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Expression and Purification of an ACE-Inhibitory Peptide Multimer from Synthetic DNA in *Escherichia coli*

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Abstract An angiotensin I-converting enzyme (EC 3.4.15.1) (ACE), which can convert inactive angiotensin I into angiotensin II, a vasoconstrictor, is one of the key enzymes in controlling hypertension. It is suggested that the inhibition of ACE prevents hypertension, and many inhibitory peptides have already been reported. In the current study, oligonucleotides encoding ACE inhibitory peptides (IY, VKY) were chemically synthesized and designed to be multimerised due to isoschizomer sites (*Bam*HI, *Bgl*II). The cloned gene named AP3 was multimerised up to 6 times in pBluescript and expressed in BL21 containing pGEX-KG. The fusion protein (GST-AP3) was easily purified with a high recovery by an affinity resin, yielding 38 mg of synthetic AP3 from a 1-l culture. The digestion of AP3 by chymotrypsin exhibited an IC_{50} value of 18.53 μM. In conclusion, the present experiment indicated that AP3 could be used as a dietary antihypertensive drug, since the potent ACE inhibitory activity of AP3 could be activated by chymotrypsin in human intestine.

Key words: Angiotensin I-converting enzyme inhibitor, repetitive artificial polypeptide

An angiotensin I-converting enzyme (EC 3.4.15.1) (ACE) is physiologically important in the regulation of blood pressure [14]; it catalyzes the conversion of inactive angiotensin I to angiotensin II, a potent vasoconstrictor, and inactivates the vasodilator, bradykinin. As such, a specific inhibitor of ACE can be of great value to prevent hypertension [8]. In the food industry, the isolation of ACE inhibitors from natural resources as a "physiologically functional food" has already been carried out.

Recently, it has also become apparent that these peptides have many additional potential functions related to other physiologically important regulations [7]. Among them, a number of functional peptides derived from maize [21], zein [18, 19], casein [20], and sake lees [17] have been identified, some of which promote Ca^{++} absorption, reduce blood pressure, and regulate cholesterol levels in serum, as well as have a variety of benefits [16]. However, these inhibitory peptides to be used as a "dietary drug" must be produced with a high yield by DNA recombinant technology [4].

The chemical synthesis of DNA provides an opportunity to construct and modify natural genes, thereby making it possible to create artificial gene coding for specific proteins. Accordingly, the current study was undertaken to investigate the expression of a gene for coding a polymer of IYVKY fused to GST from BL21 *E. coli* cells. This polymer could then be used for the production of the ACE inhibitor peptides, IY, VKY. A tandem linkage of multiple copies [5, 22] of the peptide encoding sequence was used, because the recombinant concatemer could quantitatively be converted into monomeric peptides, resulting in a high yield [11, 12]. The results demonstrated that biologically active ACE inhibitory peptides could be produced by artificial oligonucleotides.

Materials and Methods

Bacterial Strains and Plasmids

The *E. coli* strain, BL21 (Novagen, Madison, U.S.A.) was used as the host strain. The plasmid pBlue-Script II KS (+) (Stratagene) was used for the initial cloning and pGEX-KG (Pharmacia, U.S.A.) for the expression of the artificially synthesized genes in *E. coli* [9].

Chemical Synthesis of Oligonucleotides

The two 38-mers, 5'GATCCATGGATTTCATTTACGTG-AAATATGGTCCGGGC3' and 3'GTACCTAAAGTAAAT-GCACTTTATACCAGGCCCGCTGA5'; two 31-mers, 5'GA-CTTCATTTACGTGAAATATAGATCTTAAA3' and 3'AG-TAAATGCACTTTATATCTAGAATTTTCGA5'; and two 28-mers with an His codon, 5'GATCTCATCACCATCA-CCATCACTAAA3' and 3'AGTAGTGGTAGTGGTAGT-

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GATTTTCGA5' were synthesized using a DNA synthesizer Model 380 A (Applied Biosystem).

In vitro Annealing of Synthetic Oligonucleotides

Each oligonucleotide (500 pmol) was phosphorylated individually in 20 μl of kinase buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 50 mM dithiothreitol) containing 2 units of T4 polynucleotide kinase. After incubation at 37°C for 2 h, the two complementary fragments of the 38-mer and 34-mer were mixed, heated to 100°C for 10 min, and cooled to room temperature for annealing. To ligate the double-stranded DNA fragments, the annealed 38-mer and 34-mer linkers were mixed in same molar ratio and incubated with 1 unit of T4 DNA ligase at 16°C for 4 h.

Cloning of Assembled Synthetic DNA into pBlue-Script II KS(+) and pGEX-KG

The plasmid pBlue-Script II KS(+) was linearized with *Hin*dIII and *Bam*HI digestion. The digested plasmid (500 ng) and synthetic DNA (200 ng) were incubated with 1 unit of T4 DNA ligase at 16°C overnight. The ligated DNA mixture was used for the transformation of *E. coli* DH5α. The transformed bacteria were selected on an ampicillin-containing LB medium. For repeated conjugation of the synthetic DNA, the pBlue-Script KS(+) plasmid containing the synthetic DNA was digested with *Bgl*II and *Bam*HI [19], and the isolated synthetic DNA (200 ng) and *Bam*HI-digested vector (500 ng) containing the synthetic DNA were ligated together with 0.5 units of the T4 DNA ligase at 16°C overnight. The orientation of the insert was confirmed by digestion with *Bam*HI and *Bgl*II. The vectors containing the monomer, dimer, and trimer of the synthetic genes were selected and named pBL-AP1, -AP2, and -AP3, respectively. To express the multimeric synthetic genes, pBL-AP1, -AP2, and -AP3, were subcloned into the vector pGEX-KG, which was digested with *Bam*HI and *Hin*dIII, resulting in pGST-AP1, -AP2, and -AP3, respectively.

Expression of Synthetic AP DNA

A duplicated 1-ml culture of the transformants in an LB medium containing ampicillin was grown to the mid-log phase. Isopropyl-β-thiogalactopyranoside (IPTG) was then added to one of the cultures and its final concentration was adjusted to 0.4 mM. After induction at 37°C for 4 h, the cells were collected by centrifugation, lysed and then boiled in SDS-PAGE sample buffer. Approximately one-

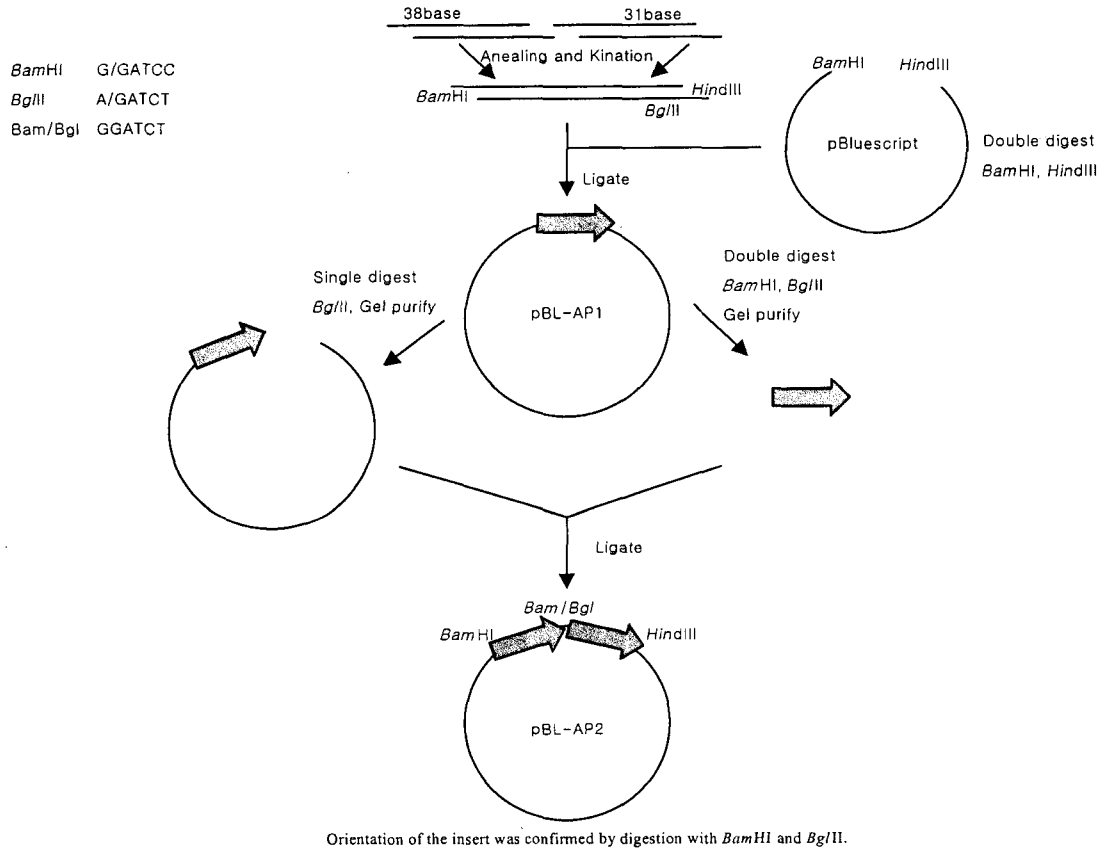

Fig. 1. Strategy for constructing multimer of artificial polypeptide (AP).
The top panel shows the construction of the AP1 "unit" using chemically synthesized artificial DNAs, as described in Materials and Methods. The lower panel illustrates how the insert was multiplied to form AP2 and also shows that such an insert could be multiplied to 3 units length by reiteration.

tenth of each sample was analyzed by 12% SDS PAGE. The gel was stained with Coomassie Brilliant Blue [9].

Isolation and Purification of Artificial Polypeptide (AP)

For the purification of the recombinant artificial polypeptide, *E. coli* harboring the expression vector pGST-AP3, including twelve copies of the ACE inhibitory peptide (IY, VKY), was cultured in 1-l of an LB medium at 37°C. After IPTG induction, the cells were harvested by centrifugation at 400 ×g for 15 min at 4°C, resuspended in 1/20 of the culture volume of a sonication buffer, (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4), and lysed by sonication (Labsonic U, B. Braun Biotech International GmbH, Melsungen, Germany). After sonication, the inclusion bodies were recovered by centrifugation at 10,000 ×g at 4°C for 30 min and washed with 50 mM Tris/HCl buffer (pH 8.0). The inclusion bodies were denatured and solubilized in 8 M urea, 300 mM KCl, and 50 mM Tris/HCl (pH 8.0) containing 10% glycerol [1]. TALON Metal affinity resin (CLONTECH, U.S.A.) was added to the solution, which was then mixed gently for 2 h at room temperature and centrifuged at 500 ×g for 3 min. The polypeptide-bound resin was washed four times with buffers containing a decreasing concentration of urea in the order of 6 M, 4 M, 2 M, and 0 M to remove all the urea. The fusion partner was removed by 0.04 U thrombin in 20 mM Tris-HCl, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$, and 1% TritonX-100 at 22°C for 22 h. The AP3-bound resin was then washed to completely remove any thrombin and GST. Finally, the AP3 was eluted with 100 mM EDTA. The resulting purified AP3 was then applied to a 3.9×300 mm Delta pak C18 column (Waters Associates, Milford, MA, U.S.A.) and further purified with a linear gradient of 10% to 80% buffer A at 0.5 ml/min for 1 h [buffer A: acetonitrile (ACN) containing 0.1% (v/v) trifluoroacetic acid (TFA)]. All peaks were monitored for the ACE inhibitory activity and amino acid sequence analyses.

Assay of ACE Inhibitor

The activity of the ACE inhibitor was measured by the modified method of Cheung and Chushman [2]. Five millimolars hippuryl-L-histidyl-L-leucine and the ACE inhibitor were mixed in 100 mM sodium borate buffer (pH 8.3) containing 300 mM NaCl, and the mixture was incubated with 8 mU of ACE at 37°C for 1 h. The concentration of the ACE inhibitor needed to inhibit 50% of the ACE activity was defined as the IC_{50} value. The ACE used in this experiment was a crude extract of rabbit-lung acetone powder.

RESULTS AND DISCUSSION

Designing of Artificial Gene

When designing the artificial gene, the following factors were taken into consideration: The amino acid sequence of the synthetic oligonucleotides should be repeated as a decapeptide, Asp-Phe-Ile-Tyr-Val-Lys-Tyr-Gly-Pro-Gly.

All degenerative codons for amino acids should be used to avoid a limited supply of the corresponding charged tRNA in the host cell. The introduction of the Asp-Phe codon, the precursor of aspartame, in the repeated sequence may counteract the positive charge of lysine, thereby providing a more stable artificial protein with less toxic effect on the host cell. The presence of Phe and Tyr in the peptide should provide cleavage sites for chymotrypsin.

Cloning of Synthetic AP DNA

The assembling of a double-stranded 38-mer in the presence of an equimolar 31-mer produced two repeated sequences. The assembled synthetic DNA on a 2% agarose gel exhibited a mobility shift toward a higher molecular region. To select the correct orientation of the oligonucleotide sequence with the desired reading frame, the synthetic DNA was cloned into a pBL vector. Since the twice-repeated Asp-Phe-Ile-Try-Val-Lys-Tyr-Gly-Pro-Gly was not long enough to study the expression system of the artificial protein, it was necessary to elongate the repeating unit. Therefore, the 61 bp fragment to be inserted was isolated from the plasmid pBL-AP1 by digestion with *Bam*HI and *Bgl*II, and recloned in the *Bgl*II-digest plasmid, pBL-AP1 (Fig. 1). As a result,

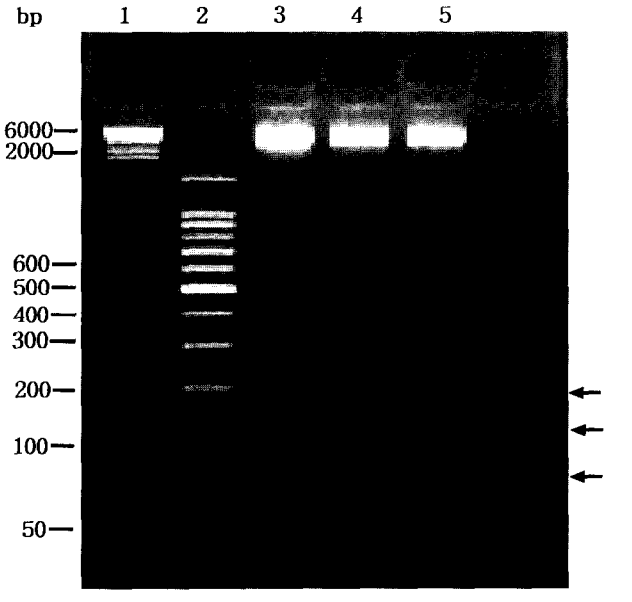

Fig. 2. Electrophoretic analysis of multimeric peptide genes.
The number of peptide genes cloned in the gene amplification vector pBL was determined by cleaving the vectors with *Bam*HI and *Hin*dIII. The digested DNAs were electrophoresed on 2% agarose gel in TAE buffer for 15 min at 50 V/cm, and the DNA bands were stained with ethidium bromide. Lanes 1–2, size marker; Lane 3, the pBL-AP1 vector digested with *Bam*HI and *Hin*dIII; Lanes 4–5, *Bam*HI and *Hin*dIII-digested pBL-AP2, three of which contained 1, 2, and 3 copies of the AP1 monomer, respectively. The arrows indicate the AP1, -AP2, and -AP3 genes.

the repeated sequence from pBL-AP1 was concatenated using head-to-tail repetition while maintaining the correct reading frame. By repeating this concatemerization successively, clones containing genes encoding up to 6 repeats of the decapeptide were constructed (pBL-AP1, -AP2, -AP3, etc.). To express the artificial genes, *Bam*HI, and *Hin*dIII digested fragments of AP1, -2, and -3 with repeating units of 2, 4, and 6, respectively, were subcloned into pGEX-KG (Fig. 2).

Expression and Purification of Artificial Gene

E. coli BL21 cells carrying the recombinant plasmid pGEX-AP1, -AP2, and -AP3 were examined for the expression of the respective polypeptides. The molecular weights of the artificial proteins encoded by pGEX-AP1, -AP2, and -AP3 were calculated from the DNA sequence to be 28,000, 30,000, and 32,000 Da, respectively (Fig. 3). The apparent sizes of the artificial proteins were in good agreement with those of the calculated molecular weights. When the fusion protein was expressed as inclusion bodies with the GST fusion protein in *E. coli* BL21, a hexahistidine codon and stop codon were inserted between the *Bgl*II and *Hin*dIII of the pGEX-AP3, which resulted in a GST-AP3-6His fusion protein. The protein was easily purified due to the His-tag, under denaturation conditions of 8 M urea with affinity chromatography. The urea was removed by stepwise elution of the column with low concentration of urea. The washing steps also removed any loosely bound unwanted proteins, and the elution buffer finally eluted the pure polypeptide, as seen in Fig. 4. The resin-bound fusion protein (GST-AP3-His) was cleaved with thrombin to

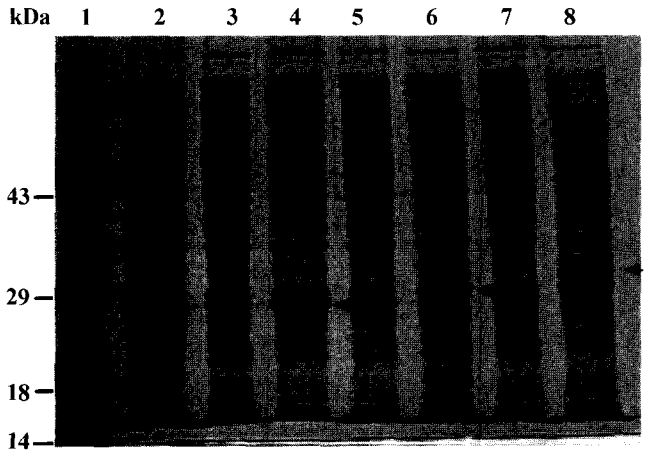

Fig. 3. Expression of multimers of AP1 and GST-APs.
Lane 1, molecular weight markers; Lane 2, BL21 containing pGEX-KG; Lanes 4, 6, and 8, BL21 harboring pGEX-AP1, -AP2, and -AP3, respectively; Lanes 3, 5, and 7, no-induced BL21 harboring pGEX-AP1, -AP2, and -AP3, respectively. The total cell proteins were analyzed by 12% SDS-PAGE. Samples were applied to the gel after boiling for 10 min. The proteins were stained with Coomassie Brilliant Blue. The arrows indicate GST-AP1, -AP2, and -AP3.

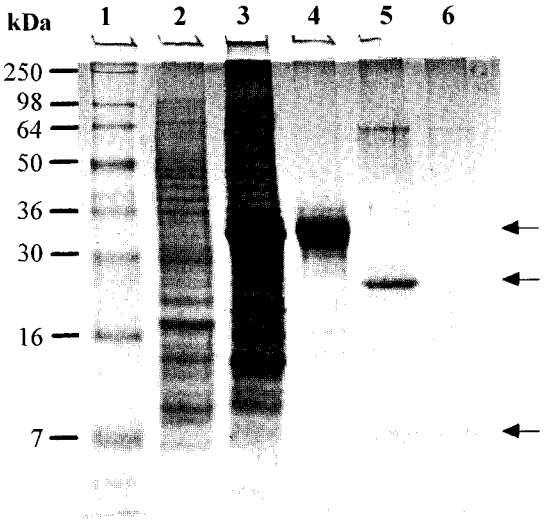

Fig. 4. SDS-PAGE analysis of purified recombinant AP3 expressed in BL21 containing pGEX-AP3.
SDS-PAGE (15%) containing 22% glycerol was used to analyze purified AP3. Lane 1, molecular weight markers; Lanes 2 and 3, total cell proteins before and after induction, respectively; Lane 4, GST-AP3 isolated from inclusion body by TALON affinity chromatography; Lane 5, purified GST-AP3 cleaved by thrombin; Lane 6, AP3 isolated by TALON affinity chromatography of eluted fraction by 100 mM EDTA.

prepare a lower molecular peptide (AP3-His) and applied to a C18 reverse-phase HPLC for further purification (Figs. 4, 5). About 38 mg of AP3 with over 95% purity was obtained from a 1-l *E. coli* culture after TALON-Metal affinity chromatography and C18 reverse-phase HPLC (Table 1).

Analysis of ACE Inhibitory Activity

To test the inhibitory activity of ACE, the purified AP3 (Fig. 5A) was cleaved with 0.25 U chymotrypsin in 50 mM Tris-HCl (pH 7.5) buffer containing 150 mM NaCl and 25 mM $CaCl_2$. The IC_{50} value of AP3-hydrolysate was estimated by plotting the inhibitory activities against the peptide concentrations of the reaction mixture. AP3-hydrolysate inhibited the angiotensin I-converting enzyme with an IC_{50} of 18.53 μM (Table 2). As shown in Fig. 5B, the HPLC elution patterns of 6 fractions were in good agreement with the expected chymotrypsin cleavage pattern (IY, VKY, GSMDF, RSMDF, RSHHHHHH, GPGDF). All fractions were assayed for the ACE inhibitory activity. Two fractions with retention time at 3.647 min and 5.213 min had the most potent ACE inhibitory activities. Their amino acid sequences were Val-Lys-Tyr (peptide a) and Ile-Tyr (peptide b), with IC_{50} values of 7.2 μM and 3.7 μM, respectively, as previously reported by Yoshiyuki *et al.* [23] and Susumu *et al.* [21]. These two fractions constituted approximately 35% of the total AP3-hydrolysate. Therefore, AP3 peptides, which had ACE inhibitory activity, appeared to have several advantages: 1) AP3 hydrolysate was made

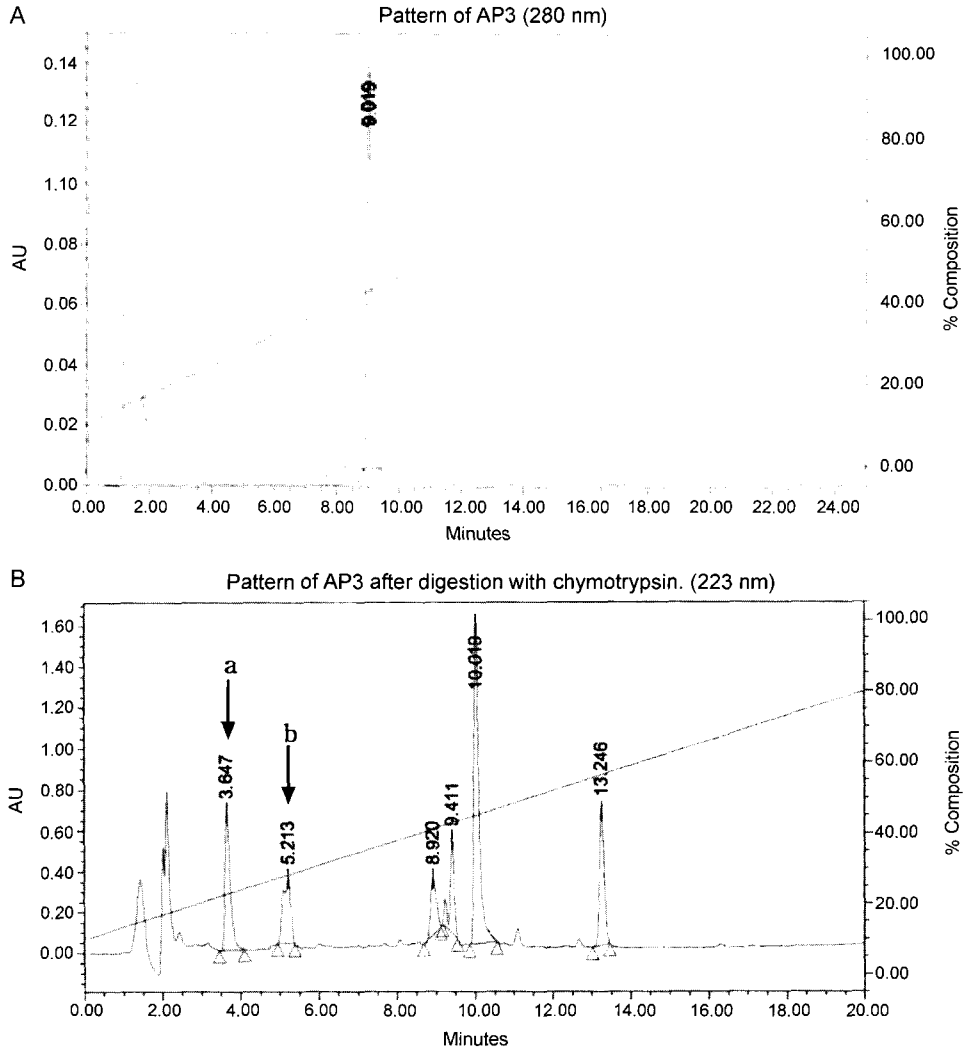

Fig. 5. Reverse phase HPLC pattern of purified AP3 (A) and AP3-hydrolysate after digestion with chymotrypsin (B).
The purified AP3 and AP3-hydrolysate were detected using TALON affinity column in Deltapack C18 column (4.6×300 mm); gradient of ACN from 10% to 80% in 0.1% TFA solution at a flow rate of 5 ml/min at 4°C. In the elution profile of the C18 reverse phase HPLC of the AP3 hydrolysate, strong inhibitors (IY, VKY) appeared in two fractions (a and b).

of 35% ACEIs, whereas natural sources yielded very low concentration of ACEIs. 2) Since AP3 could possibly release ACEIs in human intestine by chymotrypsin digestion, AP3 could be used as an antihypertensive drug. 3) Since multimeric peptides could quantitatively be converted to monomeric peptides, AP3 could be used for the mass production of ACEIs for health care foods or drugs.

Table 1. Purification of recombinant AP3 from *E. coli.*

Purification step	Total protein[1] (mg)	Protein of interest[2] (mg)	Yield (%)[3]
Crude extract	1,128.0	480.8	100.0
Inclusion bodies	650.2	343.7	71.5
TALON metal chromatography (after thrombin cleavage)	52.9	47.6	9.9
HPLC	39.0	38.0	7.9

[1]The total protein concentration was determined by a Bradford assay using bovine serum albumin as the standard.
[2]The amount of the fusion protein in the crude extract and inclusion bodies as well as the amount of AP3 purified by TALON metal chromatography and HPLC were determined by quantifying the optical density at 600 nm in each gel lane (Bio/Profile Image Analysis Software TINA, U.S.A.)
[3]The starting material was the crude extract from lysis of 1 l of an induced *E. coli* culture, as described in Materials and Methods.

Table 2. IC_{50} of AP3 digested by chymotrypsin.

AP3	IC_{50} (μM)
None	≫400
Chymotrypsin	18.53

The activity of the ACE inhibitor was assayed by the modified method of Cheung and Chushman [2], as described in Materials and Methods. The IC_{50} value was determined by the linear regression method using Sigmaplot. (Sigmaplot 2000 software for FlyingRaiche ver 6.0, U.S.A.).

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