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An angiotensin-I converting enzyme inhibitor from buckwheat (*Fagopyrum esculentum* Moench) flour

Yasuo Aoyagi *

Kagawa Nutrition University, 3-9-21 Chiyoda, Sakado, Saitama 350-0288, Japan

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Abstract

A compound that inhibited angiotensin-I converting enzyme (ACE) activity was isolated from buckwheat powder. This compound is thought to be the hydroxy derivative of nicotianamine and its chemical structure is 2″-hydroxynicotianamine. This compound showed a very high inhibitory activity toward ACE, and the IC_{50} was 0.08 μM. Only this hydroxy analog was found in buckwheat powder, at about 30 mg/100 g, and no nicotianamine was detected. However, nicotianamine was detected in the buckwheat plant body. 2″-hydroxynicotianamine was also found in other polygonaceous plants.

Keywords: Buckwheat; *Fagopyrum esculentum*; Polygonaceae; ACE inhibitor; Hypotension; Non-protein amino acid; Phytosiderophore; Nicotianamine; 2″-Hydroxynicotianamine

1. Introduction

Buckwheat (*Fagopyrum esculentum* Moench) flour is made into noodles and commonly eaten in Japan. Many nutraceutical compounds such as proteins, flavonoids, and other rare compounds have been reported in buckwheat seeds (Kayashita et al., 1996, 1999; Tomotake et al., 2001; Ikeda, 2002). Buckwheat protein in humans shows serum cholesterol-lowering activity, hypotensive activity, constipation-improvement and other effects. These physiological effects are due to its nature as a dietary fiber and the low digestibility of buckwheat protein (Kayashita et al., 1997). Furthermore, the anti-oxidative functions of its polyphenols protect against renal dysfunction caused by ischemia-reperfusion in rats (Yokozawa et al., 2001).

Angiotensin-I converting enzyme (ACE) plays an important role in the renin-angiotensin system, which regulates blood pressure. Inhibitors of this enzyme lower blood pressure and many antihypertensive drugs such as captopril and enapril are potent ACE inhibitors. It has been reported that the water extract of buckwheat flour strongly inhibits ACE (Suzuki et al., 1983). Furthermore, it has been shown that the hydrolyzate of buckwheat protein by a protease shows strong ACE interference (Kawakami et al., 1995), and biologically active peptides have been identified (Li et al., 2002). However, the bioactive plant compound in the aqueous extract has still not been identified. This study was performed to determine its identity.

2. Results

2.1. Isolation of ACE inhibitor

The results of a preliminary experiment showed that the ACE-inhibitory principle of buckwheat was soluble in water and the major ACE-interference activity was in the dialysis extracellular fluid. Therefore, it was thought that the active components were of comparatively low molecular weight. Extraction with 70% ethanol was then carried out to avoid elution of the starch by gelatinization.

* Tel./fax: +81 49 282 3709.
E-mail address: aoyagi@eiyo.ac.jp.

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The ACE inhibitor in the extract was not extracted by hexane or EtOAc. Most of the inhibitory activity remained in the water layer, and the IC_{50} value was estimated to be 31.5 μg ml^{-1}. Based on separation of this aqueous fraction by a coupled column of cation- and anion-exchange resins, the bioactive compound was found to exist in the positive ion fraction, and not in other fractions. The IC_{50} value of the fraction was estimated to be 3.1 μg ml^{-1}. In addition, this positive ion fraction was fractionated into P-1, P-2, P-3 and P-4 using an anion-exchange resin [Dowex 1×4 (AcO^-)] column. While ACE-inhibitory activity was observed in both P-1 ($IC_{50} = 23.6$ μg ml^{-1}) and P-2 ($IC_{50} = 0.37$ μg ml^{-1}), most of the activity was in P-2. The active compound **1** (57 mg from 2 kg of buckwheat flour) was isolated from P-2 following several more purifications by chromatography and recrystallization. Compound **1** showed a single ninhydrin-positive spot in TLC, and a single peak in the vicinity of valine on the chromatogram in an automatic amino acid analyzer.

2.2. Chemical structure of *1*

Compound **1** was shown to be $C_{12}H_{21}O_7N_3$, with a molecular weight of 319 based on MS analysis. The ninhydrin reaction of **1** was completely masked by Cu^{2+}, suggesting that it had an α-amino group. In addition, **1** was estimated to be an amino acid which is easily decomposed by heating in 6 N HCl, since a complicated fragment was present without the formation of a usual amino acid by hydrolysis. The ^{13}C NMR spectrum including DEPT measurement showed that **1** had three carbonyl, four methine and five methylene carbon atoms. A detailed examination of the ^1H NMR spectra by measurements of the H–H COSY and C–H COSY spectra, showed that **1** had one –CH–CH–CH_2– and two –CH_2–CH_2–CH– moieties. These data suggested that **1** was a hydroxy derivative of nicotianamine (Sugiura and Nomoto, 1984). The positive product ion spectrum of the MS/MS measurement of **1** is shown in Fig. 1. The prominent ions at *m/z* 56 and 114 were attributable to an azetidine ring moiety, as seen in the spectra of mugineic acid and nicotianamine (Kenny and Nomoto, 1994). The position of the hydroxyl group was deduced from ions at *m/z* 128, 186, 201, 219 and 245, and was determined to be at the 2″ position. Therefore, **1** was shown to be 2″-hydroxynicotianamine.

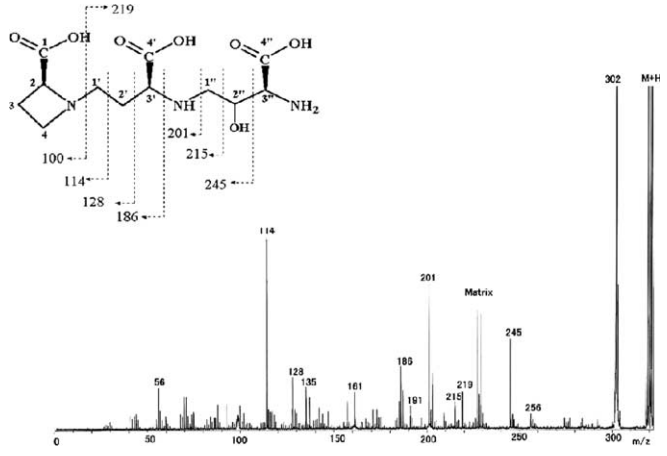

Fig. 1. Product ion spectra and proposed fragmentation scheme of the $[M + H]^+$ ion at *m/z* 320 of **1**.

2.3. Quantitative analysis of *1* in buckwheat and other polygonaceae plants

Nicotianamine and **1** were well-separated by ion-pair HPLC on an ODS column using sodium dodecyl sulfate as the counter ion.While only **1** was found in buckwheat powder, both **1** and nicotianamine were found in the buds of buckwheat and other polygonaceous plants (Table 1).

2.4. ACE-inhibitory activities of *1* and some related compounds

The ACE-inhibitory activities of **1**, azetidin-2-carboxylic acid, 2(S),3′(S)-*N*-(3-amino-3-carboxylpropyl)azetidine-2-carboxylic acid, nicotianamine, mugineic acid (Takemoto et al., 1978) and 2′-deoxymugineic acid (Sugiura and Nomoto, 1984) are shown in Table 2. The IC_{50} value of **1** was as low as 0.08 μM, and its inhibitory effect was equivalent to that of nicotianamine. The interference activities of mugineic acid, 3′2(S),3′(S)-*N*-(3-amino-3-carboxylpropyl)azetidine-2-carboxylic acid and 2′-deoxymugineic acid were considerably lower and azetidine-2-carboxylic acid did not show any inhibition.

Table 1
1 and nicotianamine contents of the *polygonaceous* plants

	1 (mg/100 g dry)	Nicotianamine (mg/100 g dry)
Fagopyrum esculentum, flour	30	–
Fagopyrum esculentum, sprout	48	18
Rheum rhaponticum, stem	6	11
Rumex acetosa, young bud	3	34
Polygonum hydropiper, sprout	52	–

Table 2
Angiotensin-I converting enzyme inhibition of **1** and azetidin-2-carboxiric acid derivatives

Compound	ACE inhibition IC_{50} (μM)
(2S)-azetidine-2-carboxylic acid	(–)
(2S)-1-((3S)-3-amino-3-carboxypropyl) azetidine-2-carboxylic acid	3.3
Nicotianamine	0.085
1	0.08
Mugineic acid	0.28
2′-Deoxymugineic acid	>99

3. Discussion

Nicotianamine occurs in all plants and chelates metal cations. It is thought to play a role in the internal transport of Fe and other metals in plants (Scholz et al., 1992). Concerning the hydroxyl derivative of nicotianamine, although there has been a report of the synthesis of 2′-hydroxynicotianamine (Matuura et al., 1994), this is the first report of 2″-hydroxynicotianamine (**1**) in a natural product. The physiological role of **1** in plants, while intriguing, is still unclear.

Compound **1** was isolated from buckwheat powder, and has also been shown to exist in some polygonaceous plants. It is thought to be widespread in polygonaceous plants. Some other families plants so far analyzed contained no detectable amounts of **1**. Thus, **1** may be a useful chemotaxonomic marker of polygonaceous plants. Attempts to detect **1** in other plants using she LCMS technique are also in progress from the viewpoint chemotaxonomic interest.

Nicotianamine shows a strong ACE-inhibitory effect, and has been shown to have a blood pressure-lowering effect in SHR (Kinoshita et al., 1993; Shimizu et al., 1999). Compound **1** shows an almost equal ACE-inhibitory effect, and a similar blood pressure-lowering effect can be expected. Studies along these lines are now in progress, and the results will be reported soon.

4. Experimental

4.1. Materials

Buckwheat flour was made in Hokkaido, Japan. ACE (from rabbit lung) was purchased from Sigma Chemical Co. (St. Louis, MO, USA) and hippuryl-L-histidyl-L-leucine (HHL) was obtained from the Peptide Institute (Osaka, Japan). Azetidine-2-carboxylic acid was isolated from the fruiting bodies of *Clavulinopsis pulchra* (Peck) Corner. 2(S),3′(S)-*N*-(3-amino-3-carboxylpropyl)azetidine-2-carboxylic acid was prepared from the azetidin-2-carboxylic acid according to the method of Kristensen and Larsen (1974). Nicotianamine was separated by the method reported for Morokheiya (*Corchorus olitorius*) (Kimoto et al., 1998). Mugineic acid and 2′-deoxymugineic acid were donated by Dr. S. Mori and Dr. N.-K. Nishizawa of The University of Tokyo.

4.2. General

TLC was performed on a Merck silica gel 60 F254 plate in *n*-BuOH–HOAc–H_2O (4:1:2) (solvent 1) and *n*-PrOH–conc. NH_3 (7:3) (solvent 2). MS spectra were measured using a JEOL MS 700V spectrometer. NMR spectra were recorded using a JEOL EX-270 spectrometer with TMSP as the internal standard. AA analysis was recorded using a Hitachi 835 automatic amino acid analyzer.

4.3. Assay for ACE-inhibitory activity

ACE-inhibitory activity was assayed by the modified method of Cushman and Cheung (1971). A reaction mixture containing 500 μl of 7 mM HHL in pH 8.3 borate buffer (200 mM), 400 μl of 2 M NaCl, 40 μl of H_2O, 30 μl of the test sample solution or H_2O and 30 μl of 150 U/ml of ACE (in the pH 8.3 buffer) was incubated at 37 °C for 30 min. The reaction was stopped by adding 500 μl of 1 N HCl. The hippuric acid liberated from the HHL by ACE was extracted with EtOAc (3 ml). An aliquot of the extract (2 ml) was evaporated to dryness, and the residue was dissolved in H_2O (1 ml). The hippuric acid concentration was determined by measuring the UV absorbance at 228 nm. The inhibitory activity was calculated as [100(Ec − Es)/(Ec − Eb)], where Es is the absorbance of the test sample added to the reaction mixture, Ec is with water (instead of the test sample), and Eb is a blank without ACE. The activity of the ACE-inhibitory principle was shown by the molarity in the reaction mixture, which showed interference of 50% (IC_{50}) under these conditions.

4.4. Preliminary survey of ACE inhibitor

Boiling H_2O (100 ml) was added to buckwheat flour (10 g), and the mixture was heated in a bath at 100 °C 30 min. The aqueous extract was obtained by centrifuging at 8000 rpm for 15 min. The ACE-inhibitory activity was measured using part of this extract. The remaining extract was transferred to a dialysis tube, and dialyzed against 1 l of H_2O for 24 h. The dialysis internal fluid and extracellular fluid were concentrated, and then diluted to the original amount. The ACE-inhibitory activity of both of these liquids was also measured.

4.5. Isolation of ACE inhibitor

Buckwheat flour (2.1 kg) was homogenized with EtOH–H_2O (8 l, 7:3), and stored overnight. The homogenate was filtered through filter paper, and the residue was similarly extracted two times. These filtrates were collected and conc. to ca. 2 l (165 g) using a rotary evaporator. This concd. extract was defatted with hexane (3 × 500 ml). The aqueous layer was extracted three more times with 500 ml EtOAc, and separated into an EtOAc layer (6.75 g) and an aqueous layer (95.5 g). The aqueous layer was then passed through columns of a cation-exchange resin Amberlite CG-120 (H^+) column (8 cm × 60 cm) and an anion-exchange resin Amberlite CG-400 (OH^-) column (5 cm × 70 cm) in series. The columns were washed with distilled H_2O (10 l) and the wash liquid was conc. to 100 ml as the non-ionic fraction (35.2 g). From the separation column with the cation-exchange resin, the positive-ion fraction was eluted using 2 N aqueous ammonia (101, 15.0 g). From the anion-exchange resin column, the negative-ion fraction was

eluted with 2 N aqueous ammonia (71, 7.8 g). These fractions were each conc. to 100 ml and the ACE-inhibitory activity was determined. The positive-ion fraction, which only showed ACE-inhibitory activity, was applied to a column of Dowex 1×4 resin (AcO^-, 3×120 cm). This column was eluted in sequence with 0.1 N AcOH (1 l) to give a fraction of basic and neutral amino acids (P-1, 7.9 g), with 0.5 N AcOH (1 l) to give a fraction of neutral amino acids (P-2, 1.9 g), with 1.0 N AcOH (1 l) to give a fraction of weakly acidic amino acids (P-3, 4.0 g) and with 2 N AcOH (2 l) to give a fraction of strong acidic amino acids (P-4, 1.4 g). Only the P-2 fraction showed ACE-inhibitory activity, and it was then applied to a column of Dowex 1×4 (AcO^-, 3×120 cm). Elution was performed with 0.3 N AcOH and 10 ml fractions were collected. Each fraction was monitored by the ninhydrin reaction and assay of the ACE-inhibitory activity. The active fractions were collected and conc. to dryness using a rotary evaporator. Recrystallization of the residues from aqueous EtOH gave a colorless crystalline **1** (57 mg).

4.6. Identification of ACE inhibitor

Compound **1**: m.p. 275–280 °C (decomp.), $[\alpha]_D^{235}$ −40 (*c* 0.2, H_2O), $[\alpha]_D^{235}$ −34.5 (*c* 0.1, 1 N HCl), ^1H NMR (D_2O) δ 2.05–2.3 (2H, m, H-2′), 2.4–2.85 (2H, m, H-3), 3.25–3.53 (4H, m, H-1″ and H-1′), 3.82–3.87 (1H, m, H-3′), 3.92–4.15 (2H, m, H-4), 4.01 (1H, d, 3.3 Hz, H-3″), 4.42–4.48 (1H, m, H-2″), 4.77 (1H, t, 9.57 Hz, H-2), ^{13}C NMR (D_2O) δ 23.9 (C-3, CH_2), 27.6 (C-2′, CH_2), 51.2 (C-1″,CH_2), 53.4 (C-4, CH_2), 54.1 (C-1′, CH_2), 60.5 (C-3″, CH), 62.4 (C-3′, CH), 68.3 (C-2″, CH), 69.7 (C-2, CH), 173.3 (C-4″, CO), 174.9 (C-4′, CO), 176.0 (C-1, CO), ESI-MS *m*/*z*: 320 (M + H), HRFABMS *m*/*z*: 320.1457 (M + H); Calcd. for $C_{12}H_{22}O_7N_3$: 320.1458.

4.7. Quantitative analysis of the active compound

A sample (about 2 g) was extracted three times with 20 vol. of EtOH–H_2O (7:3) by refluxing. The combined extract was concentrated to a small volume and defatted by EtOEt. The aqueous layer was applied to a column (2×10 cm) of Dowex 1×4 (AcO^-) resin, washed with H_2O (50 ml) and eluted with 0.5 N AcOH (100 ml). The eluate was conc. to dryness and dissolved in a suitable amount of HPLC eluting solvent. The HPLC conditions were as follows. Equipment: Shimadzu LC-7 with a fluorescence detector and a post-column reaction instrument, Column: Toso ODS 80TM, solvent: 0.05 M sodium dodecyl sulfate (pH 2.3) containing 30% CH_3CN, flow rate: 0.75 ml min^{-1}; post-column labeling agent: 900 ml of 0.1 M borate buffer (pH 9.0), 100 ml of EtOH containing 100 mg of *o*-phthalaldehyde and 0.5 ml of 2-mercaptoethanol; flow rate 1 ml min^{-1}. Fluorescence detector: Ex 365 nm, Em 455 nm.

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