Note

1,1-Diphenyl-2-picrylhydrazyl Radical-scavenging Compounds from Soybean Miso and Antiproliferative Activity of Isoflavones from Soybean Miso toward the Cancer Cell Lines

Akira HIROTA,* Shoji TAKI, Satoru KAWAI,** Masamichi YANO,** and Naoki ABE

Laboratory of Applied Microbiology, School of Food and Nutritional Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan
**National Institute of Fruit Tree Science, Okitsu-naka, Shimizu 424-0204, Japan

Received August 10, 1999; Accepted December 28, 1999

Guided by their DPPH radical-scavenging activity, nine compounds were isolated from soybean miso. Of these, 8-hydroxydiazoein, 8-hydroxyngeoisethine and syringic acid had high DPPH radical-scavenging activity as that of α-tocopherol. The antiproliferative activity of four of the isolated isoflavones toward three cancer cell lines was examined. 8-Hydroxyngeoisethine showed the highest activity (IC₅₀ = 5.2 μM) toward human promyelocytic leukemia cells (HL-60).

Key words: soybean miso; 8-hydroxyngeoisethine; DPPH radical-scavenging activity; antiproliferative activity; cancer cell

As a continuation of our study on food factors with tertiary functions from fermented foods, we attempted to isolate antioxidants from miso, a traditional fermented soybean food in Japan which is stable against peroxidation, by a simple method using the DPPH radical.2)

DPPH is used as a reagent for evaluating α-tocopherol,3 and our research group has recently isolated several new DPPH radical scavengers of microbial origin.4,5

Since soybean foods have been reported to prevent several types of cancer,6 we measured the antiproliferative activity of several compounds isolated from soybean miso toward cancer cell lines.

We report here the isolation and structural elucidation of the DPPH radical scavengers from miso, their DPPH radical-scavenging activities, and their antiproliferative activities toward cancer cell lines.

Soybean miso (2 kg), which was produced by Marusan-Ai Co. Ltd., Okazaki, Japan, was extracted with boiling water and then defatted with n-hexane (Hex). The aqueous layer was extracted with ethyl acetate (EtOAc), and the resulting EtOAc extract was applied to silica gel column chromatography (Wako gel C-200) with a Hex-EtOAc solvent system. The fraction which contained several DPPH radical scavengers (Hex-EtOAc, 40:60) was subjected to reversed-phase MPLC with a Yamazen BLPC-600-FC chromatograph connected to a YMC ODS-AQ 120-S50 column (25 × 340 mm). The obtained fractions were further applied to reversed-phase HPLC with a Jasco UV-970 spectrometer (254 nm) connected to a Shiseido Capcellpak C18 SG120 column (4.6 × 150 mm). Six compounds (I–VI) were finally isolated from the original fraction (Hex-EtOAc, 40:60). The 1H- and 13C-NMR and MS spectra of four of the six compounds identified them as p-coumaric acid (I, 2.6 mg), ferulic acid (II, 2.0 mg), daidzein (V, 28.7 mg), and genistein (VI, 10.5 mg). These compounds are known to be antioxidants originally present in soybeans,7 and the latter two compounds were aglycones of the isoflavone glycosides, daidzin and genistin. These aglycones are mainly produced by hydrolysis during the fermentation process.8

The other two compounds, III (2.3 mg) and IV (2.9 mg), were more polar than V and VI. The molecular formula of compound III was determined to be C₁₅H₂₀O₄ by its high-resolution EIMS spectrum, and the 1H- and 13C-NMR, HMQC and HMBC spectral data determined III to be 8-hydroxyngeoisethine (Fig. 1).

1H-NMR (CD₃OD): δ 6.85 (2H, d, J = 8.4 Hz), 6.96 (1H, d, J = 8.8 Hz), 7.36 (2H, d, J = 8.4 Hz), 7.59 (1H, d, J = 8.4 Hz), 8.17 (1H, s). 13C-NMR (CD₃OD): δ 115.0, 115.8, 117.0, 118.5, 124.0, 125.0, 131.0, 133.7, 148.1, 153.9, 156.0, 158.2, 178.2.

In a similar manner, compound IV, C₁₅H₁₉O₄, was determined to be 8-hydroxyngeoisethine. 1H-NMR ((CD₃)₂CO): δ 6.35 (1H, s), 6.90 (2H, d, J = 8.4 Hz), 7.45 (2H, d, J = 8.8 Hz), 8.17 (1H, s), 12.4 (1H, br s). 13C-NMR ((CD₃)₂CO): δ 99.5, 105.8, 116.0, 123.2, 123.8, 125.6, 131.3, 146.8, 153.6, 154.1, 155.9.

* To whom correspondence should be addressed. Fax: +81-54-264-5099; E-mail: hirotaa@u-shizuoka.kcn.ac.jp

Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; MPLC, medium-pressure liquid chromatography; HPLC, high-performance liquid chromatography; HMQC, 1H-detected multiple quantum coherence; HMBC, 1H-detected heteronuclear multiple-bond connectivity
Table 1. DPPH Radical-scavenging Activity of Compounds I-IX

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration for 50% Radical-scavenging Activity (μM)</th>
<th>Reaction time (hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-coumaric acid (I)</td>
<td>380</td>
<td>20.0</td>
</tr>
<tr>
<td>ferulic acid (II)</td>
<td>59</td>
<td>0.5</td>
</tr>
<tr>
<td>8-OH-daidzein (III)</td>
<td>22</td>
<td>0.5</td>
</tr>
<tr>
<td>8-OH-genistein (IV)</td>
<td>21</td>
<td>0.5</td>
</tr>
<tr>
<td>daidzein (V)</td>
<td>330</td>
<td>144.0</td>
</tr>
<tr>
<td>genistein (VI)</td>
<td>390</td>
<td>72.0</td>
</tr>
<tr>
<td>syringic acid (VII)</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>vanillic acid (VIII)</td>
<td>65</td>
<td>0.5</td>
</tr>
<tr>
<td>p-hydroxybenzoic acid (IX)</td>
<td>&gt;400</td>
<td>144.0</td>
</tr>
<tr>
<td>α-tocopherol</td>
<td>24</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Fig. 1. Structures of I-IX.

158.4, 182.0.

These two isoflavones, III and IV, have recently been isolated as potent antioxidants from soybean fermented with *Aspergillus satoi*, and III has also been isolated as a potent antioxidative and anti-UV-B compound from the fermentation of *Asp. niger*, but this is the first report on the isolation of 8-hydroxyisoflavones from soybean miso.

Since isoflavones III and IV were not detected in the aqueous acetone extract of soybean by the HPLC analysis (data not shown), it could be concluded that daidzein and genistein had been hydroxylated at C-8 during the fermentation process of miso.

The fraction (100% EtOAc) from silica gel column chromatography which contained the DPPH radical scavengers was subjected to reversed-phase MPLC, and the obtained fractions were further applied to reversed-phase HPLC. Finally, VII (6.2 mg), VIII (16.9 mg) and IX (2.0 mg) were isolated from the original fraction (EtOAc 100%). The 1H- and 13C-NMR and MS spectra identified compounds VII, VIII and IX as syringic acid, vanillic acid and p-hydroxybenzoic acid, respectively.

The DPPH radical-scavenging activity of these nine compounds was measured (Table 1) by the method described earlier, and among them, III, IV and VII each showed activity as high as that of α-tocopherol. II and VIII also had high DPPH radical-scavenging activity, while daidzein and genistein had low activity. The high radical-scavenging activity of 8-hydroxyisoflavones III and V is assumed to have been due to the ortho dihydroxyl catechol structure.

Fig. 2. Antiproliferative Activity of the Isoflavones from Miso toward the HL-60 Cell Line.

Each point represents the mean of triplicated experiments. Vertical bars indicate standard deviation of each point. (n=3).

pounds, excepting the isoflavones, had good correlation with the antioxidative activities measured and reported in another study. Considering the antioxidative activity and yield of the isolated antioxidative compounds, not only the 8-hydroxyisoflavones, but also syringic acid, ferulic acid and vanillic acid contribute to the antioxidative activity of soybean miso.

The antiproliferative activity of the four isoflavones isolated from miso toward the three cancer cell lines, human promyelocytic cell (HL-60), human lung carcinoma (A549), and melanin pigment-producing mouse melanoma (B16 melanoma 4A5), was examined. These cancer cell lines were purchased from the Riken Gene Bank, Tsukuba, Japan, and the cell proliferative assay was done as described by Kawai et al. The results are shown in Fig. 2. 8-Hydroxygenistein (IV) showed the highest activity (IC50 = 5.2μM), while 8-hydroxydaidzein (III) and genistein (VI) demonstrated IC50 values of 26μM and 33 μM toward HL-60 cells, respectively. These results indicate that a C-4 ketone group and C-5 hydroxyl group, as well as a catechol-type structure (7,8-diol structure) contributed to the suppressive activity against the HL-60 cell line.
It is interesting that the structure necessary for the antiproliferative activity of 8-hydroxyisoflavones was different from that for the DPPH radical-scavenging activity.

Toward the other two cell lines, A549 and B16 melanoma 4A5, all the isoflavones, even IV, had weak antiproliferative activity at a concentration of 40 μM. It has already been reported that chemically synthesized III showed an increase in the life span of S180 mouse sarcoma-bearing mice, but our result is the first evidence of the antiproliferative activity toward HL-60, A549 and B16 melanoma 4A5 cell lines of III and IV.

Although it has been reported that soybean foods prevented several cancers, and that genistein and daidzein might play an important role in preventing several types of cancer, 8-hydroxyisoflavone might inhibit cancer growth rather than genistein and daidzein in the case of soybean miso, judging from some in vitro cell cultures. Studies to examine the antiproliferative activities of 8-hydroxyisoflavones toward other cancer cell lines are now in progress.

Acknowledgments

We express our thanks to Mr. A. Yagi of Faculty of Agriculture at Shizuoka University, and Mr. T. Mizuoka of Molecular Science Lab. at Medicinal Research Laboratories of Taisho Pharmaceutical Co. Ltd., for measuring the high- and low-resolution mass spectra.

References