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FRAGMENTATION OF ANGIOTENSIN-I CONVERTING ENZYME INHIBITORY PEPTIDES FROM BONITO MEAT UNDER INTESTINAL DIGESTION CONDITIONS AND THEIR CHARACTERIZATION

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Kinetics of fragmentation of angiotensin-I converting enzyme inhibitory peptides obtained by digestion in gastric juice were studied under intestinal digestion conditions and their inhibitory activities were determined. A fragment IKYGD produced by digestion, as well as IKWGD synthesized, showed similar inhibitory activity to the original peptides. These peptides somehow were resistant to tryptic and/or chymotryptic digestion, and IK + aromatic amino acid might be important functional parts in some kinds of ACE inhibitory peptides.

Keywords: angiotensin-I converting enzyme; inhibitory peptide; anti-peptide antibody; intestinal digestion; bonito; competitive inhibitor.

INTRODUCTION

Many inhibitory peptides against the angiotensin-I converting enzyme (ACE), which generates a vasoconstrictor angiotensin-II (DRVYIHPF) by removing the C-terminal dipeptide from the precursor decapeptide angiotensin-I (DRVYIHPFHL) and also degrades a vasodilator bradykinin (RPPGFSPFR), have been separated from hydrolysates of food proteins as bio-functional peptides for preventing hypertension (Maruyama and Suzuki, 1982; Kohama *et al.*, 1988, 1991; Yokoyama *et al.*, 1992; Kuba *et al.*, 2005). Certain ACE inhibitory peptides provided clear positive health effects, and can be used as ingredients in functional foods. In spite of many reports on amino-acid sequences and inhibitory characteristics of these ACE inhibitory peptides, there are very few studies on the kinetics of production and fragmentation of ACE inhibitory peptides from parent proteins in the enteric cavity. In the previous work (Hasan *et al.*, 2006), we studied production kinetics of ACE inhibitory peptides from bonito in an artificial gastric juice by use of an affinity column recognizing an ACE inhibitory peptide PTHIKWGD, which was discovered by Kohama *et al.* (1988) from the thermal hydrolysate of tuna meat. Several peptides, which contained the sequence of PTHIKWGD and showed the ACE inhibitory activity, were relatively stable in the gastric juice. In the enteric cavity, these peptides will be further digested before adsorption through intestine villus. Relatively small and stable peptides among ACE inhibitory peptides can be absorbed (Yang *et al.*, 1999). In this point of view, two peptides were selected from ACE inhibitory peptides in a previous work (Hasan *et al.*, 2006), and further fragmentation of the peptides under intestinal digestion conditions and the ACE inhibitory activities of the fragmented peptides were characterized in order to study the behaviour and functionality of bonito-derived peptides in the enteric cavity.

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MATERIALS AND METHODS

Materials

Trypsin (EC 3.4.21.4, from bovine pancreas, TPCK treated, 14 400 unit mg-solid^{-1}), α-chymotrypsin (EC 3.4.21.1, from bovine pancreas, 54 unit mg-solid^{-1}) and ACE (EC 3.4.15.1, from rabbit lungs) were obtained from Sigma Chemical Co. (St Louis, MO, USA). CNBr-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ, USA) and an HPLC column of 4.6 mm × 150 mm (Cosmosil 5C_{18}-MS-II, nacalai tesque, Kyoto, Japan) were used in column chromatography. Hippuryl-L-histidyl-L-leucine (HHL, nacalai tesque) was used as an ACE substrate for activity measurements. All other reagents were of analytical grade unless otherwise specified.

Preparation of Anti-peptide Antibody Column

The synthesized antigen peptide (PC-IACE, KKPTHIKWGD) was conjugated with keyhole limpet hemocyanin (KLH) using glutaraldehyde (Katoh *et al.*, 1996) and was used for immunization. Rabbits were immunized with a mixture of an antigen solution and Freund's complete adjuvant (1 ml each). A booster injection was repeated twice in a similar manner at an interval of 7 days. A specific anti-peptide antibody (Anti-PC-IACE antibody) was purified from pooled sera by affinity chromatography using PC-IACE-coupled Sepharose 4B (Kumada *et al.*, 2005). The purified anti-PC-IACE antibody was coupled to CNBr-activated Sepharose 4B.

Preparation of ACE Inhibitory Peptides from Bonito Protein in Gastric Juice

Water extracts of bonito meat were freeze-dried, redissolved in Milli-Q water at a concentration of 25 mg ml^{-1} and stored at $-20^{\circ}C$, as previously reported (Hasan *et al.*, 2006). The water extract obtained was digested for 24–48 h at 37° C in a modified artificial gastric juice, 1.76 mg ml^{-1} NaCl solution containing 312 $\mu g\ ml^{-1}$ pepsin (pH 2.0). The hydrolysis reaction was terminated by neutralizing the pH value of the reaction mixture to 7.5 with 2 M Tris-HCl. The supernatant was recovered after centrifugation at 7200× *g* for 10 min.

Digested samples (5.0 ml) were applied to the anti-PC-IACE coupled affinity column equilibrated with PBS buffer pH 7.4. After washing, adsorbed peptides were eluted with 0.1 N HCl. Eluted fractions (4.0–5.5 ml) were dried under vacuum and redissolved in 200 μl of pure water. The sample was applied to a reverse phase column (Cosmosil $5C_{18}$-MS-II) and was eluted by gradient elution with solvent A (Milli-Q water-0.1% trifluoroacetic acid) and solvent B (acetonitrile-0.1% trifluoroacetic acid). The gradient elution condition was 0% of solvent B to 30% for 30 min, and then 30% to 50% for 5 min at a constant total flow rate of 1 ml min^{-1}. Eluted peptide peaks were detected at 215 nm with a photodiode array detector (SPD-6AV, Shimadzu, Kyoto, Japan). Several peptide peaks were collected and lyophilized with a freeze centrifugal dryer and their inhibitory activity was measured.

Two peptides, peptide A (PNRIKYGD) and peptide B (HERDPTHIKWGD), were synthesized by the solid-phase method for further study on fragmentation under intestinal digestion conditions.

Digestion of Peptides Under Intestinal Digestion Conditions

Peptides were digested in 50 mM sodium phosphate buffer (pH 7.8) containing 50–400 $\mu g\ ml^{-1}$ of trypsin and/or 50–500 $\mu g\ ml^{-1}$ of chymotrypsin for 2–12 h at 37°C. The digested sample was applied to the reverse phase column (Cosmosil $5C_{18}$-MS-II) and was eluted by gradient elution, as stated above.

Measurement of Peptide Concentration and Sequencing

The peptide concentration was measured using Fluorescamine (Wako Pure Chemical Ltd, Tokyo, Japan). Phosphate buffer solution (1.49 ml, pH 8.0) was mixed with 0.50 ml of Fluorescamine solution (30 mg in 100 ml of 1,4-dioxane), and 0.01 ml of sample was added and vigorously mixed with a vortex mixer. The fluorescent intensity was measured at an excitation wavelength of 390 nm and emission wavelength of 475 nm. The peptide B for peptides longer than six amino acids and IKWGD for peptides shorter than five amino acids were used as standards. Amino acid sequences of the purified peptides showing ACE inhibitory activity were determined by a Protein Sequencing System (Procise 492, Applied Biosystems Japan, Tokyo, Japan).

Determination of ACE Inhibition by Peptides

The ACE inhibitory activity of peptides was assayed by a modified method of Cushman and Cheung (1971). To a mixture of 150 μl of HHL (5 mM containing 0.3 M NaCl), 135 μl of KH_2PO_4 solution (50 mM, pH 8.3) and 10 μl of ACE solution (5 mU), 5 μl of sample was added and reacted in a glass tube kept at 37°C under constant shaking. The reaction was terminated after 30 min by adding 0.25 ml of 1 N HCl. Liberated hippuric acid (HA) was extracted with 1.5 ml ethyl acetate by vortex mixing for 30 s. After centrifugation (400× *g*, 20 min), an ethyl acetate layer (1 ml) was transferred to a clean glass tube and evaporated at 100°C for 30 min. After dissolving generated hippuric acid in 3.0 ml Milli-Q water, absorbance at 228 nm was measured by a spectrophotometer (UV1600, Shimadzu). One unit of ACE activity was defined as the amount catalyzing the formation of 1 μmol of hippuric acid from HHL in 1 min at 37°C. The activity of each sample was measured in triplicate. The concentration of peptide needed to reduce the angiotensin-I converting enzyme activity by 50% was defined as the IC_{50} value (50% inhibitory concentration). The IC_{50} values were determined by use of the peptides purified by the above method.

The inhibited reaction rate of HHL hydrolysis (V_{in}) was measured at different peptide concentrations, and $[(V_m - V_{in})/V_{in}]$ was plotted against (peptide concentration) on a logarithmic scale, where V_m represented the reaction rate without peptides.

ANALYSES OF RESULTS

Digested Fragments Stable in Gastric Juice

From HPLC analysis of samples purified by the affinity column coupled with anti-PC-IACE antibody, several homological peptides to the antigen peptide KKPTHIKWGD (PC-IACE), such as HERDPTHIKWGD (peptide B in the previous work), PNRIKYGD (peptide A) and REIVPGD, were separated. This means that an anti-peptide antibody is very useful to separate homological peptides from complicated mixture. During long time digestion (48 h) in the modified gastric juice containing 312 $\mu g\ ml^{-1}$ pepsin (pH 2.0) corresponding to one tenth that in gastric juice, only three major peptides were remaining, as shown in an HPLC chromatogram of the affinity purified sample (Figure 1). Although the major peptide after 24 h digestion was HERDPTHIKWGD (peptide B, retention time 24.1 min), it started separating into two close peaks after 48 h and the sequence of the newly produced peak was confirmed as PTHIKWGD (peptide B′, retention