【Short Communication】

Purification and Antihypertensive Activity of a Novel Angiotensin-I Converting Enzyme Inhibitory Peptide from Fish Sauce, Ishiru

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[ABSTRACT]
We purified a novel angiotensin-I converting enzyme (ACE) inhibitor from fish sauce Ishiru prepared from squid, and identified it as the tripeptide Leu-Ala-Arg (LAR). IC50 of this ACE inhibitor was 2.5 µM, demonstrating high potency among peptides previously purified from fish sauces. Moreover, LAR acted as an antihypertensive peptide, reducing systolic blood pressure in spontaneously hypertensive rats.

[Key words]
fish sauce, angiotensin-I converting enzyme inhibitory peptide, antihypertensive effect

INTRODUCTION
Fish sauce is an ancient seasoning that is produced and used worldwide. At present, it is produced and consumed mainly in Southeast Asia, particularly in nam-pla of Thailand, nuoc-mam of Vietnam, and patis in the Philippines. Although it is not a major sauce, fish sauce is also produced in Japan and is used as a seasoning for various processed foods. Ishiru is a representative fish sauce in Japan and is traditionally produced in the peninsula area of Ishikawa prefecture. Ishiru is mainly prepared from squid or sardine, and fish sauce prepared from squid in particular is unique in the world. We previously reported that Ishiru contains large quantities of peptides and free amino acids, and it has strong health-promoting properties, including angiotensin-I converting enzyme (ACE) inhibitory and antioxidant activities.1,2

ACE inhibitory activity is an important function of food because this activity produces a drop in blood pressure and relief from hypertension. Substances that inhibit ACE prevent production of the potent vasoconstrictor angiotensin-II from angiotensin-I.3,4 Hypertension causes cardiovascular diseases, renal disease, and stroke.5 Thus, ACE inhibitors are used in the treatment of essential hypertension and cardiac failure in humans. However, they are believed to cause a number of side effects such as cough, skin rashes, and angioedema.6,7 Many researchers have purified and identified ACE inhibitory peptides from enzymatic digests of various proteins from food such as fish8-12, meat13,14, grain15,16, and other foods17-19. Some of these peptides have been utilized as functional food
supplements for hypertension\textsuperscript{20}. Fish sauce is expected to contain ACE inhibitory peptides because it is produced by natural fermentation of fish protein. Therefore, desalted fish sauce and isolated ACE inhibitory peptides from fish sauce might be the source of antihypertensive functional food or medical drug. Some ACE inhibitory peptides were isolated from fish sauces prepared from salmon\textsuperscript{21}, anchovy\textsuperscript{22}, blue mussel\textsuperscript{23}, and oyster\textsuperscript{24}, whereas no such ACE inhibitory peptides have been isolated from fish sauce prepared from squid. Thus, we aimed to isolate a new and strong ACE inhibitory substance from \textit{Ishiru} prepared from squid, paying special attention to low-molecular-weight peptides because such peptides are readily absorbed in the body. In this study, we isolated and purified an ACE inhibitory peptide from \textit{Ishiru} and investigated the antihypertensive action of this peptide by administering it orally to spontaneously hypertensive rats (SHRs).

**MATERIALS AND METHODS**

The \textit{Ishiru} prepare from squid was purchased from a local market in Ishikawa prefecture. Fluorescence spectrometry (F-2500; Hitachi High-Technology Co. Ltd., Tokyo, Japan) was performed according to the method of Nakano et al.\textsuperscript{25} to calculate the ACE inhibitory ratio (%) from ACE activity assays using ACE from rabbit lung and hippuryl-L-histidyl-L-leucine (Sigma Chemicals Co. Ltd., MO, USA). One hundred milliliters of \textit{Ishiru} was extracted with 200 ml of acetonitrile, and the extract was evaporated. The residue was dissolved in ultrapure water and filtered using an ultrafiltration membrane (Vivaspin 15R; Sartorius Stedim Japan, Tokyo, Japan) with a molecular weight cutoff of 2000 Da. The filtrate was dialyzed between a dialysis tube (Spectra/Por Float-A-Lyzer; Spectrum Laboratories Inc., CA, USA) and a membrane (Spectra/Por Cellulose Ester Membrane; Spectrum Laboratories Inc.) with 1000 Da and 100 Da molecular weight cutoffs, respectively. The dialyzed sample was lyophilized and purified using the Sephadex LH-20 column (18 \times 700 mm, Amersham Biosciences AB, Uppsala, Sweden) eluted with ultrapure water at a flow rate of 10–20 ml/h. Subsequently, 4.0 ml fractions were collected using a fraction collector (Ecosystem, Bio-Rad Laboratories Inc., CA, USA). ACE inhibition was assayed and elution was monitored at 216 and 280 nm. The fractions with the highest ACE inhibitory activity were lyophilized and further purified by HPLC (Gilson Inc., WI, USA) on a C\textsubscript{18} column (20 \times 250 mm, Shiseido Capcell Pak C18; Shiseido Co., Ltd., Tokyo, Japan) with a linear gradient of acetonitrile (0–50% for 60 min) containing 0.1% trifluoroacetic acid at a flow rate of 5.0 ml/min. Absorbance of each fraction was measured at 216 nm, and the ACE inhibitory ratio (%) was calculated.

The amino acid sequence of the purified peptide was characterized using an automated protein sequencer (Procise 491 HT; Applied Biosystems, CA, USA), and a peptide with the same structure as the purified peptide was synthesized by Life Technologies (Tokyo, Japan). The half maximal inhibitory concentration (IC\textsubscript{50}) of the synthesized ACE inhibitory peptide was determined with ACE from rabbit lung and hippuryl-L-histidyl-L-leucine by HPLC, according to the method of Horiuchi et al.\textsuperscript{26} and Ohta et al.\textsuperscript{27}.

Antihypertensive activity of the synthesized peptide was evaluated by measuring the change in systolic blood pressure (SBP) at 0, 2, 4, 6, 12, and 24 h after oral administration. Male 8-week-old SHRs were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan) and divided into three groups of six. These groups were randomly assigned for treatment with the synthesized peptide, valyl-tyrosine (VY) peptide (Sigma Chemicals Co. Ltd.) as a positive control, or the same volume of saline solution (control). The rats were housed at 24 ±1°C with a 12:12 light–dark cycle and had free access to food (Funahashi SP diet; Funahashi Farm, Chiba, Japan) and saline. The tail-cuff method was used to measure SBP using a sphygmonanometer (BP-98A; Softron Corp., Tokyo, Japan). Differences in SBP before and after administration were analyzed using the Student’s t-test.

**RESULTS AND DISCUSSION**

An ACE inhibitory peptide was purified from Ishiru by acetonitrile extraction, dialysis, and ultrafiltration. The filtrate was applied to a Sephadex LH-20 column, and the chromatogram is shown in Fig. 1a. Fraction 48 exhibited the strongest ACE inhibitory activity. Poor correspondence of peak ACE inhibitory activity and absorbance at 280 nm indicated the absence of aromatic groups in the ACE inhibitory substance. Fraction 48 was lyophilized and applied to a C\textsubscript{18} column. As shown in Fig. 1b, there were four major absorbance peaks at 216 nm. Fractions eluted at 38 min exhibited strong activity (82% inhibition) and were pooled and lyophilized immediately. The weight of the resulting powder was approximately 3 mg.

The amino acid sequence of the purified peptide was determined to be Leu-Ala-Arg (LAR), which has never been reported as an ACE inhibitory peptide. By searching the amino acid sequence of LAR in previous reports, it was found that
LAR exists in major lens proteins of squid. Squid eyeballs are one of the ingredients of Ishiru. Hence, LAR derived from major lens proteins might be found in Ishiru. IC$_{50}$ of LAR was determined to be 2.5 μM and that of peptides purified from other fish sauces was reported to be 1.8–147 μM. Hence, the ACE inhibitory activity of LAR is high among peptides previously purified from fish sauce.

We isolated the peptide LAR showing strong ACE inhibitory activity from Ishiru and then investigated the antihypertensive action of the synthetic peptide with the same structure as LAR by administering it orally to SHRs. Fig. 2 shows the change in SBP after oral administration of the ACE inhibitor in SHRs. The antihypertensive activity of LAR was evaluated in comparison to that of VY (a positive control). It is well known that VY, which was purified from sardine hydrolysates by protease digestion, exhibits strong ACE inhibitory activity in SHRs. SBP in LAR-treated SHRs was reduced from 4 h to 12 h after administration. It is expected that LAR, as a small peptide (di- or tri-peptide), is easily absorbed in its intact form in the intestine and then exhibits a strong suppressive effect on SBP in SHRs. On the other hand, a reduction in SBP in VY-treated SHRs was only observed at 4 h. Therefore, the antihypertensive activity of LAR was found to be higher than that of VY. This result suggests the promising application of LAR as an antihypertensive functional food. Moreover, consumption of Ishiru may help to maintain blood pressure within the normal range. Our future focus will be to evaluate the blood pressure-lowering effect of LAR in human clinical trials.

**REFERENCES**

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イカ魚醤油（いしる）に含まれるアンジオテンシンⅠ変換酵素阻害ペプチドの単離と血圧降下作用

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イカ魚醤油（いしる）より新規のアンジオテンシンⅠ変換酵素 (ACE) 阻害ペプチド Leu-Ala-Arg (LAR) を単離した。LAR の ACE 阻害活性 IC_{50} は2.5 μM であり、これまでに報告されている魚醤油中の ACE 阻害活性ペプチドの中でも高い阻害活性を示した。LAR を高血圧自然発症ラットに口径摂取した結果、血圧上昇抑制作用を示した。

キーワード: 魚醤油、アンジオテンシンⅠ変換酵素阻害ペプチド、血圧上昇抑制作用