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Angiotensin I-converting enzyme inhibitory peptides in red-mold rice made by *Monascus purpureus*

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ABSTRACT

The ACE inhibitory activity in red-mold rice extracts, prepared from 24 strains of the genus *Monascus*, was measured. The most effective strain for ACE inhibition was *Monascus purpureus* IFO 4489 (IC_{50} = 0.71 mg/ml). Although the antihypertensive substance γ-amino butyric acid was detected in the red-mold rice (85.2 mg/kg), it did not contribute to ACE inhibition. Four ACE inhibitory peptides were isolated from the extract and identified as Ile-Tyr (IC_{50} = 4.0 μM), Val-Val-Tyr (22.0 μM), Val-Phe (49.7 μM) and Val-Trp (3.1 μM) by protein sequencing. The ACE inhibitory activity of these peptides was almost completely preserved after successive *in vitro* digestion by pepsin, chymotrypsin and trypsin. These results suggest that red-mold rice made by *M. purpureus* could be useful in alleviating hypertension.

1. Introduction

Red-mold rice [1] made by *Monascus* species has traditionally been used in East Asia as a foodstuff in the production of natural colorants and fermented foods like red rice wine and tofuyo (fermented tofu) [2]. It has also been used as a Chinese herbal medicine in promoting absorption in the digestive system and mobilizing blood circulation. Moreover, in recent studies, it has been confirmed that red-mold rice has some medicinal properties, such as being an antihypertensive, hypocholesterolemic, antimicrobial, antioxidative and anticarcinogenetic agent [1]. One of the antihypertensive substances in red-mold rice has been isolated and identified as γ-amino butyric acid (GABA) [3], which blocks peripheral autonomic ganglia followed by vasodilation [4].

Angiotensin I-converting enzyme (ACE, EC3.4.15.1.) is a dipeptidyl carboxy peptidase that plays an important role in the regulation of blood pressure. It converts angiotensin I into a powerful vasoconstrictor, angiotensin II, and also inactivates the vasodilator bradykinin [5,6]. ACE inhibitors in various types of foods have been studied and show the ability to prevent and alleviate hypertension. Physiologically functional foods, enriched with ACE inhibitors, such as tripeptide (Val-Pro-Pro, Ile-Pro-Pro) from sour milk [7], dipeptide (Val-Tyr) from a sardine muscle hydrolysate [8,9] and dodecapeptide (Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys) from a casein hydrolysate [10], are used as supplements to improve hypertension. We have also reported on ACE inhibitory peptides, Ile-Phe-Leu and Trp-Leu, isolated from tofuyo as well as Leu-Ala-Ile-Pro-Val-Asn-Lys-Pro and Trp-Leu produced from soybean protein hydrolysates [11,12]. Although the antihypertensive effect of GABA in red-mold rice has already been elucidated, ACE inhibitory activity has not yet been reported. Red-mold rice, which includes ACE inhibitory peptides, is an essential material for developing health food to reduce the risk of hypertension.

In this study, we selected the most favorable *Monascus* strain with which to obtain red-mold rice exhibiting high ACE inhibitory activity. Furthermore, we isolated and identified the ACE inhibitory peptides in red-mold rice, as well as characterizing the *in vitro* digestive stability of the ACE inhibitors against gastrointestinal proteases.

2. Materials and methods

2.1. Materials

The enzymes used throughout this study were angiotensin I-converting enzyme (rabbit lung, 0.25 units), pepsin (porcine gastric mucous membrane, 3900 units/mg of solid), chymotrypsin (bovine pancreas, 40–60 units/mg of protein) and trypsin (bovine pancreas, 8600 units/mg of solid) from Sigma–Aldrich Co. (Saint Louis, USA). Hippuryl-L-histidyl-L-leucine (Hip-His-Leu) was obtained from Peptide

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Institute Inc. (Osaka, Japan). Ile-Tyr, Val-Val-Tyr, Val-Phe and Val-Trp were synthesized by Nikka Techno Service Co. (Ibaraki, Japan). SEPABEADS SP825 and Sephadex G-25 resin were obtained from Mitsubishi Chemical Co. (Tokyo, Japan) and GE Healthcare UK Ltd. (Buckinghamshire, UK), respectively. All other chemicals used were of analytical grade.

2.2. Preparation of red-mold rice extracts

Of the 24 *Monascus* strains used in making red-mold rice, 12 were obtained from the Institute for Fermentation (Osaka, Japan) and the other 12 had been previously isolated from a varied collection of red-mold rice in our laboratory. Non-glutinous rice was soaked in tap water for 18 h and then autoclaved. After pre-cultivation at 30 °C for 7 days on potato-glucose agar slants, each strain was cultured in autoclaved non-glutinous rice at 30 °C for 7 days. The resulting culture was red-mold rice.

Red-mold rice was suspended in 3 volumes (w/v) of distilled water, homogenized using a labo-stirrer (Yamato L-35) and then shaken in an incubator (Yamato BT-21) at 25 °C for 1 h. The mixture was then centrifuged at 12,000 × *g* for 15 min and the resulting precipitate was removed. The turbid supernatant was filtered, and the filtrate was boiled for 20 min. The filtrate was used as the red-mold rice extract.

2.3. ACE inhibitory assay

ACE inhibitory activity was assayed with ACE from rabbit lung and Hip-His-Leu as previously described [11]. The IC_{50} value was defined as the sample concentration required to inhibit 50% of ACE activity under the assay conditions.

2.4. Purification of ACE inhibitors in red-mold rice extract

One of the *Monascus* strains which exhibited high ACE inhibitory activity was selected and used for making red-mold rice. Fifty milliliters of red-mold rice extract, containing 5.75 mg protein, was mixed with 30 ml of adsorptive resin SEPABEADS SP825. After washing the resin with approximately 50 volumes of distilled water, the ACE inhibitors were fractionated by successive elution with 10%, 15%, 20%, 25%, 40% and 70% ethanol (100, 80, 150, 50, 60 and 75 ml, respectively). Each fraction was evaporated in a rotary evaporator (Yamato RE-46), and the resulting residue was dissolved in distilled water. The active fraction was applied to a Sephadex G-25 column (Ø 1.2 cm × 142.5 cm) and eluted with distilled water. Proteins were identified by monitoring absorbance at 220 nm. Subsequently, the active fraction was subjected to a reverse-phase HPLC with a Cosmosil $5C_{18}$-AR-300 column. The elution was carried out with a linear gradient of 0–50% acetonitrile in 0.05% trifluoroacetic acid (TFA) for 50 min at 0.5 ml/min and monitored at 220 nm. The fractions corresponding to the active peaks were rechromatographed in the same column or Cosmosil 5Ph-AR-300 column, and eluted by moderate acetonitrile gradient in 0.05% TFA at 0.25 ml/min.

2.5. Amino acid sequence analysis of ACE inhibitors

The amino acid sequences of the ACE inhibitors were determined by automated Edman degradation [13] with a gas/liquid phase protein sequencer (PE Applied Biosystems 473A).

2.6. Digestion test

The *in vitro* stability of each purified ACE inhibitory peptide against gastrointestinal proteases was examined to predict their *in vivo* antihypertensive effects. Four inhibitor solutions (10 mM Ile-Tyr, Val-Val-Tyr, Val-Phe and Val-Trp, 0.2 ml each) were incubated with 0.2 ml of a 0.05% (w/v) pepsin solution (0.1 M HCl at pH 2.0) and 0.2 ml of 0.1 M HCl for 6 h at 37 °C. After boiling the reaction mixture for 5 min to stop the digestion, it was evaporated in a centrifugal concentrator. The residue was then redissolved in 0.6 ml of a solution containing both 0.025% (w/v) chymotrypsin and 0.025% (w/v) trypsin. The reaction mixture was incubated for 6 h at 37 °C and then boiled for 5 min to stop the digestion. After the enzymatic treatment, each sample was centrifuged, and the ACE inhibitory activity of the supernatants was measured.

3. Results and discussion

3.1. ACE inhibitory activity of red-mold rice extracts

The ACE inhibitory activity in red-mold rice extracts, prepared from 24 strains of genus *Monascus*, was measured (data not shown), and the 5 strains with the most potent activity were *Monascus purpureus* IFO 4478, IFO 4489, *M. sp.* C-1-1, C-3 and C-4. The ACE inhibition IC_{50} values for the extracts from the 5 strains are provided in Table 1. *M. purpureus* IFO 4489 had the highest ACE inhibitory activity, and was therefore chosen for the production of red-mold rice. The IC_{50} value was in the range seen in a variety of other foods (0.028–3.03 mg/ml) [8,11,12,14–21], indicating that the ACE inhibitory activity of red-mold rice made from *M. purpureus* was relatively strong.

Table 1
ACE inhibitory activity in red-mold rice extracts made by various strains of genus *Monascus*.

	IC_{50} (mg/ml)
M. purpureus IFO 4478	1.64
M. purpureus IFO 4489	0.71
M. sp. C-1-1	1.66
M. sp. C-3	1.22
M. sp. C-4	1.68

GABA, one of the antihypertensive substances in red-mold rice, has already been identified and its mechanism of blood pressure reduction studied in detail [4]. The concentration of GABA in red-mold rice extract was quantified by amino acid analysis and found to be 85.2 mg per kg of red-mold rice, which is consistent with other reports [3,22,23]. The ACE inhibitory activity of a synthetic GABA solution, prepared at the same concentration as that found in red-mold rice extract, was less than 10% of that of the red-mold rice extract. This result indicates that GABA may not contribute to the ACE inhibitory activity of the red-mold rice made from this strain. Red-mold rice contains not only GABA, but also ACE inhibitory peptides as antihypertensive substances, and is therefore useful as a nutraceutical for the prevention and treatment of hypertension.

3.2. Isolation and identification of ACE inhibitory peptides in red-mold rice extract

The red-mold rice extract obtained from *M. purpureus* IFO 4489 was mixed with a resin of SEPABEADS SP825 and separated into seven fractions. The ACE inhibitory activity of the fractions is shown in Fig. 1. The most potent activity was found in the 10% and 15% ethanol fractions. We have previously isolated hydrophobic peptides, WL and IFL, as ACE inhibitors in a 20% ethanol fraction from tofuyo extract. In addition, Matsui et al. [8] reported that 10% ethanol fraction of sardine hydrolysate separated by ODS column had strong ACE inhibitory activity and would contain polar peptides with potent inhibitory activities. In this study, we carried out narrow stepwise gradation from 10% to 25% ethanol, and the 10% and 15% ethanol fractions seemed to contain abundant peptides with preferable hydrophobicity and polarity for ACE inhibition. Because the 10% fraction had a much greater amount of

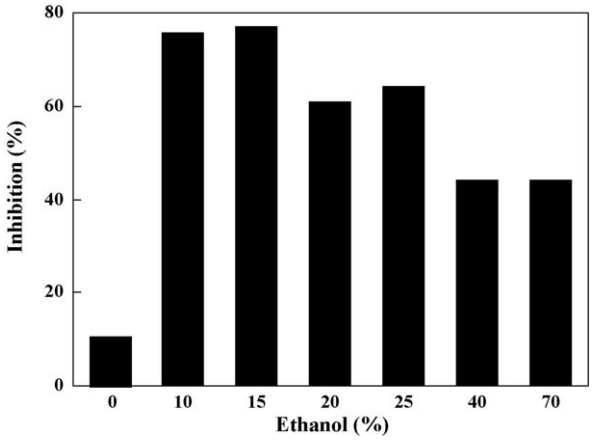

Fig. 1. Separation of ACE inhibitory peptides in red-mold rice by SEPABEADS SP825. The final concentration of protein used in the ACE inhibition assay was 1 mg/ml for each fraction.