High-level expression of the angiotensin-converting-enzyme-inhibiting peptide, YG-1, as tandem multimers in Escherichia coli

Abstract

To produce a large quantity of the angiotensin-converting-enzyme (ACE)-inhibiting peptide YG-1, which consists of ten amino acids derived from yeast glyceraldehyde-3-phosphate dehydrogenase, a high-level expression was explored with tandem multimers of the YG-1 gene in Escherichia coli. The genes encoding YG-1 were tandemly multimerized to 9-mers, 18-mers and 27-mers, in which each of the repeating units in the tandem multimers was connected to the neighboring genes by a DNA linker encoding Pro-Gly-Arg for the cleavage of multimers by clostripain. The multimers were cloned into the expression vector pET-21b, and expressed in E. coli BL21(DE3) with isopropyl \(\beta\)-D-thiogalactopyranoside induction. The expressed multimeric peptides encoded by the 9-mer, 18-mer and 27-mer accumulated intracellularly as inclusion bodies and comprised about 67%, 25% and 15% of the total proteins in E. coli respectively. The multimeric peptides expressed as inclusion bodies were cleaved with clostripain, and active monomers were purified to homogeneity by reversed-phase high-performance liquid chromatography. In total, 105 mg pure recombinant YG-1 was obtained from 1 l E. coli culture harboring pETYG9, which contained the 9-mer of the YG-1 gene. The recombinant YG-1 was identical to the natural YG-1 in molecular mass, amino acid sequence and ACE-inhibiting activity.

Introduction

Angiotensin-converting enzyme (ACE, peptidyl-dipeptide hydrolase, EC 3.4.15.1) generates the powerful vasocostritor angiotensin II by removing the C-terminal dipeptide from the precursor decapeptide angiotensin I (Skeggs et al. 1954). The enzyme also inactivates the vasodilator bradykinin (Yang et al. 1970). Even though small molecules with strong antihypertensive activity are being synthesized and used as ACE inhibitors, food-derived peptide ACE inhibitors have recently received increasing attention because of the development of functional foods contributing to homeostasis (Ondetti et al. 1977; Patchett et al. 1980; Ariyoshi 1993). Since the discovery of an ACE-inhibiting peptide in the snake venom (Cheung and Cushman 1973), many such peptides have been identified from various natural sources such as gelatin, bovine casein, porcine plasma, tuna, fig-tree latex, bonito and sardine, etc. (Oshima et al. 1979; Maruyama et al. 1985, 1989; Hazato and Kase 1986; Kohama et al. 1988; Yokoyama et al. 1992; Ukedo et al. 1992). The structure and ACE-inhibiting activity of these peptides differ depending on the isolation techniques and their sources (Ariyoshi 1993). The ACE-inhibiting peptides isolated from bovine casein (Maruyama et al. 1985) and fig-tree latex (Maruyama et al. 1989) consist of 5–12 amino acids and 3–6 amino acids respectively, and the median inhibitory concentrations (IC\textsubscript{50}) of these peptides are 6.0–77 \(\mu\)M and 4.5–14 \(\mu\)M respectively. YG-1, which consists of 10 amino acids (Gly-His-Lys-Ile-Ala-Thr-Phe-Gln-Glu-Arg) derived from the glyceraldehyde-3-phosphate dehydrogenase of baker’s yeast, has a lower IC\textsubscript{50} value than any other ACE-inhibiting peptides (Kohama et al. 1990). Therefore, YG-1 would be one of the most promising ACE-inhibiting peptides for treating hypertension.

For functional foods or pharmaceutical applications, a large quantity of YG-1 needs to be produced economically. Chemical synthesis is not practical because of its high cost and safety issues. Therefore, a biological
expression system, if developed successfully, would be the most cost-effective method for the mass production of the ACE-inhibiting peptide. With the progress in molecular biology and genetic engineering techniques, many methods for high-level gene expression have been developed for the production of enzymes or proteins (Komai et al. 1997; Gustafsson et al. 1997). However, few expression systems have been used for the production of small peptides because of the low production yield and high cost of purification (Yabuta et al. 1995).

In this study, a gene-amplification vector (Lee et al. 1996) has been used to develop a high-level gene-expression system in Escherichia coli, in which the genes of YG-1 were tandemly multimerized and the multimeric peptides expressed were cleaved quantitatively into monomeric forms, achieving the mass production of YG-1.

**Materials and methods**

Bacterial strains, vectors, enzymes and chemicals

_E. coli_ strain XLI-Blue (Stratagene, La Jolla, Calif., USA) was used as a host for subcloning, and _E. coli_ BL21(DE3) (Novagen, Madison, Wis., USA) was used for gene expression. pBBS1 (Lee et al. 1996) and pET-21b (Novagen) respectively were used as vectors for the multimerization and expression of peptide genes. Restriction enzymes and other modifying enzymes, including _BbsI_, were purchased from New England Biolabs (Beverly, Mass., USA) and used according to the recommendations of the supplier. Clostripain (EC 3.4.28.8) was purchased from Sigma (St. Louis, Mo., USA) and acetonitrile for high-performance liquid chromatography (HPLC) from Burdick & Jackson Inc. (Muskegon, Mich., USA). A mini-scale preparation of plasmid DNA was carried out using the alkaline lysis method (Maniatis et al. 1982) and a large quantity of plasmid DNA was prepared by the polyethylene glycol precipitation method (Sambrook et al. 1989). Other recombinant DNA techniques were exploited as described by Maniatis et al. (1982) and Sambrook et al. (1989).

Tandem multimerization of a gene encoding YG-1 using a gene-amplification vector

For the construction of the DNA encoding YG-1 and a 3-amino-acid linker (Fig. 1), two complementary deoxyoligonucleotides: A (5’CCCCGGTGTCACAAAATCGTACCTTTCCAGGAAACGT GGC3’) and B (5’GGGGGCGCCAGGTTCTGGAAAGGTTAGGCGA TTTGTTGACCAC3’), were synthesized, annealed, and then ligated into the _BbsI_-digested pBBS1, resulting in pBBSYG1 (Fig. 1). The DNA sequence was confirmed by the dideoxy-DNA chain-termination method (Sanger et al. 1977) with Sequenase (version 2.0, U.S. Biochemicals, Cleveland, Ohio, USA). The pBBSYG1, which contained a monomer of the YG-1 gene and linker, was digested with _BbsI_ to produce the monomeric DNA fragments with asymmetric cohesive ends: 5’-GGGG-3’.

Production and purification of the recombinant YG-1

_E. coli_ cells harboring the vector pETYG9, which contained the 9-mer of the YG-1 gene, were grown in LB medium supplemented with ampicillin (50 μg/ml) in a 2-l bioreactor. At _A_600 = 0.4, isopropyl β-D-thiogalactopyranoside was added to the culture (1 l) to a final concentration of 0.4 mM for induction. The cells were harvested 3 h after induction by centrifugation at 6000 g for 10 min. After lysis of cells by a microfluidizer (Microfluidics Co., Newton, Mass., USA), the inclusion bodies were recovered by centrifugation at 10000 g for 20 min at 4°C and washed twice with the washing buffer consisting of 30 mM Tris·HCl (pH 7.5), 1 mM EDTA, 100 mM NaCl, 5% glycerol, 0.1% Triton X-100 and 1 mM dithiothreitol. The washed inclusion bodies were then resuspended in 60 ml washing buffer. A 5-mg sample of the inclusion bodies, which was resuspended in 15 ml washing buffer containing 2.5 mM dithiothreitol and 1.0 mM CaCl2, was cleaved by incubating for 1.5 h at 37°C with 100 μg clostripain. The clostripain was activated for 3 h at room temperature just before use in 100 mM MOPS buffer (pH 7.6) containing 2.5 mM dithiothreitol and 1.0 mM CaCl2. After the reaction mixture had been deskilled by a Sep-Pak C18 cartridge, the cleaved inclusion bodies were applied to a 3.9 × 300-mm Delta Pak C18 column (Waters associates, Milford, Mass., USA) with a linear gradient of 0% buffer A to 80% buffer A at 1 ml/min for 1 h [buffer A: acetonitrile containing 0.1% (v/v) trifluoroacetic acid]. The peak, which had the same retention time as the synthetic YG-1, was collected and used for characterization after lyophilization.

Characterization of the recombinant YG-1

The molecular mass of the recombinant YG-1 was determined by matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Kratos Kompact MALDI, Manchester, England). The purified and lyophilized peptides were dissolved in 50% acetonitrile containing 7% (w/v) sinapinic acid and mixed with a Pt probe. After removal of the solvent in warm air, the peptides absorbed to the Pt probe were applied to the vacuum chamber of the MALDI mass spectroscope and analyzed. Amino acid sequencing was performed by the automated Edman degradation method on an Applied Biosystem gas-phase sequencer, model 447 (Foster City, Calif., USA). The ACE activity was assayed by measuring the amount of hippuric acid liberated from hippuryl-His-Leu as described by Cushman and Cheung (1971). The reaction mixture contained 5.0 mM hippuryl-His-Leu, 2 μl ACE and various concentrations of the recombinant YG-1 in 250 μl 0.1 M potassium phosphate buffer (pH 8.3) containing 0.3 M NaCl. After incubating at 37°C for 30 min, the enzymatic reaction was stopped by the addition of 0.5 ml 5% (w/v) TCA. The reaction products were analyzed by high-performance liquid chromatography (HPLC) using a 7.8 × 300-mm Delta-Pak C18 column (Waters associates, Milford, Mass., USA) in which the genes of YG-1 were tandemly multimerized and the multimeric peptides expressed were cleaved quantitatively into monomeric forms, achieving the mass production of YG-1.

**Expression of multimeric peptides genes in _E. coli_**

To construct an expression vector containing the multimeric peptide gene under the control of the T7 promoter, the _EcoRI-HindIII_ fragments containing the 9-mer, 18-mer or 27-mer of the YG-1 gene, which were isolated from pBBSYG9, pBBSYG18 or pBBSYG27, were cloned into the _EcoRI_- and _HindIII_-digested pET-21b, producing pETYG9, pETYG18 or pETYG27 respectively (Fig. 1). Each expression vector contained a start codon, ATG, and a ribosome-binding site, AAGGAG, derived from pET-21b next to the T7 promoter. The expression vectors were transformed into _E. coli_ BL21(DE3). Each transformant was cultured into 3 ml Luria broth (LB), supplemented with ampicillin to a final concentration of 50 μg/ml, and grown at 37°C with shaking for 9–12 h. Each culture was then diluted 1:100 into 50 ml fresh LB medium supplemented with ampicillin. At _A_600 = 0.4, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.4 mM for induction. The cells were harvested 3 h after induction and whole-cell lysates from the induced cultures were analyzed by 15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli 1970). The relative amount of expressed multimeric peptides was analyzed by a densitometer (Bio-profile image analysis software, Bio-ID, Vilber Lourmat, France). The total protein concentration was determined with a Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Calif., USA), using bovine serum albumin as a standard.