Applications of ELISA, Western blotting and immunoaffinity concentration for survey of ginsenosides in crude drugs of Panax species and traditional Chinese herbal medicines

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A combination of ELISA, Western blotting and immunoaffinity concentration using an anti-ginsenoside Rb1 monoclonal antibody was applied for qualitative and quantitative surveys of ginsenoside Rb1 and related ginsenosides in roots and traditional Chinese herbal medicines. To improve the low correlation between ELISA and HPLC analysis, the crude extract of roots was immunoaffinity concentrated to make evident the effect of malonyl ginsenoside Rb1. Immunoaffinity column chromatography also concentrated an unknown ginsenoside which had the same cross-reaction with ginsenoside Rb1.

Introduction

Ginseng, the crude drug of *Panax ginseng*, is one of the most important Chinese medicines. It is used to enhance stamina and the capacity to cope with fatigue and physical stress and as a tonic against cancers, disturbances of the central nervous system (memory, learning and behavior), hypothermia, carbohydrate and lipid metabolism, immune function, the cardiovascular system and radioprotection. Major active components are ginsenosides, which are mainly triterpenoid dammarane derivatives. Reports are often contradictory, perhaps because the ginsenoside content of ginseng root or root extracts can differ, depending on the method of extraction, subsequent treatment or even the season of its collection. Therefore, the use of standardized, authentic ginseng root both in research and by the public is to be advocated.

In our ongoing studies on the formation of monoclonal antibodies (MAbs) against naturally occurring bioactive compounds, we have established MAbs against forskolin, solamargine, opium alkaloids, *m*arihuana compounds and crocin. Also, we previously prepared the MAb against the major active component, ginsenoside Rb1 (G-Rb1), using ELISA, and established a new Western blotting method for ginsenosides because there was no assay system using MAb except polyclonal antibodies. We report here the application of ELISA for the qualitative analysis of G-Rb1 as a marker component of ginseng, immunoaffinity concentration and Western blotting for the determination of ginsenosides in the crude drug of *Panax* species and traditional Chinese herbal medicines.

Experimental

Chemicals and immunochemicals

Ginsenosides (G) -Rb1, -Rc, -Rd, -Re, -Rf and -Rg1 were purchased from Millipore (Bedford, MA, USA). Glass microfiber filter sheets (GFA) were purchased from Whatman International (Maidstone, UK). All other chemicals were standard commercial products of analytical-reagent grade.

Anti-G-Rb1 MAb was purified using a Protein G FF column (11 × 0.46 cm id) (Pharmacia Biotech, Uppsala, Sweden) as reported previously. The cultured medium (500 ml) containing the IgG was adjusted to pH 7 with 1 M TRIS solution and loaded onto the column, which was washed with 10 mM phosphate buffer (pH 7). Adsorbed IgG was eluted with 100 mM citrate buffer (pH 3). The eluted IgG was neutralized with 1 M TRIS solution, then dialyzed against phosphate buffered saline of pH 7.4 (PBS) three times, and finally lyophilized.

G-Rb1–carrier protein conjugates used for ELISA were synthesized by a modification of the procedure already used for solamargine, which is based on the method of Erlanger and Beiser.

The immunoaffinity column was prepared as follows. Purified anti-G-Rb1 MAb (9.1 mg) in Bio-Rad (Richmond, CA, USA) Affi Gel Hz coupling buffer (diluted) was dialyzed twice against the coupling buffer. To the anti-G-Rb1 MAb solution (1 ml), 100 μl of NaIO4 solution (25 mg in 1.2 ml of water) was added and stirred gently at room temperature in the dark for 1 h. After the reaction, glycerol was added to the reaction mixture to 20 mM and stirred for 10 min to inactivate the NaIO4, then dialyzed against the coupling buffer. After washing Affi-Gel Hz hydrazide gel (Pharmacia Biotech, 3 ml) with the coupling buffer, the buffer was removed. To this gel anti-G-Rb1 MAb dissolved in the coupling buffer (5 ml) was added and stirred gently at room temperature for 24 h. The immunoaffinity gel was packed into a plastic mini-column in volumes of 3 ml. The columns were washed with 20 mM phosphate buffer containing 0.5 M NaCl (pH 7.0). The immunoaffinity gel was washed with PBS until the ELISA value was equal to the background. The column was stored at 4 °C in PBS containing 0.02% sodium azide.

Plant and drug materials

The crude drug materials of *Panax* species were purchased from Nakai Koshindo (Kobe, Japan). Traditional Chinese medicine...
extracts were kindly provided by Mr T. Somehara, Saga Medical School.

Sample preparation

Dried samples (50 mg) of various ginsengs were powdered, extracted with MeOH (5 ml) under sonication five times, filtered and then evaporated. For the elimination of the malonyl group from malonylated ginsenosides, the extracts were treated with 0.1% KOH in MeOH at room temperature for 1 h, and assayed by ELISA. G-Rb1 contaminated with malonyl G-Rb1 was also treated under the same conditions as above.

Determination of G-Rb1 by HPLC

HPLC analysis was performed as reported previously. The recovery was 98.99% for G-Rb1 as reported previously.

Recovery of G-Rb1 by ELISA

For recovery experiments the extract of white ginseng root was used. From each level three samples were analyzed. The recovery was calculated from the added G-Rb1 in the same concentration ranges.

Western blotting

Western blotting was performed as reported previously, as follows. Ginsenosides, the extracts of Panax species and the traditional Chinese herbal medicines were applied to TLC plates and developed with BuOH–EtOAc–H$_2$O (15 + 1 + 4). One TLC plate was sprayed with H$_2$SO$_4$ and another TLC plate was blotted on the PVDF membrane with the blotting solution with heating. The blotted PVDF membrane was immersed in water containing NaIO$_4$ for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA was added and stirred for 3 h. The PVDF membrane was stained by standard protocols of Western blotting using MAb.

Competitive ELISA

Competitive ELISA was performed as described previously. GRb1-HSA (five molecules of GRb1 per molecule of HSA) (100 µl, 1 µg ml$^{-1}$) was adsorbed in the wells of a 96-well immunoplate (Nalge NUNC, Roskilde, Denmark), then treated with 300 µl of 5% skim milk (S) in PBS for 1 h to reduce non-specific adsorption. A 50 µl volume of solutions of various concentrations of ginsenoside and samples dissolved in 20% MeOH solution was incubated with 50 µl of 0.418 µg ml$^{-1}$ IgG solution for 1 h. After washing the plate three times with TPBS, 100 µl of substrate solution [0.1 M citrate buffer (pH 4) containing 0.003% H$_2$O$_2$ and 0.3 mg ml$^{-1}$ 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid diammonium salt] was added to each well and incubated for 15 min. The absorbance was measured with a micro plate reader at 405 nm.

Separation of G-Rb1 from the crude extracts of P. ginseng roots by immunoaffinity column chromatography

The extracts of ginseng roots were redissolved in PBS and then filtered with a 0.45 µm MILEX-HV filter (Millipore) to remove insoluble portions. The filtrate was loaded on the immunoaffinity column and allowed to stand overnight at 4 °C. The column was washed with the washing buffer solution (40 ml) and then eluted with 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0). The G-Rb1 containing fraction was concentrated and subjected to TLC with CHCl$_3$–MeOH–H$_2$O (7 + 4 + 1) and BuOH–AcOEt–H$_2$O (15 + 1 + 4), as developing solvents, followed by Western blotting.

Separation of an unknown ginsenoside from the crude extractions of P. japonicus root by immunoaffinity column chromatography was carried out as described above.

Results and discussion

Determination of G-Rb1 by ELISA

As we reported previously, the cross-reactivities of G-Rc and -Rd with anti-G-Rb1 MAb are very weak, 0.024 and 0.020%, respectively, and those of other steroidal compounds are < 0.005%. Fig. 1 shows the structure of G-Rb1 and its calibration curve obtained by ELISA. Under these conditions, the full measuring range of the assay extends from 20 to 400 ng ml$^{-1}$.

Recovery of G-Rb1 by ELISA

Table 1 indicates that the recoveries are good with additions of 50 and 100 µg. However, for the addition of 150 µg the recovery was 88%, suggesting that ELISA should be performed at suitable concentration ranges of G-Rb1.

From these results, it is evident that ELISA using anti-G-Rb1 MAb can be used routinely for phytochemical investigations involving crude plant extracts without any pre-treatment.

Fig. 1 Calibration curve for G-Rb1. Various concentrations of G-Rb1 were incubated with MAb in wells pre-coated with G-Rb1 HSA (1 µg ml$^{-1}$). After washing with PBS containing 0.05% of Tween 20, the wells were incubated again with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.
Correlation of G-Rb1 in crude extracts of white ginseng root between HPLC and ELISA

ELISA is more sensitive than TLC\(^{19}\) or HPLC methods.\(^{20,21}\) Yamaguchi \textit{et al.}, Samukawa \textit{et al.}\(^{23}\) and Chang \textit{et al.}\(^{24}\) reported the comparative contents of ginsenosides in various commercial ginseng radixes analyzed by HPLC. We have also determined the ginsenoside contents in various ginsengs using HPLC.\(^{16}\) Therefore, here we calculated the correlation coefficient from fitting the straight line obtained by ELISA and HPLC methods. Fig. 2(a) shows the correlation of G-Rb1 concentration in crude white ginseng between HPLC and ELISA, indicating scattering (\(\gamma = 0.910\)), especially at higher concentrations.

Kitagawa \textit{et al.} isolated malonyl ginsenosides from white ginseng and found that non-processed ginsengs contain higher concentrations than processed ginseng.\(^{25}\) Yamaguchi \textit{et al.} also pointed out that after heating, malonyl G-Rb1, -Rb2 and -Rc in ginseng would be converted into G-Rb1, -Rb2 and -Rc, respectively.\(^{22}\) Hence malonyl G-Rb1 may affect the correlation between the two assay systems since it is probable that malonyl G-Rb1 has a similar cross-reactivity with G-Rb1. To examine this aspect, the crude extracts were immunoaffinity concentrated.

The extract of white ginseng roots was loaded on the immunoaffinity column. After washing with washing buffer solution, the column was eluted with 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH. The G-Rb1 containing fraction was concentrated and subjected to Western blotting as reported previously.\(^{26}\) Fig. 3 shows the immunoaffinity concentrated G-Rb1 fraction containing an unknown band with an \(R_f\) value similar to that of malonyl G-Rb1. Therefore, this fraction was treated with a mildly alkaline solution as reported previously\(^{25}\) to give pure G-Rb1. From this result, since it becomes evident that the reason for the scattering of the correlation between HPLC and ELISA is the effect of malonyl G-Rb1, the crude extract of ginseng roots was treated with a mildly alkaline solution before the assay by ELISA. Fig. 2(b) shows the correlation of G-Rb1 concentration in crude white ginseng between the two assay systems, the correlation ratio being improved to \(\gamma = 0.988\). In addition to this result, we determined the cross-reactivity of malonyl G-Rb1 against anti-G-Rb1 MAb, being 60% of the concentration of malonyl G-Rb1 determined by HPLC and ELISA. Since various kinds of acylated natural products such as flavonoids,\(^{27}\) anthraquinones,\(^{28}\) anthocyanins\(^{29}\) and triterpenoid saponins\(^{30}\) have been found, it can be suggested that a mild alkaline treatment is required before ELISA analysis, as discussed above.

Concentrations of G-Rb1 in various ginseng species

Table 2 gives the concentrations of G-Rb1 in various ginseng roots analyzed by ELISA and HPLC after treatment under mildly alkaline conditions. The fibrous ginseng that is made from the active growing part in \textit{P. ginseng} showed the highest G-Rb1 concentration, 64.44 ± 3.64 \(\mu g\) mg\(^{-1}\) dry wt., \textit{P. notoginseng} and \textit{P. quinquefolium} also showed higher concentrations, 47.08 ± 3.34 and 48.51 ± 1.79 \(\mu g\) mg\(^{-1}\) dry wt., respectively. These results were in good agreement with previous reports.\(^{21,22}\) White ginseng was prepared from \textit{P. ginseng} simply by drying. The concentration of G-Rb1 was 5.49 ± 0.75 \(\mu g\) mg\(^{-1}\) dry wt., higher than that in red ginseng, but lower compared with the other samples. Chuang \textit{et al.}\(^{31}\) pointed out that white ginseng showed wide differences among the samples.

### Table 1 Results of recovery experiment

<table>
<thead>
<tr>
<th>Added amount/(\mu g) per 50 mg dry weight</th>
<th>Measured amount/(\mu g) per 50 mg dry weight</th>
<th>RSD ((n = 3)) (%)</th>
<th>Expected amount/(\mu g) per 50 mg dry weight</th>
<th>Recovery (%)(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>345.41 ± 16.06</td>
<td>4.7</td>
<td>—</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
<td>420.09 ± 41.05</td>
<td>9.8</td>
<td>395.41</td>
<td>106</td>
</tr>
<tr>
<td>100</td>
<td>449.04 ± 72.46</td>
<td>16.1</td>
<td>445.41</td>
<td>101</td>
</tr>
<tr>
<td>150</td>
<td>436.86 ± 32.49</td>
<td>7.4</td>
<td>495.1</td>
<td>88</td>
</tr>
</tbody>
</table>

\(a\) Recovery (%) = (measured G-Rb1/expected G-Rb1) \times 100.
studied, but they all contained G-Rb1 and G-Rg1 as the most abundant constituents. Red ginseng is prepared by processing, steaming and drying the roots of *P. ginseng*. Western blotting analysis indicated that this sample contained almost no malonyl G-Rb1, which is a prominent feature with this material. The concentration of G-Rb1 was lowest in these samples, suggesting that the steaming step also decreases the concentration of G-Rb1 by conversion into C-20 hydroxyl free ginsenosides.  

*P. japonicus* is distributed in Japan and China and is morphologically different from the other *Panax* species. Yahara *et al.* reported that no G-Rb1 was found in *P. japonicus*, and isolated oleanane-type saponins named as chikusetsusaponins and elucidated their structures. 33 Morita *et al.* examined the varieties of *P. japonicus* by chemical analysis of saponins. 34 From these results, the concentration of G-Rb1 might be at trace levels. However, we determined it by ELISA and found higher concentrations compared with previous reports22,23 (Table 2), although approximately half the concentration of G-Rb1 was found by HPLC analysis compared with ELISA. In order to confirm these differences, we used immunoaffinity column chromatography for immunoaffinity concentration of G-Rb1. The crude root extract of *P. japonicus* was loaded on to the immunoaffinity column and washed with the washing solvent and then with elution solvent, as already indicated. Fig. 4 shows the H$_2$SO$_4$ staining (A) and the Western blotting (B) profiles of the two fractions separated by the immunoaffinity column. Fraction 1 eluted with the washing solvent showed many spots, including chikusetsusaponins, similar to the original extract of *P. japonicus*. A clear unknown band appeared in fraction 2 eluted with the elution solvent. However, G-Rb1 was not detected by Western blotting although it was determined by TLC as indicated. It can be suggested that the unknown compound has a similar molecular structure and a similar cross-reaction with G-Rb1, and seems to be a new ginseng saponin having panaxadiol as an aglycone. Moreover, this compound might have the same sugar fragments in the molecule, but possess an additional sugar moiety compared with G-Rb1, as indicated by their $R_F$ values. From this it becomes evident that *P. japonicus* contains no G-Rb1. We concluded that the combination of immunoaffinity concentration and ELISA is suitable for samples containing lower concentrations of G-Rb1 since the immunoaffinity column contains 20 $\mu$g mL$^{-1}$ gel (data not shown). The isolation and structure elucidation of the unknown ginsenoside are now being investigated and the results will be presented elsewhere.

Quantitative and qualitative analysis of ginsenosides by Western blotting and ELISA in traditional Chinese herbal medicines

Fig. 5 shows H$_2$SO$_4$ staining (A) and Western blotting (B) of traditional Chinese herbal medicines with and without *P. ginseng*. The TLC profile stained by H$_2$SO$_4$ showed many unclear spots, probably including sugars, making it difficult to detect G-Rb1. Lanes 1 and 2 were Kikyo-to and Daiokanzo-to extracts, which do not contain *P. ginseng*, indicating no band by Western blotting. On the other hand, the band of G-Rb1 in the other extracts clearly appeared on Western blotting (B) together with a trace amount of G-Rc, which is in the same family as G-Rb1 having panaxadiol as an aglycone as reported previously. The pattern of the constituents of ginsenoside was simple, indicating G-Rb1 as a major and G-Rc as a minor ginsenoside. Although malonyl G-Rb1 was detected in the non-processed *Panax* species as discussed above, it became evident that the traditional Chinese herbal medicines contain very low concentrations of malonyl G-Rb1 owing to degradation during processing in drug production. From this, the G-Rb1 concentration in traditional Chinese herbal medicines can be determined directly without alkaline treatment. This combination of the Western blotting method can be utilized for the assay of G-Rb1, and therefore it is possible to study a large number of drugs containing ginseng.

Since many traditional Chinese herbal medicines consist of the white ginseng with other herbal medicines, the major component, G-Rb1, was determined by ELISA as a marker compound in order to confirm the quantity and quality of traditional Chinese herbal medicines, and Table 3 gives the concentrations of G-Rb1 found. The concentration of G-Rb1 is proportional to the content of ginseng in individual prescrip-

Table 2  Ginsenoside Rb1 contents in various ginseng sample (after alkaline treatment)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Content/μg mg$^{-1}$ dry wt.</th>
<th>ELISA</th>
<th>RSD (n = 3) (%)</th>
<th>HPLC</th>
<th>RSD (n = 3) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ginseng (<em>P. ginseng</em>)</td>
<td>5.49 ± 0.75</td>
<td>0.2</td>
<td>4.96 ± 0.05</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>Red ginseng</td>
<td>3.57 ± 0.62</td>
<td>0.2</td>
<td>3.93 ± 0.34</td>
<td>0.1</td>
<td></td>
</tr>
<tr>
<td>Fibrous ginseng (<em>P. notoginseng</em>)</td>
<td>64.44 ± 3.64</td>
<td>1.2</td>
<td>69.75 ± 1.45</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>San-chi ginseng (<em>P. quinquefolium</em>)</td>
<td>47.08 ± 3.34</td>
<td>1.1</td>
<td>42.39 ± 1.39</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>American ginseng (<em>P. quinquefolium</em>)</td>
<td>48.51 ± 1.79</td>
<td>0.6</td>
<td>47.96 ± 1.04</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td>Japanese ginseng (<em>P. japonicus</em>)</td>
<td>1.37 ± 0.34</td>
<td>0.1</td>
<td>0.63 ± 0.06</td>
<td>0.0</td>
<td></td>
</tr>
</tbody>
</table>
Table 3  G-Rb1 contents in various Chinese traditional herbal medicines

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Sample</th>
<th>Composition ratio</th>
<th>Amount determined/ mg g⁻¹ dry weight</th>
<th>Expected content/ mg g⁻¹ dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chorei-to</td>
<td>3:40</td>
<td>0.42 ± 0.05</td>
<td>0.41</td>
</tr>
<tr>
<td>2</td>
<td>Choto-san</td>
<td>2:28</td>
<td>0.38 ± 0.01</td>
<td>0.39</td>
</tr>
<tr>
<td>3</td>
<td>Byakko-ka-ninjin-to</td>
<td>1.5:31:5</td>
<td>0.25 ± 0.01</td>
<td>0.26</td>
</tr>
<tr>
<td>4</td>
<td>Hange-shashin-to</td>
<td>2.5:18:5</td>
<td>0.71 ± 0.01</td>
<td>0.74</td>
</tr>
<tr>
<td>5</td>
<td>Sho-saiko-to</td>
<td>3:24</td>
<td>0.64 ± 0.01</td>
<td>0.69</td>
</tr>
<tr>
<td>6</td>
<td>Hotyu-ekki-to</td>
<td>4:24</td>
<td>1.14 ± 0.18</td>
<td>0.92</td>
</tr>
<tr>
<td>7</td>
<td>Saiboku-to</td>
<td>3:34</td>
<td>0.60 ± 0.01</td>
<td>0.48</td>
</tr>
<tr>
<td>8</td>
<td>Shakunshi-to</td>
<td>4:15</td>
<td>1.71 ± 0.13</td>
<td>1.46</td>
</tr>
<tr>
<td>9</td>
<td>Rikkusen-to</td>
<td>4:21:5</td>
<td>0.90 ± 0.12</td>
<td>1.02</td>
</tr>
<tr>
<td>10</td>
<td>Ninjin-to</td>
<td>3:12</td>
<td>0.93 ± 0.07</td>
<td>1.37</td>
</tr>
<tr>
<td>11</td>
<td>Saiko-keishi-to</td>
<td>2:22</td>
<td>0.34 ± 0.03</td>
<td>0.50</td>
</tr>
<tr>
<td>12</td>
<td>Uni-kei-to</td>
<td>2:27</td>
<td>0.28 ± 0.01</td>
<td>0.41</td>
</tr>
<tr>
<td>13</td>
<td>Daio-kanzo-to</td>
<td>0:6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>Kikyo-to</td>
<td>0:5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Composition ratio expresses the contents of ginseng (g) used in each prescription.

The content of G-Rb1 was calculated from the composition of ginseng and the standardized G-Rb1 content (5.49 mg g⁻¹ dry weight) determined by ELISA as indicated in Table 2.

In conclusion, a combination of Western blotting, ELISA and immunoaffinity concentration could be used to survey low concentrations of G-Rb1 in samples of plant origin and/or in experimental animals and humans. Moreover, it is possible to study a large number of herbal medicines containing white ginseng for quality control purposes, and also regenerated plantlets cultured in the laboratory.

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