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Expression and purification recombinant antihypertensive peptide ameliorates hypertension in rats with spontaneous hypertension

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ABSTRACT

A highly efficient *Escherichia coli* expression system was established to obtain an appreciable quantity of antihypertensive peptide. The DNA-coding sequence for the Gly-Val-Tyr-Pro-His-Lys peptide was chemically synthesized and linked to form a ten-copy in tandem. It was cloned into the vector pET-15b and expressed in *E. coli* BL21 (DE3). The optimal conditions for maximal expression were verified and included the induction time and the concentration of isopropyl-β-D-thiogalactopyranoside. The recombinant protein was purified by affinity chromatography to greater than 95% purity, and further purification was achieved by High-performance Liquid Chromatography after cleavage with trypsin. The product was identified by Electrospray Ionization-Mass Spectrometry. The antihypertensive effects of the recombinant AHP were investigated in spontaneously hypertensive rats. The *in vivo* results demonstrated that a single oral administration of this peptide in spontaneously hypertensive rats resulted in a significant reduction of systolic blood pressure at 2 h. Systolic blood pressure was stabilized 4 h later and remained at a low level for 24 h. This study provides a practical method to develop the peptide into functional foods or drugs for the prevention and treatment of hypertension.

Introduction

Hypertension is a worldwide health issue and is one of the most important preventable causes of premature morbidity and mortality. It has gained extensive public and scientific concern due to its prevalence and the associated risk of cardiovascular disease [1–3]. Acting as a key regulator of blood pressure, angiotensin converting enzyme (ACE)[1] has become the major medical target, and some ACE-inhibitors have been developed as antihypertension drugs and are widely used in the clinical therapy of hypertension such as captopril, enalapril, lisinopril, tenormin, and temocapril. However, various side effects arise during prolonged use of such drugs [4,5]. Therefore, it is desirable to search for a side-effect-free ACE inhibitor and develop a more efficient drug for the prevention and treatment of hypertension.

In recent years, many peptides have been derived from food proteins and have exhibited good antihypertensive effects [6–8]. Due to their low side-effects in humans, these antihypertensive peptides (AHPs) have garnered increased attention and have the potential to be useful hypertensive agents [9]. However, the content of AHPs in natural food proteins is very low, and the processes for purification are often time consuming, laborious and costly. Industrial production of proteins by DNA recombinant technology has gained great success. However, most AHPs are very short and not easy to be directly expressed [10–12]. In our present study, an AHP peptide Gly-Val-Tyr- Pro-His-Lys (GVYPHK) is expressed as a ten-copy tandem in *Escherichia coli* and further processed into small antihypertensive peptide. Its bioactivity is studied in rats with spontaneous hypertension.

Materials and methods

Strains, plasmid and chemicals

E. coli DH5α (maintained in our laboratory) was used for subcloning and plasmid amplification. The pET-15b vector and BL21 (DE3) were obtained from Invitrogen. *Nde*1, *Bam*H1, T4 DNA ligase, and Taq DNA polymerase were purchased from Promega. DNA ladder was purchased from Beyotime. Super low protein marker was purchased from Shanghai Chaoyan Biotech. isopropyl-β-D-thiogal

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[1] *Abbreviations used:* GVYPHK, Gly-Val-Tyr- Pro-His-Lys; IPTG, isopropyl-β-D-thiogalactopyranoside; Amp, ampicillin; HPLC, High-performance Liquid Chromatography; TFA, trifluoroacetic acid; ESI-MS, Electrospray Ionization-Mass Spectrometry; WKY, Wistar-Kyoto rats; SHRs, spontaneously hypertensive rats; SBP, systolic blood pressure; AHPM, antihypertensive peptide multimers.

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actopyranoside (IPTG), lysozyme and other molecular biology chemicals were obtained from Sigma.

Plasmid construction

To construct a ten-copy fusion peptide, the single peptide (GVYPHK) was joined at the specific trypsin cleavage site. The target gene fragment and primer were synthesized by Shanghai Sangon Biotechnological Co. Ltd. (Shanghai, China). The sequence of DNA and protein is shown in Fig. 1. The target gene fragment was inserted into the pET-15b vector between the *Nde*1 and *Bam*H1 sites. The prepared vector and inserted fragment were ligated overnight at 16 °C through a standard T4 DNA ligase procedure. After transformation into *E. coli* strain DH5α competent cells, the clones were confirmed by enzymatic digestion, PCR and DNA sequencing. The PCR primers were as follows: forward: TAATACGACTCACTA TAGGGG, reverse: CAAAAAACCCCTCAAGACCCG. The constructed plasmid was designated as pET-15b-AHP10.

Expression of recombinant protein in E. coli

The *E. coli* BL21 containing the pET-15b-AHP10 recombinant plasmid was inoculated into LB liquid medium with 100 μg/mL ampicillin (Amp). After incubation at 37 °C overnight with a shaking speed of 200 rpm, the bacteria were transferred to new LB liquid medium containing 100 μg/mL of Amp. When the OD600 reached 0.8–1.0, IPTG was added at different final concentrations (0.4, 0.6, 0.8, 1.0, 1.2 mM) to induce the fusion protein for different periods (2, 3, 4, 5, and 6 h) at 30 °C. The expression level of recombinant protein was evaluated by Tricine–SDS–PAGE and stained by Coomassie blue R-250. The content of the target protein was evaluated by the software Bandscan 5.0.

Tricine–SDS–PAGE

Tricine–SDS–PAGE was used to analyze the recombinant protein [13]. The molecular mass of the protein was calculated using a calibration curve of log molecular weight versus relative mobility of standard molecular weight markers. Recombinant protein was separated by Tricine-gels with 5% acrylamide stacking and 18% acrylamide separating gels.

Purification of fusion protein

The induced cells were harvested by centrifugation at 4000*g* for 10 min, and the pellet was suspended to a concentration of 3 mg of cell/mL in lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) with lysozyme at a concentration of 100 μg/mL. After incubation on ice for 30 min, 100 mL of the lysates was sonicated with a power of 200 W for 10 min on ice (on-time of ultrasonic pulse was 2 s and off-time was 2 s). The soluble cell fractions were collected by centrifugation at 12,000*g* for 10 min at 4 °C and incubated with 5 ml Ni–NTA resin (QIAGEN) in the binding solution (20 mM sodium phosphate, 20 mM Imidazole, 500 mM NaCl, PH 8.0) for 30 min at room temperature. The protein-resin complex was then packed into a column (15 × 50 mm), which was washed with 10 bed volumes of buffer with 50 mM imidazole to remove the nonspecific binding proteins and then later eluted with 10 bed volumes of elution buffer with 500 mM imidazole to obtain the target protein. All buffer was added at a rate of 1 mL/min. The products were monitored by Tricine–SDS–PAGE.

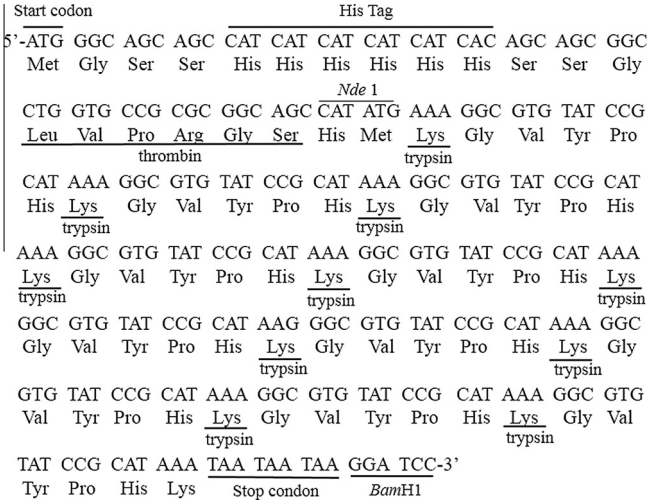

Fig. 1. The sequence of DNA and protein.

Production and identification of peptide GVYPHK

Two milliliters of purified recombinant protein (1 mg/mL) was mixed with 0.2 ml trypsin reaction buffer (500 mM PBS, 1 mg/ml trypsin, pH 8.0) for 24 h at 37 °C. The reaction was stopped by boiling for 10 min. The supernatant was recovered after centrifugation at 4000*g* for 10 min and then purified by High-performance Liquid Chromatography (HPLC). The mobile phase consisted of 1 g/L trifluoroacetic acid (TFA) in water (A) and 1 g/L TFA in acetonitrile (B). The flow rate was maintained as 1.0 mL/min using a linear gradient of A –B (10–50%). Analysis was performed at a wavelength of 220 nm. The molecular mass of purified peptide was determined using Electrospray Ionization-Mass Spectrometry (ESI-MS). The synthesized peptide GVYPHK (>98% purity, by DgPeptides Co. Ltd., Hangzhou, China) was used as the standard to measure the amount of purified AHP. The purified recombinant AHP was made as a powder and stored at −80 °C for further study.

Effects of the peptide GVYPHK on SHRs

SHRs and Wistar-Kyoto rats (WKY) were obtained from Shanghai Slaccas Laboratory Animal Co. Ltd. (Shanghai, China), housed individually in steel cages, and fed with a standard laboratory diet in a room kept at 25 °C. The rats were divided into five groups: a water control group ($n = 6$), a captopril group at a dose of 10 mg/kg body weight ($n = 6$), and three peptide inhibitor groups at doses of 200 μg/kg (low dose, $n = 6$), 400 μg/kg (middle dose, $n = 6$) and 800 μg/kg (high dose, $n = 6$) body weight. The water, captopril and peptide were orally administered to spontaneously hypertensive rats (SHRs). Rats were held at 40 °C for 10 min, and the systolic blood pressure (SBP) was measured by tail cuff with a programmed noninvasive blood pressure controller (model ML125, AD Instruments Pty Ltd., Australia).

Statistical analysis

All results were expressed as means ± SEM ($n = 6$). The significance of the differences against the control was analyzed using the Student's *t*-test and was considered to be significant when $P < 0.05$.

Results

Construction and Identification of Recombinant Expression Vector pET15b-AHP-10

The aimed DNA sequence containing *Nde*1 and *Bam*H1 restriction sites was subcloned into the pET15b expression vector. To confirm that the targeted gene had been correctly inserted into the vector, enzymatic digestion, PCR, and gene sequencing were used. Fig. 2

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