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Expression of Antihypertensive Peptide, His-His-Leu, as Tandem Repeats in *Escherichia coli*

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Abstract His-His-Leu (HHL), a tripeptide derived from a Korean soybean paste, is an angiotensin-I-converting enzyme (ACE) inhibitor. We report here a method of producing this tripeptide efficiently by expressing tandem multimers of the codons encoding the peptide in *E. coli* and purifying the HHL after hydrolysis of the peptide multimers. The HHL gene, tandemly multimerized to a 40-mer, was ligated with ubiquitin as a fusion gene (UH40). UH40 was inserted into vector pET29b; the UH40 fusion protein was then produced in *E. coli* BL21. The recombinant UH40 protein was purified by cation-exchange chromatography with a yield of 17.3 mg/l and analyzed by matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) mass spectrometry and protein N-terminal sequencing. Leucine aminopeptidase was used to cleave a 405-Da HHL monomer from the UH40 fusion protein and the peptide was purified using reverse-phase high-performance liquid chromatography (HPLC) on a C18 HPLC column, with a final yield of 6.2 mg/l. The resulting peptide was confirmed to be HHL with the aid of MALDI-TOF mass spectrometry, glutamine-TOF mass spectrometry, N-terminal sequencing, and measurement of ACE inhibiting activity. These results suggest that our production method is useful for obtaining a large quantity of recombinant HHL for functional antihypertensive peptide studies.

Keywords: Antihypertensive peptide, HHL, tandem repetitive multimers, expression, ubiquitin

Hypertension is a major independent risk factor for cardiovascular diseases such as heart disease and stroke. Angiotensin-I-converting enzyme (kininase II; EC 3.4.15.1; ACE) is the key enzyme in the renin-angiotensin system and a central regulator of cardiovascular and renal diseases [7, 25]. ACE increases blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II and inactivating the vasodilator bradykinin. In order to prevent the disease, therapeutic agents such as ACE inhibitors can be used to decrease high blood pressure to within the normal range. One of these ACE inhibitors is an antihypertensive peptide that originates in food such as soybean paste, milk, and fish, and it has been extensively studied [1, 12, 20, 22]. Moreover, the various ACE inhibitory peptides have been shown to be effective in altering blood pressure *in vivo* [15, 20, 24]. Therefore, ACE inhibitory peptides derived from food proteins would be useful in the development of a novel functional food additive for preventing hypertension as well as for therapeutic purposes.

It is necessary to be able to produce a large quantity of antihypertensive peptides economically for widespread food and pharmaceutical applications, with chemical synthesis being impractical owing to its high cost and safety issues. The expression of heterologous proteins in bacteria is by far the simplest and most inexpensive means to produce large amounts of the product of interest. However, it is difficult both to express small peptides at high levels in engineered bacteria and to recover them from the expression systems. Several biological expression systems have been developed for the production of antihypertensive peptides [11, 14, 16]. However, the few expression systems that have been used for the production of small peptides are far from satisfactory for mass production because of the low production yield and high cost of purification.

In a previous study, we isolated and identified the strong ACE inhibitory peptide His-His-Leu (HHL) from fermented soybean paste [19, 20] and confirmed both its ACE inhibitory and blood-pressure-lowering activities *in vivo* [20]. The present study demonstrates the effectiveness of our new approach to highly express antihypertensive peptide HHL as tandem repeats in *Escherichia coli*.

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MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions

E. coli DH5α was used for plasmid construction, and *E. coli* BL21 was used for the expression of antihypertensive peptide and the fusion of ubiquitin and 40-mer HHL (UH40). These two strains of *E. coli* were grown in Luria-Bertani (LB) broth containing appropriate antibiotics. The antibiotics were employed as ampicillin (100 μg/ml), and kanamycin (10 μg/ml).

pUC18 (NEB, Beverly, MA, U.S.A.) was used as a vector for subcloning and amplification of peptide genes. pET29b (Novagen, Madison, WI, U.S.A.) was used as an expression vector for expressing the HHL multimer and UH40, and pUC18K6Ub (AP Tech, Ansan, Korea) was used as a donor plasmid for the ubiquitin gene.

Plasmid Construction

The DNA fragments encoding the HHL dimer were tandem multimerized using pUC18, as described in Fig. 1. Plasmid

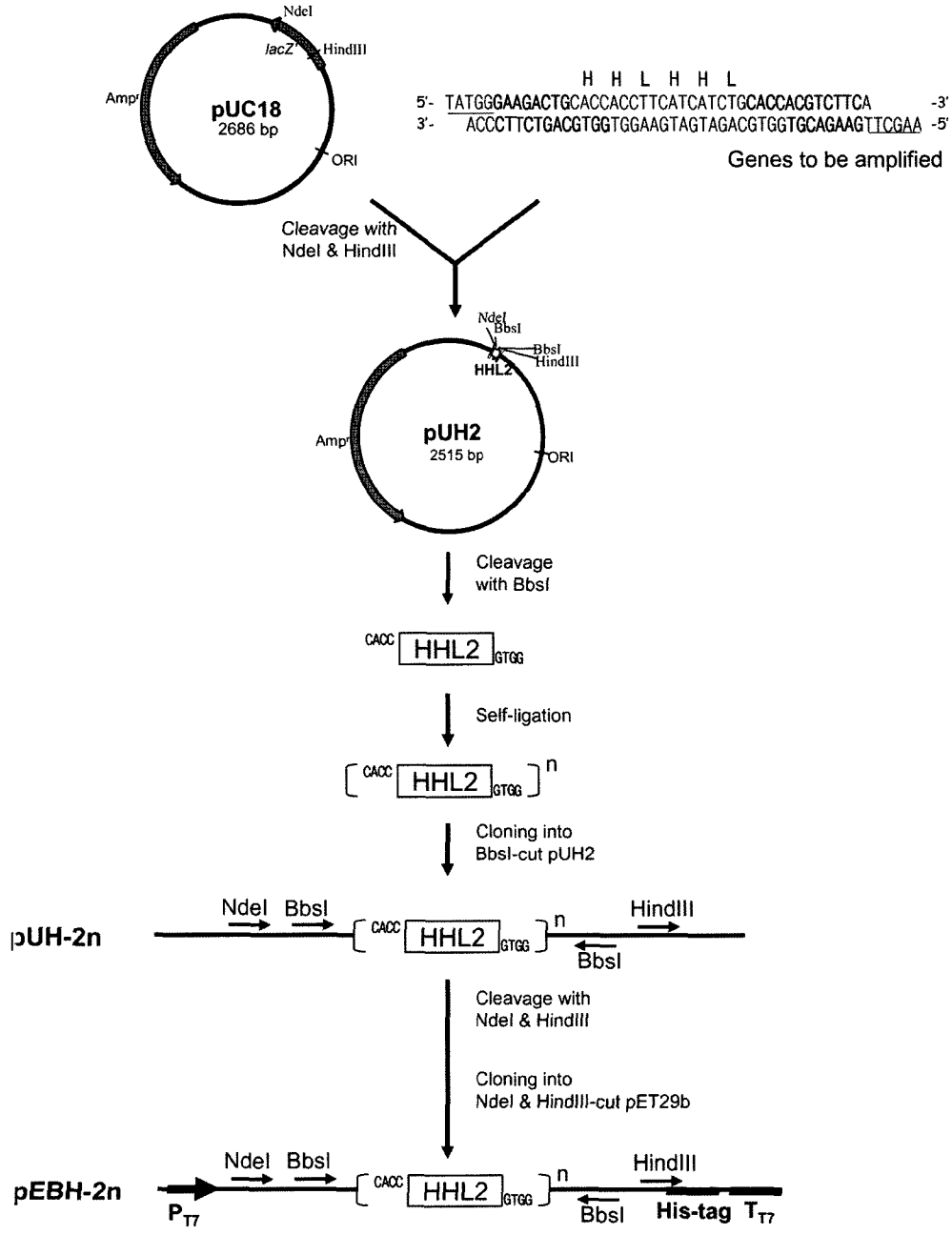

Fig. 1. Schematic representation of the tandem multimerization of the HHL dimer gene.
The gene amplification cassette contains two inversely oriented BbsI sites (in boldface) and also the cohesive ends of NdeI and HindIII.

constructs were verified by enzyme digestion and DNA sequencing.

For the construction of the gene encoding two-copy HHL, two complementary deoxyoligonucleotides, A (5'-TATGGGAAGACTGCACCACCTTCATCATCTGCACC-ACGTCTTCA-3') and B (5'-AAGCTTGAAGACGTGG-TGCAGATGATGAAGGTG-GTGCAGTCTTCCCA-3'), were synthesized, annealed, and then inserted into pUC18 digested with NdeI and HindIII, resulting in pUH2 containing two-copy HHL (Fig. 1).

To produce a dimeric HHL gene with asymmetric cohesive ends of 5'-CCCC/5'-GGGG, pUH2 was digested with BbsI. The resulting dimeric fragments were self-ligated for 12 h at 16°C and tandemly multimerized by cloning into BbsI-digested pUH2. The number of dimers in the multimeric DNA was determined by cleaving the vector with NdeI and HindIII, whose sites flank the multimer, and by nucleotide sequencing.

To construct an expression vector containing the multimeric peptide gene under the control of the T7 promoter, the NdeI-HindIII fragment containing the 20- or 40-mer HHL gene was isolated from pUH-2n and inserted into the same sites of pET29b, producing pEBH20 or pEBH40, respectively. Moreover, to enable stable overexpression of the multimeric gene, the 40-mer HHL was fused to ubiquitin in the following manner: pEBH40 was digested with BglII, made blunt ended by Klenow treatment, and digested with HindIII. This fragment was then fused with the ubiquitin gene in pUC18K6Ub, resulting in plasmid pKlubH40. The NdeI/HindIII fragment containing the UH40 from pKlubH40 was inserted into the same sites of pET29b. The resulting plasmid was pEUH40.

Production and Purification of Recombinant UH40

E. coli BL21 harboring pEBH20, pEBH40, or pEUH40 was cultured at 37°C in 400 ml of LB broth containing kanamycin until the optical density at 600 nm was 0.5. The multimeric gene was induced by isopropyl-beta-D-thiogalactopyranoside (IPTG, 1.0 mM) for 4 h at 28°C and the cells were harvested by centrifugation (Hanil Science, Suwon, Korea) at 4,000 ×*g* for 10 min at 4°C. The pellets of recombinant *E. coli* were resuspended in 40 ml of 50 mM sodium phosphate buffer containing 8 M urea (pH 7.0) and disrupted by an ultrasonic oscillator (Sonics and Materials, Newton, CT, U.S.A.). The cell debris was removed by centrifugation (14,000 ×*g*, 30 min, 4°C), and cell lysates were stored at 4°C.

An ÄKTA explorer (Amersham Pharmacia Biotech, Uppsala, Sweden) was used to analyze the cation-exchange chromatography separation of *E. coli* BL21 harboring pEUH40 cell extracts. The column (1 ml; HiTrap-SP FF, Amersham Pharmacia Biotech) packed with SP Sepharose Fast Flow was used as a cation exchanger. Sodium phosphate buffer (50 mM) containing 8 M urea (pH 7.0) was fed at a flow rate of 1 ml/min, and the fusion protein containing UH40 was eluted through an NaCl gradient (0.0–1.0 M).

UH40 Cleavage and Recovery of the HHL Monomer

The purified UH40 was cleaved by leucine aminopeptidase (Sigma-Aldrich, St. Louis, MO, U.S.A.) at 37°C for 12 h in 50 mM sodium phosphate buffer (pH 7.0). Using reverse-phase high-performance liquid chromatography (HPLC; model HP1100, Hewlett Packard, Palo Alto, CA, U.S.A.) with a Discovery C18 HPLC Column (Sigma-Aldrich), the HHL monomer was purified from the hydrolysate of the recombinant UH40. A 10% acetonitrile solution containing trifluoroacetic acid (0.1%, v/v) was used as the mobile phase at 1 ml/min. The elution was monitored at 216 nm with an ultraviolet detector.

The ACE inhibitory activity was assayed by measuring the amount of hippuric acid liberated from hippuryl-His-Leu, as described by Cushman and Cheung [6]; this measurement was made in triplicate. The reaction mixture contained 5.0 mM hippuryl-His-Leu, 8.0 mM ACE, and the recombinant HHL at various concentrations in 300 µl of 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl. After incubating at 37°C for 30 min, the enzymatic reaction was terminated by adding 500 µl of 0.1 N HCl. The hippuric acid formed by the action of ACE on hippuryl-His-Leu was extracted from the acidified solution into 3 ml of ethyl acetate by mixing for 15 s. After brief centrifugation, a 2.5-ml aliquot of the ethyl acetate layer was transferred to a clean tube and dried by heating to 100°C for 3 h. The resulting dried sample was dissolved in 2.5 ml H_2O and the amount of hippuric acid was determined by measuring the absorbance at 228 nm.

SDS-PAGE and Western Blot Analysis

The expression and purification level of UH40 were analyzed using 15% Tris-glycine gel or Novex 16% tricine gel (Invitrogen, Carlsbad, CA, U.S.A.). The protein bands were detected by Coomassie blue staining or Western blotting. For Western blotting, proteins were transferred from SDS-PAGE gels onto nitrocellulose membranes (0.45 µm pore size; Life Sciences, Pensacola, FL, U.S.A.). The protein concentration was determined using the DC assay kit (Bio-Rad, Hercules, CA, U.S.A.). Bands corresponding to the fusion protein containing UH40 were detected with monoclonal anti-polyhistidine (Sigma-Aldrich) and monoclonal anti-ubiquitin (Sigma-Aldrich) antibodies. Protein bands were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech).

Mass Spectrometry

Masses were analyzed using matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF) and glutamine (Q)-TOF mass spectrometry. MALDI-TOF mass spectrometry was performed using a Voyager-DE STR Biospectrometry

workstation with delayed extraction and linear capability (Applied Biosystems, Foster City, CA, U.S.A.). It was equipped with a 337-nm nitrogen laser and a 2-m flight tube. Mass spectra were obtained in the positive-ion mode, with an accelerating voltage of 25 kV. A 1-μl aliquot of the protein was mixed with 1 μl of matrix (10 mg sinapinic acid and 0.1% 4-hydroxy-α-cyanocinnamic acid in 1 ml of distilled water). A 0.3-μl aliquot of this mixture was applied to the Teflon coating plate. Bovine serum albumin was used for calibration. Q-TOF mass spectrometry was performed using a Micromass Q-TOF Ultima Global Mass Spectrophotometer (Micromass, Manchester, U.K.) equipped with a nano-ESI ion source (National Center for Inter-University Research Facilities, Seoul National University, Seoul, Korea).

Amino Acid Sequence Analysis

N-terminal sequences of UH40 and HHL monomer were identified with a precise protein sequencer (model 491, Applied Biosystems) after desalting with a sample preparation cartridge (Prosorb, Applied Biosystems) at the Korea Basic Science Institute (Seoul, Korea).

RESULTS

Construction of the HHL Gene and Multimerization as Tandem Repeats

An oligonucleotide coding for the HHL dimer and its complementary oligonucleotide were synthesized based on *E. coli* codon usage and hybridized to create a double-stranded DNA with asymmetric ends, having NdeI and HindIII sites, 5'-TA and 5'-AAGCT, respectively. The annealed double-stranded DNA fragment encodes the 2-mer HHL and contains a BbsI site located immediately adjacent to either end (Fig. 1). The BbsI sites were introduced for amplification of the HHL gene. The hybridized DNA fragments were ligated into the NdeI and HindIII sites of pUC18, resulting in the formation of pUH2. DNA fragments containing a dimer of the HHL gene with BbsI cohesive ends, 5'-CCCC/5'-GGGG (Fig. 1), were isolated from pUH2 after digestion with BbsI. The isolated DNA fragments were tandemly repeated multimers and ligated with the BbsI-digested pUH2 (Fig. 1). Clones containing the 20- and 40-mer HHL were then selected and named pUH20 and pUH40, respectively.

Expression of Recombinant UH40

To construct an expression vector, DNA fragments containing the 20- and 40-mer HHL were inserted downstream of the T7 promoter in pET29b, resulting in plasmids pEBH20 and pEBH40, respectively. The inserted HHL multimer was fused to the His-tag in the pEBH series (Fig. 2A); the expression level of the multimer under the T7 promoter was low in *E. coli*, as shown in Fig. 2B.

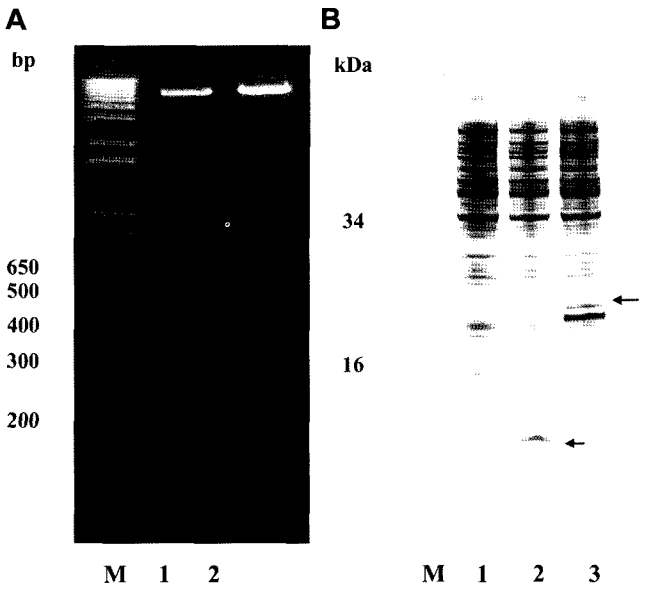

Fig. 2. Electrophoretic analysis and expression of multimeric peptide genes in *E. coli* BL21 harboring pEBH20 or pEBH40.
A. Electrophoretic analysis of the multimeric peptide gene. Lanes: M, 1,000-bp ladder; 1 and 2, NdeI- and HindIII-digested pEBH20 and pEBH40, which contain 20- and 40-copies of HHL monomer, respectively. **B.** Expression of HHL multimers fused to His-tag. Lanes: M, molecular mass marker; 1, *E. coli* BL21 (pET29b); 2 and 3, *E. coli* BL21 harboring pEBH20 and pEBH40, respectively. Proteins were stained with Coomassie brilliant blue.

To increase the level of gene expression in *E. coli*, the 40-mer HHL gene was fused with the ubiquitin gene to give pEUH40. To determine whether pEUH40 could be used to express the fusion protein UH40, *E. coli* BL21 cells harboring the plasmid were treated with 1 mM IPTG. A dominant protein band was induced in a time-dependent manner. Using antibodies raised against ubiquitin or His-tag, which were located at the N-terminus and C-terminus, respectively, of recombinant UH40, it was determined that the protein from the dominant band was the fusion protein UH40 (data not shown). The amount of UH40 expressed was estimated to be 22.3% of the total cell protein in *E. coli* BL21 harboring pEUH40; UH40 was a soluble protein (Fig. 3A, Table 1).

Purification of Recombinant UH40

UH40 was purified by cation chromatography (Fig. 3). UH40 constituted 22.3% of the crude extract based on protein concentration (Table 1). Approximately 17.3 mg of UH40 was purified from 359.9 mg of the total cell protein. MALDI-TOF mass spectrometry and N-terminal sequencing were used to identify the purified UH40. The molecular mass of the extracted UH40 was determined to be 28,635 Da, which is the expected molecular mass of UH40 (Fig. 3C). N-terminal sequencing of the purified UH40 confirmed that the first 15 residues were identical to those deduced from the

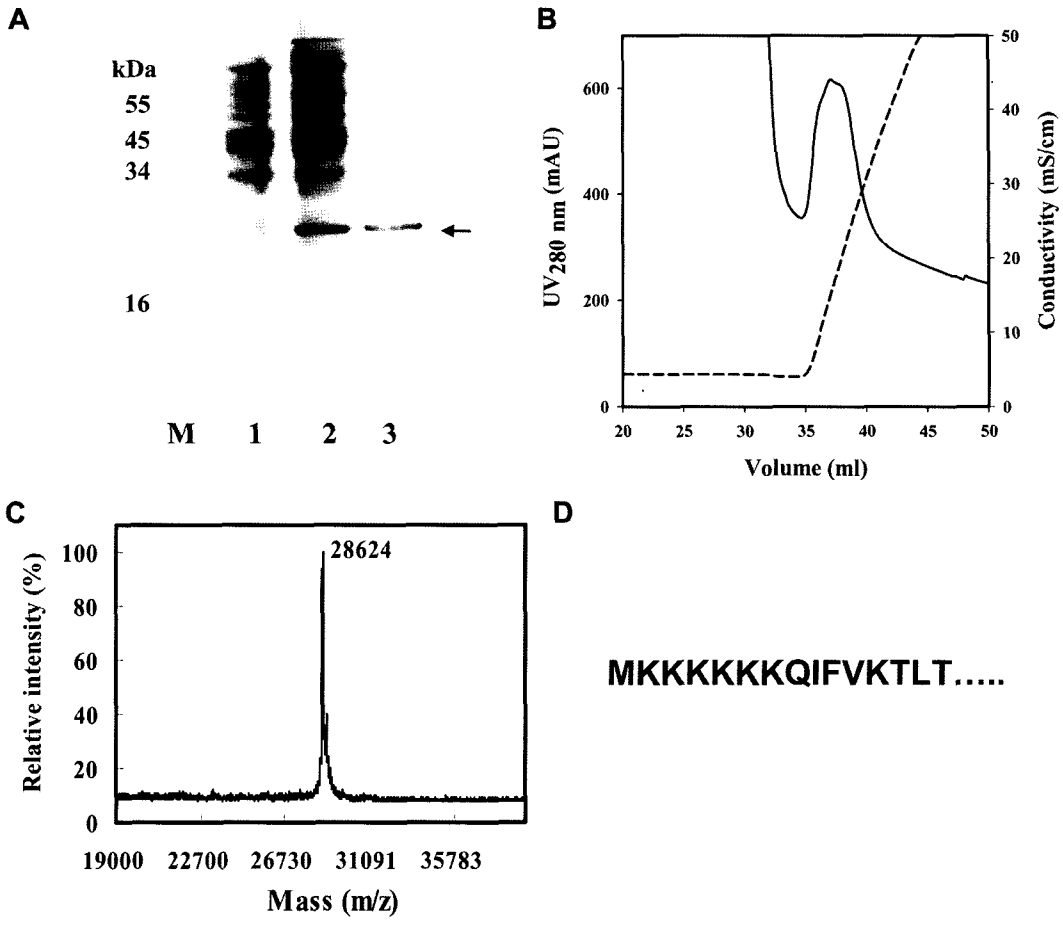

Fig. 3. Purification of the UH40 fusion protein by cation-exchange chromatography.
A. Tricine SDS-PAGE analysis of purified recombinant UH40. Lanes: M, molecular mass marker; 1 and 2, total cell protein before and after induction, respectively; 3, HiTrap-Sepharose Fast Flow (SP FF) column-purified fraction of fusion protein UH40. **B**. Chromatogram of HiTrap-SP FF column for analysis of recombinant UH40. **C**. Molecular mass analysis of UH40 by MALDI-TOF mass spectrometry. **D**. N-terminal amino acid sequence analysis of UH40.

DNA sequence of UH40 (*i.e.*, MKKKKKKQIFVKTLT; Fig. 3D).

Purification of Recombinant HHL

The recombinant HHL monomer peptide (obtained as a result of cleavage with leucine aminopeptidase) was purified by applying the cleavage product to a reverse-phase HPLC column (Discovery C18; Fig. 4A). The identity of the main eluted fraction was determined by MALDI-TOF mass spectrometry (Fig. 4B), where a singly protonated molecular ion of purified HHL was observed at m/z=406 Da. The identification of purified HHL was confirmed by Q-TOF mass spectrometry and N-terminal sequencing. The molecular mass of the main eluted fraction was determined to be

Table 1. Purification of recombinant HHL from *E. coli*.

Purification step	Total protein (mg)[a]	Protein of interest (mg)[b]	Yield (%)[c]
Crude extract	359.9	77.7	100
Soluble protein	263.4	51.9	66.8
Cation-exchange chromatography	18.7	17.3	22.3
HPLC (after hydrolysis by leucine aminopeptidase)		6.2	8.0

The starting material was crude extract from the lysis of 1 l of induced *E. coli* culture.
[a]Total protein concentration was determined by the DC assay kit.
[b]The amount of fusion protein in crude extract, as soluble protein, and after cation-exchange chromatography were determined by quantifying the amount in each gel lane by the ImageJ densitometer program. The amount of monomer HHL after HPLC was determined by area analysis using synthesized peptide His-His-Leu as a standard.
[c]The purification yields are calculated based on the amount of protein of interest.

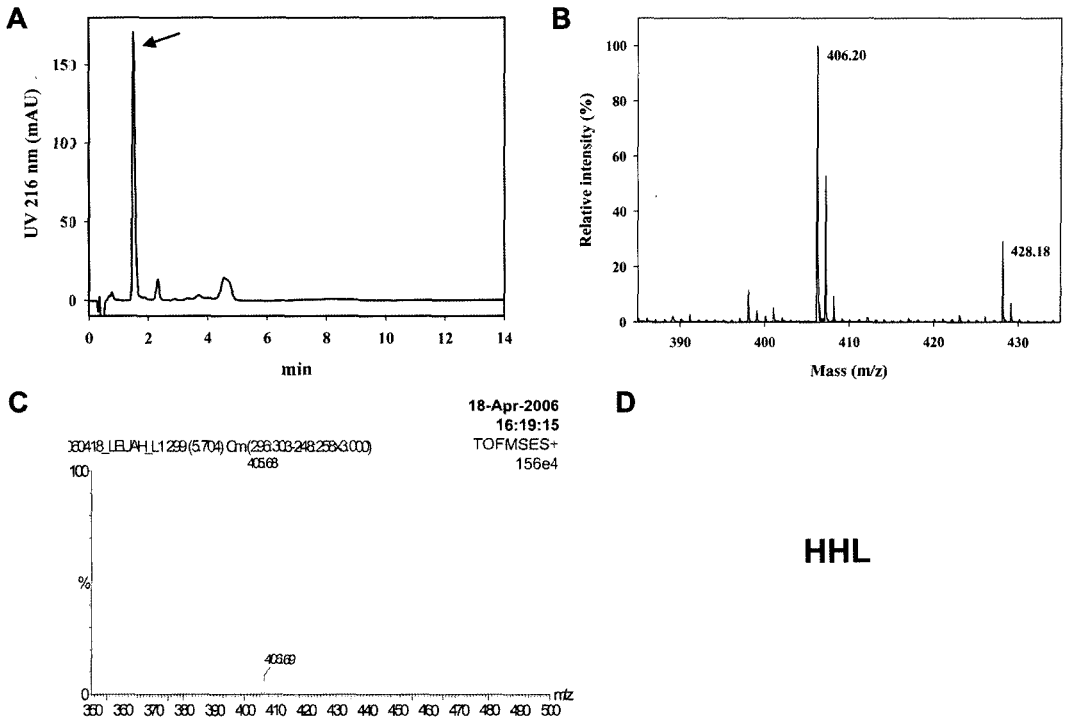

Fig. 4. Purification of recombinant HHL by reverse-phase HPLC.
A. Chromatogram of reverse-phase HPLC for analysis of the recombinant HHL monomer. Molecular mass analysis for recombinant HHL by (**B**) MALDI-TOF mass spectrometry and (**C**) Q-TOF mass spectrometry. **D.** N-terminal amino acid sequence analysis of the recombinant HHL monomer.

405 Da, as would be expected for HHL (Fig. 4C). N-terminal sequencing of the purified HHL confirmed the presence of the three amino acid residues (HHL; Fig. 4D). The purified recombinant HHL was tested for its antihypertensive activity: the recombinant HHL exhibited ACE inhibitory activity (IC_{50}=3.4 μg/ml) similar to that of synthetic HHL (IC_{50}=3.2 μg/ml).

The amount of purified HHL from 400 ml of culture was approximately 6.2 mg (Table 1). The total recovery of the peptide was 8%. The apparently low protein yield was due to removal of the fusion partner and, in part, to incomplete digestion of the UH40 fusion protein by leucine aminopeptidase.

DISCUSSION

Antihypertensive peptides have been studied extensively because of their possible clinical applications as agents for the treatment of cardiovascular diseases [21, 23]. Among these ACE inhibitory peptides, HHL is a very promising candidate because it originates from fermented food and has a low IC_{50} activity. For potential clinical applications, it is necessary to produce biologically active HHL in large scale by using an efficient and low-cost method. Because of this requirement, we tested a method of producing HHL utilizing *E. coli*, as it yields a high level of pure peptide. We thus constructed a highly efficient system for the expression and purification of human HHL in *E. coli*, using a fusion of the peptide with ubiquitin driven by the T7 promoter in the pET29b expression vector.

Ubiquitin, which is comprised of 76 amino acid residues, is a small, highly conserved eukaryotic protein that acts as a degradation marker for a wide spectrum of cellular proteins and is a unique molecule of intracellular proteolysis [9]. In addition, ubiquitin is a useful fusion protein for the production of recombinant proteins in *E. coli* [2, 10]. As part of a fusion protein, ubiquitin acts as a chaperone, and is able to enhance the expression level and folding efficiency of the protein of interest [4, 8]. In *E. coli* BL21 harboring pEBH20 and pEBH40, the amount of 20- and 40-mer HHL expressed was low at 3.9–2.3% of the total cell protein; however, in *E. coli* BL21 harboring pEUH40, which contains ubiquitin fused to the 40-mer HHL, the expression of HHL was enhanced to approximately 22% of the total cell protein. In addition, the expressed protein was soluble. Ubiquitin was cleaved at the C-terminal glycine residue with ubiquitin-specific protease or ubiquitin C-terminal hydrolase [3, 22]. Since the HHL monomer was isolated from UH40 by cleaving with leucine aminopeptidase, it was not necessary to use ubiquitinase.

Recombinant fusion proteins usually need to be cleaved to release the peptide of interest for clinical and biological studies [5, 17]. To purify the monomer peptide from the

multimer peptide efficiently, specific amino acid sequences representing protease cleavage sites were inserted into tandem multimeric expression systems for small peptides [13, 16, 18]. In the present study, we constructed an HHL multimer that was composed only of tandem HHL. Pepsin and leucine aminopeptidase were used to obtain the HHL monomer from UH40. Pepsin is an endopeptidase that preferentially cleaves at the C-terminus of leucine. Leucine aminopeptidase is an exopeptidase that also cleaves at the C-terminus of leucine. The HHL monomer was obtained efficiently using leucine aminopeptidase (data not shown); this enzyme was thus used routinely. The mechanism underlying this efficient cleavage of the HHL monomer from UH40 by leucine aminopeptidase remains to be established.

In conclusion, this study demonstrated that a relatively high amount of the recombinant UH40 fusion protein could be obtained in soluble form. The final yield of the purified HHL monomer, 6.2 mg per l of culture, could be improved by fusing with ubiquitin to increase the solubility and expression. It may be possible to improve the yield using other recombinant procedures for producing small antihypertensive peptides in bacteria. These results clearly open a door to the mass production of the ACE-inhibiting peptide within a biological expression system. In addition, HHL peptides, which can be used as antihypertensive agents, were originally extracted from fermented food and soybean pastes, so they can be safely utilized as functional peptides in the food and medical industries.

Acknowledgments

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