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Screening of ACE-inhibitory peptides from a random peptide-displayed phage library using ACE-coupled liposomes

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Abstract

Angiotensin I converting enzyme (ACE)-inhibitory peptides were screened from a random peptide-displayed phage library using ACE-coupled liposomes. Among four kinds of inhibitory peptides selected by biopanning with two different elution strategies, a peptide (LSTLRSFCA) showed the highest inhibitory activity with an IC_{50} value of 3 μM. By measuring inhibitory activities of fragments of the peptide, it was found that the RSFCA region was a functional site to inhibit strongly the ACE catalytic activity, and particularly both Arg and Cys residues were essential for the strong inhibitory activity. The inhibitory activity of RRFCA was slightly increased, while that of the RSFRA, in which the Cys residue was replaced by Arg, was decreased to greater extent in comparison with the inhibitory activity of RSFCA. Taking into account the results obtained from the SPOT analysis, it was suggested that the Arg and Phe residues in RSFCA were important for a specific interaction with ACE, and the Cys residue inhibited the ACE activity. The cystein-based ACE-inhibitory peptides have not been isolated from processed food materials. These findings suggested that the biopanning method utilizing protein-coupled liposomes and random peptide libraries might have a possibility to screen new functional peptides that are not found in processed food materials.

Keywords: Angiotensin I converting enzyme; Inhibitory peptide; Biopanning; Liposome; Phage display; SPOT

1. Introduction

Recently, many bio-functional peptides such as antihypertensive, antioxidant, antimicrobial, antithrombotic and immunomodulatory peptides, which were either chemically synthesized or isolated from foods, have been reported. Among these peptides, antihypertensive peptides (angiotensin I converting enzyme inhibitors) are of particular interest for prevention and treatment of hypertension. In the renin–angiotensin system (RAS), inhibition of angiotensin I converting enzyme (ACE, EC 3.4.15.1) is important to regulate blood pressure. Angiotensin II that is produced from angiotensin I by ACE is a vasoconstrictor, and thus ACE inhibitors have considerable importance as antihypertensive agents. Synthetic ACE inhibitors, such as captopril, lisinopril and enalapril, have been used as a drug to decrease blood pressure but may show some side effects (Antonios and Macgregor, 1995). Therefore, ACE-inhibitory peptides produced from food materials, such as soy beans, milk and fish, have been studied by many researchers because of little or no side effects (Maruyama et al., 1985; Kohama et al., 1988; Kohama et al., 1991; Yokoyama et al., 1992; Kuba et al., 2005; Hasan et al., 2006a,b, 2007).

In these days, phage display systems, in which peptides consisting of randomized amino acids are displayed on the surfaces of filamentous M13 phage particles, have been widely used for screening of affinity ligands specific to target proteins and epitope mapping for monoclonal antibodies (Yao et al., 1996; Muhle et al., 2004; Fack et al., 1997). This system will be useful as an alternative screening method in order to select ACE-inhibitory peptides, since it should be possible to screen

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inhibitory peptides, which amino acid sequences are not found in processed food materials. However, any ACE-inhibitory peptide screened from a phage library has not been reported yet, although Huang et al. reported the screening of inhibitory peptides for angiotensin converting enzyme 2 (ACE 2), which has been categorized as angiotensin I converting enzyme showing different specificities to both substrates and inhibitors in comparison with the original ACE (Huang et al., 2003). One possible difficulty in screening ACE-inhibitory peptides from a random peptide library might be attributed to a relatively broader catalytic specificity of ACE as a carboxydipeptidase than ACE 2. ACE can cleave not only angiotensin I and bradykinin, but also many other bioactive peptides such as substance P, neurotensin, and enkephalin (Corvol et al., 1995). Another difficulty will be caused by denaturation of ACE that was immobilized on the plastic surface. Denatured ACE may show low or different substrate specificities from those of native ACE.

In the previous studies, we have reported an efficient method to screen affinity peptides from a phage library using protein-coupled liposomes (Kumada et al., 2005, 2006). By use of liposomes as adsorbents, proteins can be immobilized on the surface of phospholipid membrane with minimum denaturation. Furthermore, the hydrophilic nature of the biological membrane surface can reduce non-specific adsorption of phages without blocking operations. Therefore, a biopanning method using protein-coupled liposomes can be useful to select peptides showing specific affinity toward a target protein in native state, compared with the conventional method using protein-immobilized polystyrene tubes.

In this study, ACE-inhibitory peptides were screened from an octapeptide-displayed phage library by use of ACE-coupled liposomes and their ACE-inhibitory activities and a functional site of one of the peptides were determined.

2. Experimental

2.1. Materials

ACE (EC 3.4.15.1, from rabbit lung) and its substrate N-hippuryl-L-histidyl-L-leucine (HHL) were obtained from Sigma–Aldrich (MO). Dipalmitoylphosphatidylcholine (DPPC) (Nacalai Tesque, Kyoto, Japan), dicetylphosphate (DCP) (Sigma–Aldrich, MO) and 4-(*p*-maleimidophenyl)-butyl-dipalmitoylphosphatidylethanolamine (MPB-DPPE) (Avanti Polar Lipid, Inc., AL) were used for liposome preparation. 2-Iminothiolane·HCl (Pierce, IL) was used for coupling of ACE on liposome membrane. N-Ethyl maleimido was purchased from Nacalai Tesque. All other reagents were of analytical grade unless otherwise specified. The concentrations of antibiotics used to supplement 2× YT medium were as follows—ampicillin (Amp): 50 mg/L and tetracycline (Tet): 12.5 mg/L.

2.2. Escherichia coli strain and random peptide-displayed phage library

Escherichia coli XL1-Blue, which has the Tet^R F plasmid and the chromosomal genotype containing *endA1*, *hsdR17*, *supE44*, *thi1*, *recA1*, *gyrA96* and *relA1*, was purchased from Stratagene (CA) and used as a host for generation of random octapeptide-displayed M13 phage particles. Random octapeptide-displayed M13 phages were prepared as reported previously (Kumada et al., 2005), and the repertory of peptide-displayed phages in this library was 1.2×10^5, which was determined by counting colonies appeared on a selective agar plate after transformation.

2.3. Preparation of ACE-coupled liposomes

The ACE-coupled liposomes which formed multilamellar vesicles (ACE-MLVs) were prepared according to the method reported previously (Kumada et al., 2005). Briefly, a lipid mixture of 10 μmol DPPC, 1 μmol DCP and 0.5 μmol MPB-DPPE in 5 mL chloroform was dried at 50 °C under reduced pressure to make a thin lipid film on the inside wall of a pear-shaped flask. Then, 3 mL of phosphate buffered saline (PBS, pH 7.2) was added, and the flask was shaken at around 50 °C to peal off the lipid film to form multilamellar vesicles (MLVs). The MLVs were washed twice with PBS by repeated centrifugation, and then, resuspended in 500 μl of PBS containing 0.62 mg/mL ACE stock solution, which was coupled on liposomes by use of 2-iminothiolane hydrochloride. After washing with PBS, the ACE-coupled liposomes (ACE-MLVs) were stored at 4 °C until use. The ACE concentration in liposome suspensions was measured by use of DC-protein assay kit using bovine serum albumin as a standard protein, and the DPPC amount in ACE-MLVs was measured by a kit, phospholipid C Test WAKO (Wako Pure Chemicals Industries Ltd., Osaka, Japan).

2.4. Biopanning selection of inhibitory peptides by use of ACE-coupled liposomes

Biopanning selections of ACE-inhibitory peptides were performed basically according to the method reported previously (Kumada et al., 2005). One millilitre of PBS containing ACE-MLVs at an ACE concentration of 37 μg/mL was prepared in a sterile plastic tube and then centrifuged at 15,000 × *g* for 2 min. After supernatant was discarded, ACE-MLVs were washed twice with PBS. ACE-MLVs sedimented were then resuspended with 1 mL of PBS containing 10% blocking one and 2.5×10^{11} cfu/mL of octapeptide-displayed M13 phages and transferred to a new sterile plastic tube. After incubation at 25 °C for 30 min, the suspension was transferred into a new plastic tube. ACE-MLVs were washed nine times by repeated centrifugation using PBS as a washing buffer. AB-MLVs washed were transferred into a new plastic tube, and then the supernatant was removed by centrifugation. The phages bound to ACE-MLVs were eluted with 0.2 mL of 0.1N HCl (pH 1.0). The eluate was recovered by centrifugation and immediately neutralized with 0.1 mL of 2 M Tris–HCl (pH 8.0).

The collected phages were infected with precultured XL1-Blue, and the infected cells were grown in 50 mL of 2× YT medium (Amp, Tet) until OD at 600 nm reached 0.5–1.0. After superinfection with helper phage VCSM13 (MOI = 20), the recovered phages were amplified in fresh 2× YT medium (Amp,