Design and Expression of Recombinant Antihypertensive Peptide Multimer Gene in *Escherichia coli* BL21

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The design and expression of an antihypertensive peptide multimer (AHPM), a common precursor of 11 kinds of antihypertensive peptides (AHPs) tandemly linked up according to the restriction sites of gastrointestinal proteases, were explored. The DNA fragment encoding the AHPM was chemically synthesized and cloned into expression vector pGEX-3X. After an optimum induction with IPTG, the recombinant AHPM fused with glutathione S-transferase (GST–AHPM) was expressed mostly as inclusion body in *Escherichia coli* BL21 and reached the maximal production, 35% of total intracellular protein. The inclusion body was washed, dissolved, and purified by cation-exchange chromatography under denaturing conditions, followed by refolding together with size-exclusion chromatography and gradual dialysis. The resulting yield of the soluble GST–AHPM (34 kDa) with a purity of 95% reached 399 mg/l culture. The release of high active fragments from the AHPM was confirmed by the simulated gastrointestinal digestion. The results suggest that the design strategy and production method of the AHPM will be useful to obtain a large quantity of recombinant AHPs at a low cost.

Keywords: Antihypertensive peptide, antihypertensive peptide multimer, GST fusion protein, expression, design

Hypertension is a major risk factor for cardiovascular diseases such as heart failure, stroke, coronary heart disease, and myocardial infarction [11]. Chemically synthesized hypotensive drugs, such as captopril, hydrochlorothiazide, propranolol, nifedipine, losartan, and so on, are still broadly used to treat and prevent hypertension. Although having obvious antihypertensive effect, these drugs are reported to have many side effects such as dry cough, taste disturbances, skin rashes, and many other dysfunctions of human organs [7, 12]. Recently, food protein-derived antihypertensive peptides (AHPs) have received considerable attention, to their good antihypertensive effects, safety, mild effects on humans, and potential use as health foods and pharmaceutical preparations. These peptides usually have multifunctional properties and are easily absorbed. Therefore, these food protein-derived AHPs show great promise in the development of a novel physiologically functional food for preventing hypertension as well as for therapeutic purposes [15].

By far, the preparation methods of AHPs mainly include enzymatic hydrolysis, microbial fermentation, and gene expression. Among these methods, enzymatic hydrolysis is most frequently used to prepare antihypertensive peptides derived from food protein sources, and secondly microbial fermentation [22]. However, it is very difficult to prepare antihypertensive peptides industrially by enzymatic hydrolysis and microbial fermentation, because of low yield, high cost, and multilink of separation and purification processes [4, 5, 20, 21, 31, 33]. These issues need to be solved step by step. Although the production of antihypertensive peptides by genetic engineering means has been developed in recent years, there is still much work to be conducted. Because of the peptide sequence of AHP being very short, usually only several amino acids, it is susceptible to degradation by protease or peptidase, and the expression products may be harmful to the host, impacting the high-level expression of the gene, so it is difficult to be expressed directly by genetic engineering means. Luckily, this shortcoming has been conquered by expressing AHP in the forms of a fusion protein [12, 16, 17] or a tandem gene [25]. Athough the AHPs such as HHL [13], KVLPVP [16], CEI12 (FFVAPFPEVFGK) [17] and YG 1 (GHKIATFQER) [25] have been expressed successfully in *Escherichia coli*, the expressed multimer products need to be hydrolyzed by the corresponding special proteases (leucine aminopeptidase

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[13], clostripain and carboxypeptidase B [16], trypsin [17] and clostripain [25], respectivily) to release the target active peptides. Thus, it increases the cost of separation and purification after enzymatic hydrolysis.

In this paper, different AHP monomers were tandemly multimerized to an antihypertensive peptide multimer (AHPM) using one to three amino acids or not, according to the restriction sites of gastrointestinal proteases. The target polypeptide AHPM was expressed as a glutathione S-transferase (GST) fusion protein in *E. coli* BL21. The active fragments with potent activity of lowering blood pressure can be released from the AHPM by hydrolysis recurring to natural gastrointestinal proteases in the human body, and absorbed in an intact form or converted into stronger ones by gastrointestinal proteases or angiotensin I-converting enzyme (ACE) *in vivo*, and then reach a target organ or tissue and exert an antihypertensive effect after oral administration. This method solved the technical difficulty of unstable expression of small molecule peptides in bacteria, overcame the shortcomings of low yield, and reduced the cost of separation and purification of AHP. The expressed AHP does not need to be released by hydrolysis with a special protease, and the purified AHPM can be regarded as the final product to treat and prevent hypertension. This work provided a new strategy to produce a large quantity of antihypertensive peptides.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Media
E. coli BL21 (Novagen, Germany) was used as the host strain. Plasmid pGEX-3X (Amersham Biosciences, Sweden) was used as the expression vector. *E. coli* BL21 was grown in Lurea–Bertani (LB) medium (pH 7.2) consisting of 1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl. The fermention broth, which consisted of 1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 0.2% (w/v) KH_2PO_4, and 1.6% (w/v) $K_2HPO_4 \cdot 3H_2O$, was used for induction expression of GST–AHPM.

Enzymes and Reagents
All restriction enzymes and the Expand High Fidelity PCR system were purchased from Takara (Japan). Pepsin, pancreatin, angiotensin I-converting enzyme (ACE), and substrate peptide hippuryl-histidyl-leucine (HHL) were purchased from Sigma (U.S.A.). All reagents utilized in this study were purchased from Sigma (U.S.A.) unless indicated otherwise.

Design of Recombinant Antihypertensive Peptide Multimer Gene
Based on the reported achievements, the principles of choosing AHP for further study were determined as follows: (i) high antihypertensive activity *in vivo* and *in vitro*; (ii) it can be absorbed efficiently through the intestine in an active form without degradation, or decomposed into the higher active fragment by gastrointestinal proteases or ACE; (iii) easy to be released from the AHPM with gastrointestinal proteases, and the link fragments can improve the release rates of active peptide fragments.

Table 1. The chosen antihypertensive peptides according to the design principles described in the text.

Peptide sequence	IC_{50} (μM)	Dose (mg/kg)	SBP (mmHg)	Reference
VWIS	30	12.5	−12.5±2.9[a]	[19]
VW	3.3	1	−22[d]	[27]
	1.6	7.5	−10.8±2.7[a]	[19]
RIY	28	7.5	−11.3±1.8[b]	[19]
IY	6.1	1	−21[d]	[27]
	3.7	7.5	−9.8±2.1[a]	[19]
LW	6.8	60	−22[a]	[8]
	23.6	1	>−10[d]	[27]
IKW	0.21	60	−17[b]	[8]
LKPNM	2.4	10.5	−10	[9]
	2.4	60	−23[c]	[8]
LKP	0.32	4.2	−10	[9]
	0.32	60	−18[b]	[8]
RPLKPW	-	0.1	>−15[b]	[24, 32]
NMAINPSK	60	-	-	[30]
IPP	5	1	−15[c]	[23]

[a]Changes of systolic blood pressure in SHR at 2 h after oral administration.
[b]Changes of systolic blood pressure in SHR at 4 h after oral administration.
[c]Changes of systolic blood pressure in SHR at 6 h after oral administration.
[d]Changes of systolic blood pressure in SHR at 9 h after oral administration.

According to the principles above, different AHP monomers, as shown in Table 1, were chosen and tandemly multimerized to the AHPM with the link fragments QR, QER, QK, QY, and W. The amino acid sequence of the target polypeptide, with many restriction sites of gastrointestinal proteases, is shown in Fig. 1B. On the basis of the target favored codons of *E. coli*, the amino acid sequence of the AHPM was translated into the polynucleotide sequence (Fig. 1A), the BamHI cleavage site GGATCC was added to the 5' end of this sequence, and the EcoRI cleavage site GAATTC was added to the 3' end of this sequence. The final sequence was named as the Ahpm target gene.

Construction of the Recombinant Plasmid
The polynucleotide sequence Ahpm (Fig. 1A) encoding the tandem multimer AHPM (Fig. 1B) was chemically synthesized in light of the *E. coli* preferred codons by the "partially overlapping primer-based PCR" method (data not shown). The sequence contained BamHI and EcoRI restriction sites at the 5' and 3' ends, respectively. All recombinant DNA techniques were performed as described by Sambrook *et al.* [26]. According to the manufacturer's instructions, the chemically synthesized target gene Ahpm was cloned into the BamHI and EcoRI sites of pGEX-3X expression vector, and then the recombinant expression vector was confirmed by restriction analysis and sequencing (Biosune, China). The correct recombinant prokaryotic expression vector was named as pGEX-3X-Ahpm.

Expression and Recovery of GST and GST Fusion Protein
A single colony of *E. coli* BL21 transformed with pGEX-3X or pGEX-3X-Ahpm was inoculated into 20 ml of LB medium supplemented with ampicillin (100 μg/ml), and cultured at 37°C with sharp shaking (250 rpm) overnight. The overnight culture was diluted 1:50 into fresh
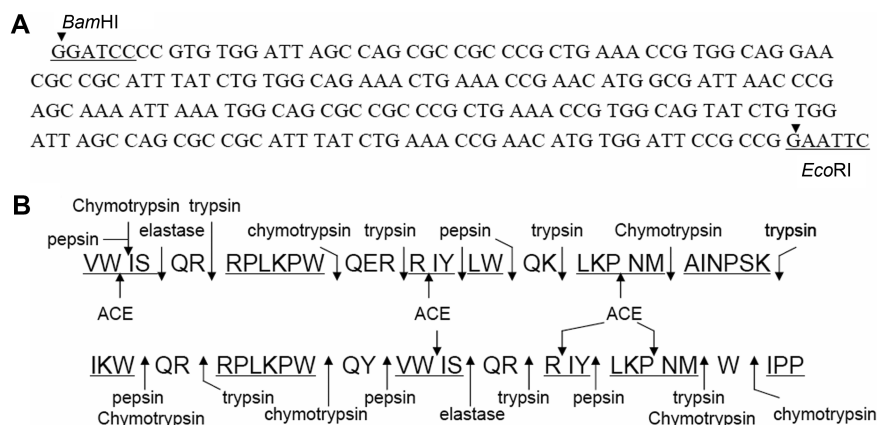
Fig. 1. The oligonuleotides sequence and amino acids sequence of the designed AHPM gene.
A. The oligonucleotides sequence of the designed AHPM gene containing *Bam*HI and *Eco*RI restriction sites at the 5' and 3' ends, respectively. **B**. The corresponding amino acids sequence of the designed AHPM gene. Arrows indicate potential restriction sites of gastrointestinal enzyme.

fermentation broth containing 100 µg/ml ampicillin, and grown at 30°C with vigorous shaking (250 rpm) until OD_{600} reached 0.6–0.8. Then, isopropyl-beta-D-thiogalactopyranoside (IPTG) was added to the culture to a final concentration of 0.4 mM, and the induced culture was grown for an additional 5 h. The cells were harvested and resuspended in 20 mM sodium phosphate buffer (PBS, pH 7.3) and collected by centrifugation at 10,000 ×g for 20 min. The cell pellets were weighed and stored at –20°C.

Pellets of the harvested cells were resuspended in lysis buffer A (50 mM Tris-HCl, 0.15 M NaCl, 1 mM EDTA, 0.5% Triton X-100). Subsequently, freezing and thawing were carried out at –20°C and 37°C, respectively, for three cycles. After that, the cells were homogenized by ultrasonic disruption for a total of 10 min at 300 W with pulse and interval times of 5 s and 5 s, respectively, for each duty cycle. The whole disruption process was operated in an ice bath. Disrupted cells were centrifuged at 16,000 ×g for 20 min at 4°C to separate soluble proteins and insoluble pellets. After centrifugation, the clear lysates and insoluble pellets were obtained and stored at –20°C, respectively, and analyzed by 12% SDS–PAGE.

Affinity Purification of GST
Affinity purification of GST was performed as described by Smith and Johnson [28]. After pre-equilibration three times with PBS, 1 ml of a 50% glutathione-agarose (Sigma, U.S.A.) beads suspension was added to 10 ml of the supernatant containing GST, and incubated with gentle agitation at room temperature for 30 min. After being centrifuged at 500 ×g for 5 min, the sedimented matrix was packed into a column and washed with three bed volumes of PBS. Then, GST was eluted with 15 mM reduced glutathione in 50 mM Tris-HCl (pH 7.3) and collected.

Purification of GST Fusion Protein Expressed as Inclusion Body
Frozen pellets (0.6 g wet weight) containing inclusion bodies were thawed and suspended in 25 ml of washing buffer B [50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 20 mM dithiothreitol (DTT), 4 M urea] and washed three times, and then resuspended in 25 ml of PBS (20 mM, pH 7.5). Partially purified inclusion bodies were dissolved in solution buffer C (20 mM PBS, pH 7.5, 8 M urea, 1 mM EDTA, 5% glycerol). After the addition of DTT to a final concentration of 0.1 M, the mixture was kept stirring continuously to solubilize proteins

at 4°C overnight. The suspension was centrifuged at 12,000 ×g for 20 min at 4°C to remove insoluble materials, and the supernatant was subjected to a 1-ml CM Sepharose Fast Flow column (Pharmacia Biotech, Sweden) pre-equilibrated with buffer C. After being rinsed with buffer C until no materials appeared in the effluent, the column was eluted with buffer D (1 M NaCl in buffer C) and the elution peak was collected. Fractions containing high pure target protein were pooled, and concentrated to about 10 mg/ml by ultrafiltration using Amicon Ultra-15 centrifugal filter devices (10 kDa NMWL; Millipore). Size-exclusion chromatography (SEC) was performed using a HiPrep 16/60 column packed with Sephacryl S-100 gel media (Pharmacia Biotech, Sweden). After pre-equilibration with buffer E (20 mM PBS, pH 7.5, 4 M urea, 0.15 M NaCl, 1 mM EDTA, 5% glycerol), the concentrated protein solution was applied to the column and eluted with the same equilibrating buffer as the sample volume of 2 ml. Subsequently, to remove urea and other materials, the eluted peak containing GST fusion proteins was dialyzed gradually at 4°C against a series of buffer E containing 3, 2, 1, and 0 M urea in sequence, and finally against 0.9% NaCl with a pH of 2.0 adjusted by adding 1 M HCl. After dialysis, the protein sample should immediately be subjected to the next-step two-stage hydrolysis process. The final protein concentration was about 1 mg/ml. All chromatography operations were carried out on an AKTA Purifier Workstation (Pharmacia, Sweden).

Simulated Gastrointestinal Digestion
After the purified GST (about 50 mg) and GST fusion protein (about 50 mg) had been dialyzed against 0.9% NaCl buffer, pH 2.0, the process of simulated gastrointestinal digestion was carried out according to Alting *et al.* [1] with some modifications. Two samples were first hydrolyzed with pepsin (enzyme: substrate ratio of 1:50, w/w) for 90 min at 37°C and pH 2.0, followed by hydrolysis with pancreatin (enzyme: substrate ratio of 1:25, w/w) for 240 min at 37°C and pH 7.5. Hydrolysis was carried out in a shaking bath, and the reactions were stopped by heating at 95°C for 10 min in a water bath, followed by cooling to room temperature. Subsequently, the reaction mixtures were centrifuged at 10,000 ×g for 20 min and the supernatants were subjected to ultrafiltration centrifugation using Amicon Ultra-15 centrifugal filter devices (3 kDa NMWL; Millipore, U.S.A.). The 3-kDa NMWL permeates were freeze-dried and kept at –20°C until further analysis.

Digestibility of Peptide by ACE

After peptic and pancreatic hydrolyses, the 3-kDa NMWL permeates (final concentration 1.2 mg/ml) were subjected to the incubation of ACE, with the method described by Fujita *et al.* [10]. A 25-μl ACE (3 mU) solution was added to 2 ml of sample. The mixture was incubated at 37°C for 4 h and the reaction was stopped by boiling for 10 min. After cooling in ice, the ACE inhibitory activity of the solution was assayed.

Measurement of ACE Inhibitory Activity

ACE inhibitory activity was determined by the spectrophotometric assay of Cushman and Cheung [6] with some modifications by Yamamoto *et al.* [34]. Briefly, the pH of each sample was adjusted to 8.3 by adding 1 M NaOH. A volume of 10 μl of each sample was added to 180 μl of 0.1 M borate buffer (pH 8.3) containing 0.3 M NaCl and 5.5 mM HHL preincubated at 37°C for 3 min, and then 10 μl of ACE (4 mU) (E.C. 3.4.15.1, 5.1 U/mg) solution (containing 0.1 M borate and 0.3 M NaCl, pH 8.3) was added, and the mixture was incubated at 37°C for 30 min. Borate buffer (0.1 M, pH 8.3) was used instead of ACE solution for the blank determination. The reaction was stopped by adding 0.2 ml of 1 M HCl. Then, 0.8 ml of ethyl acetate was added, mixed, and centrifuged at 6,000 ×g for 5 min. Ethyl acetate extraction solution was transferred to a test tube and dried at 80°C for 45 min. The hippuric acid was redissolved in 0.8 ml of distilled water. The absorbance was determined at 228 nm. The ACE inhibitory activity was calculated as follows:

Inhibition (%)=(C−S)×100/(C−B)

S : Absorbance of sample.

C: Absorbance of control (buffer for samples).

B: Absorbance of blank (hydrochloric acid was added before ACE).

The ACE-inhibitory activity was also calculated as the protein concentration to inhibit 50% of the original ACE activity (IC_{50}).

SDS−PAGE and Protein Assay

Protein analysis was performed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) as described by Laemmli [14]. Protein samples were boiled for 5 min under denaturing conditions and then centrifuged at 10,000 ×g for 5 min prior to loading the gel. Protein concentrations in the samples were determined by the Bradford method [3] with bovine serum albumin as a standard. Protein purity was estimated by densitometric analysis of protein bands separated by 12% SDS−PAGE after staining with Coomassie brilliant blue R-250.

Results

Construction of the Recombinant Plasmid and Expression Identification of the Recombinant AHPM

The Ahpm gene was inserted into the downstream of GST-encoding DNA in the same open reading frame at the BamHI and EcoRI restriction sites in the multiple cloning sites of pGEX-3X. Between GST and AHPM, there was a site-specific recognition sequence of Factor Xa for the release of intact AHPM.

The correct expression vector was confirmed by PCR, sequencing, restriction enzyme analysis, and SDS−PAGE analysis. The universal primers for the amplification of the Ahpm gene in the constructed expression vector were designed on the basis of nucleotide sequences of pGEX-3X, which are located at the upstream of the BamHI restriction site and the downstream of the EcoRI restriction site in the multiple cloning sites of pGEX-3X. The PCR product that was amplified by the above primers was 346 base pairs (bp), as shown in Fig. 2A, lane 1. After the PCR product was digested by BamHI and EcoRI, a fragment with a size of 206 bp (Fig. 2A, lane 2), which was the same as the predicted size of the target gene, was detected. The result indicated that the exogenous fragment of Ahpm was inserted into multiple cloning sites of pGEX-3X. Furthermore, this was further verified by DNA sequencing of the recombinant plasmid (data not shown).

E. coli BL21 transformed with the pGEX-3X vector produced a protein of 26 kDa (GST protein) after IPTG induction, as shown in Fig. 2B, lane 2. Similarly, *E. coli* BL21 transformed with the pGEX-3X-Ahpm produced a protein of around 34 kDa (GST−AHPM fusion protein) after IPTG induction (Fig. 2B, lane 4). The size of the fusion protein matched well with its theoretical molecular mass. In contrast, non-induced *E. coli* BL21 cells did not express this protein (Fig. 2B, lane 3). These results indicated that the recombinant AHPM was successfully expressed in the constructed *E. coli* BL21, as expected.
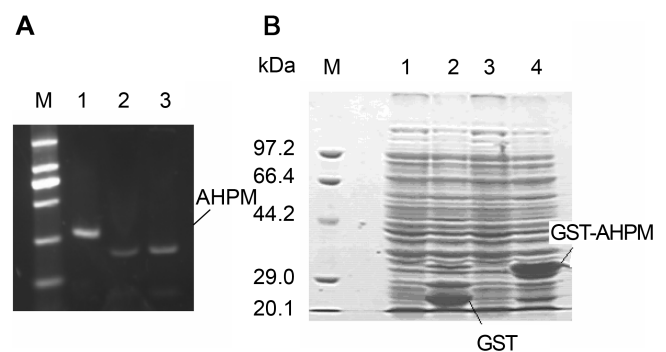

Fig. 2. Restriction enzyme analysis and expression identification of recombinant Ahpm gene.

A. Restriction enzyme analysis of the recombinant plasmid. M, DNA marker (DL2000: 2,000, 1,000, 750, 500, 250, 100); Lane 1, PCR product containing Ahpm nucleotide sequence (the universal primers of the pGEX vector used are follows: forward primer: 5'-GGGCTGGCAAGCCACGTT TGGTG-3'; reverse primer: 5'-CCGGGAGCTGCATGTGTCAGAGG-3'); Lanes 2, 3, cleavage of the PCR product by BamHI and EcoRI, target gene Ahpm is indicated. **B**. 12% SDS−PAGE analysis of the lysates obtained from *E. coli* BL21 after induction with 1 mM IPTG at 37°C. M, protein molecular mass marker; Lanes 1, 3, uninduced total protein of *E. coli* BL21 containing pGEX-3X and pGEX-3X-Ahpm, respectively. Lane 2, total protein of *E. coli* BL21 containing pGEX-3X induced with IPTG for 2 h. GST is indicated; Lane 4, induced total protein of *E. coli* BL21 containing pGEX-3X-Ahpm induced with IPTG for 2 h. GST−AHPM is indicated.

Expression and Purification of GST Fusion Protein

After 5 h of induction with 0.4 mM IPTG at 30°C, recombinant *E. coli* BL21 was grown by high-density and high-expression culture, and the cells were harvested when the wet weight of cells reached 9.5 g wet weight/l culture. As illustrated by the SDS–PAGE (Fig. 3), densitometric scanning of the stained gel demonstrated that GST–AHPM fusion protein accounted for approximately 35% of total cell proteins (Fig. 3, lane 2). SDS–PAGE analysis of soluble and insoluble fractions of the induced *E. coli* cell extracts indicated that the fusion proteins were mostly expressed as insoluble inclusion bodies (Fig. 3, lane 3, and lane 4). In this fraction, the yield of the target protein reached about 681.5 mg/l fermentation broth, as shown in Table 2. After being washed three times, the purity of the aimed protein in inclusion bodies was up to 70%, whereas it was 58% before washing. The washed inclusion bodies were mostly solubilized in buffer C, and purified by IEC under denaturing conditions. In this step, GST fusion proteins were obtained with 89% purity in the pooled fractions (Fig. 3, lane 8). After being further purified by SEC and dialyzed against 0.9% NaCl, pH 2.0, about 399 mg of GST fusion proteins with above 95% purity were obtained from a 1-l culture of induced cells (Fig. 3, lane 9). The fusion protein yield and purity during purification are summarized in Table 2.
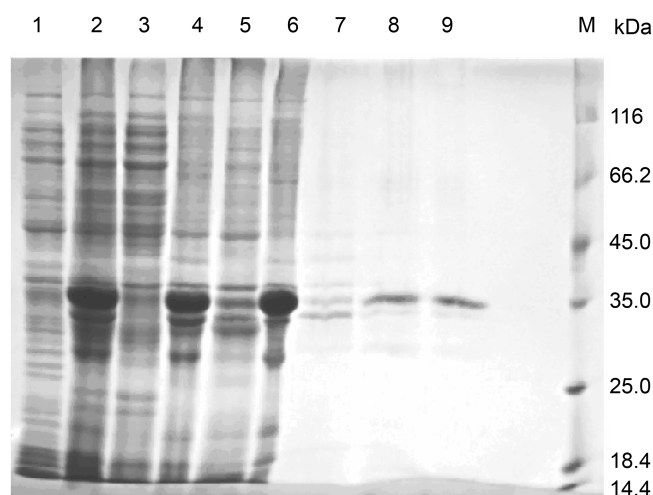

Fig. 3. SDS–PAGE analysis of the expression and purification of the GST fusion protein expressed as an inclusion body.
M, protein molecular mass marker (sizes are indicated in kDa at the right); Lane 1, Total cell proteins from uninduced *E. coli* BL21 containing pGEX-3X-AHPM; Lane 2, Total cell proteins from induced *E. coli* BL21 containing pGEX-3X-AHPM; Lane 3, Supernatant from centrifugation of induced sonicated cells; Lane 4, Pellet from centrifugation of induced sonicated cells; Lane 5, Soluble fraction separated after washing of inclusion bodies; Lane 6, Washed inclusion bodies; Lane 7, Pass-through fraction from cation-exchange chromatography; Lane 8, Pooled fractions from cation-exchange chromatography containing purified GST–AHPM; Lane 9, Pooled fractions from size-exchange chromatography containing purified GST–AHPM.

Table 2. Summary of purification of the GST fusion protein expressed as inclusion bodies.

Procedure	Total protein (mg)	Purity (%)	Target protein (mg)	Recovery (%)
Inclusion bodies (3 g wet weight)	1,175	58	681.5	100
Washed inclusion bodies	860	70	602	88.3
IEC	525	89	467.5	68.6
SEC	435	95	413.5	60.6
Dialysis	420	95	399	58.6

The wet weight of cells after IPTG induction for 5 h at 30°C was 9.5 g/l culture. The 3 g of wet weight inclusion bodies came from 1 l of fermentation broth.

Release of Antihypertensive Peptides in the AHPM

Whether the target active fragments in the AHPM can be released by gastrointestinal digestion enzyme and ACE *in vivo* was studied. Firstly, purified GST (data not shown) and purified GST fusion protein were subjected to a two-stage hydrolysis process that simulated gastrointestinal digestion, respectively. The 3-kDa NMWL permeate of each aliquot was obtained by ultrafiltration centrifugation after gastrointestinal digestion of protein sample. The 3-kDa NMWL permeate from the digestion hydrolysate of GST protein was acted as control. During the simulated physiological digestion of GST protein, the ACE inhibitory activity of the 3-kDa NMWL permeate was 12% after the hydrolysis with pepsin, and no significant change was observed after the hydrolysis with the pancreatic extract (14%), as shown in Table 3. In comparison, the ACE inhibitory activity of the 3-kDa NMWL permeate from the peptic hydrolysate of GST fusion protein reached 85%, which was much higher than the corresponding sample from GST. After further hydrolysis of peptic hydrolysate with pancreatin, the ACE inhibitory activity of the 3-kDa NMWL permeate increased to 91%, which indicated that higher molecular mass peptides in the retentate fraction or precursor peptides in the permeate contributed to the ACE inhibitory activity. The results also showed that the ACE inhibitory activity of the 3-kDa NMWL permeate from the gastrointestinal digestion hydrolysate of GST fusion protein reached 0.1 mg/ml (IC_{50} value). After the two-phase

Table 3. ACE inhibitory activities of the 3-kDa NMWL permeates from the digestion hydrolysates of GST and GST fusion protein, respectively.

Enzyme used for hydrolysis on stage	ACE inhibitory activity of sample (%)	
	GST	GST fusion protein
Pepsin	12	85
Pancreatin	14	91
ACE	15	95

hydrolysis, the 3-kDa NMWL permeate from GST–AHPM was preincubated with ACE before measurement of ACE inhibitory activity. The results indicated that the ACE inhibitory activity of the 3-kDa NMWL permeate from GST–AHPM increased from 91% to 95% as ACE occurring cleavage action. Data suggested that prodrug-type ACE inhibitory peptides in the permeate were hydrolyzed by ACE and released active fragments with more high activities. It can be concluded that the active fragments in the AHPM can be released by enzymatic proteolysis during gastrointestinal digestion and ACE incubation.

DISCUSSION

As shown in Table 1, it has been confirmed that the chosen AHPs can remarkably lower the blood pressure of spontaneously hypertensive rats (SHRs) at a very low dose after oral administration [8, 9, 19, 23, 24, 27, 32]. In our recombinant polypeptide sequence, both the Trp–Gln bond and the Arg–Arg bond were easy to be cleaved by chymotrypsin and trypsin in pancreatin, respectively [24]. Analogously, QR and QER were chosen as link fragments in this research. The link fragments QR and QER were designed to locate between one peptide with Trp residue on the C-terminal and another peptide with Arg on the N-terminal. For example, QR was set between IKW and RPLKPW, and similarly QER was set between RPLKPW and RIYLW, so that the C-terminal or N-terminal of these peptides was easily exposed to the degradation of trypsin and chymotrypsin in pancreatin. In the same way, we designed QK, QY, and W as link fragments to easily release the target active peptides. VWIS, RIY, and LKPNM are regarded as prodrug-type ACE inhibitory peptides, which can be respectively hydrolyzed by ACE to produce VW, IY, and LKP, exerting more potent antihypertensive activity *in vivo* [8, 19]. Perhaps the active fragments can not be entirely released from the recombinant AHPM as expected, but according to our design principles, the designed polypeptide possesses a huge potential to lower the blood pressure after being orally administrated. In fact, the entire AHPM can also be regarded as a precursor of multiple kinds of AHPs. It can be explained that the AHPMs have a high content of AHPs with potent antihypertensive activities and the restriction sites of digestive enzyme and ACE. Our results also verified that many ACE inhibitory peptides were released from the AHPM after simulated gastrointestinal digestion and ACE hydrolysis.

It is well known that short peptide fragments are easily degraded by proteases in the engineered strains when produced by recombinant DNA technology [18]. In this study, we adopted *E. coli* BL21 as host bacteria, a protease-deficient strain [29], and chose the pGEX-3X expression

vector containing the GST expression system to improve the expression level of the AHPM. Although the GST expression system is designed for inducible, high-level intracellular expression of genes as soluble and stable fusion proteins [28], many factors influence the expression of the fusion protein. In our research, GST fusion proteins mostly accumulated in the form of inclusion bodies. In order to reduce the formation of insoluble aggregates, shorter induction periods, lower induction temperature, lower IPTG concentration, and other strategies were performed, but the expression level of soluble fusion proteins was still very low (data not shown). This phenomenon may be partly due to the strong hydrophobic nature of the AHPM, which contains a high ratio of hydrophobic amino acids such as Ile, Leu, and Pro. The hydrophobic nature of GST fusion proteins may contribute to the formation of inclusion bodies [2]. GST fusion proteins expressed as inclusion bodies accounted for approximately 35% of total cell proteins in this work (Fig. 3, lane 3), so we considered performing the denaturation and renaturation of inclusion bodies. According to the amino acids sequence, we forecasted that GST–AHPM is a cation protein with isoelectric point of 9.29, by "Compute pI/Mw tool" (http://www.expasy.ch/tools/pi_tool.html). Therefore, in the first step, cation-exchange chromatography was used for separation and purification of GST fusion proteins in inclusion bodies under denaturing conditions, and the fusion proteins were obtained with 89% purity and 68.6% recovery in the pooled fractions (Fig. 3, lane 8). Secondly, size-exclusion chromatography was performed for refolding in part and further purification, followed by gradual dialysis for further renaturation. During the process of dialysis refolding, a part of the GST fusion proteins formed flocculent precipitate. After adjusting the pH to 2 by adding HCl, the precipitate redissolved, and then soluble GST fusion proteins were obtained with 95% purity.

In conclusion, we have provided a new strategy and established a procedure to obtain a large quantity of antihypertensive peptides by DNA recombinant technology. The main advantage of this approach concerns the capacity to produce high yields of AHPs with potent antihypertensive activities. Furthermore, the purified GST–AHPM or AHPM can be directly used as food additives in functional foods as well as drugs for treating and preventing hypertension. In fact, it is a precursor peptide containing a lot of AHP monomers, which can be released by digestive enzymes and ACE in the human body after administration. Thus, AHP monomers do not need to be released by a special protease, and the cost used to separate and purify the AHP can be greatly reduced. Further animal trials are required to determine the safety and potential antihypertensive effect of GST-AHPM or AHPM. Efforts are currently under way to evaluate the release rate of the active peptides from AHPM by digestive enzymes and ACE by qualitative and quantitative analysis.

Design and expression of a new AHPM, from which active peptides would be released with higher efficiency by digestive enzymes and ACE, are now in progress. Further experiments will focus on directly secretory expression of the new AHPM in food-grade expression systems, as well as separation and purification of the expression product AHPM using routine means such as ultrafiltration and ion exchange chromatography. These studies will greatly promote industrial production of AHPs.

Acknowledgments

REFERENCES

1. Alting, A. C., R. J. Meijer, and E. C. van Beresteijn. 1997. Incomplete elimination of the ABBOS epitope of bovine serum albumin under simulated gastrointestinal conditions of infants. *Diabetes Care* **20:** 875–880.

2. Bettadapura, J., K. K. Menon, S. Moritz, J. Liu, and C. C. Bernard. 1998. Expression, purification, and encephalitogenicity of recombinant human myelin oligodendrocyte glycoprotein. *J. Neurochem.* **70:** 1593–1599.

3. Bradford, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

4. Byun, H. G. and S. K. Kim. 2001. Purification and characterization of angiotensin I converting enzyme (ACE) inhibitory peptides from Alaska pollack (*Theragra chalcogramma*) skin. *Process Biochem.* **36:** 1155–1162.

5. Chen, G. W., J. S. Tsai, and B. S. Pan. 2007. Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. *Int. Dairy J.* **17:** 641–647.

6. Cushman, D. W. and H. S. Cheung. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. *Biochem. Pharmacol.* **20:** 1637–1648.

7. FitzGerald, R. J., B. A. Murray, and D. J. Walsh. 2004. Hypotensive peptides from milk proteins. *J. Nutr.* **134:** 980–988.

8. Fujita, H., K. Yokoyama, and M. Yoshikawa. 2000. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *J. Food Sci.* **65:** 564–569.

9. Fujita, H. and M. Yoshikawa. 1999. LKPNM: A prodrug-type ACE-inhibitory peptide derived from fish protein. *Immunopharmacology* **44:** 123–127.

10. Fujita, H., K. Yokoyama, and M. Yoshikawa. 2000. Classification and antihypertensive activity of angiotensin I-converting enzyme inhibitory peptides derived from food proteins. *J. Food Sci.* **65:** 564–569.

11. Glasser, S. P. 2001. Hypertension syndrome and cardiovascular events. *Postgrad. Med.* **110:** 29–36.

12. Israaili, Z. H. and W. D. Hall. 1992. Cough and angioneurotic edema associated with angiotensin converting enzyme inhibitor therapy: A review of the literature and pathophysiology. *Ann. Intern. Med.* **117:** 234–242.

13. Jeong, D. W., D. S. Shin, C. W. Ahn, I. S. Song, and H. J. Lee. 2007. Expression of antihypertensive peptide, His-His-Leu, as tandem repeats in *Escherichia coli*. *J. Microbiol. Biotechnol.* **17:** 952–959.

14. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.

15. Li, G. H., G. W. Le, and Y. H. Shi. 2004. Shrestha, angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. *Nutr. Res.* **24:** 469–486.

16. Liu, D., H. Y. Sun, L. J. Zhang, and S. M. Li. 2007. High-level expression of milk-derived antihypertensive peptide in *Escherichia coli* and its bioactivity. *J. Agric. Food Chem.* **55:** 5109–5112.

17. Lv, G. S., G. C. Huo, and X. Y. Fu. 2003. Expression of milk-derived antihypertensive peptide in *Escherichia coli*. *J. Dairy Sci.* **86:** 1927–1931.

18. Makrides, S. C. 1996. Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiol. Rev.* **60:** 512–538.

19. Marczak, E. D., H. Usui, H. Fujita, Y. J. Yang, M. Yokoo, A. W. Lipkowski, and M. Yoshikawa. 2003. New antihypertensive peptides isolated from rapeseed. *Peptides* **24:** 791–798.

20. Megias, C., M. D. M. Yust, J. Pedroche, H. Lquari, J. Giron-Calle, M. Alaiz, F. Millan, and J. Vioque. 2004. Purification of an ACE inhibitory peptide after hydrolysis of sunflower (*Helianthus annuus* L.) protein isolates. *J. Agric. Food Chem.* **52:** 1928–1932.

21. Megias, C., J. Pedroche, M. D. M. Yust, M. Alaiz, J. Giron-Calle, F. Millan, and J. Vioque. 2006. Affinity purification of angiotensin converting enzyme inhibitory peptides using immobilized ACE. *J. Agric. Food Chem.* **54:** 7120–7124.

22. Murray, B. A. and R. J. FitzGerald. 2007. Angiotensin converting enzyme inhibitory peptides derived from food proteins: Biochemistry, bioactivity and production. *Curr. Pharm. Des.* **13:** 773–791.

23. Nakamura, Y., N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki, and T. Takano. 1995. Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk. *J. Dairy Sci.* **78:** 777–783.

24. Onishi, K., N. Matoba, Y. Yamada, N. Doyama, N. Maruyama, S. Utsumi, and M. Yoshikawa. 2004. Optimal designing of beta-conglycinin to genetically incorporate RPLKPW, a potent anti-hypertensive peptide. *Peptides* **25:** 37–43.

25. Park, C. J., J. H. Lee, S. S. Hong, H. S. Lee, and S. C. Kim. 1998. High-level expression of the angiotensin-converting-enzyme-inhibiting peptide, YG-1, as tandem multimers in *Escherichia coli*. *Appl. Microbiol. Biotechnol.* **50:** 71–76.

26. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, U.S.A.

27. Sato, M., T. Hosokawa, T. Yamaguchi, T. Nakano, K. Muramoto, T. Kahara, K. Funayama, A. Kobayashi, and T. Nakano. 2002. Angiotensin I-converting enzyme inhibitory peptides derived from wakame (*Undaria pinnatifida*) and their antihypertensive effect in spontaneously hypertensive rats. *J. Agric. Food Chem.* **50:** 6245−6252.

28. Smith, D. B. and K. S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67:** 31−40.

29. Studier, F. W. and B. A. Moffatt. 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189:** 113−130.

30. Tauzin, J., L. Miclo, and J. L. Gaillard. 2002. Angiotensin-I-converting enzyme inhibitory peptides from tryptic hydrolysate of bovine alpha(S2)-casein. *FEBS Lett.* **531:** 369−374.

31. Wang, J. P., J. E. Hu, J. Z. Cui, X. F. Bai, Y. G. Du, Y. Miyaguchi, and B. C. Lin. 2008. Purification and identification of a ACE inhibitory peptide from oyster proteins hydrolysate and the anti hypertensive effect of hydrolysate in spontaneously hypertensive rats. *Food Chem.* **111:** 302−308.

32. Yamada, Y., N. Matoba, H. Usui, K. Onishi, and M. Yoshikawa. 2002. Design of a highly potent anti-hypertensive peptide based on ovokinin(2-7). *Biosci. Biotech. Biochem.* **66:** 1213−1217.

33. Yamamoto, N., M. Maeno, and T. Takano. 1999. Purification and characterization of an antihypertensive peptide from a yogurt-like product fermented by *Lactobacillus helveticus* CPN4. *J. Dairy Sci.* **82:** 1388−1393.

34. Yamamoto, S., I. Toida, and K. Iwai. 1980. Re-examination of the spectrophotometric assay for serum angiotensin-converting enzyme. *Nippon Kyobu Shippei Kaishi* **18:** 297−303.