ⅳ (5'-GGATCCCCGCG-TTTACC-3', *Bam*H I site was underlined) were used, and for ACEI, which was containing of the trypsin cleavable region, primer I (5'-GGTGTTT-ACCCGCACAAAGGTAAACGC-3') and primer II (5'-TTTGTGCGGGTAAACACCGCGTTTACC-3') were used. The detail procedure was described in Fig. 1.

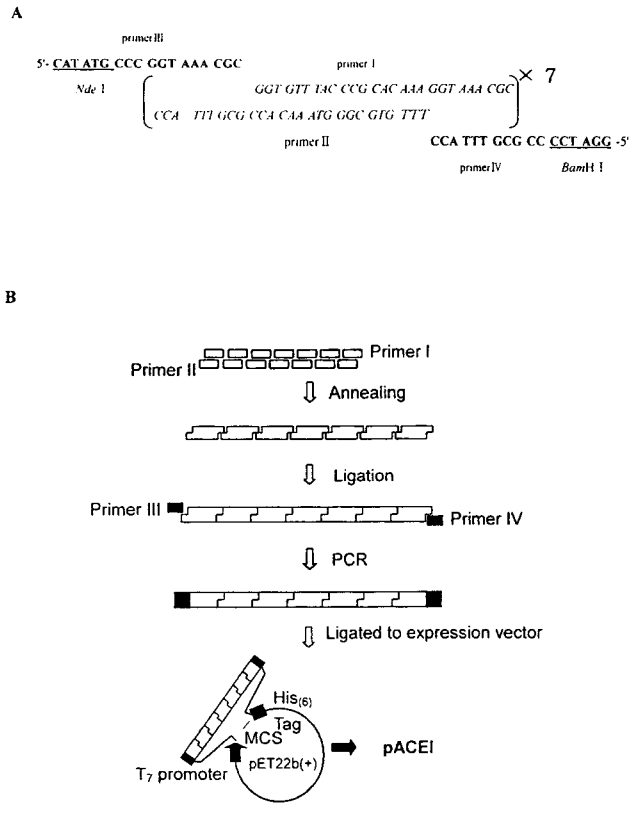

Fig. 1. Schematic representation of the multimerization of the ACEI gene, and construction of the expression vector.
A) Oligonucleotides were synthesized and annealed to make the left and right adaptors synthetic DNAs. B) Construction of the expression plasmids containing of seven copy basic units of the ACEI synthetic DNA. First, a cohesive 3' overhanging ACEI synthetic DNA was ligated and then adaptors synthetic DNAs were made a clonable cassette by PCR. The adaptor cassette mixture was cloned into the *Nde* I /*Bam*H I site of the pET22b (+) and transformed into *E. coli* XL1-blue for screening.

Expression and purification of multimeric ACEI in *E. coli*

The competent cells, XL1-blue and *E. coli* BL21 (DE3), were prepared on the method of Hiroaki et al. The obtained plasmid pACEI was introduced into *E. coli* BL21 (DE3) for overexpression. *E. coli* BL21 (DE3) harboring pACEI was grown at 37℃ in Luria-Bertani (LB) medium supplemented with 50 μg/mL ampicillin. When the cultures reached an optical density (OD_{600}) of 0.5, isopropyl β-D-thiogalactose (IPTG) was added to a final concentration of 1 mM. After 4 hrs, the cells were harvested by centrifugation at 12,000 rpm for 30 min at 4℃ and resuspended in 20 mM Tris-HCl buffer (pH 8.0). After lysated by sonification on ice, the soluble fraction was applied to Ni^{2+}-NTA agarose (Novagen Co.) equilibrated with 50 mM $NiSO_4$, and eluted with 100 mM imidazole. The purified fractions were analyzed by 20% tricine sodium-dodecyl-sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) and cleaved by trypsin (0.1 mg/mL) at 25℃ for 12 hrs. The digested fragments were chromatographed by HPLC on the Capcell pak MG C18 column (10×250 mm, Shiseido, Japan). The separation was performed at a flow rate of 2 mL/min with a linear gradient from 10 to 45% acetonitrile (in 0.1% trifluoroacetic acid) over 35 min. Absorbance was monitored at 220 nm for the detection of peptides.

Results and Discussion

Construction of the pACEI gene and multimerization as concatametric peptides

The synthetic oligonucleotides (primer I, primer II), which were synthesized on the basis of the reported amino sequence of Bonito ACEI, were hybridized with asymmetric cohesive ends. The hybrid DNA fragment codes for ACEI which was sourced of the Bonito (GVYPHK) (Rubin et al., 1978) and contains trypsin cleavable linker peptide (GKR).

The basic unit of the 3' overhanging cohesive end of trypsin cleaved linker synthetic DNA was ligated with T4 DNA ligase to make a tandem repeated ACEI and trypsin cleaved linker synthetic DNA. The oligonucleotides (primer Ⅲ, primer Ⅳ) were synthesized and annealed to make for the left and

right adaptors. The adaptors were added to the opposite side of a tandem repeated ACEI and trypsin cleaved linker synthetic DNA. Resultant DNA cassette was amplified by PCR with primer Ⅲ and primer Ⅳ. Then PCR product (Fig. 2) was ligated with the *Sma* I site of pGEM4Z and isolated with restriction endonucleases *Nde* I and *Bam*H I, and the ACEI fragment was transferred into the *Nde* I and *Bam*H I site of pET22b (+) vector to generate pACEI. This expression vector was confirmed 7 copy basic units of ACEI and 282 bp as an open reading frame was identified by nucleotide sequencing.

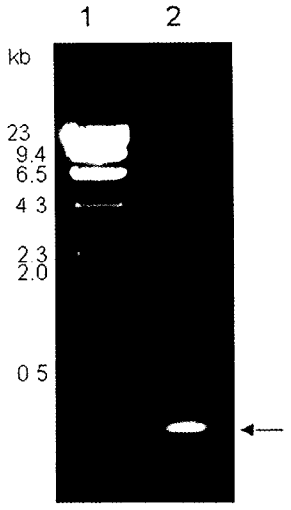

Fig. 2. Agarose gel electrophoresis of PCR products by primer III and primer IV.
Lane 1. Molecular marker; Lane 2. ACEI (224 bp, 7 copy).

Expression and cleavage of the ACEI gene as tandem repeats

To examine the expression of the ACEI multimer, the *E. coli* BL21 (DE3) harboring pACEI was grown in the LB medium. The expression of ACEI multimers were induced by adding IPTG to final concentration of 1 mM. After 4 hrs, the cells were harvested and lysated by sonication. After lysis of host cell, purification was performed as described in materials and methods. The purified recombinant ACEI multimers were analyzed on 20% tricine SDS-PAGE. The expressed protein having a molecular mass of 14 kDa (Fig. 3). The expressed

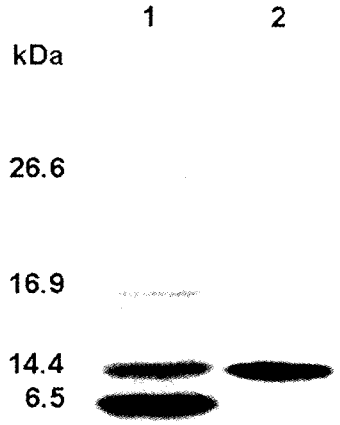

Fig. 3. SDS-PAGE analysis of multimerized ACEI.
The tandem multimers were purified by Ni^{2+}-NTA affinity chromatography and then analyzed by 20% tricine SDS-PAGE. Lane 1. polypeptide marker; Lane 2. purified tandem multimer.

multimers were digested with trypsin and analyzed by reverse phase HPLC with Capcell pak MG C18. The chromatogram showed the same retention time with chemically synthetic Bonito ACEI (Fig. 4).

Production of peptide in *E. coli* was limited by several disadvantage. Post translational modification, such as amidation and pyroglutamic acid formation, was not achieved in *E. coli*. By contrast, this ACEI from Bonito has not any modification in their sequence, and has a lower IC_{50} value than any other ACEI (Yamamoto et al., 1997). This study is one of the most simple and effective methods for the production of short peptide multimers by cloning the constructed basic unit of the short synthetic DNA repetitively. The method can be easily applied for producing many short peptides for laboratory research and industrial purposes.

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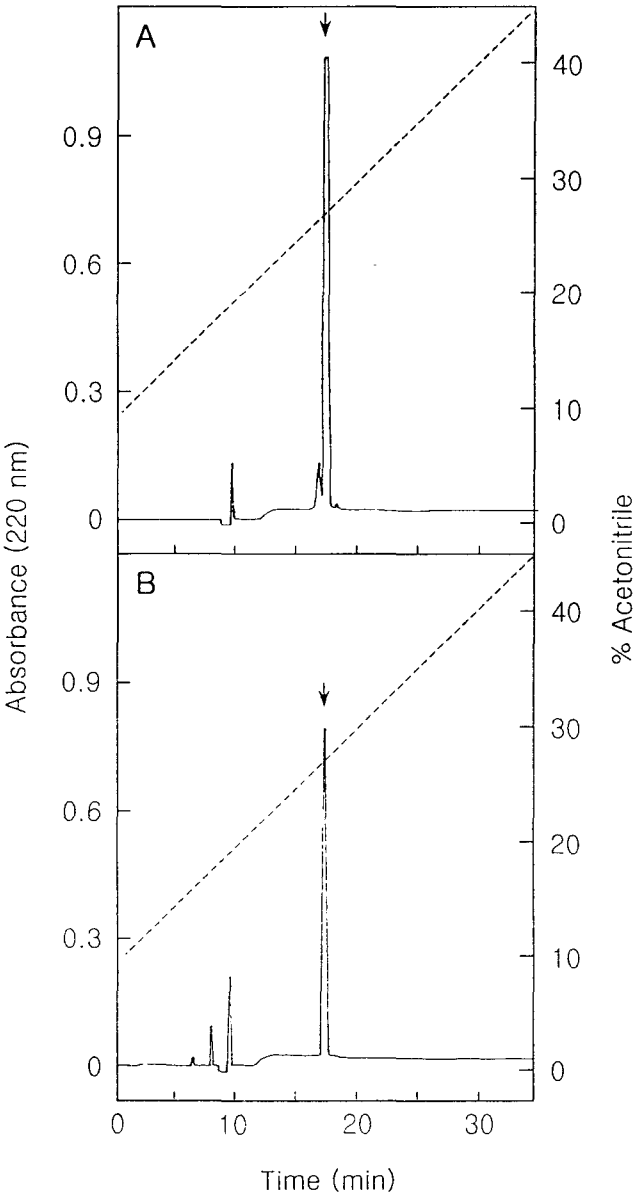

Fig. 4. Reverse-phase HPLC chromatogram of ACEI at the same condition.
A) The chemically synthetic Bonito ACEI
B) The purified ACEI from transformant *E. coli* BL21 (DE3) harboring pACEI.

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