A New Method for the Detection and Quantitative Measurement of the Contents of Trichosanthes Root Component Composing Chinese Traditional Medicines

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A new method was developed to estimate the content of Trichosanthes root (TR) component in two Chinese traditional medicines. Characteristic antigens of TR were separated from TR extract using rabbit antisemur specific for TR, a dried root tuber of Trichosanthes kirilowii. Two selected antibody enzyme immunoassay (SAEIA) methods, the SAEIA A for assay of TR extract and the SAEIA B for assay of karasurin A were used as detection methods for the separation of TR antigens. Using several column chromatographies, two kinds of TR antigens, a protein component and a glycan component, were separated from TR extract. A SAEIA C method for assay of TR glycan component was developed using biotinylated second antibody and peroxidase-labeled avidin as the detection method. The SAEIA C was also found applicable for specific assay of TR extract as well as for the contents of TR component present in two Chinese traditional medicines, prescriptions of which contained TR. Content of trichosanin, an abortifacient protein component of T. kirilowii, in both the medicines were also measured by applying the SAEIA B for assay of karasurin A. Little trichosanin was found in either medicine and the reason for this was determined.

Key words enzyme immunoassay; Trichosanthes root; glycan antigen; protein antigen; quantitative analysis; Chinese traditional medicine

A Chinese traditional medicine (CTM) consists of an extract from a mixture of many kinds of crude drugs, the prescription of which is defined for each CTM.1,2 Little study on scientific analysis of any CTM has been reported, and no method has been available to confirm that a specific CTM is properly prepared according to its prescription. Consequently, an easy method for measurement of the content of a specific herb extract composing a CTM should provide us with a useful new tool for quality control of CTMs.

We have been attempting to develop a new method applicable for the quality control of CTMs by immunoassay. The strategy for developing the method was as follows: if we were able to prepare an antisemur highly specific to a crude drug, we could find and separate the antigen specific to that drug using the antisemur. The development of an immunoassay method for specific assay of the antigen should be possible using the antisemur and the antigen as immunological reagents. Because extract of the crude drug would contain the antigen in a dose response manner, an immunoassay method should be applied to detect and quantitatively measure the crude drug extract so that its content in a CTM can be measured. We thus undertook development of a new serological method for this purpose.

This new immunological technique is called selected antibody enzyme immunoassay (SAEIA) and was developed to identify microbial species.3–7 A crude drug contains a number of constituents and antisemur elicited against the drug consists of a variety of polyclonal antibodies specific for various epitopes contained in molecules of the constituents. When we use a solid-phase antigen in an ELISA type assay, only the antibodies specific for epitopes contained on the surface of the solid-phase antigen are selected from the antisemur and used in serological reaction of the immunoassay, thus the name SAEIA.8 Use of a specific antigen of a crude drug as solid-phase antigen in this immunoassay means that selective use of antibodies specific for the antigen contained in the polyclonal antibodies should be possible; a specific assay method for the antigen is thus anticipated. The assay method should therefore be applicable for the detection and quantitative measurement of the crude drug component of a CTM.

We report here studies to realize the above hypothesis using as a model Trichosanthes root (TR), a dried root of Trichosanthes kirilowii.

MATERIALS AND METHODS

Materials Horseradish peroxidase (HRP)-labeled Fab' of goat anti-rabbit IgG was bought from MBL Co., Nagoya, biotin-labeled goat anti-rabbit IgG from Nitirei Co., Tokyo and HRP-labeled strept-avidin from Vector Lab. Inc, Burlingame, CA, U.S.A. Phenol reagent was obtained from Wako Pure Chemical Ind., Ltd. Osaka, and Schiff's reagent solution from Nacalai Tesque Inc., Kyoto. Rabbit anti-TR used is the antisemur reported.8 Karasurin A9,10 was kindly provided by Dr. Y. Ogihara, Nagoya City University. Commercial crude drugs were obtained from the Osaka market of crude drugs. Other chemicals used were of reagent grade.

Assay Methods Used: SAEIA Procedure The SAEIA A for assay of TR extract using solid-phase TR extract and anti-TR,8 and the SAEIA B for assay of karasurin A which used solid-phase karasurin A and anti-TR,11 were performed by the procedures reported.

SAEIA C for Assay of Glycan-b Component Wells of 96-well polystyrene microplates were coated with 100 μl/ well of glycan-b component (1 μg/ml in 10 mM Tris·HCl

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buffer, pH 8.5, containing 0.1 M NaCl and 10 mm Na$_2$CO$_3$) at 25°C for 1 h. Each well was washed twice with 200 μl of 60 mm sodium phosphate buffer, pH 7.4, containing 10 mm ethylenediamine tetraacetate and 1% bovine serum albumin (buffer I), and non-specific binding sites were blocked with 200 μl of buffer I at 25°C for overnight. After blocking, 100 μl of anti-TR diluted 10000-fold in buffer I was incubated in the component b-coated well at 25°C for 3 h with 100 μl of either component b solution, a sample solution, or buffer I as a control. The wells were washed four times with 10 mm phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% Tween 20 (PBST), and then incubated at 25°C for 2 h with 200 μl/well of biotin-labeled goat anti-rabbit IgG diluted 1000-fold in PBST. Each well was washed four times with PBST, after which, 200 μl of HRP-labeled avidin diluted 2000 times with PBST was added to each well and incubated at 25°C for 2 h. After four washes with PBST, 200 μl/well of freshly prepared substrate solution containing 0.1 μl of 35% H$_2$O$_2$ and 0.1 μl of 0.5 mg/ml of 0.1 M citrate-phosphate buffer, pH 5.2, was added. Following incubation for 10 min, the enzymatic activity was stopped by the addition of 50 μl/well of 2 N H$_2$SO$_4$ and absorbance was read at 492 nm with an ELISA analyzer (SLT Lab. Instruments, Salzburg, Austria).

**Phenol–H$_2$SO$_4$ Method** The method of Dubois *et al.* was applied; briefly, aqueous 5% phenol (100 μl) was mixed well with 100 μl of each chromatographic fraction, followed by the addition of 500 μl of conc. H$_2$SO$_4$. Ten minutes after the addition, absorbance at 490 nm was measured.

**Lowry Method** The method of Lowry *et al.* was applied; briefly, 100 μl of each fraction was incubated with 1 ml of 0.01% CuSO$_4$ in 1 N NaOH containing 2% Na$_2$CO$_3$ at 25°C for 20 min, and then the solution was incubated with 100 μl of two-fold diluted phenol reagent at 25°C for 20 min and absorbance at 660 nm was measured.

**PAS Stain** The method of Pearse was applied; after electrophoresis, the gel was incubated in 0.5% periodate for 2 h, washed twice with 0.5% NaAsO$_2$ in 5% acetic acid, then kept in Schiff reagent overnight. Next day the gel was washed four times with 0.5% Na$_2$S$_2$O$_3$, twice with water and was then kept in 7% acetic acid.

**Western Blot** Western blotting was carried out by the method of Laemmli with a slight modification. TR antigens contained in an extract of TR were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run on 10% polyacrylamide gel. Separated proteins were then transferred electrophoretically to an IPVH filter (Japan Millipore, Ltd., Tokyo). The filter was blocked with 1% skim milk (pH 7.4) at 37°C for 1 h, and then was incubated with 1000-fold diluted antisera in buffer I at 4°C overnight. The filter received six washes with PBST, and was then incubated with 2000-fold diluted HRP-labeled Fab' of goat anti-rabbit IgG at 25°C for 30 min. After four washes with PBST, the HRP-labeled secondary antibody bound on the filter was developed to brown bands with a substrate solution at 25°C for 5 min.

**TR Extract** The powdered TR (5 g) was extracted three times with 100 ml of boiling water for 1 h. The powder was removed by filtration. Combined filtrate was lyophilized to obtain hot TR extract (663 mg) as amorphous. When the powdered TR (16 g) was extracted three times with 1000 ml of cold water over 5 h, 3.08 g of cold TR extract was obtained from the combined extracts.

**Separation of TR Antigens** TR cold extract (2 g) dissolved in 10 ml of water was dialyzed five times with 1000 ml of water from which TR antigens (TRA) were separated by chromatography using the SAEIA A method to detect the antigen.

i) Sepharose 6B Column: After dialysis, TR extract was subjected to gel filtration on Sepharose 6B column (2 × 44 cm) which was previously swollen and eluted with 0.02 M phosphate buffer (pH 7.5). Fraction nos. (2 ml/tube) 25—44, positive to the SAEIA A were combined, dialyzed against water and then lyophilized to give 180 mg of a component (TRA1).

ii) Sephacryl S-200 Column: TRA1 (180 mg) was subjected to gel filtration on Sephacryl S-200 column (2 × 40 cm) previously swollen and eluted with 0.02 M phosphate buffer (pH 7.5). Fraction nos. 27—37 (2 ml/tube) positive to the SAEIA were combined, dialyzed, lyophilized and 163 mg of a lyophilized product (TRA2) was obtained.

iii) Sephacryl S-300 Column: TRA2 (50 mg) was purified with Sephacryl S-300 column (0.6 × 50 cm) previously swollen and eluted with 0.02 M phosphate buffer (pH 7.5). The phenol–H$_2$SO$_4$ method, Lowry method, SAEIA A for assay of TR extract and SAEIA B for assay of karasarin A were applied for each fraction (1 ml/tube).

iv) DEAE-Sephacryl CL-6B Column: TRA2 (100 mg) was subjected to DEAE-Sephacryl CL-6B column (1.5 × 10 cm). The column was washed with 0.02 M phosphate buffer (pH 8.0) to elute component which was not adsorbed (component c), then eluted with 0.02 M phosphate buffer (pH 8.0) containing 0.3 M NaCl.

**CTMs** Two medicines, Saiko-seikan-to and Saiko-keishi-kanyakou-to

<table>
<thead>
<tr>
<th>Table 1. Prescriptions of the crude Drugs in Preparations in Adult Dose of Two Chinese Traditional Medicines, Saiko-seikan-to and Saiko-keishi-kanyakou-to</th>
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<tbody>
<tr>
<td><strong>Saiko-seikan-to</strong> (柴胡清肝湯)</td>
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<tr>
<td>Japanese Angelica root (當歸)</td>
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<tr>
<td>Peony root (芍藥)</td>
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<tr>
<td>Chuanxiong rhizoma (川芎)</td>
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<tr>
<td>Rehmannia root (地黃)</td>
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<tr>
<td>Forsythia fruit (連翹)</td>
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<tr>
<td>Platycodon root (桔梗)</td>
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<tr>
<td>Arctium fruit (牛蒡子)</td>
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<tr>
<td>Trichosanthes root (瓜 TURN)</td>
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<tr>
<td>Mentha herb (薄荷)</td>
</tr>
<tr>
<td>Glycyrrhiza (甘草)</td>
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<tr>
<td>Copis rhizome (黄連)</td>
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<tr>
<td>Scutellaria root (黄芩)</td>
</tr>
<tr>
<td>Phellodendron bark (黄柏)</td>
</tr>
<tr>
<td>Gardenia fruit (山橘子)</td>
</tr>
<tr>
<td>Bupleurum root (柴胡)</td>
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<td><strong>Total 23.0 g</strong></td>
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keishi-kanyakou-to were prepared by the extraction of specific mixtures of crude drugs with boiling water for 1 h, and then the extracts were evaporated to dryness in vacuo. Prescriptions for a one day dose of both medicines before the extraction are shown in Table 1. Two TR deficient medicines, pseudo-Saiko-seikan-to and pseudo-Saiko-keishi-kanyakou-to were also prepared in similar ways from the mixtures of crude drugs in which TR was removed from their corresponding prescriptions.

RESULTS

**SAEIA A for TR Extract** A competitive SAEIA for assay of TR extract was developed using a rabbit anti-TR serum and solid-phase TR extract which was prepared by adsorbing soluble components of a TR fragment suspension on the surface of wells of titer plates according to the method reported. The antibody bound to the solid-phase antigen on the surface of wells was then reacted with HRP-labeled Fab' of goat anti-rabbit IgG as a second antibody. The enzymatic activity of the bound HRP-label in wells was measured, and the binding of antibody to the antigen was decreased by competition with free TR extract in a dose response manner.

**Separation of Antigens** Antigens contained in TR extract specific to anti-TR were separated by chromatography and the SAEIA A for assay of TR extract was used to detect TR antigen. After dialysis of 2 g of TR extract, TR antigen in the aqueous solution was partially purified by using Sepharose 6B column (data not shown). A 180 mg of partially purified TR antigen (TRA1) was obtained from the SAEIA active fractions (nos. 25—44). TRA1 (180 mg) was then subjected to gel filtration on a Sephacryl S-200 column. The elution profiles of the chromatogram were also determined by the SAEIA (data not shown).

The fractions (nos. 27—37) positive to the SAEIA assay were combined, dialyzed and lyophilized and 163 mg of TRA2 was obtained.

**Detection of Glycan and Protein as TR Antigen Components** Fifty milligrams of TRA2 was subjected to gel filtration chromatography on Sephacryl S-300 column. Elution profiles of the chromatogram determined by Lowry method for the detection of protein, phenol—H$_2$SO$_4$ method for the detection of glycan, the SAEIA A for assay of TR antigen and the SAEIA B for assay of karasurin A were used to detect components of TR antigens (Fig. 1). Glycan rich fractions reacted positively to phenol—H$_2$SO$_4$ method were eluted first, followed by the elution of protein rich fractions which were reacted positively to Lowry method. Two peaks were observed for the elution profiles determined by the SAEIA for assay of TR extract which coincided with both protein and glycan rich fractions.

**Western Blot** Western blot analysis was chosen as the method of TR antigens specific to anti-TR. Each fraction (no. 25, 30, 35, 40, 45, 50, 55 or 60) from Sephacryl S-300 column was separated using SDS-PAGE under non-reducing conditions. Western blot analysis was performed using anti-TR, α-Lactalbumin (14400), soybean trypsin inhibitor (20100), carbonic anhydrase (30000), ovalbumin (43000), bovine serum albumin (67000), and phosphorylase b (94000) were used as reference proteins. Coomassie brilliant blue R-250 (CBB) staining was used for the detection of proteins, and molecular weights are shown by arrows in figures.

High molecular glycan bands were observed between fraction nos. 30—50, while 28 kDa protein was observed for fraction nos. 45, 50 and 55 (Fig. 2).

**Separation of Protein and Glycan Antigens** TRA2 was

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![Fig. 1. Sephacryl S-300 Column Chromatography of TR Extract Monitored in Terms of the SAEIA A for Assay of TR Extract, the SAEIA B for Assay of Karasurin A, and Absorbance at 660 and 490 nm Developed by Lowry Color Reaction and Phenol—H$_2$SO$_4$ Method, Respectively](image)

Crude TR antigen (TRA2, 50 mg) was put on a column of Sephacryl S-300 (2 × 44 cm) which was previously equilibrated and eluted with 0.02 M phosphate buffer (pH 7.5; absorbance at 660 and 490 nm (left vertical axis) which was developed by the Lowry method (□) and the phenol—H$_2$SO$_4$ method (○), respectively, were measured. TR antigen activities (right vertical axis) were assayed by the SAEIA A (●) and the SAEIA B (■) for each fraction (2 ml/tube). Each data point of antigen content is the mean of three replicated experiments.
subjected to DEAE-Sepharose CL-6B column chromatography, the elution profiles of which were determined by phenol–H₂SO₄ method, SAEIA A and absorbance at 280 nm (Fig. 3). Glycan-c component (50 mg) was obtained from fraction nos. 1—6, and protein-a component from fraction nos. 18—30. Protein-a component was further purified to a single band on Western blot by Sephacryl S-200 column chromatography (Fig. 5).

Glycan-c component (50 mg) was subjected to chromatographic purification with Sephadex G-200 column eluted with water (Fig. 4). Glycan-b component was obtained from fraction nos. 8—12.

Component b was hardly stained by CBB but was clearly stained by the PAS method (Fig. 5). It consisted of 90.7% glycan component as calculated by phenol–H₂SO₄ method using glucose as the standard, and 6% protein component as determined by the Lowry method using bovine serum albumin as the standard.

**SAEIA Methods for Assays of TR Antigens**

i) SAEIA C method for assay of glycan-b component was developed using solid-phase glycan-b component. For assay of protein-a component, the SAEIA B method for assay of karasurin A was found applicable.

The optimal conditions of doses of reagents and incubation time for each procedure of SAEIA C for assay of glycan-b component were established, and the working range was between 10 ng and 1 μg per ml (Fig. 6). Applications of the SAEIA C for assays of TR hot extract, TR cold extract, and the two CTMs, Saiko-seikan-to and Saiko-kei-kangyou-to were performed with satisfactory results (Fig. 6). Very little inhibition was observed for both TR deficient CTMs, pseudo-Saiko-seikan-to and pseudo-Saiko-kei-kangyou-to. The SAEIA C was highly specific to glycan-b component and other herb extracts contained in both TR deficient medicines did not disturb the specific assay even at the total concentration of 10 mg/ml (Fig. 6).

ii) Content of Trichosanat: The content of an abortifacient protein trichosanat, a karasurin A analog, in hot and cold TR extracts, two CTMs, and also both of their pseudo-medicines were measured by the SAEIA B method with the working range between 10 ng and 10 μg per ml for assay of karasurin A (Fig. 7).

None of the four medicines at concentrations of less than 1 mg/ml showed inhibition on the SAEIA B for assay of karasurin A (Fig. 7). TR cold extract showed a dose response inhibition curve by the SAEIA B for assay of karasurin A but not with hot TR extract (Fig. 7). It was concluded that trichosanat was extracted with cold water.

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**Fig. 2.** Western Blot Analyses of TR Antigens Contained in Fractions of Sephacryl S-300 Column Chromatography

Western blot analysis: antigens separated by SDS-PAGE were transferred to the PVDF filter and incubated with anti-TR. The rabbit IgG bound to antigens was reacted with HRP-labeled second antibody, and the bound HRP-labeled antibody was reacted with substrate to give brown protein bands on the filter. CBB staining was used for determination of molecular weights of standard proteins shown by arrows (data not shown).

**Fig. 3.** DEAE-Sepharose CL-6B Column Chromatography of TR Antigens Monitored in Terms of the SAEIA A for TR Extract, Absorbance at 280 and 490 nm, the Latter Being Developed by Phenol–H₂SO₄ Color Reaction

A crude TR antigen (TRA2; 100 mg) was put on a column of DEAE-Sepharose CL-6B (1.5 × 10 cm), which was previously equilibrated and eluted with 0.02 M phosphate buffer (pH 8.0) containing 0.3 M NaCl; absorbance at 280 nm (●; left vertical axis) and 490 nm which was developed by phenol–H₂SO₄ method (○; middle vertical axis) were measured. TR antigen content (●; right vertical axis) was assayed by the SAEIA A method using 100 μl/well of 100-fold diluted solution of each fraction (2 ml/tube). Each data point of antigen content is the mean of three replicated experiments.
DISCUSSION

The value of annual CTM production of pharmaceutical industries in Japan is more than two hundred billion yen and these medicines have been given to patients for whom modern drugs have been ineffective. The little scientific analysis done on CTMs, however, may be because of the following difficulties: A) quality of a crude drug is heterogeneous because conditions for planting and harvesting of the mother plants are multifarious: B) extract of a single crude drug contains many unidentified components so that composition of a CTM is also undefined: C) when a specific low molecular compound of a crude drug is available, detection of the crude drug component in CTMs is possible by analyzing the compound. Heterogeneous properties of a crude drug, however, make difficult for quantitative analysis of the component: D) no method has been available to prove that a CTM is prepared according to its prescription.

We began the serological study on crude drugs under the working hypothesis that the SAIEA method, which we originally developed for assay of microbes, could be modified to make it applicable for the detection and quantitative measurement of the content of a specific crude drug extract.

To confirm the hypothesis, we had to overcome six difficult items: a) we prepared an antiserum highly specific to a crude drug; b) despite use of the same antiserum specific to the crude drug, SAIEA methods for assays of different components of the drug should be possible by selecting a solid-phase antigen; c) a SAIEA for assay of a crude drug extract was developed using the solid-phase of the crude drug extract; d) the SAIEA method for assay of a crude drug extract should possess sufficient specificity.

but not with hot water.

**Heat Stability Test** TR glycan-b component and TR protein-a component, with and without heat treatment at 100℃ for 1 h, were subjected either to the SAIEA B or the SAIEA C assay (Fig. 8).

Trichosanチン rapidly lost its antigenic property, while the glycan-b component was stable in heat so that it was applicable to the assay of the content of the TR component in CTMs.
for assay of the content of a particular crude drug component in a CTM; e) CTMs are prepared using crude drugs of heterogeneous qualities. The SAEIA should be modified so as to be an applicable general method for assay of a crude drug extract of such qualities; f) the modified SAEIA should be specific so as to be applicable for the general assay of the component of a specific crude drug in CTMs prepared by using such crude drug.

We have overcome these six difficulties one at a time and in the preceding paper we reported with regard to item a), that we had succeeded in preparing five rabbit antisera specific for each of five corresponding crude drugs including TR, a dried root of *Trichosanthes kirilowii*. Items b), c) and d) were studied using anti-TR. SAEIA A for assay of TR extract and SAEIA B for assay of karasurin A were used to detect and isolate characteristic antigens of TR. Using several column chromatographies, we were able to separate two types of specific TR antigens from a TR extract: protein antigen and glycan antigen (Figs. 1—5). Characterizations of both antigens were performed by Western blot method and color reactions, phenol–H₂SO₄ method and PAS staining for glycan and Lowry method and CBB staining for protein (Figs. 1—6).

The protein antigen was identified as trichosantin, a karasurin A analog, judging from the response to the SAEIA B for assay of karasurin A. The third SAEIA
method, SAEIA C for the assay of TR glycan-b, was developed using solid-phase glycan-b component and anti-TR as immunological reagents. To sensitize this method, biotinylated secondary antibody and HRP-labeled avidin were used in the assay. The working range of the assay for TR glycan is between 10 ng and 1 μg. Application of the SAEIA C for the detection and quantitative measurement of the content of TR extract was successful (Fig. 6).

The specificity of the SAEIA C for assay of TR extract was established by measuring the contents of TR component in the two CTMs Saiko-seikan-to and Saiko-keishi-kankyou-to, which contain TR in their prescriptions. The two medicines and their corresponding TR deficient medicines were prepared and their dose response inhibition curves were measured with the SAEIA C for assay of TR glycan. The TR deficient medicines showed little displacement in the antibody binding assay of the SAEIA C (Fig. 6). Accuracy of the determined contents was confirmed by good recovery of TR extract (Table 2).

Table 2. Contents of Trichosanthes root extract (TRE) in Solutions of Two Kinds of Chinese Traditional Medicines, Saiko-seikan-to (0.1 ml of 100 μg/ml) and Saiko-keishi-kankyou-to (0.1 ml of 20 μg/ml) with Recovery Percentages of 1 μg of Added TRE

<table>
<thead>
<tr>
<th></th>
<th>Mean ± S.D.</th>
<th>CV (%)</th>
<th>Added TRE</th>
<th>B—A (μg)</th>
<th>Recovery (%)</th>
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<tr>
<td><strong>Saiko-seikan-to</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No addition (A)</td>
<td>1.4 ± 0.16</td>
<td>11.4</td>
<td></td>
<td>1.0</td>
<td>102.0</td>
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<tr>
<td>With addition (B)</td>
<td>2.42 ± 0.14</td>
<td>6.0</td>
<td>1.0</td>
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<tr>
<td><strong>Saiko-keishi-kankyou-to</strong></td>
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<tr>
<td>No addition (A)</td>
<td>1.07 ± 0.035</td>
<td>3.2</td>
<td>1.0</td>
<td>1.06</td>
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<tr>
<td>With addition (B)</td>
<td>2.13 ± 0.045</td>
<td>2.1</td>
<td>1.0</td>
<td>1.06</td>
<td>106.0</td>
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</table>

a) Standard deviation of five replicated experiments. b) Coefficient of variation.

Fig. 8. Heat Stability Tests of TR Protein-a and TR Glycan-b Measured Either with SAEIA B for Assay of Karasurin A or SAEIA C for Assay of TR Glycan

Both antigens with or without the heat treatment at 100 °C for 1 h were used as assay specimens.

A heat treatment of TR protein-a component made it insoluble with loss of its antigen activity.

We have reported here on items b), c) and d) among the six difficult items to be overcome. SAEIA methods A and B were applied to isolate TR antigens. The SAEIA C method for assay of TR glycan-b was developed and used for the assay of TR component in CTMs. We earlier reported that a SAEIA A type method for assay of a crude drug extract is applicable for assay of contents of crude drug components in CTMs. The mechanism of the SAEIA A assay, which was quite recently speculated, was proved by developing an improved SAEIA C method, which is more sensitive and specific for assay of TR extract than is the SAEIA A method. This provided us with a useful new tool for quality control of CTMs. Studies on items e) and f) are in progress.

REFERENCES