Introduction

Royal jelly (RJ) contains a wide variety of substances, including proteins, lipids, carbohydrates, minerals, and vitamins. Past studies have shown that RJ possesses a broad range of pharmacological effects, including antibacterial action, antitumor action, suppression of diabetes mellitus, sex hormone action, increase in blood flow, body weight gain, growth promotion, survival prolongation against radiation disorders, and suppression of arteriosclerosis. RJ is widely utilized in nutritional supplements, cosmetics, quasi-drugs, pharmaceuticals, and other items. As a healthful food, RJ has long been utilized by many people; it is very safe for living organisms with a low incidence of side effects. Any side effect is likely to be due to additives.

When heated, RJ produces a glycation product of proteins and carbohydrates contained therein. In this study, we examined the anti-oxidative capacity of the glycation product of RJ.

Methods

Preparation of materials

In this study, intact RJ, and dry RJ prepared by lyophilizing intact RJ, were used. Since the components of RJ are likely to be denatured by light, heat, and oxygen, RJ was immediately frozen after collection and stored in a dry state under freezing conditions to maintain adequate storage stability.

Samples prepared by lyophilizing intact RJ were heated at a specified temperature (60-100°C) in a water bath for 1 hour to yield heated RJ (HRJ). HRJ, 1.0 g, was dissolved in 6 mL of 0.2 M NaOH, and 4 mL of 0.2 M phosphate buffer solution (pH 7.4) was added to yield a test solution, which was diluted with the same buffer solution to desired dilution rates. The resulting dilutions were assayed to evaluate the anti-oxidative capacity in the following experiments listed below.
Parameters

DPPH radical eliminating activity

The absorbance of 1,1-diphenyl-2-picryl hydrazyl (DPPH), a radical species as potent as active oxygen in oxidizing capacity, is lost upon a reaction with a substance having anti-oxidative capacity. In this experiment, the anti-oxidative power of each sample was determined by measuring the loss of the absorbance of DPPH using a spectrophotometer. RJ was heated at 100°C for 20, 40, 60, and 180 minutes, and the DPPH radical eliminating activity was determined using a spectrophotometer (wavelength: 517 nm).

An evaluation test was performed using a reaction system comprising 1.95 mL of 0.05 M Tris-HCl buffer solution (pH 7.4) supplemented with 1.0 mL of 0.1 mM DPPH and 0.05 mL of HRJ sample (5-fold dilution). The reaction mixture was centrifuged, and the resulting supernatant fraction was assayed 8 minutes later. The DPPH radical eliminating activity was calculated as a DPPH retention ratio (%) using the following equation:

\[
\text{DPPH retention ratio} \times 100 = \frac{A1}{A2} \\
\text{A1: Absorbance after HRJ addition} \\
\text{A2: Absorbance before HRJ addition}
\]

Superoxide anion radical eliminating activity

This activity was determined by trapping superoxide anion radicals generated from *in vivo* xanthine-xanthine oxidase system (a system for producing superoxide anion radicals) using the spin trapper 5,5-dimethyl-1-pyrroline-N-oxide (DMPO), and detecting the resulting adduct using an electron spin resonance (ESR) system (JES-FR30, JEOL Ltd., Akishima-shi, Tokyo). The radical eliminating activity was evaluated on the basis of the ESR signal attenuation caused by the addition of HRJ.

An evaluation test was performed using a reaction system comprising 47 µL of 0.2 M phosphate buffer solution (pH 7.4) supplemented with 3.0 µL of 9.0 M DMPO, 5 mM hypoxanthine, HRJ (treated at 100°C for 1 hour), and 60 µL of 1.0 U/mL xanthine oxidase. The aforementioned buffer solution used to dissolve and dilute HRJ was used as the control.

Leukocyte-derived radical eliminating activity

Leukocytes produce radicals upon stimulation with phorbol 12-myristate 13-acetate (PMA). Such radicals were reacted with the sensitizer luminol, and the resulting chemiluminescence was measured over time using a luminescence detector (CLD-110, Tohoku Electronic Industrial Co., Ltd., Taihaku-ku, Sendai-shi, Miyagi, Japan). The radical eliminating activity was evaluated from the luminescence intensity attenuation with the addition of a sample having radical eliminating capacity to the system.

An evaluation test was performed using a reaction system comprising Hanks’ buffer solution (pH 7.4) supplemented with leukocytes from rat blood (1x10⁸ cells/mL), 0.56 mM luminol, an HRJ sample (treated at 100°C for 1 hour), and 100 mM PMA. The aforementioned buffer solution used to dissolve and dilute HRJ was used as a control in place of the sample.

Chemiluminescence suppression rate (%) = \(\frac{1-D1}{C2} \times 100\)

A1: Absorbance before sample addition
A2: Absorbance after sample addition

C1: Chemiluminescence level (photons) after HRJ addition
C2: Chemiluminescence level (photons) before HRJ addition

Results

DPPH radical eliminating activity

Results for DPPH radical eliminating activity are shown in Table 1. The DPPH radical suppression rate increased with increasing heat treatment time, but did not change with heating for 1 hour or longer. Therefore, a heating time of at least 60 minutes was required to prepare HRJ with higher anti-oxidative action by 100°C heat treatment.

Subsequently, samples were prepared at various dilution rates (1-160 fold) using HRJ that had been previously heated at 100°C for 1 hour, and DPPH radical suppression rates were determined in the same manner using non-heated RJ (1-, 2-, 4-, and 8-fold dilutions) as the control.

Results showed that the highest DPPH radical eliminating activity was observed at a dilution rate of 1, with the activity decreasing with increasing dilution rate (Table 2). Hence, the radical eliminating activity was found to depend on the concentration of HRJ. The radical eliminating activity of HRJ was significantly higher than that of RJ (p < 0.05).

Superoxide anion radical eliminating activity

When examining the ESR signal attenuation curve, HRJ was shown to eliminate superoxide anion radicals (Fig. 1). The elimination ratio increased according to the concentration of HRJ; it was remarkably higher with HRJ than with non-heated RJ (Table 3).
Table 1. Relationship between the heating time and DPPH radical suppression rate with HRJ

<table>
<thead>
<tr>
<th>Heat treatment time (100°C)</th>
<th>(Minutes)</th>
<th>0</th>
<th>20</th>
<th>40</th>
<th>60</th>
<th>180</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH radical suppression rate (%)</td>
<td></td>
<td>40.0</td>
<td>65.1</td>
<td>70.5</td>
<td>85.6</td>
<td>86.5</td>
</tr>
</tbody>
</table>

HRJ, heated royal jelly; DPPH, 1,1-diphenyl-2-picryl hydrazyl.

Table 2. Relationship between the sample dilution rate and DPPH radical suppression rate with HRJ or RJ

<table>
<thead>
<tr>
<th>HRJ dilution rate</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td>DPPH radical suppression rate (%)</td>
<td>87.9</td>
<td>85.6</td>
<td>61.9</td>
<td>40.0</td>
<td>24.8</td>
<td>16.8</td>
<td>9.8</td>
</tr>
<tr>
<td>RJ dilution rate</td>
<td>1</td>
<td>2</td>
<td>4</td>
<td>8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DPPH radical suppression rate (%)</td>
<td>75</td>
<td>66</td>
<td>45</td>
<td>21</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HRJ, heated royal jelly; RJ, royal jelly; DPPH, 1,1-diphenyl-2-picryl hydrazyl. Results are expressed as mean values (n = 3). p < 0.05, significantly different in slopes between HRJ and RJ by Mann–Whitney U test. Standard error means are not presented due to a missing data problem of the computer.

Fig 1. ESR signal attenuation curves: superoxide anion radical eliminating action of HRJ

Measured using the JES-FR30 analyzer (JEOL Ltd.). ESR, electron spin resonance; HRJ, heated royal jelly.

Table 3. Superoxide anion radical suppression by HRJ and RJ

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HRJ (%)</td>
<td>74</td>
<td>60</td>
<td>52</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>RJ (%)</td>
<td>44</td>
<td>22</td>
<td>17</td>
<td>–</td>
</tr>
</tbody>
</table>

HRJ, heated royal jelly; RJ, royal jelly; –, no inhibitory effect.
Leukocyte-derived radical eliminating activity

Measurements of chemiluminescence from leukocyte-derived radicals in 1-fold dilution of HRJ and control are shown in Fig. 2. The radical-induced increase in the luminescence intensity in the leukocytes was suppressed by HRJ. Hence, HRJ was shown to also suppress radicals produced in cells.

Subsequently, the suppression rate with HRJ was compared with that with RJ at various dilution rates (1, 10, and 50 fold). The suppression rate for leukocyte-derived radicals was found to have concentration dependency like the radical-inhibitory activity described above, and the suppression rate was higher with HRJ than with RJ (Table 4).

Aldose reductase inhibiting activity

HRJ and RJ both exhibited high aldose reductase inhibiting activity (Table 5). The bioactivity of conventional RJ was not affected by heat treatment.

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**Table 4. Suppression of chemiluminescence from leukocyte-derived radicals by HRJ and RJ**

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>1</th>
<th>10</th>
<th>50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemiluminescence suppression rate</td>
<td>HRJ (%)</td>
<td>94.3</td>
<td>74.9</td>
</tr>
<tr>
<td></td>
<td>RJ (%)</td>
<td>83.4</td>
<td>55.5</td>
</tr>
</tbody>
</table>

HRJ, heated royal jelly; RJ, royal jelly.

**Table 5. Aldose reductase inhibiting activities of HRJ and RJ**

<table>
<thead>
<tr>
<th>Dilution rate</th>
<th>5</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldose reductase suppression rate</td>
<td>HRJ (%)</td>
<td>84.7</td>
</tr>
<tr>
<td></td>
<td>RJ (%)</td>
<td>81.5</td>
</tr>
</tbody>
</table>

HRJ, heated royal jelly; RJ, royal jelly.
Discussion

In this study, HRJ, prepared by heating RJ, was shown to exhibit higher anti-oxidative action than RJ in terms of DPPH radical eliminating activity, superoxide anion radical suppressing activity, and leukocyte-derived radical eliminating activity. Because the heat treatment did not alter the aldose reductase activity, HRJ was confirmed to possess increased anti-oxidative function while avoiding the loss of the bioactivity of conventional RJ.

Usually, heating alters food component proteins, lipids, and carbohydrates, and causes them to interact with each other, resulting in a wide variety of changes. Caramelization is a phenomenon in which saccharides change their colors to brown without reacting with amino compounds when heated to 100°C or higher 14. Caramelization and glycation are strictly differentiated. Because food composition is diverse, in reality, coexisting components work catalytically during heating, resulting in the concurrent occurrence of caramelization and glycation. Although general foods rarely undergo caramelization only, the contribution of caramelization is high in cacao and coffee bean roasting, meat grilling, bakery, and other processes.

When heated alone to about 60-150°C, reducing sugars (glucose and fructose) undergo anomerization and isomerization, concurrently producing disaccharides with 1,6-glucoside bonds, such as isomaltose and gentiobiose, 1-6-anhydro sugars, oligosaccharides, and other substances 15-19. Furthermore, a dehydration condensation reaction produces 3-deoxyglucosone (3DG), 5-hydroxymethylfurfural, and 2-hydroxyacetyl furfural. Non-volatile acids such as succinic acid, tartaric acid, pyruvic acid, levulinic acid, and furanecarboxylic acid are also produced concurrently. Thus, the products produced due to heating are diverse.

A brown substance produced upon glycation of food is known as melanoidin. Although the chemical structure of melanoidin remains unidentified, it is thought to be an assembly of many coloring substances 20. Melanoidin is a brown final product produced via pyrrole aldehyde and other substances following the conversion of D-glucosides from saccharides to amadori compounds via enaminol, and condensation-polymerization with amino compounds involving osones and furfurals as intermediates.

Food melanoids have been suggested to have favorable effects on the body. Those produced from amino acids such as glycine, serine, isoleucine, and glutamic acid, in particular, are active in hydroxy radical elimination and exhibit anti-oxidative action 20-23. With regard to coffee, advanced research has been conducted on the glycation product melanoidin, resulting in a number of reports on its functionality with favorable effects on human health, including anti-oxidative action 23,24. When roasted, coffee beans have increased melanoidin contents, and exhibit enhanced anti-oxidative action 25,26.

RJ is composed of 60-70% water, 9-18% amino acids (including glycine, serine, isoleucine, and glutamic acid) and proteins (royalisin 27, royalactin 28, apisin 29), 11-23% carbohydrates (glucose, fructose, sucrose, oligosaccharides), and 3-8% fatty acids (decenoic acid 30), and it also contains small amounts of water-soluble vitamins and minerals 31-33. RJ has a wide variety of effects, including anti-oxidative action 34-38.

The above-described facts suggest that the amino acids and reducing sugars contained in RJ underwent glycation due to heating to produce melanoidin, and that the anti-oxidative action of HRJ might be enhanced as an add-on effect of the anti-oxidative action of melanoidin on the anti-oxidative action of the starting material RJ.

Conclusion

HRJ, produced upon heat treatment of RJ, contains the glycation product melanoidin, and was shown to exhibit higher anti-oxidative action than conventional RJ, suggesting a potential for use as a new anti-oxidative food.

State of Conflict of Interest

Non contributory.

Acknowledgments

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References