ISOLATION AND PURIFICATION OF ACE INHIBITORY PEPTIDE DERIVED FROM CHICKEN BONE EXTRACT

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Background

The angiotensin I-converting enzyme (ACE) catalyses the formation of angiotensin II, a strong vasopresser, from angiotensin I, together with inactivation of bradykinin possessing hypotensive activity (Ondetti et al. 1977). Such dual effects of ACE inhibitors are contributory to their potent antihypertensive activity. Commercially available antihypertensive drugs such as captopril and enarapril are very potent ACE inhibitors. Recently, many ACE inhibitory peptides have been isolated from casein (Maruyama et al. 1985), sardine muscle (Matsui et al. 2000), dried bonito (Yokoyama et al. 1992), and porcine skeletal muscle (Arihara et al. 2001, Katayama et al. 2003 & 2004). However, there are few reports on deriving ACE inhibitory peptides derived from chicken bone extract.

Objectives

The objective of this study is to isolate and purify ACE inhibitory peptide derived from chicken bone extract.

Materials and methods

Materials and reagents

Chicken bone was obtained from ITOHAM FOODS Inc. Hippuryl-L-histidyl-L-leucine (HHL) was purchased from Nacalai Tesque (Kyoto, Japan). Commercial ACE (from rabbit lung) and trypsin were purchased from Sigma Chemical Co. (St. Louis, MO. U.S.A).

Preparation and hydrolysis of chicken bone extract

One kg of chicken bone was cut into about 3 cubic cm fragments using a saw, and heated under pressure at 121 ^{o}C for 30 min in an autoclave. The treated bone was suspended in 4 L of distilled water and boiled for 4 hours at 100 ^{o}C. The heated solution was filtered through a paper towel, and the filtrate was collected. Thereafter, the filtrate was centrifuged for 60 min at 10,000 x g to remove fat. The remaining solution was filtered again through a No. 5A filter paper (Toyo Roshi Kaisya Ltd., Tokyo, Japan), then the filtrate was lyophilized and used as chicken bone extract. Trypsin was used for hydrolysis of chicken bone extract. Five mg/ml of chicken bone extract was used as substrate. The ratio of enzyme to substrate was 1/100 (based on protein content). After 6 hours of hydrolysation at 37 ^{o}C (pH7.5), the solution was heated at 95 ^{o}C for 10 min to inactivate the protease. The solution was centrifuged for 10 min at 10,000g, and the supernatant was collected for experiments.

Purification of ACE inhibitory peptide

The chicken bone extract hydrolysate was fractionated by high-performance liquid chromatography (HPLC) with gel-filtration (column:TSK gel G2000SW$_{XL}$, 7.8 x 300 mm, Tosoh Co., Tokyo, Japan). Elution was performed with an isocratic elution of 0.2 M PBS (pH7.0) at a flow rate of 1.0 ml/min, after which the absorbance was detected at 225 nm (first HPLC run). The active fraction was collected, and rechromatographed under reversed-phase mode (column:Inertsil ODS-2 (octadecyl silica column), GL-Science, Inc., Tokyo, Japan). Elution was performed with a linear gradient of 0-35 % CH_3CN in 0.1 % TFA at a flow rate of 1.0 ml/min., after which the absorbance was detected at 225 nm (second HPLC run). The active fraction was again collected and rechromatographed under the same conditions except for the eluting solution. This time the elution was performed with a linear gradient of 8-14 % CH_3CN in 0.1 % TFA at a flow rate of 0.5 ml/min. Absorbance was again detected at 225 nm (third HPLC run). The active fraction was once again collected and rechromatographed under reverse-phase mode (column:Cosmosil 5PE-MS (phenyl

column), GL-Science, Inc., Tokyo, Japan). Elution was performed with an isocratic elution of 10 % CH_3CN (fourth HPLC run) and 5 % CH_3CN (final HPLC run) at a flow rate of 0.5 ml/min. This time the absorbance was detected at 215 nm.

Assay for ACE inhibitory activity

The ACE inhibitory activity was measured according to the method of Cushman and Cheung (1971) with slight modifications. This assay is based on the liberation of hippuric acid from hippuryl-L-histidyl-L-leucine (HHL) catalyzed by ACE. Six µl of sample solution was mixed with 20 µl of ACE (60 mU/ml) which dissolved in borate buffer (pH8.3), and with 50 µl of 7.6 mM HHL dissolved in 0.01 M borate buffer and 0.608 M NaCl. The mixture was incubated for 30 min at 37 ^{o}C. To terminate the enzyme activity, 554 µl of 0.1N HCl was added. To extract hippuric acid liberated from HHL by ACE, 1.5 ml of ethyl acetate was added and the tubes were vigorously shaken, and then centrifuged for 15 min at 1,000 x g. One ml of the ethyl acetate layer was dried for 10 min at 100 ^{o}C. Hippuric acid liberated by ACE was photometrically measured at 228 nm after ethyl acetate extraction. IC_{50} value was defined as the concentration of ACE inhibitors needed to inhibit 50 % of ACE activity.

Analysis of peptide

The amino acid sequence of the purified peptide was analyzed by a protein sequencer (Procise 492, Applied Biosystems, Foster City, CA, USA).

Synthesis of peptide

The peptide was synthesized by a solid phase method with a peptide synthesizer (Symphony, RAININ Instrument Co. Inc., Woburn, MA, USA).

Results and discussion

The plain chicken bone extract did not show ACE inhibitory activity, but its hydrolysate showed relatively strong activity with an IC_{50} value of 0.781 mg/ml. This level of activity is comparable to that of carnosine, which is a well-known substance with ACE inhibitory activity. The hydrolysate of chicken bone extracted by trypsin was fractionated by gel-filtration HPLC and the findings are shown in Fig. 1 (first HPLC run). Three fractions with the highest inhibitory activity (12-15 min) were further purified by reversed-phase HPLC (second and third HPLC run). Then the fraction with the highest inhibitory activity (26-27 min) was further purified by reversed-phase HPLC (fourth ie., the final HPLC run). From the single active fraction shown in Fig. 2 (final HPLC run), one peptide with a high ACE inhibitory activity was purified. The single purified peptide was sequenced by a protein sequencer. The amino acid sequence of the one ACE inhibitory peptide was determined, and the structure of the peptide was YYRA (Tyr-Tyr-Arg-Ala, MW 571.67). The IC_{50} value of this single isolated peptide was 33.9 µg/ml, and it showed a relatively high level of activity as do other ACE inhibitory peptides derived from foods. A search for sequence homology in certain databases (FASTA WWW service) demonstrated that the same sequence exists in the primary structure of Ig heavy chain V region (27-30 position) as shown in Fig. 3. Therefore, a novel ACE-inhibitory peptide not previously reported, was now introduced.

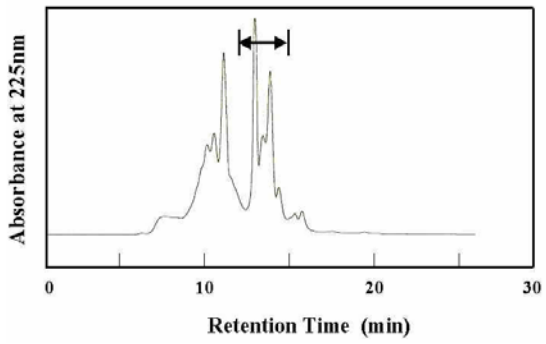

Fig. 1 Gel-filtration HPLC chromatograph (first HPLC run) Arrow indicate active fraction.

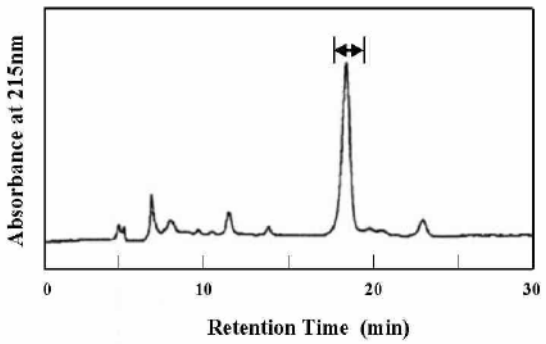

Fig. 2 Reversed-phase HPLC chromatograph (final HPLC run) Arrow indicate active fraction.

Fig. 3 Position of the peptide in the sequence (YYRA) of Ig heavy chain V region.

There are some peptides with potent inhibitory activity in vitro or when intravenously administered that are not active in vivo, when administered orally. It is thought that these peptides are digested into inactive peptide fragments or amino acids after oral administration. It is of great importance that the peptides have sufficient antihypertensive activities in vivo. Further studies are, thus, needed to measure the ACE activity using spontaneously hypertensive rats (SHR).

Conclusions

A novel ACE inhibitory peptide was isolated and purified from chicken bone extract. The amino acid sequence of the peptide was YYRA (Tyr-Tyr-Arg-Ala), which was of the origin of the Ig heavy chain V region (27-30 position). The result of this study suggests that ACE inhibitory peptides derived from chicken bone extract could be utilized in developing physiologically functional foods.

References

Arihara, K., Nakashima, Y., Mukai, T., Ishikawa, S. and Itoh, M. (2001). Peptide inhibitors for angiotensin I-converting enzyme from enzymatic hydrolysates of porcine skeletal muscle proteins. *Meat Science*, 57, 319-324.

Cushman, D.W., and Cheung, H.S. (1971). Spectrophotometric assay and properties of the angiotensin converting enzyme of rabbit lung. *Biochemical Pharmacology,* 20, 1637-1648

Katayama, K., Tomatsu, M., Fuchu, H., Sugiyama, M., Kawahara, S., Yamauchi, K., Kawamura, Y and Muguruma, M. (2003) Purification and characterization of an angiotensin I-converting enzyme inhibitory peptide derived from porcine troponin C. *Animal Science Journal*, 74, 53-58.

Katayama, K., Tomatsu, M., Kawahara, S., Yamauchi, K., Fuchu, H., Kodama, Y., Kawamura, Y and Muguruma, M. (2004) Inhibitory profile of nonapeptide derived from porcine troponin C against angiotensin I-converting enzyme. *Journal of Agricultural and Food Chemistry*, 52, 771-775.

Maruyama, S., Nakagomi, K., Tomizuka, N. and Suzuki, H. (1985). Angiotensin I-converting enzyme inhibitor derived from an enzymatic hydrolysate of casein. *Agricultual and Biological Chemistry*, 49, 1405-1409.

Matsui, T., Kawasaki, T. (2000). Antihypertensive effects of bioactive peptides derived from protein. *Journal of Japanese Society of Nutrition and Food Science*, 53, 77-85.

Ondetti, M.A., Rubin, B. and Cushman, D.W. (1977). Design of specific inhibitors of angiotensin converting enzyme: new class of orally active antihypertensive agents. *Science*, 196, 441-444.

Yokoyama, K., Chiba, H. and Yoshikawa, M. Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito. *Bioscience, Biotechnology and Biochemistry*, 56, 1541-1545.