JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# High-Level Expression of Milk-Derived Antihypertensive Peptide in *Escherichia coli* and Its Bioactivity

DONG LIU,* HAIYAN SUN, LIJUN ZHANG, SHIMIN LI, AND ZHENYU QIN

Department of Applied Biotechnology, Shenzhen Polytechnic, Shenzhen, Guangdong 518055, People's Republic of China

An optimal antihypertensive peptide (AHP), KVLPVP, was linked to form a six-copy of tandem dotetracontapeptide with the specific cleavage site (Arg-X) of clostripain. The gene of the dotetracontapeptide was synthesized and expressed in *Escherichia coli* BL21. After a 5 h induction with 1.2 mM isopropyl-$\beta$-D-thiogalactopyranoside the recombinant AHP fused with glutathione-S-transferase tag reached the maximal production, 24.6% of total intracellular protein. Following digestion with clostripain and carboxypeptidase B, the product was separated with ultrafiltration and reversed-phase HPLC, and 170 mg of pure recombinant AHP was obtained from 1 L of *E. coli* culture. The $IC_{50}$ of the recombinant AHP was 4.6 $\mu$M. The systolic blood pressure of spontaneously hypertensive rats could be decreased dramatically by the recombinant AHP in a dose-dependent manner after delivering 0.3 mg of AHP/kg of body weight (BW) or 0.6 mg of AHP/kg of BW orally. The strong antihypertensive effect was reached 4−24 h after oral administration of 0.3 mg of AHP/kg of BW, and the peak point was at the fourth hour (−21.4 ± 7.2 mm of Hg). This study overcame traditional enzymatic digestion problems in preparing AHP and established a novel approach for industrial production of AHP.

**KEYWORDS: Antihypertensive peptide; expression; bioactivity**

## INTRODUCTION

Hypertension is a major risk factor for the development of serious disease including stroke, myocardial infarction, coronary heart disease, and nephropathy. The renin angiotensin system plays a key role in blood pressure regulation, and angiotensin-converting enzyme (ACE, EC 3.4.15.1) is the pivotal enzyme of the system. ACE inhibitors can lower blood pressure by inhibiting the activity of ACE. Now some synthetic ACE inhibitors, such as captopril and enalapril, have been widely used in the clinical therapy of hypertension. In recent years, antihypertensive peptides (AHP) derived from food proteins have been paid considerable attention, due to their good antihypertensive effects, safety, mild effects on humans, and potential use as health foods and pharmaceutical preparations (*1*, *2*). By far, a number of AHP have been isolated, purified, and identified from different natural food sources (*3*−*5*). Among all the literature available, almost all the AHP was obtained by means of enzymatic hydrolysis (*6*). However, due to the low content of AHP in natural food proteins, few reports are available about the commercial production of AHP. Industrial production of proteins by DNA recombinant technology has gained great success; it could be the most promising method for mass production of AHP. But few methods for high-level gene expression have been developed for the production of small peptides because of the low production yield and high cost of purification (*7*, *8*).

In the present paper, we successfully established an efficient *Escherichia coli* expression system of AHP, and the bioactivity of the recombinant AHP in vivo and in vitro was studied systematically.

## MATERIALS AND METHODS

**Reagents and Microorganism.** *BamH*I, *Sal*I, $T_4$DNA ligase, and Taq DNA polymerase were purchased from Promega Corp., Madison, WI; QIAprep Spin miniprep kit and QIAquick gel extraction kit were obtained from QIAGEN Inc., Valencia, CA; hippuryl-L-histidyl-L-leucine (Hip-His-Leu), ACE, clostripain (EC 3.4.22.8), carboxypeptidase B, hippuric acid, and trifluoroacetic acid (TFA) were purchased from Sigma Corp., St. Louis, MO; pGEX-4T-2 expression vector [a fusion expression vector with a glutathione-S-transferase (GST) affinity tag] and *E. coli* BL21 were obtained from Amersham Biosciences Corp., Piscataway, NJ; PCR primer was synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Shanghai, P. R. China, (the forward primer was 5′GGGCTGGCAAGCCACGTTTGGTG 3′ and the reverse primer was 5′CCGGGAGCTGCATGTGTCAGAGG 3′). Other chemicals were obtained from the P. R. China domestic market.

**Experimental Animal.** Male spontaneously hypertensive rats (SHR), 12 weeks of age, 250−300 g of body weight (BW), were purchased from Shanghai Slac Laboratory Animal Co. Ltd., Shanghai, P. R. China. The rats were fed with standard low-fat chow and accessed water freely with 25 ± 1 °C of environmental temperature, 55% ± 5% of relative humidity, and regular light supply. The rats were chosen when their systolic blood pressure (SBP) was higher than 180 mm of Hg. The experiments were performed in rats after 1 week of their training in the animal facility. This experiment was approved by the animal committee of the college.

* To whom correspondence should be addressed. Phone: 0086-0755-26019170. Fax: 0086-0755-26019169. E-mail: liudongszpt@163.com.

10.1021/jf0703248 CCC: $37.00 
Published on Web 05/17/2007

**Selection of High-Bioactivity Single AHP and Design of the Multicopy Sequence.** Following the review of AHP published in the literature, the following principles were applied to choose the optimal AHP for further study: (1) high in vitro ACE inhibitory activity and high in vivo antihypertensive activity; (2) the number of amino acid residues should be higher than five since it is technically difficult to express the peptide with lower molecular weight; (3) the multicopy peptide should contain the proper cleavage site of the specific proteinase (clostripain was used in this study); however, the single amino acid sequence should not contain the same cleavage site.

On the basis of these principles, a hexapeptide, KVLPVP, was chosen as the target AHP for further study. This peptide was first purified and identified from the milk casein hydrolysate by Maeno et al. (*9*). The $IC_{50}$ of this AHP was 5 $\mu$M; after oral administration of 1 mg of peptide/kg of BW, the SBP was decreased 32.2 $\pm$ 1.4 mm of Hg at 6 h in SHR, which indicates that the peptide has high bioactivity in vivo as well as in vitro.

In order to enhance the expression yield of this AHP in the *E. coli* expression system, the single AHP peptide was linked to form a six-copy of tandem peptide (dotetracontapeptide) with the specific cleavage site (Arg-X) of clostripain, and the amino acid sequence of this dotetracontapeptide was as follows: Lys-Val-Leu-Pro-Val-Pro-Arg-Lys-Val-Leu-Pro-Val-Pro-Arg-Lys-Val-Leu-Pro-Val-Pro-Arg-Lys-Val-Leu-Pro-Val-Pro-Arg-Lys-Val-Leu-Pro-Val-Pro-Arg-Lys-Val-Leu-Pro-Val-Pro-Arg (which is equal to KVLPVPRKVLPVPRKVLPVPRKVLPVPRKVLPVPRKVLPVPR).

**Construction of Recombinant AHP Expression System.** pGEX-4T-2 is a high-efficiency expression vector which can express fusion protein higher than 20% of total intracellular protein in *E. coli*. This vector also contains the GST gene which helps in preventing the peptide degradation in host *E. coli*.

On the basis of the favored codons of *E. coli*, the amino acid sequence of the dotetracontapeptide was translated into the polynucleotide sequence. *BamH*I cleavage site GGATCC was added to the 5′ end of this sequence, and stopping codon and *Sal*I cleavage site GTCGAC were added to the 3′ end of this sequence. Therefore, the final sequence, named as the *AHP* target gene, was as follows: 5′GGATCCAAAGTGCTGCCGGTGCCGCGTAAAGTGCTGCCGGTGCCGCGTAAAGTGCTGCCGGTGCCGCGTAAAGTGCTGCCGGTGCCGCGTAAAGTGCTGCCGGTGCCGCGTAAAGTGCTGCCGGTGCCGCGTAAAGTGCTGCCGGTGCCGCGTTGAGGGTCGAC3′.

According to the protocol described by Sambrook and Russel (*10*), the *AHP* target gene (synthesized by Shanghai Sangon Biological Engineering Technology and Service Co., Ltd, Shanghai, P. R. China) was cloned into the site between the *BamH*I and *Sal*I sites of pGEX-4T-2 generating the pGEX-4T-2-*AHP* recombinant plasmid. This recombinant plasmid was then transformed into *E. coli* BL21. The colonies that grew on LB agar medium containing 100 $\mu$g/mL ampicillin (Amp) were picked to purify recombinant plasmids. The recombinant plasmids were identified by double cleavage of *Bam*HI and *Sal*I and sequenced (sequenced by TaKaRa Biotechnology Co. Ltd., Dalian, P. R. China).

**Expression of Recombinant AHP.** The *E. coli* BL21 containing the pGEX-4T-2-*AHP* recombinant plasmid was inoculated into 25 mL of LB liquid medium with 100 $\mu$g/mL of Amp. After incubation at 37 °C overnight in a shaking incubator, 2.5 mL of culture was transferred to another 25 mL of LB liquid medium containing 100 $\mu$g/mL of Amp. When the OD reached 1.5, isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) was added with different final concentrations (0.2, 0.4, 0.8, 1.0, 1.2, 1.6, 2.0, and 2.4 mM) to induce the fusion protein for different periods (1, 2, 3, 4, 5, and 6 h). The expression level of GST−AHP was evaluated using SDS−PAGE.

**Purification of Recombinant AHP.** The induced cells were harvested by centrifuging at 12 000*g* for 5 min, and the pellet was resuspended to a concentration of 0.3 mg of cell/mL in 0.1 M Tris−HCl; 200 mL of mixture was sonicated (Sonics and Materials Inc., Newtown, CT) for 40 min. Following centrifugation again at 12 000*g* for 10 min, the supernatant was collected and ultrafiltered with molecular weight cutoff 50 and 10 kDa membranes (Millipore Corp., Billerica, MA) sequentially. The filtrate (10 to ~50 kDa) was digested by clostripain as the following condition: 100 mL of filtrate containing 50 mg of protein was incubated with 0.2 mg of clostripain at 25 °C for 6 h (pH 7.6). Following heating at 100 °C for 5 min, the mixture was further ultrafiltered with a 1 kDa ultrafilter membrane. The permeate was then digested by carboxypeptidase B as the following condition: 100 mL of permeate containing 7 mg of protein was incubated with 0.2 mg of carboxypeptidase B at 37 °C for 5 h. Following heating at 100 °C for 5 min, the mixture was purified with reversed-phase HPLC (Agilent 1100, Agilent Technologies Inc., Palo Alto, CA) as the following optimized condition: reversed-phase column Everest C18 (4.6 mm × 250 mm, Thermo Electron Corp., Waltham, MA), elution solvent contained 12% (v/v) acetonitrile (ACN) and 0.05% (v/v) of TFA, the flow rate was 1.0 mL/min, and detection was at 215 nm .

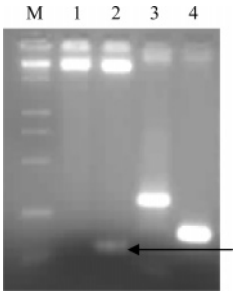


**Figure 1.** Identification of recombinant vector: M, DNA marker (15 000, 10 000, 7500, 5000, 2500, 2000, 1000, 750, 500, 250, and 100 bp); lane 1, cleavage of pGEX-4T-2 by *BamH*I and *Sal*I; lane 2, cleavage of pGEX-4T-2-AHP by *BamH*I and *Sal*I, target gene shown by arrow; lane 3, PCR of pGEX-4T-2-AHP; lane 4, PCR of pGEX-4T-2.

The synthesized peptide KVLPVP (>98% purity, by L. (Xian) Bioscientific Co., Ltd., Xian, P. R. China) was used as the standard to measure the amount of purified AHP. Following freeze-drying by ALPHA 2-4 freeze dryer (MARTIN CHRIST Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany), the purified recombinant AHP was made as a powder and stored at −80 °C for further study.

**Measurement of ACE Inhibitory Activity.** The ACE inhibitory activity was measured according to the method of Chen and Xu (*11*) with some modification. Each 200 $\mu$L of AHP solution was preincubated with the Hip-His-Leu borate buffer (5 mM Hip-His-Leu, 0.1 M borate, and 0.3 M NaCl; pH 8.3) at 37 °C for 5 min, then 20 mU of ACE was added. The reaction was performed at 37 °C for 30 min and then terminated by boiling for 5 min. The content of hippuric acid was determined by reversed-phase HPLC as the following condition: reversed-phase column Everest C18, elution by a linear gradient from 30% solvent A (99.9% ACN and 0.1% TFA) to 40% solvent B (5% ACN, 0.1% TFA, and $H_2O$) over 30 min at a flow rate of 1.0 mL/min, and detection at 215 nm. The activity of an AHP ($IC_{50}$) was defined as the amount of peptide causing 50% inhibition of ACE activity.

**Measurement of Blood Pressure.** SBP of SHR were measured as follows. Rats that had been given each peptide by oral administration were kept at 45 °C for 5 min, and the SBP were measured by the tail cuff with a programmed noninvasive blood pressure controller (model ML125, AD Instruments Pty Ltd., Australia). A 0.9% (w/w) NaCl saline solution and a 0.9% saline solution containing captopril were used as a negative control and a positive control, respectively, in SHR, and the antihypertensive effects of the purified recombinant peptide dissolved in this saline solution were measured. Data were expressed as the means $\pm$ SD and were analyzed statistically using Student's *t* test with a significance level of $P < 0.05$.

## RESULTS

**Identification of Recombinants.** To confirm the *AHP* gene had been correctly inserted into the vector, enzymatic digest, PCR, and gene sequencing were used. **Figure 1** shows that following enzymatic digestion of the recombinant plasmid pGEX-4T-2-*AHP*, a fragment with a size of 126 base pairs (bp) was detected, which was the same as the predicted size of the *AHP* gene. The sizes of the PCR amplification of the empty

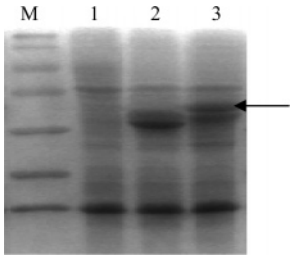


**Figure 2.** SDS–PAGE analysis of expression of recombinant protein induced by isopropyl-$\beta$-D-thiogalactopyranoside with final concentration of 0.4 mM for 3 h: M, protein molecular weight marker (14.4, 18.4, 25.0, 35.0, 45.0, 66.2, and 116.0 kDa); lane 1, *E. coli* BL21; lane 2, *E. coli.* BL21 (pGEX-4T-2); lane 3, *E. coli.* BL21 (pGEX-4T-2-*AHP*), desired fusion protein shown by arrow.

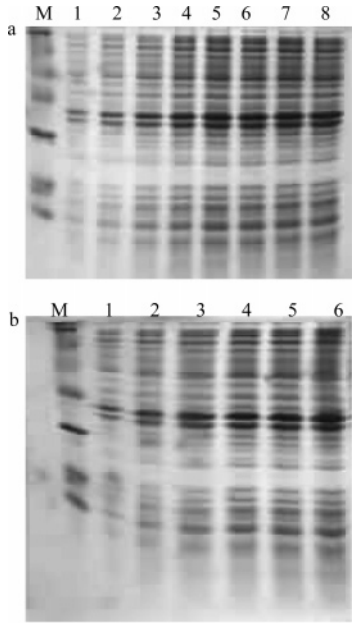


**Figure 3.** SDS–PAGE analysis of expression of the recombinant antihypertensive peptide (AHP) (fused with glutathione-S-transferase (GST)) with different inducing conditions; (**a**) expression of fusion protein induced with different isopropyl-$\beta$-D-thiogalactopyranoside (IPTG) concentrations at 4 h and (**b**) expression of fusion protein at different times after 1 mM IPTG induction. M, protein molecular weight marker (14.4, 18.4, 25.0, 35.0, 45.0, 66.2, and 116.0 kDa); 1, 2, 3, 4, 5, 6, 7, 8 in (**a**) represent induced by IPTG with final concentrations of 0.2, 0.4, 0.8, 1.0, 1.2, 1.6, 2.0, and 2.4 mM, respectively; 1, 2, 3, 4, 5, 6 in (**b**) represent induced by 1 mM IPTG at 1, 2, 3, 4, 5, and 6 h, respectively.

vector and recombinant plasmid were 173 and 300 bp, respectively, which indicated that the exogenous fragment of *AHP* had been inserted into multiple cloning sites of pGEX-4T-2. This had also been confirmed by gene sequencing of the recombinant plasmid.

**Figure 2** shows that after *E. coli* induced by 0.4 mM of IPTG for 3 h, fusion protein GST−AHP with molecular weight of 31 kDa was detected, which indicated that the recombinant AHP was successfully expressed in constructed *E. coli* BL21.

**Expression and Purification of Recombinant AHP.** To optimize the condition of expression of recombinant AHP, the recombinant *E. coli* BL21 with pGEX-4T-2-*AHP* plasmid was induced for 4 h with different concentrations of IPTG (0.2, 0.4, 0.8, 1.0, 1.2, 1.6, 2.0, and 2.4 mM). As shown in **Figure 3a**, with the increased concentration of IPTG, the expression level of fusion protein was increased and reached the plateau when the concentration of IPTG was 1.2 mM. In another experiment, the recombinant *E. coli* BL21 was induced with 1.0 mM IPTG for different time courses (1, 2, 3, 4, 5, and 6 h). As shown in **Figure 3b**, with the increased induction time, the expression of fusion protein was increased and reached the plateau after a 5 h induction. Therefore, the optimized condition for maximum yield of GST−AHP was to induce the recombinant strains with 1.2 mM of IPTG for 5 h. Under this condition, the amount of expressed fusion protein was 24.6% of total intracellular protein of *E. coli* (data not shown).

After purification as described under Materials and Methods, 170 mg of single recombinant AHP (KVLPVP), whose purity was greater than 98%, was collected from 1 L of culture medium of recombinant *E. coli*. The peptide sequence was also confirmed by sequencing by Shenzhen Hybio Engineering Co., Ltd., Shenzhen, P. R. China.

**Bioactivity of Recombinant AHP.** To confirm the bioactivity, the in vitro inhibitory activity of ACE and in vivo antihypertensive effect were measured. The $IC_{50}$ of recombinant AHP (KVLPVP) was 4.6 $\mu$M. Different doses of recombinant AHP (0.15, 0.3, and 0.6 mg/kg of BW) were given to SHR. As shown in **Table 1**, the SBP was not significantly decreased with AHP at 0.15 mg/kg of BW. However, the SBP was significantly decreased at 0.3 and 0.6 mg/kg of BW in a dose-dependent manner compared with that of the negative control group. These studies indicated that recombinant AHP developed in this study had the same in vitro ACE inhibitory activity and antihypertensive activity as AHP purified from natural milk $\beta$-casein.

At 0, 2, 4, 6, 8, 10, 12, 16, 20, 24, 28, and 32 h after oral administration of the recombinant AHP, the SBP of SHR was measured (**Figure 4**). The SBP was significantly decreased at a dosage of 0.3 mg/kg of BW at 4, 6, 8, 10, 12, 16, 20, and 24 h after administration (−21.4 ± 7.2, −20.5 ± 9.3, −18.7 ±

**Table 1.** Antihypertensive Effects of Different Dosages of Recombinant Antihypertensive Peptide ($\bar{X} \pm$ SD, $n = 8$)

| groups | dosage (mg/kg of BW) | decrease of SBP ($\Delta$mm of Hg)[a] |
|---|---|---|
| negative control | | −9.3 ± 6.6 |
| AHP | 0.6 | −29.2 ± 9.8 a |
| AHP | 0.3 | −20.6 ± 7.9 b |
| AHP | 0.15 | −12.7 ± 7.4 c |
| captopril | 10.0 | −22.4 ± 7.1 b |

[a] Letters indicate the level of significant difference from control: a, $P < 0.001$; b, $P < 0.01$; c, $P < 0.10$

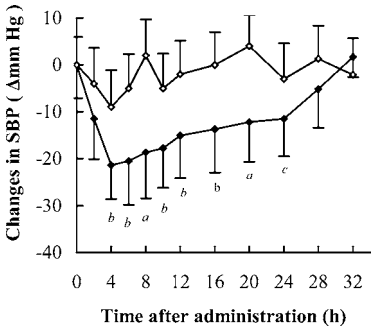


**Figure 4.** Antihypertensive effect of a single oral administration of recombinant antihypertensive peptide (AHP) in spontaneously hypertensive rats (SHR) within 32 h. Each point is the mean of the changes of systolic blood pressure (SBP) of eight SHR; the vertical bars represent standard errors. The negative control (◇) was SBP containing 0.9% NaCl; the treatment dose was 0.3 mg of the recombinant AHP/kg of BW (◆). Letters indicate the level of significant difference from control: *a*, $P < 0.001$; *b*, $P < 0.01$; *c*, $P < 0.05$.

9.7, −17.8 ± 8.4, −15.1 ± 9.0, −13.7 ± 9.2, −12.2 ± 8.5, and −11.5 ± 8.0 mm of Hg, respectively), and the effect was maximal at 4 h after oral administration. No changes of SBP of the negative control rats occurred during the 32 h after administration, and SBPs between the negative control and experimental rats were not significantly different at 2, 28, and 32 h after administration.

## DISCUSSION

The advantages of microbiological genetic engineering techniques to prepare AHP include higher protein yield and lower cost, as compared with those of the traditional enzymatic digestion method from natural food protein to prepare AHP. However, the following problems of this technique have been noticed. (1) The numbers of amino acid of the peptide which have higher activity of antihypertension is between 2 and 12 (*12*), and peptide with low molecular weight is easily degraded in the host bacteria and loses the antihypertension activity. (2) The expression of peptide with low molecular weight is lower. In our study, in order to increase the protein expression and avoid the degradation, we used GST fusion protein, multicopy expression, and *E. coli* BL21 (protease deficient strain). Our results indicated that our approach is effective.

Since there is a strong relationship between antihypertension activity of AHP and the type of C-terminal and N-terminal amino acid (*13*), when the multicopy expression technique was employed, the following questions must be considered including whether the C-terminal and N-terminal of single AHP released from expressed tandem peptide using chemical or enzymatic digestion contained additional amino acid residues, whether these additional amino acid residues could decrease the activity of AHP, and how to remove these additional amino acid residues. In order to conquer these problems, in our study, six copies of dotetracontapeptide expression sequence were constructed together using specific enzymatic cutting of clostripain to link AHP. The expressed fusion protein GST−AHP was digested enzymatically by clostripain and carboxypeptidase B sequentially, and the original AHP was released from N- and C-terminals without additional amino acid residues. Therefore, the higher activity of antihypertension was preserved. In order to industrialize this study in the near future, the following studies should be addressed through more research: (1) to enhance the expression yield through optimizing fermentation technology, for example, high-density fermentation and using lactose as inducer to substitute expensive IPTG; (2) to optimize the separation and purification technologies in large scale, such as ion-exchange technology to substitute HPLC, etc.

## ABBREVIATIONS USED

AHP, antihypertensive peptide; IPTG, isopropyl-$\beta$-D-thiogalactopyranoside; ACE, angiotensin-converting enzyme; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; GST, glutathione-S-transferase; SHR, spontaneously hypertensive rats; BW, body weight; SBP, systolic blood pressure; Amp, ampicillin; bp, base pair.

## NOTE ADDED AFTER PRINT PUBLICATION

The name of author Zhenyu Qin (Department of Cardiovascular Disease, University of Cincinnati, Cincinnati, Ohio 45267) was inadvertently omitted from the original byline published on the Web on May 17, 2007, and in the June 27, 2007, print issue (Vol. 55, Issue 13). The corrected electronic version was published on the Web on February 20, 2008, and an Addition/Correction appeared on the Web on February 1, 2008, and in the February 27, 2008, print issue (Vol. 56, Issue 4).

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**Received for review February 5, 2007. Revised manuscript received April 5, 2007. Accepted April 12, 2007. This research was financially supported by the Guangdong Science and Technology Plan Program (2003C30104) and the Shenzhen Science and Technology Plan Program (03KJb022).**

JF0703248