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Inhibitory effects of L-pipecolic acid from the edible mushroom, *Sarcodon aspratus*, on angiotensin I-converting enzyme

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Abstract The aqueous extract of the edible mushroom *Sarcodon aspratus* showed inhibitory effects against angiotensin I-converting enzyme (ACE). L-Pipecolic acid (L-piperidine-2-carboxylic acid) was isolated from a hot-water extract in a 0.02% yield as an active principle. The mode of inhibition of L-pipecolic acid was found to be competitive, whereas its D-isomer showed no significant inhibitory effects against ACE, suggesting that the configuration of the carboxyl group in the molecule plays an important role in the enzyme inhibition.

Key words Angiotensin I-converting enzyme (ACE) · Antihypertensive agent · L-Pipecolic acid · *Sarcodon aspratus*

Introduction

Angiotensin I-converting enzyme (ACE, dipeptidyl carboxypeptidase I, EC 3.4.15.1) is a zinc-laden enzyme located mainly in the endothelial lining of the vasculature of the lung, and plays an important role in regulating blood pressure.[1] ACE catalyzes the conversion of inactive decapeptide, angiotensin I, to the potent vasoconstrictor octapeptide, angiotensin II, and also degrades the vasodilator nonapeptide, bradykinin. Inhibition of ACE is, therefore, a means to cure human hypertension and heart failure. Although synthetic ACE inhibitors, such as captopril (1-[2(*S*)-3-mercapto-2-methyl-1-oxopropyl]-L-proline), have been used as antihypertensive drugs, they cause several disadvantageous side effects. Investigation on naturally occurring ACE inhibitors from foods has received great attention.

Hypertension is the most common serious chronic health problem, affecting 15%–20% of adults in many developed countries, and carries a high risk factor for myocardial infarction and stroke. It has been reported that naturally occurring peptides, such as a series of snake venom peptides,[2] possess potent antihypertensive activities. Many ACE inhibitory peptides have been isolated from food proteins.[3] Recently, several peptides having inhibitory activities against ACE have been isolated from edible mushrooms, including *Grifola frondosa*,[4] *Tricholoma giganteum*,[5] and *Mycoleptodonoides aitchisonii*.[6]

Sarcodon aspratus (Berk.) S. Ito is a wild edible mushroom species growing in Japan and Korea, and belongs to Thelephoraceae family. This mushroom has been utilized as a folk medicine for allergic diseases in a local area of Japan.[7] Recently, it was reported that certain noncyclic polyhydric alcohols present in edible mushrooms including *S. aspratus*, such as D-mannitol and D-arabitol, acted as moderate ACE inhibitors.[8,9] In this study, L-pipecolic acid was isolated from the hot water extract of *S. aspratus* as a moderate ACE inhibitor. The objectives of this study were to examine ACE inhibitory activities of the isolated compound and its enantiomer, and to evaluate their kinetics of ACE inhibition.

Experimental

Materials

Dried fruit bodies of *Sarcodon aspratus* were purchased in a market in Cheongju, Korea. Moisture content of the material was 6.9% ± 0.1% (n = 5). ACE from rabbit lung was purchased from Sigma-Aldrich (St. Louis, MO, USA).

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Hippuryl-L-histidyl-L-leucine (HHL) was obtained from the Peptide Institute (Osaka, Japan) and used as a substrate for ACE. The D- and L-isomers of pipecolic acid, bovine serum albumin (BSA), and captopril were purchased from Wako Pure Chemical (Osaka, Japan). All other reagents used in this study were reagent grade chemicals.

Isolation of ACE inhibitor

Dried fruit bodies of *S. aspratus* were pulverized and defatted with *n*-hexane and acetone successively in a Soxhlet extraction apparatus. The extracted residue was further refluxed with water for 3 h. The hot water extract was concentrated in vacuo, and then ten volumes of ethanol were added to the concentrated extract to eliminate some polysaccharides. The resulting precipitate was filtered off, and the filtrate was concentrated and lyophilized. The ethanol-soluble part was fractionated using a Sephadex LH-20 (GE Healthcare Biosciences) gel permeation column (5 × 45 cm). The column was eluted with 50% aqueous ethanol at a flow rate of 7.2 ml h^{-1}. The fraction possessing ACE inhibitory activity was further chromatographed on a silica gel (Silica Gel 60, Kanto Chemical, Tokyo, Japan) column (3 × 60 cm) with solvent mixtures of chloroform and methanol (the ratio of methanol to chloroform was increased gradually from 1:1 to 2:1) to afford a crystalline compound. The compound was subjected to preparative high-performance liquid chromatography (HPLC) on an ODS-80Ts column (20 × 250 mm, Tosoh, Tokyo, Japan) employing an increasing acetonitrile linear gradient (from 5% to 50% aqueous acetonitrile) at a flow rate of 9 ml min^{-1}. Elution peaks were monitored at 215 nm. The fraction corresponding to the main peak was concentrated and recrystallized from aqueous methanol to give colorless rhombics in 0.02% yield. The compound was identified as L-pipecolic acid by infrared (IR) and nuclear magnetic resonance (NMR) spectroscopic analysis, electrospray ionization mass spectrometry (EI-MS), and by measurement of optical rotation.

Assay of ACE inhibitory activity

ACE inhibitory activity was assayed by the method of Cushman and Cheung[10] with minor modifications. A mixture containing 0.2 M borate buffer (pH 8.3), 0.8 M NaCl, 1 mg of BSA, 4×10^{-3} units of ACE, and inhibitor solution was preincubated at 37°C for 10 min. The reaction was initiated by adding 0.02 M HHL solution, and terminated after 1 h of incubation by the addition of captopril solution (10 μg ml^{-1}). The resulting hippuric acid was determined by HPLC equipped with a CAPCELL PAK C_{18} column (4.6 × 250 mm, Shiseido, Tokyo, Japan). The mobile phase consisted of 20% acetonitrile in 10 mM phosphate buffer (pH 2.8) and was used at a flow late of 1 ml min^{-1}. Chromatograms were recorded at 240 nm. A column thermostat was set to 40°C. The IC_{50} value was defined as the concentration of inhibitor required to inhibit 50% of the ACE activity.

Determination of ACE inhibition mode

Determination of ACE inhibition mode was carried out according to the method of Bush et al.[11] with minor modifications. The enzyme activity was measured with different concentrations of the substrate. Each reaction velocity of ACE after 5-min incubation in the presence of inhibitor was calculated, and the kinetics of ACE inhibition was determined using Lineweaver-Burk plots.

Results

In our previous study,[8] the hot-water extract from the fruit bodies of *Sarcodon aspratus* showed relatively strong ACE inhibitory activity compared with those of noncyclic polyhydric alcohols. The sequential chromatographic separation of the ethanol-soluble part afforded colorless rhombics. IR, NMR, and EI-MS spectra of the compound were identical with those of an authentic specimen of L-pipecolic acid. The compound moderately inhibited the ACE activity with respect to HHL substrate (IC_{50}: 23.7 mg ml^{-1}).

To determine the mode of inhibition, the kinetics of inhibition of ACE by L-pipecolic acid was plotted according to Lineweaver and Burk, as shown in Fig. 1. The double-reciprocal plots showed a competitive mode of enzyme inhibition. The Michaelis constant (K_m) after treatment with 20 mg ml^{-1} of this inhibitor (19.5 mM) was elevated compared with that of the control group (without inhibitor, K_m: 5.4 mM), indicating that the affinity of HHL substrate to the active site of ACE was lowered by the dose of L-pipecolic acid with K_i value of 60.1 mM.

To study the relationship between structure and activity, the ACE inhibitory activity of D-pipecolic acid was examined. No significant inhibitory activity against ACE could be detected by the dose of D-pipecolic acid (IC_{50} > 230 mg ml^{-1}).

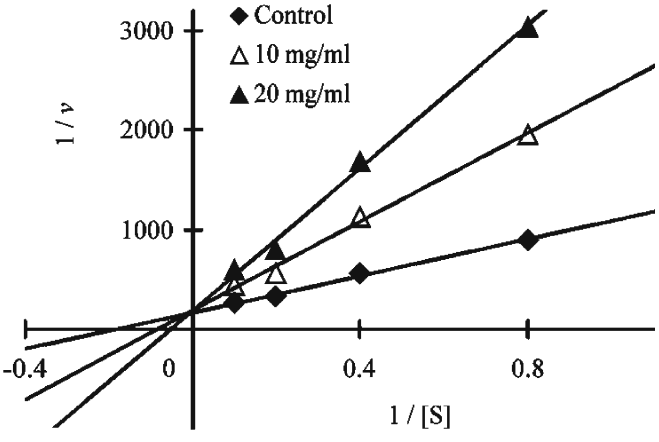

Fig. 1. Lineweaver-Burk plot for the inhibition of angiotensin I-converting enzyme (ACE) by L-pipecolic acid. K_m values in the presence of the designated concentrations of the inhibitor are as follows: control (without inhibitor), 5.4 mM; 10 mg ml^{-1}, 12.4 mM; 20 mg ml^{-1}, 19.5 mM. The K_i value of L-pipecolic acid for hippuryl-L-histidyl-L-leucine substrate was 60.1 mM

Discussion

Pipecolic acid (piperidine-2-carboxylic acid) is a metabolite of lysine, and occurs in various organisms including legumes[12] and mammals.[13,14] For mushroom species, L-pipecolic acid has been found in only a toxic mushroom, *Russula subnigricans*.[15] This nonprotein amino acid is an important biomedical marker for peroxisomal-related disorder,[16] and plays an important depressive role in the central nervous system.[17]

L-Pipecolic acid moderately inhibited the ACE activity with respect to HHL substrate in a competitive manner, whereas no significant inhibitory activity against ACE could be detected by its D-isomer. Although the inhibitory activity of L-pipecolic acid is rather weak compared with those of synthetic ACE inhibitors, the correlation between the configuration of pipecolic acid and the ACE inhibitory activity is of interest. The accommodation of the carboxyl group in L-pipecolic acid by the enzyme S'_2 subsite may be mediated by ionic interactions with basic amino acid residues in the ACE molecule.[18] Consequently, the piperidine ring seems to be stabilized by the S'_2 subsite. The configuration of the carboxyl group in D-pipecolic acid may contribute to weakening of the interaction between the piperidine ring and the S'_2 subsite. Recently, Tzakos et al.[19] studied the interaction between the *cis*- and *trans*-isomers of captopril and the binding sites of human ACE. At physiological pH, captopril exists in solution with near equal populations of the *cis*- and *trans*-isomers, with respect to the proline amide bond. However, the enzyme shows high stereospecificity for amino acid residue in the substrate and its active sites interact with only the *trans*-isomer.[19]

Although a higher proportion of L-pipecolic acid was found in several plants and food samples than its D-isomer, the existence of both the D- and L-isomers in mammalian physiological fluids and tissues has been demonstrated.[16,17,20] Fujita et al.[17] reported that the D-isomerization of L-pipecolic acid can be easily mediated by intestinal bacteria in humans, and, therefore, pipecolic acid is excreted mainly as the D-form. The ratio of its D-isomer to the L-isomer in plasma is, however, much lower than the ratio in urine. These findings strongly suggest that L-pipecolic acid from direct food intake is stable in the gastrointestinal tract and can be absorbed in its intact form into plasma.

An excessive amount of L-pipecolic acid in human plasma is often associated with some peroxisomal-related disorders including Zellweger's syndrome.[16] Although L-pipecolic acid is considered to be an important biomedical marker for these genetic diseases, a moderate antihypertensive effect can be expected by the dose of an appropriate amount of L-pipecolic acid derived from *Sarcodon aspratu*s or other foodstuffs.

References

1. Hollenberg NK (1979) Pharmacologic interrupting the renin-angiotensin system. Ann Rev Pharmacol 19:559–582
2. Cheung HS, Cushman DW (1973) Inhibition of homogeneous angiotensin-converting enzyme of rabbit lung by synthetic venom peptides of *Bothrops jararaca*. Biochim Biophys Acta 293: 451–463
3. Li G-H, Le G-W, Shi Y-H, Shrestha S (2004) Angiotensin I-converting enzyme inhibitory peptides derived from food proteins and their physiological and pharmacological effects. Nutr Res 24: 469–486
4. Choi HS, Cho HY, Yang HC, Ra KS, Suh HJ (2001) Angiotensin I-converting enzyme inhibitor from *Grifola frondosa*. Food Res Int 34:177–182
5. Lee DH, Kim JH, Park JS, Choi YJ, Lee JS (2004) Isolation and characterization of a novel angiotensin I-converting enzyme inhibitory peptide derived from the edible mushroom *Tricholoma giganteum*. Peptides 25:621–627
6. Sakamoto Y, Takeuchi A, Sato T, Obara K, Takai K, Fujino K, Hirose T, Inagaki Y (2001) Identification of antihypertensive substance in an aqueous extract from fruit body of *Mycoleptodonoides aitchisonii*. Oyo Yakuri/Pharmacometrics 61:221–229
7. Tateishi K, Hosono T, Shimazu S, Fujimoto M (2000) Anti-allergenic properties of the fruiting bodies of *Sarcodon asptatus* (in Japanese). Nippon Shokuhin Kagaku Kogaku Kaishi 47:281–286
8. Kiyoto M, Suzuki H, Hara T, Yagi Y, Cho N-S, Aoyama M (2005) Moderate angiotensin I converting enzyme (ACE) inhibitor from the fruit body of *Sarcodon aspratus*. Mushroom Sci Biotechnol 13:189–194
9. Suzuki H, Kiyoto M, Nobuyama N, Hara T, Yagi Y, Togashi I, Aoyama M (2006) Moderate angiotensin I converting enzyme (ACE) inhibitors from the edible mushroom *Pleurotus citrinopileatus* Singer (Agaricomycetideae). Int J Med Mushrooms 8:45–47
10. Cushman DW, Cheung HS (1971) Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 20:1637–1648
11. Bush K, Henry PR, Slusarchyk DS (1984) Muraceins – muranyl peptides produced by *Nocardia orientalis* as angiotensin-converting enzyme inhibitors. I. Taxonomy, fermentation and biological properties. J Antibiot 37:330–335
12. Oh C-H, Kim J-H, Kim K-R, Mabry TJ (1995) Rapid gas chromatographic screening of edible seeds, nuts and beans for non-protein and protein amino acids. J Chromatogr A 708:131–141
13. Inoue H, Sakata Y, Fukunaga K, Nishio H, Tsuruta Y (2004) Sensitive determination of pipecolic acid in serum by high-performance liquid chromatography using 4-(5,6-dimethoxy-2-phthalimidinyl)-2-methoxyphenylsulfonyl chloride as a fluorescent labeling reagent. Anal Chim Acta 511:267–271
14. Kerckaert I, Poll BT, Espeel M, Duran M, Roeleveld ABC, Wanders RJA, Roels F (2000) Hepatic peroxisomes in isolated hyperpipecolic acidaemia: evidence supporting its classification as a single peroxisomal enzyme deficiency. Virchows Arch 436:459–465
15. Kusano G, Ogawa H, Takahashi A, Nozoe S, Yokoyama K (1987) A new amino acid, (2*S*, 3*R*)-(–)-3-hydroxybaikiain from *Russula subnigricans* Hongo. Chem Pharm Bull 35:3482–3486
16. Stefan R-I, Nejem RM, Staden JF, Aboul-Enein HY (2003) Biosensors for the enantioselective analysis of pipecolic acid. Sensor Actuat B-Chem 94:271–275
17. Fujita T, Fujita M, Kodama T, Hada T, Higashino K (2003) Determination of D- and L-pipecolic acid in food samples including processed foods. Ann Nutr Metab 47:165–169
18. Ondetti MA, Cushman DW (1982) Enzymes of the renin-angiotensin system and their inhibitors. Ann Rev Biochem 51: 283–308
19. Tzakos AG, Naqvi N, Comporozos K, Pierattelli R, Theodorou V, Husain A, Gerothanassis IP (2006) The molecular basis for the selection of captopril *cis* and *trans* conformations by angiotensin I converting enzyme. Bioorg Med Chem Lett 16:5084–5087
20. Moulin M, Deleu C, Larher FR, Bouchereau A (2002) High-performance liquid chromatography determination of pipecolic acid after precolumn ninhydrin derivatization using domestic microwave. Anal Biochem 308:320–327