A General Enzyme Immunoassay for the Licorice Root Component Contained in Traditional Chinese Medicines

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Received September 27, 1996; accepted November 19, 1996

The development and application of a new enzyme immunoassay for general assay of the Glycyrrhiza radix (GR) component in Chinese traditional medicines is described. Three commercial GR-based medicines, Toboku kanzo (GRTK), Seihoku kanzo (GRSEK) and Sinkyo kanzo (GRSK) were used as GR specimens. Anti-GRSK serum was elicited from rabbits immunized with GRSK fragments. The presence of common proteins as specific antigens of GR was first established by Western blot analysis of extracts of GRSK, GRSEK or GRTK using anti-GRSK. The specific antigens were applied to develop an ELISA for the assay of GR extract. Anti-GRSK was put in competition with a sample or standard GR extract and immobilized GRSK components in microrotator plate wells. The proportion of antibody binding to the solid-phase GRSK component was detected using an enzyme-labeled second antibody. The ELISA method was specific to GRSK extract and showed low sensitivity for the assay of GRTK extract. The technique of selected antibody enzyme immunoassay (SAELA) was applied to develop a sensitive general assay method. Solid-phase GRTK extract, rather than immobilized GRSK extract, was used in the SAELA. The SAELA possessed the same quantitative working range of between 1 and 100 μg/ml for the assay of each extract of GRTK, GRSK and GRSEK. The SAELA was successful in the detection and quantitative measurement of GR component contents in Chinese traditional medicines.

Key words quantitative analysis; specific herb component; Chinese traditional medicine; general immunoassay

A Chinese traditional medicine (CTM) consists of extracts from a mixture of many crude drugs, the prescription for which is defined for each CTM. 1,2) Little scientific analysis of CTM has been reported. The following difficulties may account for this: A) the quality of the crude drugs is variable because conditions for the planting and harvesting of mother plants of the crude drugs are variable; B) the extract of a single crude drug contains many undefined components so that a CTM consists of many undefined components; C) no method has been available to prove that a CTM is properly prepared according to its prescription.

The crude drug Glycyrrhiza radix (GR, licorice root, Japanese name kanzo) has been one of the most frequently used crude drugs in CTMs. 3,4) Several species, such as Glycyrrhiza uralensis, G. glabra, G. inflata, and G. korshinskii, have been the botanical sources of GR and the quality of a GR preparation has been variable. Three kinds of licorice root, Toboku kanzo (GRTK; licorice root produced from the northeastern part of China), Seihoku kanzo (GRSEK; from the northwestern part) and Sinkyo kanzo (GRSK; from Xinjiang province), the names of which are in accord with the region of their production, have been imported and used in Japan. The botanical origin of each preparation is not defined. 3) GRTK, the botanical origin of which is said to be G. uralensis, has been most frequently used for the preparation of CTMs.

We have been attempting to develop a new method for the scientific analysis of the GR component contained in CTMs, with the strategy that the selected antibody enzyme immunoassay (SAELA) technique could be applicable for this. 3,4) Antiserum was elicited from a rabbit immunized with GRSK fragments (anti-GRSK). With the use of anti-GRSK in Western blot analysis, 3, 5) it was found that GRTK, GRSK and GRSEK contain specific protein components as common characteristic antigens. The characteristic proteins were applied to develop an ELISA for the assay of GR extract. The ELISA method was specific to the GRSK extract and showed low sensitivity in the assay of the GRTK extract. The technique of the SAELA method was applied to develop a sensitive general assay method for GR extracts. The assay method is specific for GR extract and other herb extracts did not disturb the specific assay for GR extract. This method allowed the detection and quantitative measurement of the GR component contained in three CTMs.

MATERIALS AND METHODS

Materials Microtiter plates with 96 wells (Immunoplate II) were purchased from A/S Nunc, Denmark, and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG/Fab from MBL Co., Nagoya. Three kinds of commercial licorice root, GRTK, GRSEK, and GRSK, and other medicinal herbs were obtained from the Osaka market of medicinal herbs. Four kinds of rabbit antiserum specific to crude drugs, Pinellia tuber, Hoelein, Panax ginseng, or Trichosanthes root were prepared by reported methods. Other chemicals used were of reagent grade.

GR Fragments GRTK was ground with a mortar and pestle and then filtered with a boiler (Mesh No. 48), and powdered GRSK was obtained. A suspension of powdered GRSK (1 mg/ml) in 0.5 mm phosphate-buffered saline, pH 7.0 (PBS), was homogenized by a sonic cell disrupter (Branson Sonic Power, model 185E, Danbury, CT, U.S.A.) at 60 W for 3 min in an ice-water bath, and the GRSK fragments obtained were immediately used as the immunogen and for the preparation of the solid-phase antigen.

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Antiserum Two white rabbits were immunized with GRK fragments (1 mg/ml in PBS) plus Freund’s complete adjuvant. Four booster injections of 0.5 mg of GRK fragments, plus Freund’s incomplete adjuvant, were given at biweekly intervals. The rabbits were occasionally bled from the ear vein until two weeks after the final injection. The titer of the serum was measured using the immunoassay procedures described below. The highest titered serum specific for GRK (anti-GRK) was stored at -30°C.

GR Extract Extraction was done from 1 g of powdered GRK using 20 ml of boiling water for 30 min. The residual powder underwent three additional extractions with 10 ml of hot water in a similar manner. The combined filtrates were lyophylized and the GRK extract (206 mg) was obtained as pale yellow amorphous powder. In a similar way, pale yellow amorphous extracts of GRK (335 mg) and GRTK (324 mg) were obtained from 1 g of GR powder.

SAEIA Procedure The SAEIA was performed on 96-well polystyrene microtiter plates. Wells were coated with a suspension of 50 μl/well of GRTK fragments (40 μg/ml in 10 mM Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl and 10 mM NaN3 as a coating buffer) at 25°C for 1 h. The plates were washed twice with 100 μl/well of 60 mM sodium phosphate buffer, pH 7.4, containing 10 mM ethylenediamine tetraacetate and 1% bovine serum albumin (buffer I). Non-specific binding sites were blocked with 200 μl/well of buffer I at 25°C for 2 h. A 50 μl/well sample of anti-GRK diluted 2000-fold in buffer I, was incubated in the wells at 25°C for 3 h with 50 μl/well of either a GR fragment suspension, GRK extract, a sample solution, or buffer I as a control. The plates were washed four times with 10 mM of phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% Tween 20 (PBST) and then incubated with 50 μl/well of HRP-labeled goat anti-rabbit IgG/Fab' diluted 2000-fold in PBST at 25°C for 2 h. After four washes with PBST, each well was incubated for 10 min with 100 μl/well of freshly prepared substrate solution containing 0.01% of 35% H2O2 and o-phenylene diamine (0.4 mg/ml) in 0.1 M citrate-phosphate buffer, pH 5.2. The enzymatic reaction was stopped by adding 50 μl/well of 2 N H2SO4, and the absorbance was read at 492 nm with an ELISA analyzer (SLT Lab, Instruments, Salzburg, Austria).

Western Blot Western blot analysis was carried out according to the reported method (essentially the method of Laemmli7): briefly, protein components contained in fragment suspensions of GRTK, GRK and GRK were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gel, and transferred to an IPVH filter (Japan Millipore, Ltd., Tokyo) which was then incubated with 1000-fold diluted anti-GRK. The filter was then incubated with 2000-fold diluted HRP-labeled Fab' of goat anti-rabbit IgG. The HRP-labeled second antibody bound on the filter was then developed to brown bands with a substrate solution.

CTM Three medicines, Shakuyaku-kanzo-to, Sho-saiko-to, and Boi-ogi-to, were prepared by extraction from specific mixtures of medicinal herbs with twenty times the volume of boiling water for 1 h, the extracts then being evaporated to dryness in vacuo. Prescriptions for the ingredients before extractions for a one day dose of each of the three medicines are shown in Table 1. GRTK was used as the licorice root. Three kinds of GR deficient medicines, pseudo-Shakuyaku-kanzo-to, pseudo-Sho-saiko-to and pseudo-Boi-ogi-to were also prepared in a similar way from the same mixtures of herbs without licorice root.

| Table 1. Prescriptions for the Preparation of Adult Daily Doses of Three CTMs, Shakuyaku-kanzo-to, Sho-saiko-to and Boi-ogi-to |
|-----------------|-----------------|-----------------|
| Daily dose (g)  | Shackuyaku-kanzo-to | Paenon root | 5.0 |
|                 |                  | Glycyrrhiza    | 5.0 |
|                 |                  | Total          | 10  |
| Sho-saiko-to    | Bupleurum root   | 7.0            |
|                 | Pinellia tuber   | 5.0            |
|                 | Ginseng          | 3.0            |
|                 | Scutellaria root | 3.0            |
|                 | Ginger           | 1.0            |
|                 | Jujube           | 3.0            |
|                 | Glycyrrhiza      | 2.0            |
|                 | Total            | 24             |
| Boi-ogi-to      | Sinomenium stem  | 5.0            |
|                 | Astragalus root  | 5.0            |
|                 | Atractylodes rhizome | 3.0 |
|                 | Ginger           | 0.8            |
|                 | Jujube           | 3.0            |
|                 | Glycyrrhiza      | 1.5            |
|                 | Total            | 18.3           |

A specific mixture of crude drugs was extracted with twenty times the volume of boiling water for 2 h. After filtration with a cotton cloth, the extract was lyophylized to give a CTM.

RESULTS

Specific Antigen of GR The presence of characteristic proteins of GR were shown by the Western blot method. Proteins contained in each extract of GRK, GRTK and GRK were separated using SDS-PAGE under non-reducing conditions. z-Lactalbumin (14400), soybean trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (670000), and phosphorylase (94000) were used as reference proteins. Coomassie brilliant blue R-250 staining was used for the detection of reference proteins. The molecular weights are shown by arrows in Fig. 1.

Common characteristic protein bands were observed for each extract of GRK, GRTK and GRK using anti-GRK in the blot analyses. The blot analysis is specific to GR antigens and the reference proteins did not produce bands. The proteins representing GR antigens were not stained by blot analyses using four other rabbit antisera specific to the crude drugs Pinellia tuber, Panax ginseng, Hoelen, or Trichosanthes root instead of anti-GRK.

ELISA Method A suspension of GRK fragments contained a GRK extract which was immobilized in plastic titer plate wells (solid-phase GRK fragments). A novel ELISA method for the assay of the soluble com-
Table 2. Cross-Reactivity Percent (CR %) Values of Extracts (EX) of the Crude Drugs, GRSK, GRSEK, GRTK, PT, PG, HE, TR, and AR Measured by the ELISA Method for the Assay of GRSK Extract

<table>
<thead>
<tr>
<th>Crude drug EX</th>
<th>100</th>
<th>21.0</th>
<th>2.0</th>
<th>&lt;0.01</th>
<th>&lt;0.01</th>
<th>&lt;0.01</th>
<th>&lt;0.01</th>
<th>&lt;0.01</th>
</tr>
</thead>
<tbody>
<tr>
<td>CR % value</td>
<td>100</td>
<td>21.0</td>
<td>2.0</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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Fig. 3. Typical Dose Response Curves of Three Kinds of Extracts of GRs, GRSK, GRTK, and GRSEK, Measured by the SAIEA for the Assay of GR

Each data point is the mean of three replicated experiments.

sitivity between the extracts of GRSK, GRSEK and GRTK were compared with the dose at $B/B_0$ 50% value ($X$ µg/ml). For each of the five other crude drugs, the dose of standard GRSK extract ($Y$ µg/ml) corresponding to the $B$ value at 1000 µg/ml for each of the five other crude drugs was read from the dose-response curves of the standard GRSK. The relative percent cross-reactivity value (percent) of each inhibitor was calculated from the value $X$, and the corresponding dose of each inhibitor was calculated from the values $X$ or $Y$ and the corresponding dose of the inhibitor, defining the reactivity of standard GRSK as 100% (Table 2).

The ELISA method was found to be inadequate for the assay of GRTK extract, because of its low sensitivity.

A General Assay Method We applied a SAIEA technique to develop a general method for assays of extracts of three kinds of GRs, GRTK, GRSK and GRSEK with high sensitivities and the same quantitative working ranges. In the SAIEA method, solid-phase GRTK extract was used instead of solid-phase GRSK extract. The quantitative working ranges of the SAIEA method calculated from nonlinear regression analysis of the extracts of GRTK, GRSK or GRSEK were the same, between 1 and 100 µg/ml (Fig. 3). A similar amounts of GR extract at $B/B_0$ 50% value of 18.2 µg/ml for GRSK, 17.8 µg/ml for GRSEK and 25 µg/ml ($n=10$) for GRTK were measured by nonlinear regression analysis. The intraassay
Table 3. Cross-Reactivity Percent (CR %) Values of Extracts of the
Crude Drugs, GRSK, GRSEK, GRTK, PT, PG, HE, TR, and AR
Measured by the SAEIA Method for the General Assay of GR Extract

<table>
<thead>
<tr>
<th>CR % value</th>
<th>GRSK</th>
<th>GRSEK</th>
<th>GRTK</th>
<th>PT</th>
<th>PG</th>
<th>HE</th>
<th>TR</th>
<th>AR</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>102.2</td>
<td>71.2</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
<td>&lt;0.01</td>
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</table>

Fig. 4. Typical Dose Response Curves of Three Kinds of CTMs,
Shakuyaku-kanzo-to (SKT), Sho-saiko-to (SST), and Boi-ogi-to (BOT),
and Three Kinds of GR Deficient Medicines, Pseudo-Shakuyaku-kanzo-
to (PSKT), Pseudo-Sho-saiko-to (PSST), and Pseudo-Boi-ogi-to (PBOT),
Measured by the SAEIA for the Assay of GR Extract.

Each data point is the mean of three replicated experiments.

coefficient of variation at 4 different levels of GRTK extract (1, 10, 100, and 1000 μg/ml, n = 10) was determined as 4.3—8.7% (average 8.3%).

The sensitivity limit, defined as the lowest amount of the GRTK extract distinguishable from zero at the 95% confidence level, was 1.0 ± 0.07 μg/ml (n = 10).

Specificity The specificity of the SAEIA method for the assay of GR extract was confirmed by the assay of extracts of five other medicinal herbs, Panax ginseng, Pinellia tuber, Hoelen, Aconite root and Trichosanthes root (Table 3). Cross-reactivity values of extracts of GRSEK and GRTK were similar to that of GRSK, and those of the five other herbs were less than 0.01%.

Content of GR Extract Three CTMs, Shakuyaku-kanzo-to, Sho-saiko-to, and Boi-ogi-to, and their GR deficient correlates, pseudo-Shakuyaku-kanzo-to, pseudo-Sho-saiko-to and pseudo-Boi-ogi-to, were prepared according to their prescriptions. The dose response curves of CTMs and pseudo-CTMs were measured by the SAEIA assay for GR extract (Fig. 3). All three GR deficient medicines showed less than 0.01% cross-reactivity values. Shakuyaku-kanzo-to, Sho-saiko-to, and Boi-ogi-to, at the concentrations of 345 μg/ml, 4.7 mg/ml and 13.3 mg/ml, respectively, showed 50% inhibition of antibody binding on the f-SEIA assays.

Accuracy of Contents The content of GR extract contained in 0.1 ml of either Shakuyaku-kanzo-to (0.3 mg/ml), Sho-saiko-to (5 mg/ml), and Boi-ogi-to (10 mg/ml) was measured by the f-SEIA in the presence or absence of 10 μg of GR extract (Table 4). The accuracy of the f-SEIA for the assay of the GR component content in the three CTMs was shown by good recovery percentages of the added 10 μg of GR extract: between 99.4% and 103.6%.

**DISCUSSION**

We have been attempting to develop a method for the detection and quantitative measurement of the contents of a particular crude drug component contained in CTMs, based on the strategy that the SAEIA method, which we originally developed for the assay of microbes, could be modified for this purpose. To realize this strategy, we had to overcome six difficulties, one by one, as pointed out in our previous papers.⁵,⁶ We reported studies on four of the problems in which: a) we succeeded in preparing an antiserum highly specific to a crude drug using five kinds of crude drugs as models.⁷ Other studies reported that b) despite the use of the same antiserum specific to a crude drug, SAEIA methods for assays of different components of the crude drug were possible by selecting a solid-phase antigen; c) a SAEIA for specific assay of a crude drug extract was developed using solid-phase crude drug extract, and d) the SAEIA method was applicable for assay of the content of a specific crude drug component contained in CTMs.⁸,⁹,¹⁰

Two other problems which need to be overcome are: e) CTMs are prepared with ingredients of variable qualities, so the SAEIA should be modified to be applicable as a general method for the assay of a crude drug extract of heterogeneous qualities; and f) the general method should be specific so as to be applicable for the assay of the crude drug component of CTMs prepared with ingredients of variable quality.

We used three kinds of commercial GRs, GRTK, GRSK, and GRSEK, as models for the studies on the two problems. The strategy for developing the general immunoassay method was to reduce the binding activity of the tracing antigen (tracer) to the antibody in a
competitive immunoassay. The use of low cross-reacting tobramycin instead of kanamycin as the enzyme-labeled tracer allowed us to develop a new method for the general detection of five kinds of kanamycin group antibiotics. With the new method, assay sensitivities of kanamycin and its analogs were increased from 8.5 to 191000 times that measured by the usual method using the enzyme-labeled kanamycin as the tracer. The assay method was specific to kanamycin group antibiotics. In addition, the quantitative ranges of the assay method for all kanamycin groups were the same.

The application of the principle of this finding to developing a general assay method for the three GRs by finding an adequate tracer which possesses a reduced binding activity to anti-GRSK was studied. With the use of highly titered anti-GRSK serum, the presence of common proteins as characteristic antigens of GR was first established by Western blot analyses of extracts of GRSK, GRSEK and GRTK (Fig. 1). The proteins were specific to anti-GRSK and were not detected by the blot analyses by the antiserum specific to four other crude drugs, Pinellia tuber, Hoelen, Panax ginseng, and Trichosanthes root. An ELISA method for the assay of GR extract was developed using solid-phase GRSK extract and anti-GRSK as immunological reagents. We reported that the mechanism of the ELISA method was clarified as the measurement of the specific protein of GR. It was shown that immunological properties of the three GR extracts are heterogeneous; the assay sensitivity of the GRTK extract measured by the method was only 1/50 that of the GRSK extract (Table 2). The reason for the low sensitivity for the GRTK extract could be interpreted in two ways: one probable reason could be the low content of characteristic GR protein in GRTK; the second could be the low reactivity of GRTK protein to anti-GRSK owing to a slightly different structure of the protein of GRTK than that of GRSK, so that the GRTK protein may show low cross-reactivity. In the case of the second reason, it seemed possible to apply the principle of the general assay method for kanamycin group antibiotics to develop a general assay method for the three GR extracts.

To reduce the binding activity of the tracer, we used GRTK extract as the tracing solid-phase antigen. In fact, the modified SAIEA method did prove to be a general assay method for the three kinds of GR extracts since quite similar dose response curves were obtained for them, all with high sensitivities (Fig. 3). The specificity of the SAIEA method for the assay of GR extract was established by measuring the cross reactivity values of five other herb extracts of less than 0.01% (Table 3). The specificity of the SAIEA method for the assay of GR extract was confirmed by the measurement of the GR extract contained in CTMs which consisted of extracts of a mixture of ingredients. Three CTMs, Shakuyaku-kanzo-to, Sho-saiko-to or Boi-ogi-to and three corresponding GR-deficient medicines were prepared and used as specimens. None of the three GR deficient medicines showed displacement in the SAIEA, the general assay method, at concentrations of less than 10 mg/ml. The content of the GR component contained in 0.1 ml of solutions of 100

\[ \mu g/ml \text{ of Shakuyaku-kanzo-to, } 50 \mu g/ml \text{ of Sho-saiko-to or } 100 \mu g/ml \text{ of Boi-ogi-to was measured. The accuracy of the determination was confirmed by good recoveries of GR extract added (Table 4). It was also shown that the specificity of the SAIEA was limited to GR extract, and varying amounts of a mixture of many other herb extracts contained in prescriptions of the three kinds of medicines did not disturb the measurements of the SAIEA under the conditions used.}

We reported that the principle of the general assay method for kanamycin analogs is applicable for developing general assay methods for several microbes. In the present paper we report that the principle is also applicable for developing a general method for the assay of GR extract of different qualities using three commercially available GR preparations as models.

Qualities of a particular crude drug preparation are variable, as are its immunological properties. This poses a difficult problem for the measurement of the content of the drug component in various CTMs. An immunoassay method requires the dose response curve of a standard crude drug extract from which the content of the drug component in a CTM is calculated. For accurate quantitative measurement, each standard extract used for the ELISA-type assay of the drug component in a CTM should be prepared from the crude drug of the same lot used for preparation of the CTM to avoid a large error in the assay result caused by the use of an inadequate standard extract. This posed a difficult problem in applying an ELISA-type method for accurate assay of the content of the crude drug component in a commercial CTM: it is difficult to procure a crude drug sample, which should be from the same lot used for preparing the commercial CTM, to prepare each standard drug extract. The newly proposed general assay method does not require each specific standard extract and is able to use a common standard crude drug extract for the assay of various CTMs. This general method will be useful for mass screening for the detection and quantitative measurement of the content of GR components in various CTMs.

REFERENCES