J. Biol. Macromol., **17** (1), 3-13 (2017)

Article

Inhibition of renin-angiotensin system related enzymes (renin, angiotensin converting enzyme, chymase, and angiotensin converting enzyme 2) by water shield extracts.

Saori Takahashi,*[1] Ai Satou,[1] Hiroshi Shimoda,[2] and Keishi Hata[1]

[1]Akita Research Institute of Food and Brewing, 4-26 Sanuki, Arayamachi, Akita 010-1623, Japan
[2]Oryza Oil & Fat Chemical, 1 Numata, Kitagatacho, Ichinomiya, Aichi 493-8001, Japan

Received January 25, 2017; Accepted February 21, 2017

We screened for the inhibitory activities of 19 wild vegetable and fruit extracts for renin-angiotensin system (RAS)-related enzymes, including renin, angiotensin-converting enzyme (ACE), chymase, and angiotensin-converting enzyme 2 (ACE2). Among them, a hot water extract of *Brasenia schreberi* (Water shield, Junsai) strongly inhibited renin and chymase activities and significantly inhibited ACE and ACE2 activities. We also tested the RAS-related enzyme inhibitory activities of 13 polyphenols isolated from *Brasenia schreberi*. The polyphenols mostly had little effect on ACE and ACE2 activities. Among them, hypolaetin 7-*O*-glucoside showed the strongest chymase inhibitory activity with an IC_{50} value of 3.8 μM. Some other polyphenols also inhibited renin and chymase activities.

Key words: *Brasenia schreberi*, renin-angiotensin system, inhibition, chymase.

Introduction

The renin-angiotensin system (RAS) is one of the most important blood pressure control systems in mammals (Fig. 1). Renin is a highly specific aspartic proteinase that is mainly synthesized in juxtaglomerular cells in the kidney. The enzyme catalyzes the liberation of angiotensin I (AI) from the plasma substrate angiotensinogen. The produced prohormone AI is further processed to the pressor peptide angiotensin II (AII) by angiotensin-converting enzyme (ACE) or chymase. Chymase is a chymotrypsin-like serine proteinase that cleaves C-terminal aromatic amino acids [1-3]. Using angiotensin I as the substrate, human chymase cleaves the Phe^{8}-His^{9}

*To whom correspondence should be addressed:
E-mail: saori@arif.pref.akita.jp
Fax: (+81)18-888-2008

bond of AI to produce AII. The produced AII directly acts on arterial smooth muscle cells to maintain blood pressure and increases the release of aldosterone from the adrenal cortex to increase sodium retention in the kidney. Recently, the ACE homologous carboxypeptidase angiotensin-converting enzyme 2 was discovered [4, 5]. The enzyme hydrolyzes AI and AII to produce angiotensin 1-9 (Ang 1-9) and angiotensin 1-7 (Ang 1-7), respectively. Ang 1-9 is also processed to Ang 1-7 by ACE. Ang 1-7 evokes vasodilation involving interaction with Mas [6-8] (Fig. 1).

Recently, we developed a rapid and sensitive assay method for the measurement of renin, ACE, and ACE2 activities using internally quenched (IQF) substrates [9, 10]. In the present study, we developed a novel assay method for chymase using an IQF substrate for human renin. Using these assay systems, we screened for RAS-related enzyme inhibitory activities in various foodstuffs and found strong enzyme inhibitory activities in a hot water extract of *Brasenia schreberi* (Junsai). We also tested the RAS-related enzyme inhibitory activities of polyphenols isolated from *Brasenia schreberi* (Junsai) and found hypolaetin 7-*O*-glucoside strongly inhibited chymase activity.

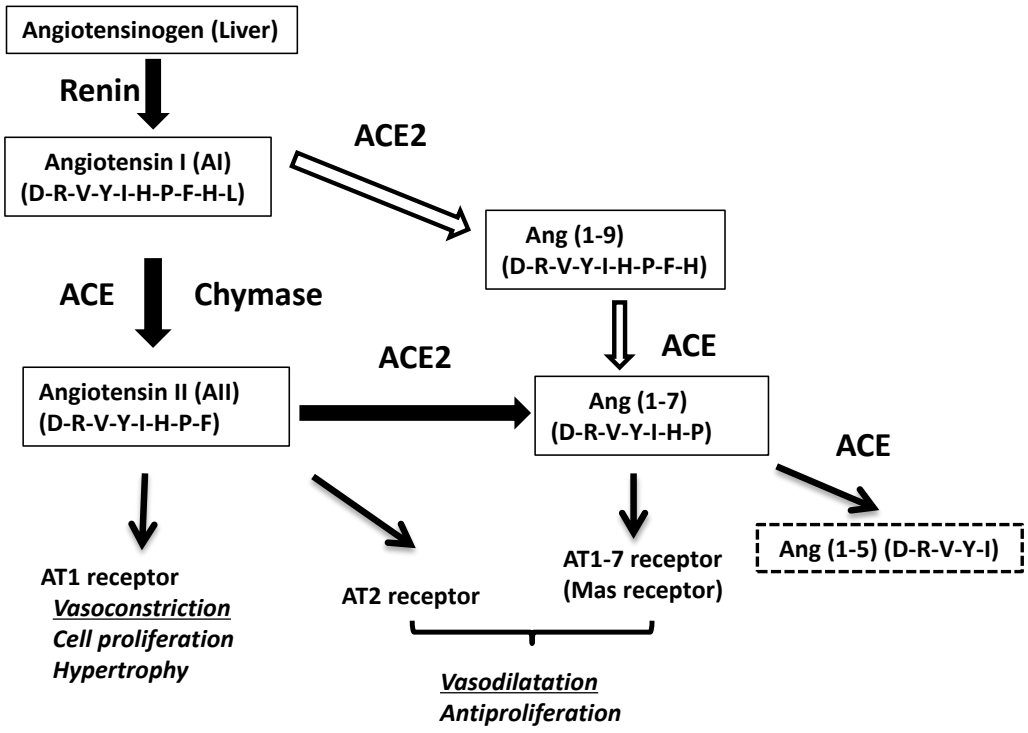

Fig. 1 Renin-angiotensin system

Renin is a rate-limiting enzyme of RAS. Renin produces AI from angiotensinogen. AI further processed to AII by ACE or chymase.

Materials and Methods

Materials

Recombinant human renin expressed in Sf-9 insect cells was prepared by the method of Takahashi *et al*. [11]. Recombinant human chymase expressed in *Pichia pastoris* was obtained from Sigma-Aldrich (St. Louis, MO, USA). Recombinant human ACE and ACE2 were from R&D Systems (Minneapolis, MN, USA). Internally quenched fluorogenic substrates were from Peptide Institute, Inc. (Saito, Osaka, Japan). Polyphenols isolated from *Brasenia schreberi* (Junsai) (13 kinds of compounds) were obtained from Oryza Oil & Fat Chemical (Aichi, Japan) [12]: compound 1, ethyl gallate; compound 2, caffeoyl glucose; compound 3, hypolaetin 7-*O*-glucoside; compound 4, quercetin 3-*O*-(6”-galloyl) glucoside; compound 5, quercetin 3-*O*-glucoside; compound 6, kaempferol 3-*O*-(6”-galloyl) glucoside; compound 7, gossypetin; compound 8, kaempferol 3-*O*-glucoside; compound 9, junsainoside A; compound 10, quercetin; compound 11, tiliroside; compound 12, kaempferol; compound 13, methyl caffeic acid.

Preparation of hot water extracts of foodstuffs

Lyophilized sample powder was added to water and heated at 105°C for 30 min. After centrifugation, the supernatant was lyophilized. The sample was dissolved in distilled water at a final concentration of 10 mg/ml.

Measurement of chymase activity

As the recombinant human chymase cleaves Phe^{8}-His^{9} of angiotensin I to yield angiotensin II, we tested various IQF substrates for ACE and renin and found that the enzyme efficiently cleaves the following IQF substrate for human renin: [2-(*N*-Methylamino)benzoyl] (Nma)-Ile-His-Pro-Phe*His-Leu-Val-Ile-Thr-His-[N^{ϵ}-(2,4-dinitrophenyl)-Lys] [Lys(Dnp)]-D-Arg-D-Arg-NH_2 (*, scissile peptide bond). The cleavage site of the enzyme was determined by LC-MS analysis (data not shown). The reaction mixture contained 40 μl of 0.1 M Glycine-NaOH, pH 9.0, 0.1 M NaCl, 25 μM IQF substrate, 0.02% NaN_3, 0.02% Triton X-100, 5 μl of chymase solution (0.4 μg/ml), and 5 μl of inhibitor solution in a total volume of 50 μl. The reaction mixture was incubated at 37°C for 30 min, and the reaction was terminated by adding 200 μl of 0.1 sodium borate buffer, pH 10.5. The increase of fluorescence intensity was measured at an emission wavelength of 440 nm upon an excitation wavelength at 340 nm.

Measurement of renin, ACE, and ACE2 activities

Renin activity was measured using (Nma)-Ile-His-Pro-Phe-His-Leu-Val-Ile-Thr-His-Lys(Dnp)-D-Arg-D-Arg-NH_2 as the substrate [11]. ACE and ACE2 activities were measured using Nma-Phe-His-Lys(Dnp) and Nma-His-

Pro-Lys(Dnp) as substrates, respectively [9, 10].

Inhibition of renin, ACE, chymase, and ACE2 activities by polyphenols

The 13 kinds of polyphenols isolated from *Brasenia schreberi* [12] were used for enzyme inhibition assays at a final concentration of 10 μM (Table 1). Data are expressed as the mean ± standard deviation (n = 3). The significance of differences was analyzed using a one-way ANOVA with Dunnett's multiple comparison tests. The sample concentrations required to inhibit 50% of enzyme activity under the assay conditions was taken as the IC_{50} value (Table 2).

Table 1 Inhibition of renin, ACE, chymase and ACE2 by Junsai polyphenols

	Relative activity (%)			
	Renin Means ± SD	ACE Means ± SD	Chymase Means ± SD	ACE2 Means ± SD
Control	100.0 ± 8.0	100.0 ± 2.8	100.0 ± 5.8	100.0 ± 3.3
Compound 1	102.7 ± 2.7	99.8 ± 0.8	56.7 ± 2.7**	101.0 ± 3.4
Compound 2	103.4 ± 3.0	99.2 ± 5.6	97.8 ± 4.0	94.9 ± 5.8
Compound 3	95.4 ± 1.3	100.8 ± 1.6	10.0 ± 2.4**	104.9 ± 1.5
Compound 4	87.7 ± 0.6**	100.7 ± 2.3	42.1 ± 0.9**	97.5 ± 6.7
Compound 5	100.1 ± 1.5	93.0 ± 4.3	68.1 ± 0.4**	95.8 ± 4.7
Compound 6	91.0 ± 2.1*	107.9 ± 5.1	49.4 ± 2.0**	95.3 ± 1.4
Compound 7	97.2 ± 1.2	99.2 ± 6.0	45.2 ± 1.2**	97.4 ± 7.8
Compound 8	101.0 ± 2.9	107.2 ± 6.6	79.1 ± 2.3**	92.6 ± 11.5
Compound 9	74.0 ± 1.9**	98.2 ± 0.3	39.1 ± 1.9**	86.9 ± 0.5
Compound 10	80.8 ± 1.9**	102.3 ± 5.3	33.6 ± 0.4**	89.2 ± 3.5
Compound 11	75.3 ± 2.6**	99.4 ± 5.2	84.5 ± 1.7**	92.1 ± 4.5
Compound 12	73.7 ± 1.0**	90.5 ± 2.9	62.4 ± 1.3**	81.0 ± 1.3*
Compound 13	106.6 ± 0.9	104.1 ± 5.8	96.3 ± 1.5	97.2 ± 6.0

*, $p<0.01$; **, $p<0.001$

Results and Discussion

Chymase activity

We determined the kinetic parameters of recombinant human chymase for the IQF substrate. The K_m, k_{cat}, and k_{cat}/K_m values of chymase for (Nma)-Ile-His-Pro-Phe-His-Leu-Val-Ile-Thr-His-Lys(Dnp)-D-Arg-D-Arg-NH_2 were 7.69 μM, 1,280 s^{-1}, and 166 μM^{-1} s^{-1}, respectively. In a previous study, endogenous chymase substrates and AI from various animals were used for the chymase assay [1]. Among them, human AI was one of the most suitable substrates for human chymase, with K_m, k_{cat}, and k_{cat}/K_m values of 60 μM, 198 s^{-1}, and 3.3 μM^{-1} s^{-1}, respectively. The k_{cat}/K_m value of the IQF substrate is about 50-fold higher than that of human AI, indicating that (Nma)-Ile-His-Pro-Phe-His-Leu-Val-Ile-Thr-His-Lys(Dnp)-D-Arg-D-Arg-NH_2 is an excellent substrate for human chymase.

Chymase inhibitory activities of foodstuff extracts

We tested the chymase inhibitory activity of hot water extracts from 19 wild vegetables and fruits (Fig. 2, open bars). Among them, the *Brasenia schreberi* (Junsai), *Aralia cordata* (Udo), *Matteuccia struthiopteris* (Kusasotetsu), and *Glycine max* (Soy) extracts strongly inhibited chymase activity. The IC_{50} value of the *Brasenia schreberi* (Junsai) extract was 12.5 μg/ml. This is the first demonstration of chymase inhibitory activity in foodstuffs, although there are several report of chymase inhibitors from microorganisms [13] and synthetic compounds [14, 15].

Renin inhibitory activity of foodstuff extracts

Our previous studies demonstrated the occurrence of renin inhibitory activities in ethanol extracts of various foodstuffs [16-18]. Moreover, we have identified the renin inhibitors as soyasaponin I from soybean [19], oleic acid and linoleic acid from rice [20], and (-)-kaur-16-en-19-oic acid, pimara-8(14), 15-dien-19-oic acid, and pimara-9(11), 15-dien-19-oic acid from *Aralia cordata* (Udo) [21]. In the present study, we also tested the renin inhibitory activity of hot water extracts from 19 wild vegetables and fruits (Fig. 2, closed bars). As already mentioned, *Aralia cordata* (Udo) and *Glycine max* (Soy) extracts inhibited renin activity. Moreover, *Brasenia schreberi* (Junsai) extracts strongly inhibited renin activity, and *Matteuccia struthiopteris* (Kusasotetsu), *Corchorus olitorius* (Nalta jute), *Oenanthe stolonifera* (Seri), and *Bassia scoparia* (Tonburi), *Chrysanthemum morifolium* (Chrysanthemum) extracts effectively inhibited renin activity. The identification of renin inhibitors from these vegetables is necessary to understand the structure-function relationship of renin inhibitors from foodstuffs.

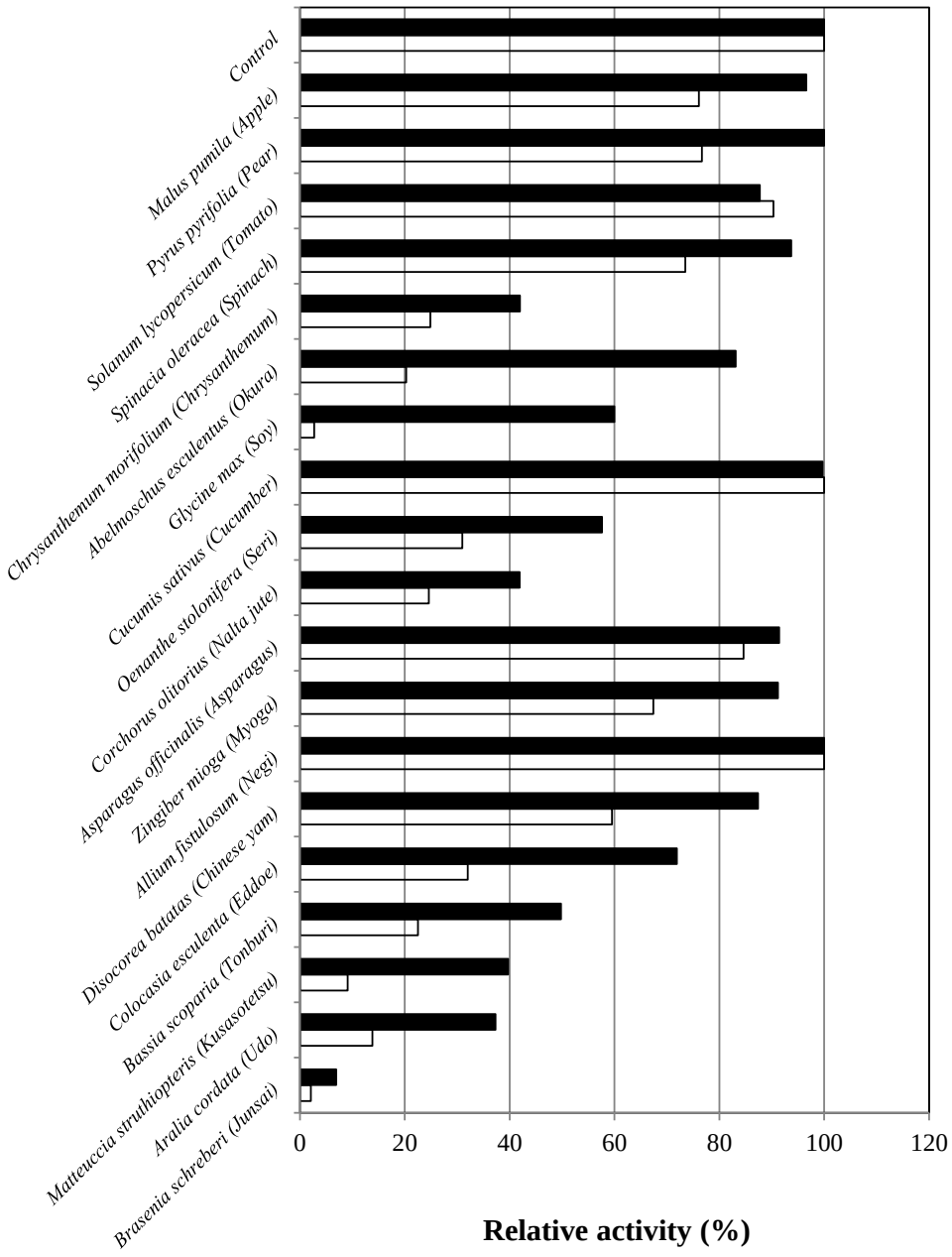

Fig. 2 Inhibition of renin and chymase activities by extracts of fruits and vegetables.

The reaction was carried out at 37°C for 30 min with 500 ng/ml of renin or 40 ng/ml of chymase and hot-water extracts of fruits and vegetables at a final concentration of 0.1 mg/ml. Each result is the mean value of triplicate determination. Open bars indicate chymase activities, and filled bars indicate renin activities.

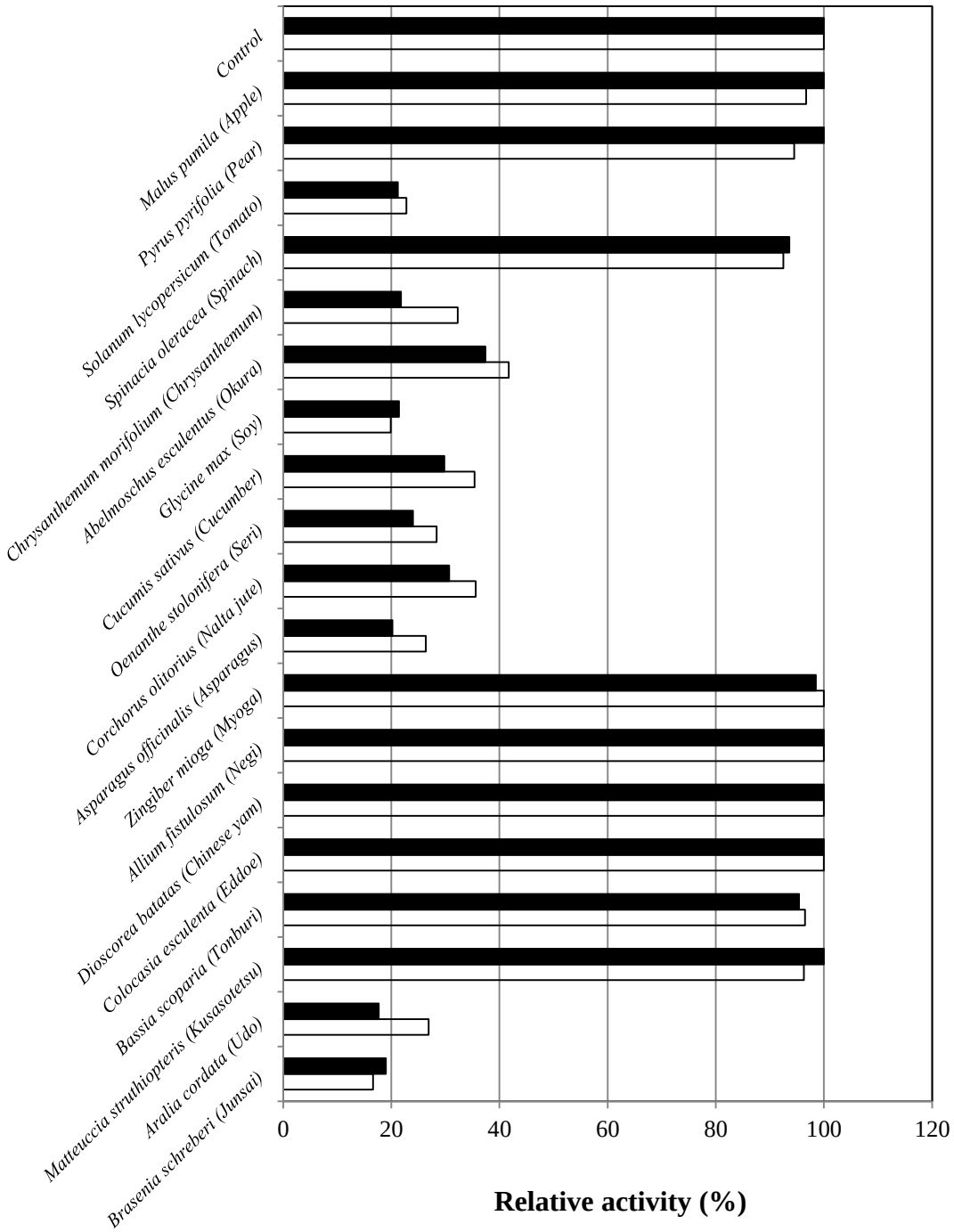

Fig. 3 Inhibition of ACE and ACE2 activities by extracts of fruits and vegetables

The reaction was carried out at 37°C for 30 min with 20 ng/ml of ACE or 10 ng/ml of ACE2 and hot-water extracts of fruits and vegetables at a final concentration of 0.1 mg/ml. Each result is the mean value of triplicate determination. Open bars indicate ACE2 activities, and filled bars indicate ACE activities.

ACE and ACE2 inhibitory activity of foodstuff extracts

Our recent study demonstrated the occurrence of an ACE2 inhibitor in soybean, and the inhibitor was identified as nicotianamine [10]. In the present study, we screened the ACE and ACE2 inhibitory activities of hot water extracts of 19 wild vegetables and fruits (Fig. 3, open bars for ACE2 and closed bars for ACE). The extracts of *Brasenia schreberi* (Junsai), *Aralia cordata* (Udo), *Asparagus officinalis* (Asparagus), *Corchorus olitorius* (Nalta jute), *Oenanthe stolonifera* (Seri), *Cucumis sativus* (Cucumber), *Glycine max* (Soy), A*belmoschus esculentus* (Okura), *Chrysanthemum morifolium* (Chrysanthemum), and *Solanum lycopersicum* (Tomato) equally inhibited ACE and ACE2 activities. These results suggest that both enzymes are inhibited with the same inhibitor compound. In connection with this matter, the ACE2 inhibitor isolated from soybean, nicotianamine, inhibited both ACE and ACE2 activities [11]. The isolation of an ACE and/or ACE2 inhibitor in these extracts is necessary to clarify the problems.

Table 2 IC_{50} values (μM) of Junsai polyphenols on renin and chymase

	Renin	Chymase
Compound 1	N.D.	11.6
Compound 2	N.D.	N.D.
Compound 3	N.D.	3.8
Compound 4	58.0	5.4
Compound 5	N.D.	18.5
Compound 6	74.0	6.1
Compound 7	N.D.	7.7
Compound 8	N.D.	>50.0
Compound 9	23.0	5.8
Compound 10	64.0	5.4
Compound 11	>100	>50.0
Compound 12	38.0	16.2
Compound 13	N.D.	N.D.

N.D., not determined

RAS-related enzyme inhibitory activities of polyphenols from *Brasenia schreberi* (Junsai)

Recently, various kinds of polyphenols were isolated from *Brasenia schreberi* produced in Akita Prefecture, Japan [12]. We tested the renin, ACE, chymase, and ACE2 inhibitory activities of 13 kinds of compounds as described in "Materials and Methods". Table 1 shows the results of the inhibition studies. None of the compounds inhibited ACE and ACE2 activities except compound 12. Compound 12 inhibited ACE2 activity weakly, with an IC_{50} value of about 120 μM. On the other hand, compounds 4, 6, 9, 10, 11, and 12 significantly inhibited renin activity. The IC_{50} values of compounds 4, 6, 9, 10, and 12 ranged from 23.0 μM to 74.0 μM (Table 2). The IC_{50} value of compound 11 was estimated to be >100 μM. Moreover, compounds 1 and 3 to 12 significantly inhibited chymase activity (Table 1). The IC_{50} values are also summarized in Table 2. Among these compounds, compound 3 was the strongest inhibitor for human chymase with an IC_{50} value of 3.8 μM. As far as we know, this is the first demonstration of chymase inhibitory polyphenols isolated from *Brasenia schreberi*. On the other hand, further elaborate studies are necessary to understand the structure and functions relationship of Junsai polyphenols.

Recently, we screened for the inhibitory activity of various vegetables for antihyperlipidemic activities and found that *Brasenia schreberi* extracts had strong inhibitory activities against triglyceride and cholesterol secretions from human HepG2 hepatoma cells [22]. Moreover, *Brasenia schreberi* extracts inhibited human immunodeficiency virus type 1 (HIV-1) reverse transcriptase activity and HIV-1 replication [23, 24]. The present study showed a novel physiological function of *Brasenia schreberi* extracts. *Brasenia schreberi* will be a good source for anti-hypertensive functional foods.

References

[1] Urata, H., Kinoshita, A., Misono, K. S., Bumpus, F. M., and Husain, A. (1990) *J. Biol. Chem.*, 265, 22348-22357.

[2] Okunishi, H., Oka, Y., Shiota N., Kawamoto, T., Song K., and Miyazaki, M. (1993) *Jpn. J. Pharmacol.*, 62, 207-210.

[3] Takai, S. and Jin, D. (2016) *Clin. Exp. Pharm. Phys.*, 43, 387-393.

[4] Tipnis, S. R., Hooper, N. M., Hyde, R., Karran, E., Christie, G., and Turner, A. J. (2000) *J. Biol. Chem.*, 275, 33238-33243.

[5] Donoghue, M., Hsieh F., Baronas, E., Godbout, K., Gosselin, M., Stagliano, N., Donovan, M., Woolf, B., Robison, K., Jeyaseelan, R., Breitbart, R. E., and Acton, S. (2000) *Circ. Res.*, 87,

e1-e9.

[6] Kuba, K., Imai, Y., and Penninger, J. M. (2013) *Circ. J.*, 77, 301-308.

[7] Marian, A. J. (2013) *Circ. Res.*, 112, 1307-1309.

[8] Santos, R. A. (2014) *Hypertension*, 63, 1138-1147.

[9] Takahashi, S., Ono, H., Gotoh, T., Yoshizawa-Kumagaye, K., and Sugiyama, T. (2011) *Biomed. Res.*, 32, 407-411.

[10] Takahashi, S., Yoshiya, T., Yoshizawa-Kumagaye, K., and Sugiyama, T. (2015) *Biomed. Res.*, 36, 219-224.

[11] Takahashi, S., Hata, K., Kikuchi, K-I, and Gotoh, T. (2007) *Biosci. Biotechnol. Biochem.*, 71, 2610-2613.

[12] Shimoda, H., Nakamura, S., Hitoe, S., Terazawa, S., Tanaka, J., Matsumoto, T., and Matsuda, H. (2014) *Nat. Prod. Chem. Res.*, 2, (an open access journal) (http://dx.doi.org/10.4172/2329-6836.1000146))

[13] Umezawa, H. (1976) *Methods Enzymol.*, 45, 676-695.

[14] Nakajima, M. and Naya, N. (2001) *Jpn. J. Pharmacol.*, 90, 206-209.

[15] de Garavilla, L., Greco, M. N., Sukumar, N., Chen, Z-W., Pineda, A. O., Mathews, F. S., Cera, E. D., Giardino, E. C., Wells, G. I., Haertlein, B. J., Kauffman, J. A., Corcoran, T. W., Derian, C. K., Eckardt, A. J., Damiano, B. P., Andre-Gordon, P., and Maryanoff, B. E. (2005) *J. Biol. Chem.*, 280, 18001-18007.

[16] Takahashi, S., Ogasawara, H., Watanabe, T., Kumagai, M., Inoue, H., and Hori, K. (2006) *Biosci. Biotechnol. Biochem.*, 70, 2913-2918.

[17] Takahashi, S., Hori, K., Kumagai, M., and Wakabayashi, S. (2007) *J. Biol. Macromol.*, 7, 49-54.

[18] Takahashi, S., Tokiwano, T., Suzuki, N., Kodama, I., Yoshizawa, Y., and Gotoh, T. (2010) *J. Biol. Macromol.*, 10, 83-91.

[19] Takahashi S., Hori, K., Shinbo, M., Hiwatashi, K., Gotoh, T., and Yamada, S. (2008) *Biosci. Biotechnol. Biochem.*, 72, 3232-3236.

[20] Takahashi, S., Tokiwano, T., Hata, K., Kodama, I., Hokari, M., Suzuki, N., Yoshizawa, Y., and Gotoh, T. (2010) *Biosci. Biotechnol. Biochem.*, 74, 1713-1715.

[21] Takahashi, S., Tokiwano, T., Hata, K., Hokari, M., Yoshizawa, Y., and Gotoh, T. (2011) *J. Biol. Macromol.*, 11, 83-89.

[22] Takahashi, J., Toshima, G., Matsumoto, Y., Kimura, F., Kikuchi, T., Hamada, K., and Hata, K. (2011) *J. Nat. Med.*, 65, 670-674.

[23] Hisayoshi, T., Shinomura, M., Konishi, A., Tanaka, J., Shimoda, H., Hata, K., Takahashi, S., and Yasukawa, K. (2014) *J. Biol. Macromol.*, 14, 59-65.

[24] Hisayoshi, T., Shinomura, M.,

Yokokawa, K., Kuze, I., Konishi, A., Kawaji, K., Kodama, E. N., Hata, K., Takahashi, S., Nirasawa, S., Sakuda, S., and Yasukawa, K. (2015) *J. Nat. Med.*, 69, 432-440.

Communicated by Kiyoshi Yasukawa