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Original Article

Expression of recombinant angiotensin I-converting enzyme-inhibitory heptapeptide gene in *Escherichia coli* BL21

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ABSTRACT

The angiotensin I-converting enzyme (ACE) plays a crucial role in regulating blood pressure and its inhibition is considered a treatment for hypertension. Cys-Met-Try-Leu-Ala-Ser-Gly (CMYLASG), a heptapeptide derived from *Gnetum gnemon* L. seeds, is an ACE-inhibitory peptide. This study aimed to investigate the expression of the tandem repeat heptapeptide gene (Gg-7pAH) and its ACE-inhibitory activity. The heptapeptide gene was cloned and expressed into the pBT7–N–His vector and synthesized as the recombinant protein Gg-7pAH. Sequencing results of the recombinant plasmid showed high similarity with the sequence of the heptapeptide genes inserted. The recombinant protein was expressed mostly as a soluble protein in *Escherichia coli* BL21 after 8 h of induction using isopropyl-β-D-thiogalactoside (IPTG) at room temperature around 26-28 ºC. Purification was achieved using Ni^{2+}-chelate (Ni-NTA) affinity chromatography, which produced a 12 kDa recombinant protein. Hydrolysis of the recombinant protein with 2% formic acid released the heptapeptide as a target molecule. The hydrolyzed recombinant protein exhibited excellent ACE-inhibitory activity with an IC_{50} of 8.64 μM, which was lower than that of captopril (IC_{50} = 11.68 μM). Inhibitory kinetics showed that the heptapeptide was a non-competitive inhibitor of ACE and functioned suitably at low substrate concentrations. These findings revealed the high potency of the recombinant Gg-7pAH to produce ACE-inhibitory peptides that may be used as hypotensive agents and nutraceuticals for hypertension treatment.

KEYWORDS: recombinant Gg-7pAH, ACE-inhibitory, heptapeptide, hypertension, *E. coli* BL21.

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INTRODUCTION

Hypertension is a global health problem in both developed and developing countries. It is a primary risk factor for cardiovascular diseases (CVDs) such as heart failure, coronary heart disease, stroke, and kidney malfunction. The WHO has predicted that by 2020, CVDs would surpass infectious diseases and become the leading cause of disability and death [1]. Hypertension is known as a lethal disease and 90-95% of the cases occur due to unknown causes. Implementation of healthy lifestyles such as regular exercise, eating healthy foods, not smoking, decreasing sodium intake, and reducing stress could prevent hypertension [2]. ACE-inhibitory substances are considered as the first-line medicine in hypertension management because of their effectiveness in reducing blood pressure. ACE is a type of peptidyl dipeptide hydrolase that deactivates bradykinin and hydrolyzes angiotensin I to angiotensin II. ACE is also a strong vasoconstrictor that causes high blood pressure. Clinical hypotensive drugs such as captopril, enalapril, and lisinopril are used to manage and prevent high blood pressure. However, these drugs can have side effects such as allergic reactions, cough, skin rashes, taste function disorders, and other dysfunctions of human organs [3, 4].

ACE-inhibitory peptides from natural food protein derivatives have received considerable attention for their health benefit effects, safety, and potential use as healthy foods [5]. In recent studies, bioactive peptides from several food sources such as milk, egg yolk, fermented soybean, microalga, fish, and yeast have been reported to possess nutraceutical properties such as ACE-inhibition, as well as antioxidant and antibacterial activities [6-11].

Gnetum gnemon L. (melinjo) is a gymnosperm plant, which has become one of the important agroforestry species in Melanesia and Southeast Asia. These plants are tolerant to extensive rainfall, have fairly large temperature ranges, but are greatly influenced by salinity [12]. *G. gnemon* is commonly used for its seeds, leaves, and timber. In Indonesia, the seeds are used as food ingredients such as vegetables, flour, and chips. Melinjo seeds contain carbohydrates (58%), lipids (10.7%), protein (19%), minerals (2.2%), water (9.1%), and phenolic compounds (1%) [13, 14]. Previous studies have shown that the high levels of protein in melinjo seeds are a potential source of antioxidants for human health [15]. Earlier studies reported that hydrolyzed protein from melinjo seeds exhibit high antioxidant, antidiabetic, and antihypertensive activities [16-18]. However, the method used to obtain the bioactive peptide from food sources has several disadvantages such as high cost, low yield, and complicated separation and purification processes [4, 19]. There are no previous studies on the preparation of bioactive peptides from melinjo seeds based on recombinant proteins. Heptapeptide is a short-peptide consisting of seven amino acids derived from *Gnetum gnemon* L. seeds. These short-peptides are sensitive to degradation by proteases, which decreases gene expression levels; protein expression can be hazardous to cells [20, 21]. Therefore, the peptides were produced by genetic engineering combined with tandem repeat genes to solve the problem of unstable expression in the host cell and reduce purification costs [22, 23]. In this study, the heptapeptide gene was cloned and expressed as the recombinant protein Gg-7pAH to produce large quantities of the bioactive peptide to treat hypertension.

MATERIALS AND METHODS

Construction of recombinant plasmid Gg-7pAH

The polynucleotide sequences encoding the heptapeptide genes were tandemly repeated and cloned in the open reading frame (ORF) between two EcoRI restriction sites of the pBT7-N-His vector (Bioneer Co., Korea). Furthermore, the recombinant plasmid was transformed into *Escherichia coli* BL21 (Novagen, USA) by the heat shock method according to the manufacturer's instructions. A single colony of transformed *E. coli* was amplified using the T7 primer (Takara Ex Taq™, Japan) through polymerase chain reaction (PCR). Restriction enzyme analysis using EcoRI (Promega, USA) was also conducted to ensure that the heptapeptide genes were inserted as well as to confirm their size. The correct expression of the recombinant Gg-7pAH was verified through sequencing analysis (Bioneer Co., Korea) using the recombinant plasmid in two ORFs. The polypeptide sequence of the amino acid is shown in Fig. 1A, and the amino acid sequences with their cleavage in the proline-aspartic acid (DP) site are described in Fig. 1B.

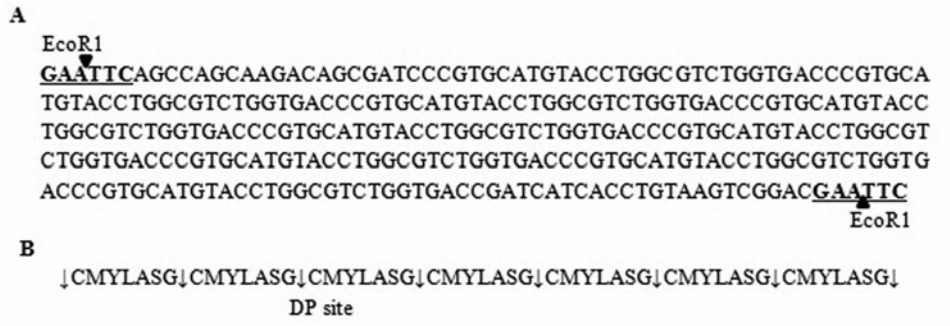

Fig. 1. A: Tandem repeat of the heptapeptide gene in the recombinant Gg-7pAH containing EcoRI restriction sites at the 5 'end and 3' ends. B: The corresponding amino acid sequence of the recombinant Gg-7pAH. Arrows indicate potential restriction sites in DP.

Expression of the recombinant protein Gg-7pAH

Competent cells were prepared from a glycerol stock of *E. coli* BL21 and then the recombinant plasmids were used to transform the cells. A single colony of transformed *E. coli* was inoculated into 5 mL of Luria-Bertani (LB) broth containing 75 µg/mL ampicillin for the rapid selection of transformed cells. The cells were cultured overnight in an orbital shaker incubator with vigorous shaking (200 rpm) at 37 °C. A total of 1 mL of the overnight culture was inoculated into 100 mL LB broth supplemented with ampicillin and then grown at room temperature around 26-28 °C with shaking at 150 rpm. Gene expression was induced using 0.5 mM IPTG (Isopropyl β-d-1-thiogalactopyranoside) (Wako Pure Chemical, Japan) when the culture reached OD_{600} 0.5-0.6. Bacterial cells were harvested after 8 h of incubation by centrifugation at 5,000 rpm at 4 °C for 12 min. The cells were resuspended in extraction buffer, sonicated on ice, and centrifuged at 12,000 rpm for 15 min to extract the recombinant protein. Sample buffer (2X) was added to the supernatant and pellet in a 1:1 ratio and diluted five times with extraction buffer. The Bradford method was used to determine the total concentration of the recombinant protein at $OD_{595\ nm}$ using bovine serum albumin (BSA) as a standard (Sigma-Aldrich, USA) [24]. All samples were transferred to 95 °C dry heat blocks for 5 min before being analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli [25]. Patterns of the recombinant protein were compared with those of competent cell protein patterns as a control of the expression.

Recombinant protein purification

The recombinant protein was purified using affinity chromatography with a nickel-nitrilotriacetic acid resin (HiTrap™ HP-GE Healthcare, USA) followed by fractionation on an AKTA purifier workstation fast protein liquid chromatography (FPLC) system (Amersham Pharmacia Biotech, Sweden). The recombinant protein extract was injected into the column matrix and then incubated for 1 h with moderate agitation at room temperature. Subsequently, the affinity column was rinsed with 20 mL of washing buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 20-200 mM imidazole (pH 8.0). The elution buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, and 250-500 mM imidazole (pH 8.0) was applied onto the column to elute the fraction of the his-tagged recombinant protein. The fraction was collected at a peak absorbance signal at 280 nm using the fractionation system. The protein fractions were visualized with Coomassie brilliant blue R-250 and their molecular weight were determined through 15% SDS-PAGE.

Digestion of purified recombinant protein Gg-7pAH

The purified recombinant protein was chemically digested according to Long *et al*. [26]. Formic acid (2%) was added to all samples that were further incubated at 50 °C for 4 h to degrade the protein in DP sites and release the heptapeptide (Fig. 1B). The mixture was neutralized with NaOH and the ACE-inhibitory activity subsequently determined.

Determination of the ACE-inhibitory activity and kinetics

The ACE-inhibitory activity was determined according to Lie *et al*. [27]. The standard of this test, hippuric acid, is generated through the reaction of the hippuryl-histidyl-leucine (HHL) substrate (Sigma-Aldrich, Singapore) with the ACE from rabbit lung extracts. HHL (5 mM in 0.1 M sodium borate buffer, pH 8.3) containing 0.3 M NaCl was added to the hydrolyzed recombinant protein and pre-incubated at 37 °C for 5 min. The reaction was initiated with 50 mU/mL ACE at 37 °C for 30 min. The reaction was stopped with 100 µL of 1.0 M HCl. Ethyl acetate was added to the mixture and centrifuged at 1,200 rpm for 10 min to extract the hippuric acid fraction from the top layer. The fraction was dried using an evaporator (Genevac, Germany) at 45 °C for 15 min and then dissolved with distilled water. The absorbance was measured at 228 nm using a spectrophotometer (Hitachi type U-2900 UV-Vis, Japan) and the ACE-inhibitory activity (%) was calculated with the following equation: (%) = [(AC-AS)/(AC-AB] x 100, where AC, AS, and AB are the absorbance of the control, samples, and blank, respectively. IC_{50} values are obtained by regression analysis of the inhibitory activity (%) at various sample concentrations and it is defined as the concentration of the recombinant protein that inhibits 50% of the original ACE activity. The purified recombinant protein, captopril, and the synthetic heptapeptide (Peptron, Inc., Korea) were also determined for ACE-inhibitory activity.

The type of inhibition was estimated through ACE kinetics and the Lineweaver-Burk plot. The ACE-inhibitory activity of the hydrolyzed recombinant protein compared with captopril and the synthetic heptapeptide was determined at various substrate concentrations (2.5; 5.0; 10; 20 and 40 mM). Inhibition kinetics and parameters such as K_M and V_{max} were estimated by linear regression between ACE rates at various substrate concentrations.

Data analysis

All data are presented as the mean ± standard deviations from triplicate tests. The variance was conducted using analysis of variance (ANOVA) followed by Duncan's test and the differences were considered significant at $p < 0.05$.

RESULTS

Expression identification of the recombinant plasmid Gg-7pAH

The polynucleotide sequence encoded by the tandem repeat heptapeptide gene was inserted in the same ORF between EcoRI restriction sites of the recombinant Gg-7pAH. The DP bond identified specific cleavage sites from the sequences of amino acids to release the desired peptide. The correct expression of the transformed *E. coli* was verified by colony PCR, restriction enzymes, and sequencing analysis. PCR of the transformed cells using T7 promoter and T7 terminator was amplified as a single band of 500 bp using 100 bp DNA Ladder for sizing and is depicted in Fig. 2A, lane 1. The EcoRI-released fragment of inserted genes at around 325 bp is shown in Fig. 2A, lane 3. Further, sequencing analysis of the recombinant Gg-7pAH showed high similarity with the heptapeptide sequence. Thus, the exogenous DNA of the heptapeptide was successfully cloned and expressed in the recombinant plasmid Gg-7pAH.

Expression of the recombinant protein Gg-7pAH and its purification

Recombinant *E. coli* BL21 was grown after 8 h of induction using IPTG at room temperature and yielded 4 g/L of cell culture. SDS-PAGE revealed that the recombinant protein was clearly expressed in the supernatant as a soluble protein with a molecular weight of 12 kDa (Fig. 2B, lane 2). In contrast, the expression was absent in competent cells, non-transformed *E. coli,* and the pellet (Fig. 2B, lane 1). The results confirmed that the recombinant Gg-7pAH was successfully expressed as a soluble protein.

The his-6tag marker in the recombinant Gg-7pAH was used as an affinity tag to mediate the detection and purification of the recombinant protein.

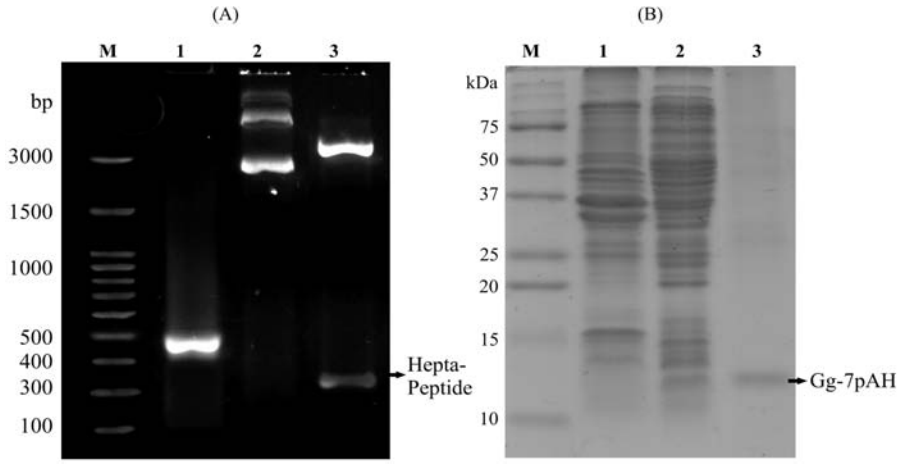

Fig. 2. Restriction enzyme analysis and expression identification of recombinant Gg-7pAH. A: Restriction enzyme analysis of the recombinant plasmid. M: 100 bp DNA Ladder; lane 1: PCR product containing T7 primer; lane 2: recombinant plasmid; lane 3: restriction analysis using EcoRI. B: Protein pattern analysis of the supernatant recombinant protein using 15% SDS-PAGE. M: Dual Color Bio-Rad protein^{TM} marker; lane 1: competent cells; lane 2: transformed *E. coli* after induction with IPTG; lane 3: pool of elution fractions and purified recombinant protein.

Polyhistidine ligands of the recombinant protein were strongly bound to the affinity column matrix to produce a highly tagged protein. The Ni-NTA affinity column has Ni^{2+} ions as electron donors immobilized to the imidazole ring for the his-tagged protein selection. The elution buffer containing a high imidazole concentration may weaken the protein ligand-column matrix binding, thus enabling purification. The molecular weight analysis of the purified recombinant protein on 15% SDS-PAGE gel showed a single protein band of 12 kDa (Fig. 2B, lane 3) appropriate for the design of the recombinant Gg-7pAH. During purification, the purified fractions were collected in a volume of 5 mL and a concentration reaching 1 mg/mL.

Digestion of the recombinant protein

Gene expression yielded a long-chain protein resulting from the tandem repeat of heptapeptide genes with DP as a specific site of cleavage. The procedure aims at reducing the rapid degradation of the recombinant protein by proteases in the host cell. The recombinant protein digestion was conducted at 50 °C for 4 h using 2% formic acid to cleave the protein at the DP site and release the heptapeptide. The degree of hydrolysis reached up to 57% by releasing free amino acids as shown in Fig. 3A.

ACE-inhibitory activity and kinetics evaluation *in vitro*

Several studies of ACE-inhibitory peptides have been shown to be effective in lowering blood pressure. The hydrolyzed recombinant protein showed an increase in the ACE-inhibition activity (Fig. 3B) and the effect of recombinant protein digestion released small peptides. The peptides are likely to be bound to the active substrate and induce ACE-inhibition activity. Captopril was used as a standard ACE-inhibitor drug by intermolecular interaction at Zn^{2+} sites of ACE with the sulfur groups inside the captopril molecule. Determination of the ACE-inhibitory activity of captopril showed an IC_{50} of 11.68 μM, higher than the IC_{50} for the hydrolyzed recombinant protein and the synthetic heptapeptide, which were 8.64 and 6.77 μM, respectively. In addition, the IC_{50} of the purified recombinant protein, 29.65 μM, was the highest IC_{50}. Thus, the hydrolyzed recombinant protein provides more effective ACE-inhibition than captopril.

The identification pattern of the ACE-inhibition by the hydrolyzed recombinant protein was obtained using the Lineweaver-Burk plot. The inhibition produced by the hydrolyzed recombinant protein was compared with the one produced by the synthetic heptapeptide and the control solution in the absence of ACE-inhibitory peptide. The

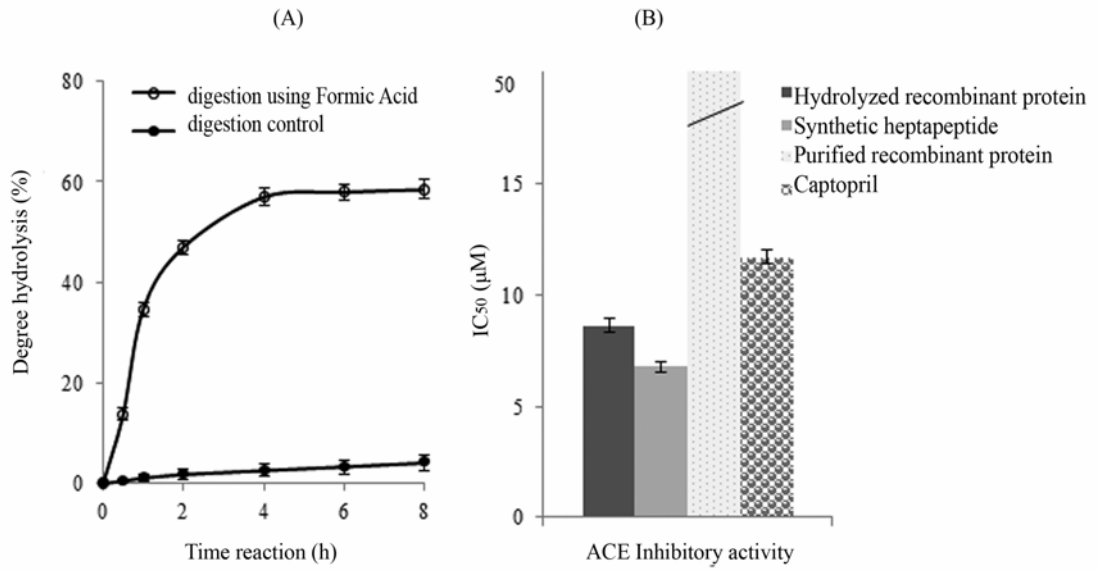

Fig. 3. A: Degree of hydrolysis of the purified recombinant protein at 50 ºC. B: The IC_{50} (μM) for ACE-inhibitory activity is shown as the mean ± SD of three replicates.

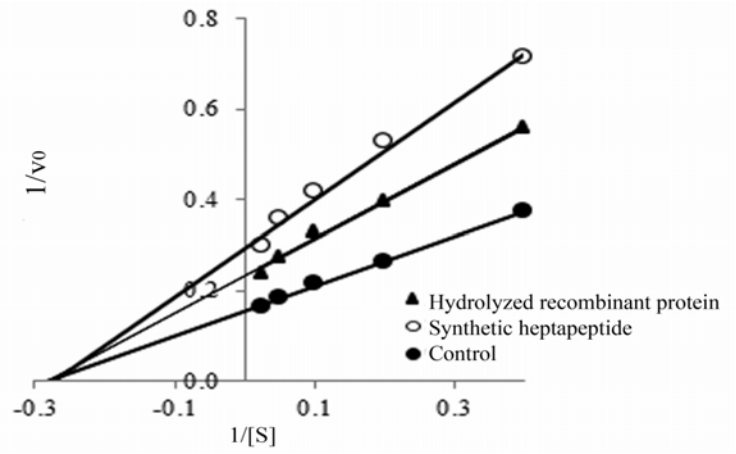

Fig. 4. Lineweaver-Burk plot of ACE-inhibitory heptapeptide. The ACE-inhibitory activity was measured in the absence (control) and the presence of the ACE-inhibitory heptapeptide and compared to the synthetic heptapeptide.

Table 1. Inhibition kinetics of an ACE-inhibitory activity.

Samples	K_M (μM)	V_{max} (μM/min)
Control	3.49 ± 0.41	6.41 ± 0.12
Hydrolyzed recombinant protein	3.55 ± 0.24	4.30 ± 0.28
Synthetic heptapeptide	3.63 ± 0.12	3.39 ± 0.04

reciprocal plot for the ACE-catalyzed reaction in the control solution, hydrolyzed recombinant protein, and synthetic heptapeptide is shown in Fig. 4. The plot indicated a mostly non-competitive type of inhibition. The heptapeptide might be bound to the ACE structure to produce complex dead ends regardless of the substrate concentration. ACE-inhibition might be caused by conformational changes, which blocked the catalytic reaction of the ACE enzyme and HHL to produce hippuric acid, thereby highly inhibiting the enzyme at low substrate concentration. Nevertheless, captopril showed a competitive inhibition type in which the active site of ACE might be bound to the drug molecule and prevents the binding of the substrate, resulting in a different type of inhibition. K_M for the recombinant protein is relatively similar to the one of the control solution and V_{max} is lower in the presence of heptapeptide (Table 1).

DISCUSSION

Heptapeptide is a short-peptide consisting of seven amino acids that are derived from *Gnetum gnemon* L. seeds. A previous study reported that a heptapeptide isolated from a hydrolyzed protein of *G. gnemon* seeds exhibits high ACE-inhibitory activity *in vitro* [18]. This study investigated the expression of heptapeptide genes to produce large quantities of an ACE-inhibitory peptide and reduce the complexity of purification and costs. In addition, the short-peptide is difficult to express directly in *E. coli* and is easily degraded by proteases in the host cell. *E. coli* BL21 was used as a host bacterium to increase the levels of gene expression and the stability of the recombinant protein. This strain lacks the ompT and Lon (La) protease, a type of endoprotease that rapidly degrade mis-folded and recombinant proteins [22, 28]. *E. coli* BL21 contains a chromosomal copy of the T7 RNA polymerase gene and has the ability to transcribe and translate about 60,000 polypeptide chains per minute. Therefore, this strain is the most widely-used promoter system for protein expression. The tandem repeat gene was combined with the recombinant expression in pBT7-N-His vector to solve problems with gene expression [21, 22]. Plasmid pBT7-N-His carrying the T7 promoter

was used for gene expression and to produce large amounts of small peptides with minimum risks of unstable expression [23]. The presence of an ampicillin tag in the plasmid helps in the rapid selection of recombinant cells.

Several factors are optimized to facilitate the folding and increase the solubility of the synthesized protein [26]. The time point for induction, incubation time, temperature, and concentration of the inducer were optimized. Although a high inducer concentration is applied to increase gene expression, it might reduce the rates of transcription and/or translation. The expression system was designed to be induced in the supernatant by an IPTG concentration ranging from 0.1-1.0 mM [29]. The expression of recombinant Gg-7pAH was induced with 0.5 mM of IPTG in the log phase culture when OD_{600} reached 0.5-0.6 after 2 h incubation at room temperature. The recombinant protein was clearly expressed as a soluble protein after inducing expression for 8 h at room temperature.

The heterologous his-tagged protein consisting of six tandem histidine residues not only increased the expression and solubility, but also mediated the detection and purification of the protein in the matrix of the Ni^{2+}-chelate affinity chromatography column. The Ni-NTA affinity column has a high selectivity for his-tagged proteins by chelating immobilized Ni^{2+} ions in the column matrix. The his-tagged protein as a specific ligand could be completely released by eluting with a buffer containing a high concentration of imidazole that weakens the ligand protein-matrix interaction. Furthermore, heptapeptide release was specifically designed at the parent protein DP sites. A previous study reported that DP sites of the recombinant protein were engineered with the formic acid cleavage to release the target peptide [30].

The potential of ACE-inhibition as the first-line therapy for hypertension management is widely researched on natural food-derived compounds such as peptides, polyphenols, and terpenes [2]. In the renin-angiotensin system (RAS), the enzyme circulates in the bloodstream and breaks down angiotensinogen and a peptide secreted by the liver into angiotensin I. Furthermore, angiotensin I is cleaved in the lungs by ACE into angiotensin II, which plays a powerful role as a hormone and has potential for vasoconstriction in smooth muscle [1]. In addition, angiotensin II promotes aldosterone release which facilitates homeostatic blood pressure and other harmful effects of hypertension. The ACE-inhibitory activity causes the accumulation of bradykinin to support blood vessel dilation. In recent studies, the correlation between structure and activity has shown strong ACE bonds with active ACE sites that are influenced by inhibitor peptides carrying different residues [31].

The location of N-terminal or C-terminal amino acid residues and the different peptide sequences are critical factors in determining their biological activity, including the ACE-inhibitory ability. Besides, the peptide sequence, amino acid composition, and extend and/or coil structure of the inhibitor might be related to the ACE-inhibitory activity [32]. The hydrophobicity of the amino acid residues, including branched or aromatic side chains such as Tyr and Phe at the N-terminal and C-terminal of peptides, may favor the ACE [14]. In addition, Leu and Val with hydrophobic branched side chains are preferably bound to catalytic sites of ACE, hence acting as strong competitive ACE-inhibitors [26, 27]. A strong ACE-inhibitory peptide from casein (Phe-Phe-Val-Ala-Pro), dried bonito (Ala-Leu-Pro-His-Ala), soy peptide (His-His-Leu), and fish protein (Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu) were reported to have an IC_{50} of 5.0, 10.0, 4.98, and 22.3 μM, respectively [9, 10, 33, 34]. Based on the results, the hydrolyzed recombinant protein consisting of a heptapeptide (Cys-Met-Tyr-Leu-Ala-Ser-Gly) showed an IC_{50} of 8.64 μM. The recombinant protein had hydrophobic amino acid residues that contributed to ACE-inhibition by interfering with the access of the peptide to the ACE active site. The hydrophilic-hydrophobic residues distributed in the peptide sequence were also an important factor in the inhibition activity. The high level of hydrophobic amino acid residues such as Pro, Glu, Val, Phe, and Tyr in the structure of the peptide had a comparative low IC_{50}. Therefore, it was expected to have an excellent antihypertensive activity [35].

The inhibition of ACE was generally of the non-competitive type. Data obtained were identical to data reported in research on peptide-like fermented soybean food (Ile-Phe-Leu and Trp-

Leu) [8], *Limanda aspera* protein (Met-Ile-Phe-Pro-Gly-Ala-Gly-Gly-Pro-Glu-Leu) [10], yeast hexapeptide (Thr-Pro-Thr-Gln-Gln-Ser) [11], and the peptide in human serum albumin (Ala-Phe-Lys-Ala-Trp-Ala-Val-Ala-Arg) [36]. Non-competitive inhibition occurs when the enzyme binds the substrate and the inhibitor at any given time and produces enzyme-substrate-inhibitor complexes. This pathway does not enable product formation because of an irreversible reaction resulting in enzyme-substrate or enzyme-inhibitor complexes [11]. However, there was no clear definition of the exact ACE-inhibition mechanism of the non-competitive type [8, 34]. In recent studies, the majority of peptides inhibited ACE in a competitive manner. The heptapeptide might bind at different sites from the substrate, enhance the ACE-inhibitory capacity, and reduce the efficiency of enzyme catalysis. Thus, increasing substrate concentration does not affect enzyme inhibition activity. The heptapeptide indicates the potential use of the ACE-inhibitory peptide as a hypotensive agent in low concentration [8]. K_M is the concentration of substrate which permits the enzyme to achieve half V_{max} and is a measure of the enzyme affinity for its substrate. In this study, the presence of the ACE-inhibitory peptide did not increase K_M, while V_{max} was smaller compared to the control.

CONCLUSION

The biological activity of some food-derived compounds has been previously reported, but no prior studies have focused on the bioactive peptides derived from *G. gnemon* L. seed proteins and the use of recombinant proteins. Heptapeptide (CMYLASG) has an excellent ACE-inhibitory ability at low concentrations and does so in a non-competitive inhibition manner. Thus, the use of recombinant Gg-7pAH may be a great strategy to produce large quantities of the peptide with a positive impact on hypertensive treatment. In conclusion, the heptapeptide with important bioactivity and easily accessible sources holds promising potential for the future development of functional peptides to manage hypertension.

ACKNOWLEDGMENTS

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

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