Abstract

This study was performed to purify a substance with angiotensin I-converting enzyme (ACE) inhibitory activity from tomato juice. Tomato juice was centrifuged, and the supernatant was filtered through a membrane filter and applied to a cation exchange, hydrophobic and reverse phase chromatography, sequentially. The impermeable fractions with molecular weights of about 1.0 kDa were collected. The fraction was further purified by high-performance liquid chromatography. Then, the fractions containing ACE inhibitory activity were concentrated using centrifugal ultrafiltration and lyophilized. The lyophilized sample was subjected to nuclear magnetic resonance analysis. The results of the analyses suggested that the fraction with ACE inhibitory activity from tomato juice contains a structure containing less than 10 sugar moieties of uronic acid of approximately 3.0 kDa.

Introduction

It is known that cerebral vascular disease and ischemic heart disease are two out of four major causes of death in Japanese people, and high blood pressure is a major risk factor for them. World Health Organization reported that one out of three people in the world in the age group of 25 years or older are likely to contract hypertension. One of the main mechanisms leading to hypertension is the rennin-angiotensin system. In this system, the angiotensin I-converting enzyme (ACE) converts angiotensin I to angiotensin II, which is a strong vasopressor substance and plays a crucial physiological role in blood-pressure regulation. This mechanism suggests that the inhibition of ACE is a useful therapeutic approach for hypertension remedy. It has been also shown that the ACE inhibitor dosage restrains hypertensive onset in spontaneously hypertensive rats. Several ACE inhibitor synthetic drugs are used for treatment of high blood pressure. However, several side effects of these medicines were reported: cough, taste abnormality, skin rash, and angioneurotic edema. Therefore, extensive search of safe, innovative substances, showing effective ACE inhibitory activity is carried out using various natural materials, including food.

It has been reported that some foods possess ACE inhibitory properties: herbs and phaseolus coccineus, black matpe, chenopodium quinoa, morinda citrifolia, and mushroom. Furthermore, specific substances in foods, such as polyphenol in tea, peptides from sardine meat protein, animal protein, oligopeptides in porphyra yezoensis, nicotianamine in various beans, anthocyanins from hibiscus sabdariffa, chitooligosaccharide derivatives and phlorotannins from marine
resources\textsuperscript{19}, and vegetables\textsuperscript{20}, also show ACE inhibitory effects.

Tomato juice is a popular beverage among the health-conscious people in Japan. Spontaneously Hypertensive Rats (SHR) showed significant reduction in blood pressure on ingestion of tomato juice\textsuperscript{21}. Therefore, we undertook the purification and structure determination of the fraction responsible for ACE inhibitory activity from tomato juice.

### 3 Materials and Methods

#### 3.1 Samples and reagents

Tomato juice products (salt-free and extracted juice 100\%) were purchased from KAGOME (Tokyo, Japan) products. All other chemicals were of reagent grade and were obtained from Nacalai Tesque (Kyoto, Japan).

#### 3.2 Purified procedure of ACE inhibitor substance from Tomato juice

The purification procedure is shown in Fig. 1. The steps were as follows:

**Step 1**: Tomato juice (7.2 L) was centrifuged at 8,000 \( \times \) g for 30 min at 4° C, and the obtained supernatant was centrifuged at 11,000 \( \times \) g for 60 min at 4° C. The supernatant was concentrated onto a 0.45 μm-pore size membrane filter (293 mm diameter, ADVANTEC CELLULOSE ACETATE, Toyo Roshi, Tokyo, Japan) under vacuum.

**Step 2**: The filtrate was separated on a cation exchange resin (DOWEX 50W-X2, Wako Pure Chemicals, Chiba, Japan) using an open glass column (id. 20 mm \( \times \) 300 mm), and the flow-through was collected. The solution was applied to a column packing with hydrophobic resin (Supelite DAX-8, Sigma-Aldrich; id. 40 mm \( \times \) 300 mm), and the flow-through was recovered again. The solution was applied to a column packing with reversed phase silica gel (Cosmosil 5C18-PAQ, id. 20 mm \( \times \) 250 mm, Nacalai Tesque). The eluate was detected at 280 nm. The mobile phase (MP) of HPLC consisted of 0.05% TFA. The column was operated at temperature at 25 °C, the flow rate was 3.0 mL/min, and fractions containing ACE inhibitory activity were collected. The fraction was subjected to centrifugal ultrafiltration to remove TFA, and the impermeable fraction (MWCO > 1.0 kDa) was freeze-dried.

**Step 3**: The freeze-dried sample was dissolved in 0.05% trifluoroacetic acid (TFA) and further purified using high performance liquid chromatography (HPLC) system (Waters, Massachusetts, USA) equipped with pumps with controller (600, Waters), detector UV/VIS (2487 Dualλ Absorbance Detector, Waters), differential refractometer (2410 Refractive Index Detector, refractive index (RI) detector, Waters), degasser (ERC-3415u, ERC, Saitama, Japan), autosampler (717 plus Autosampler, Waters), Cosmosil 5C18-PAQ (id. 20 mm \( \times \) 250 mm, Nacalai Tesque). The eluate was detected at 280 nm. The mobile phase (MP) of HPLC consisted of 0.05% TFA. The column was operated at temperature at 25 °C, the flow rate was 3.0 mL/min, and fractions containing ACE inhibitory activity were collected. The fraction was subjected to centrifugal ultrafiltration to remove TFA, and the impermeable fraction (MWCO > 1.0 kDa) was freeze-dried.

**Step 4**: The freeze-dried sample was dissolved in ultrapure water, and purified by HPLC using a Shodex-Ohepak SB-2002.5 column (id. 20 mm \( \times \) 300 mm, Showa Denko, Tokyo, Japan) and the eluate was monitored at 450 nm. The MP of HPLC consisted of ultrapure water, the flow rate was 3.0 mL/min, and injection volume was 2.0 mL. The HPLC system was connected to a fraction collector (SF-2120, Toyo Roshi), which was programmed to collect each 1.5 mL/min, and fractions containing ACE inhibitory activity were collected. The fraction was subjected to centrifugal ultrafiltration to remove TFA, and the impermeable fraction (MWCO > 1.0 kDa) was freeze-dried.

**Step 5**: The freeze-dried sample in Step 4 was dissolved in 5.0% acetonitrile solution with 0.1% TFA, and purified by HPLC as described in Step 4, substituting MP with 5.0% acetonitrile solution containing 0.1% TFA, and the eluate was monitored at 300 nm. The flow rate was 3.0 mL/min, and injection volume was 2.0 mL. The fractions with ACE inhibitory activity were collected, and the impermeable fraction (MWCO > 1.0 kDa) was freeze-dried.

**Step 6**: The freeze-dried sample was dissolved in 0.1 M sodium nitrate solution, and purified by HPLC as described in Step 4, substituting MP with 0.1 M sodium nitrate solution, and the eluate detected at 280 nm. The flow rate was 3.0 mL/min, and injection volume was 2.0 mL. The fractions with ACE inhibitory activity were collected and freeze-dried and used as the purified sample.

#### 3.3 Determination of the ACE inhibitory activity

ACE inhibitory activity was measured by the enzyme method (ACE kit-WST, Dojindo Molecular Technologies, Inc.), which measured the amount of 3-hydroxybutyric acid generated...
from 3-hydroxybutylyl-Gly-Gly-Gly, and expressed as an IC₅₀ value.

3.4 Measurement of molecular weight using high-pressure size exclusion chromatography (HPSEC)

The purified sample was solubilized in 0.1 M sodium nitrate solution, and its molecular weight was measured using HPSEC. The HPLC system was described in Step 3. MP was 0.1 M sodium nitrate, using polyethylene glycol (MW: 300, 400, 600, 1,000, 1,500, 2,000, 3,350, 4,000, 8,000, Sigma-Aldrich) as standard substance. Analysis of gel permeation chromatography was performed on a millennium 32-J software (Showa Denko), with a Shodex-Ohpak SB-803HQ column (id. 20 mm × 300 mm, Showa Denko) and Shodex-Ohpak SB-G guard column (id. 6.0 mm × 50 mm, Showa Denko). The exclusion limit molecular weight was determined as 4 million by pullulan (Wako Pure Chemicals, Chiba, Japan). The flow rate was 0.8 mL/min, and the eluate was monitored with an RI detector (2410 RI Detector, Waters), and the column temperature was maintained at 40°C.

3.5 Detection of protein, carbohydrate, and uronic acid

The detection of protein was carried out in accordance with ninhydrin reaction. The purified sample was dissolved as 5.0 mg/mL in 5.0% acetonitrile solution with 0.1% TFA and spotted on a thin-layer chromatography plate (Silica gel 60 F254, 5.0 cm × 10.0 cm, Merck, Darmstadt, Germany) and 0.1% glutamic acid was used as a positive control. The plate was developed for 25 min in butanol: glacial acetic acid: distilled water (2:1:2, v/v), and dried for 5 min and the samples were visualized with 0.2% ninhydrin solution.

The detection of carbohydrate was carried out in accordance using the phenol-sulfuric acid method modified by Hodge et al.²² Purified sample (500 mg) was dissolved in 1.0 mL of 0.05 M phosphate buffer (pH 7.0). 1.0 mL of the solution was dispensed in a test tube, followed by addition of 1.0 mL of 5.0% phenol solution and 5.0 mL of concentrated sulfuric acid, and incubation at 27°C for 15 min. Absorbance was measured at 485 nm with the spectrophotometer (UV-120, Shimadzu, Kyoto, Japan), using distilled water as the blank.

Presence of uronic acid in the sample was detected using the meta-hydroxydiphenyl method.²³ The purified sample (200 mg) was dissolved in 400 μL of distilled water and shaken with 2400 μL of 18 M sulfuric acid/sodium tetraborate in a vortex mixer, followed by heating at 100°C for 5 min. After cooling, 40 μL of 0.15% meta-hydroxydiphenyl in 0.5% NaOH was added, shaken, and incubated at 27°C for 5 min. Absorbance was measured at 520 nm.

3.6 Structural analysis by nuclear magnetic resonance (NMR)

The purified sample, sucrose, glucose, and cellulose were dissolved in deuterium oxide solution (20 mg/0.6 mL). The entire sample volume of 0.6 mL was transferred to 5.0 mm NMR sample tube (BMS-005B, Shigemi Inc., Tokyo, Japan). NMR measurement was performed using Varian NMR System 500 (Agilent Technologies, California, USA). ¹H spectroscopy was acquired under following conditions: delay of 1.5 s, acquisition time of 3.5 s, flip angle of 45°, and 128 scans. ¹³C spectroscopy was acquired under following conditions: delay of 1.5 s, acquisition time of 1.5 s, flip angle of 45°, and 16,834 scans. Correlation spectroscopy (COSY) was acquired with 1.0 s delay, acquisition time of 0.22 s, flip angle of 90°, and 16 scans. Heteronuclear multiple quantum correlation (HMQC) was acquired with 0.13 s delay, acquisition time of 1.0 s, flip angle of 90°, 32 scans, and heteronuclear multiple bond coherence was acquired with 1.0 s delay, acquisition time of 0.13 s, coupling constant 8 Hz, flip angle of 90°, and 128 scans.

4 Results and Discussion

4.1 Measurement of MW using HPSEC

MW of purified sample was measured using HPSEC (Fig. 2). Chromatograph of RI showed a pattern that peaks at RT of 10.6 min and showed ACE inhibitory activity. This top MW peak corresponded to 3.0 kDa on the calibration curve. So, purified sample that exhibited ACE inhibitory activity has a molecular weight of about 3.0 kDa.

![Fig. 2](image)

High pressure size exclusion chromatography (HPSEC) chromatogram of fractions. The purified fraction with ACE inhibitory activity was applied to a Shodex-Ohpak SB-803HQ column with 0.1 M NaNO₃ for 20 min at a flow rate of 0.8 mL/min. The line shows the chromatogram (relative intensities at the RI detector). The molecular weight of the highest peak (ca. 10.6 min) was calculated as 3.0 kDa from the standard curve.

4.2 IC₅₀ value of ACE inhibitory activity of purified sample

IC₅₀ value of ACE inhibitory activity of purified sample was 0.36 mg/mL, which was lower than that measured at Step 1 (2.57 mg/mL). This IC₅₀ level was comparable with that reported for hot water of mushroom (Hiratake: 0.65 mg/mL, Hatakeshimeji: 0.76 mg/mL,¹⁰ and enzymatic hydrolysate of fish meat (0.65 ~ 3.24 mg protein/mL).¹²

4.3 Detection of proteins, carbohydrates, and uronic acid
Purified sample was tested with the ninhydrin reaction to detect proteins. Glutamic acid, used as a positive control, showed orange color after the reaction, while the purified sample did not. Thus, the purified sample did not contain peptide or amino acid. Furthermore, the purified sample developed orange color on subjecting it to the phenol-sulfuric acid method to detect carbohydrates. Thus, the purified sample may contain carbohydrates. Additionally, the purified sample was tested using the meta-hydroxydiphenyl method, and the concentration of uronic acid in the purified sample was calculated as 171.0 μg/mL.

4.4 ¹H and ¹³C NMR spectroscopy of lyophilized sample of ACE inhibitor purified fraction

¹H NMR spectra of the purified sample are shown in Fig. 3. The resonances of purified sample linked to hemiacetal hydrogen in the region of δ4.6 ~ 5.4 ppm and hydrogen bound to oxy carbon (COH) in the region of δ3.4 ~ 4.6 ppm. Therefore, the sample may consist of mainly carbohydrates. The lines broadening the spectra indicate existence of iteration of similar carbohydrates (main chain: arrowed line) and other carbohydrate backbone (minor structure). ¹³C NMR spectra of purified sample showed resonance that proceeds to carboxyl in the region of δ170 ~ 175 ppm. Thus, the purified sample may contain acid carbohydrate (Fig. 4). These results indicate that this component must have iteration of acid carbohydrates.

¹H NMR spectra of pectin or de-esterified pectin were compared with that of polygalacturonic acid (poly-GalA) as the main component of pectin. The main chain resonances of

Fig. 3 ¹H NMR spectrum of the fraction with angiotensin I-converting enzyme (ACE) inhibitory activity (deuterium water, 25°C). The resonances of purified sample linked to hemiacetal hydrogen in the region of δ4.6 ~ 5.4 ppm and hydrogen bound to oxy carbon (COH) in the region of δ3.4 ~ 4.6 ppm. Therefore, the sample may consist of mainly carbohydrates. The lines broadening the spectra indicate existence of iteration of similar carbohydrates (main chain: arrowed line) and other carbohydrate backbone (minor structure).

Fig. 4 ¹³C NMR spectrum of the fraction with angiotensin I-converting enzyme (ACE) inhibitory activity (deuterium water, 25°C). ¹³C NMR spectra of purified sample showed resonance that proceeds to carboxyl in the region of δ170 ~ 175 ppm.
purified sample (arrowed line) agree with those of pectin, de-esterified pectin, and poly-GalA, but not with the resonance of methoxy group (-OCH₃). Thus, the main chain of purified sample was suggested to be polymer of GalA by α-1,4 linkage like pectin acid.

ⁱH NMR spectra showed that the signal proceeds to main and minor structure. ¹H and ¹³C NMR and measurement results of COSY, HMQC, and NOESY are shown in Table 1. Minor structure also gave the other signals, and COSY spectra, depending on the minor structure (Fig. 5), showed the correlation among three hexose skeletons (Group A ~ C). ¹³C NMR spectra showed the existence of carbonyl group in the C6 position, so the purified sample may contain uronic acids, such as glucuronic acid and mannuronic acid.

Protons on C1 ~ C2 and C2 ~ C3 of group A have a J-coupling with frequency of 8 Hz and 10 Hz, so the proton conformation on C1 ~ C2 and C2 ~ C3 is suggested to be diaxial.

![Fig. 5](image)

**Fig. 5** Correlation spectroscopy (COSY) spectra of the fraction with angiotensin I-converting enzyme (ACE) inhibitory activity (deuterium water, 25°C). COSY spectra showed the correlation among three hexose skeletons (Group A ~ C).

### Table 1  Attribution of ¹H and ¹³C spectra.

<table>
<thead>
<tr>
<th>No.</th>
<th>¹H (δ ppm)¹</th>
<th>Multiplicity²</th>
<th>J (Hz)</th>
<th>¹³C (δ ppm)³</th>
<th>Group</th>
<th>Atribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.5</td>
<td>dd</td>
<td>10, 8</td>
<td>25 A</td>
<td>Main chain</td>
<td>β-GalA*² H2, C2</td>
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<tr>
<td>2</td>
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<td>19 B</td>
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<tr>
<td>3</td>
<td>3.7-3.8</td>
<td>m</td>
<td>—</td>
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<td>α-GalA H2, C2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>3.81</td>
<td>S</td>
<td>—</td>
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<td>Main chain</td>
<td>β-GalA H3, C3</td>
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<tr>
<td>5</td>
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<td>dd</td>
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<tr>
<td>6</td>
<td>3.91</td>
<td>dd</td>
<td>11, 4</td>
<td>21 B</td>
<td>Main chain</td>
<td>α-GalA H3, C3</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>m</td>
<td>—</td>
<td>20 Main chain</td>
<td>C</td>
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<tr>
<td>8</td>
<td>4.09</td>
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<td>9</td>
<td>4.28</td>
<td>dd</td>
<td>4, 2</td>
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<td>β-GalA H4, C4</td>
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</tr>
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<td>4.41</td>
<td>d</td>
<td>3</td>
<td>29 A</td>
<td>β-GalA H4, C4</td>
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</tr>
<tr>
<td>11</td>
<td>4.4</td>
<td>m</td>
<td>—</td>
<td>30 Main chain</td>
<td>C</td>
<td>α-GalA H4, C4</td>
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<tr>
<td>12</td>
<td>4.46</td>
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<td>22 C</td>
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<tr>
<td>13</td>
<td>4.6</td>
<td>d</td>
<td>8</td>
<td>32 A</td>
<td>β-GalA H1, H1</td>
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<td>14</td>
<td>4.82</td>
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<td>16</td>
<td>5.05</td>
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<td>4</td>
<td>33 B</td>
<td>α-GalA H1, H1</td>
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<td>17</td>
<td>5.1</td>
<td>m</td>
<td>—</td>
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<td>α-GalA H1, C1</td>
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<td>18</td>
<td>5.31</td>
<td>d</td>
<td>4</td>
<td>31 C</td>
<td>α-GalA H1, C1</td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>—</td>
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<td>—</td>
<td>34 —</td>
<td>—COOH₂CH₃</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>36 A</td>
<td>β-GalA C6</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>36 Main chain</td>
<td>C</td>
<td>α-GalA C6</td>
</tr>
<tr>
<td>22</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>37 B</td>
<td>α-GalA C6</td>
<td></td>
</tr>
</tbody>
</table>

¹ Signal of HDO was δ4.77 ppm.
² Multiplicity: s (single line), d (double line), dd (dual-double line), m (multiple line), bs (wide range of single line)
³ GalA: Galacturonic acid
The proton on C4 shows doublet with narrow splitting width, and the proton on C5 has no splitting. Therefore, the proton on C3 ~ 4 and C4 ~ C5 must have a J-coupling with a frequency of under 3 Hz. These results indicate that the proton conformation on C3 is axial, but C4 proton conformation could not be determined. Group B has protons on C1 ~ C2, C2 ~ C3 and C3 ~ C4, of which a J-coupling showed frequencies of 4 Hz, 11 Hz, and 4 Hz, respectively. Each proton conformation must be equatorial, axial, and equatorial and axial position. However, among group C, the proton conformation on C1 and C2 may be equatorial and axial position, similar to that on group B. The protons on C3 and C4 present overlapped signal derived other protons, rendering it difficult to calculate their J-coupling frequencies. Therefore, the proton on C3 is suggested to be in an axial position with J-coupling between that on C2, but the conformation of the proton on C4 could not be assigned. These conformations suggest that both protons on C2 and C4 of group A and B are equatorial and axial position, and their minor component is GalA, similar to that in the main chain. Glucuronic acid and mannuronic acid are excluded as their components because the protons on C2 and C4 on the former are in axial position and the protons on C2 and C4 on the latter are in equatorial and axial position.

The proton conformation on C2 of group C is in axial position, but that on C4 is unclear. The component might be GalA or glucuronic acid. Based on the proton conformation on C1 of group A, both groups B and C should have β-structure and α-structure, respectively. GalA in main chain, of which the proton position on C1 is located next to group B, and the partially confirmable microstructure has J-coupling frequency of 4 Hz, is considered as the α-structure. Thus, the main chain may consist of GalA, which binds each other with α1,4-linkage. The signals of the minor structure are more definitive than that of the main chain. Therefore, these minor carbohydrates should be located in the sugar chain terminal. HMBC spectra showed that the proton on C1 of group A did not correlate with another carbohydrate, but the proton on its C4 correlated with carbon on C1 of group B or the main chain. The protons on C1 of group B correlate with carbon on C4 of group A and main chain, while the proton on C4 did not correlate with another carbohydrate. The proton on C1 of group C did not correlate with another carbohydrate. However, the proton signal on its C4 overlaps with that on C4 of main chain, so the linkage with another carbohydrate had not been confirmed. The result showed that hydroxyl groups on C1 of group A and C, and C4 of group B, are in the free state. Thus, each group is considered to be a sugar chain terminal. Group A and group B could link with C4 and C1 on the main chain, respectively. The component ratio of main chain and minor structure is estimated from the intensity ratio of proton on the former and the latter (Table 2). The intensity ratio of each signal showed the ratio of each building block to be main chain: group A: group B: group C = 8 : 2 : 3 : 1. The results indicate that the purified sample may consist of mainly GalA linked each other with α-1,4-bond (partially β-1,4 linkage). Comparison of integrated values of the signal for the structures of terminal and main chain indicated that the purified sample should have less than 10 uronic acid moieties. Thus, ACE inhibitor substance in tomato juice is speculated to have a molecular weight of about 3.0 kDa with less than 10 uronic acid moieties.

### 5 Conclusions

IC50 level of the purified sample showed lower ACE inhibitory activity compared to those of the enzymatic hydrolysate of fish meat and the hot water extract of mushroom. The present study indicates the fraction containing about 3 kDa of uronic acid-derivatives from the tomato juice has ACE inhibitory activity.

### Acknowledgments

We thank Ms. Chiyo Sano for supporting this work, and Drs. Y. Nakano, A. Wadano, R. Sakamoto of Osaka Prefecture University, for excellent advice and guidance. A part of this study was supported by a grant from KAGOME CO., Ltd..

### References


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**Table 2** Area intensity of main chain and minor structure (1H spectrum).

<table>
<thead>
<tr>
<th>Signal</th>
<th>Area intensity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Main Chain H1 + Group B H1</td>
<td>6.8</td>
</tr>
<tr>
<td>Main Chain H3 + Group C H3</td>
<td>5.8</td>
</tr>
<tr>
<td>Group A H1</td>
<td>1</td>
</tr>
<tr>
<td>Group A H2</td>
<td>1</td>
</tr>
<tr>
<td>Group B H3</td>
<td>2.1</td>
</tr>
<tr>
<td>Group B H4</td>
<td>1.9</td>
</tr>
<tr>
<td>Group C H1</td>
<td>0.6</td>
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