Development and Application of an Enzyme Immunoassay for Karasurin A, an Effective Protein Component of *Trichosanthes kirilowii* MAX. var. *japonicum* KITAM.

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A method was developed for specific estimation of the content of a non-enzymatic protein, karasurin A, in fractions taken during the extraction and purification processes from a natural source. Anti-karokon serum was elicited in rabbits immunized with fragments of karokon, a dried root tuber of *Trichosanthes kirilowii* MAX. var. *japonicum* KITAM. Rabbit antibody specific for karasurin A was identified in anti-karokon serum by the Western blotting method. After separation by SDS-PAGE, protein bands of purified karasurin A and extracted proteins from a medicinal herb which is a karasurin A source were reacted with anti-karokon serum followed by treatment with horseradish peroxidase (HRP)-labeled Fab′ of goat anti-rabbit IgG, and then bound HRP-labeled second antibody on protein bands was developed to brown by reaction with a substrate solution of the used enzyme. A novel selected antibody enzyme immunoassay (SAEIA) for karasurin A was developed using selective binding of anti-karasurin A antibody in anti-karokon serum to solid phase karasurin A and HRP-labeled Fab′ of the second antibody as the tracer. Specific estimation of the content of karasurin A in several fractions taken during the isolation and purification processes of the protein were possible using the SAEIA method.

Keywords selected antibody enzyme immunoassay; karasurin A; anti-karokon serum; Chinese crude drug; *Trichosanthes kirilowii*

Chinese traditional medicines consist of undefined extracts from a mixture of many kinds of solid medicinal herbs.[1] Although kind and composition of each herb before extraction is defined for the preparation of a specific medicine, the composition of none of these medicines has been reported. In addition, no method has yet been applicable to measurement of the content of the extract of a specific herb contained in such a medicine.[1,2]

To overcome this situation, we have been studying a new method named selected antibody enzyme immunoassay (SAEIA) which allows us to measure the content of an extract of a specific herb, either Pinellia tuber or Hoelen, contained in several Chinese traditional medicines.[3]

Trichosantin is an abortifacient protein isolated from the Chinese medicinal herb *Trichosanthes kirilowii*.[4] The protein is a potent inhibitor of protein synthesis[5] and it selectively kills the cells infected by human immunodeficiency virus (HIV).[6] Karasurin A isolated from the root tubers of the medicinal herb *T. kirilowii* MAX. var. *japonicum* KITAM. (TKMJK) possesses amino acid sequence and abortifacient activity similar to trichosantin.[7] The SAEIA method was used to study a protein karasurin A.

A highly titered rabbit anti-karokon serum specific to a solid karokon, a dried root tuber of TKMJK, was prepared. We developed a SAEIA method for karasurin A in which anti-karasurin A antibody contained in anti-karokon serum was selectively used for the assay of karasurin A. Application of the SAEIA method allowed us easy measurement of the karasurin A content in fractions taken during the extraction and purification processes from the fresh root tuber of TKMJK.

Experimental

Materials Microtiter plates (Immunoplate II) were bought from A/S Nunc, Roskilde, Denmark and horseradish peroxidase (HRP)-labeled Fab′ of goat anti-rabbit IgG (code 458; lot 31) from BML Co., Nagoya. Karasurin A and karasurin B used were isolated from fresh root tuber of TKMJK.[7a)] The medicinal herb, karokon, a dried root tuber of TKMJK was obtained from Kotaroh Pharmaceutical Co., Osaka. Fresh root tuber of TKMJK was harvested from the botanical garden of the Faculty of Pharmaceutical Sciences, Nagoya City University. Other chemicals used were of reagent grade.

Media PBS (10 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl); PBST (PBS containing 0.1% Tween 20); coupling buffer (10 mM Tris–HCl buffer, pH 8.5, containing 0.1 M NaCl and 10 mM NaN_3); buffer I (60 mM sodium phosphate buffer, pH 7.4, 10 mM ethylene-diamine tetraacetate (EDTA), 1% (w/v) bovine serum albumin); dilution buffer, (PBS plus 5% normal goat serum); substrate solution A (12 ml of 0.1 M citrate–phosphate buffer, pH 5.2, plus 5 mg of *o*-phenylendiamine was mixed with 11 μl of 35% H_2O_2 and immediately used); substrate solution B (for immunoblotting), (20 ml of 10 mM Tris–HCl buffer, pH 8.5, with 10 mg of 3,3′-diaminobenzidine tetra-hydrochloride were mixed with 6 μl of 35% H_2O_2 and immediately used); stop solution, (2 N H_2SO_4).

Preparation of Immunogen Karokon was ground with a mortar and pestle and then filtered with a bolter (mesh No. 48). A PBS suspension of the powdered karokon (1 mg/ml) 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.

Immunization Two white rabbits were immunized with karokon fragments (1 mg/ml PBS) emulsified with Freund's complete adjuvant. Four booster injections of 0.5 mg of karokon fragments emulsified with 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 karokon (anti-karokon) was stored at $-30\,°C$.[8)]

Preparation of Solid Phase Antigen Wells of a microtiter plate were coated by incubating with 200 μl/well of karasurin A solution (8 μg/ml)

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in a coupling buffer at 25 °C for 1 h. After the solution was removed, each well was incubated with 200 μl/well of buffer I at 25 °C for 3 h to prevent nonspecific absorption.

SAEIA Method One hundred μl/well of anti-karokon diluted 5000-fold in buffer I was incubated at 25 °C for 3 h in karasurin A-coated wells with 100 μl/well of either karasurin A, a sample solution, or buffer I as a control. The plate was then rinsed with PBST, following which 100 μl/well of 1000-fold diluted HRP-labeled Fab′ of goat anti-rabbit IgG in PBST was added and the plate was incubated at 25 °C for 2 h. After 4 washes with PBST, the enzyme conjugate bound to each well was reacted with 100 μl/well of substrate solution A at 25 °C for 10 to 30 min to produce a color change. The enzymatic activity was stopped by the addition of 50 μl/well of 2 N H_2SO_4. The color intensity in each well was measured with an ELISA analyzer (SLT Lab. Instruments, Sartzburg, Austria) set at 492 nm.

Cross-Reactivity (CR) Value The relative amount of an antigen required to reduce the initial binding of antibodies in anti-karokon serum to solid phase karasurin A by one half (B/B_0 50% value) was calculated from the dose response curves assayed by the SAEIA method in which the mass of karasurin A was arbitrarily taken as 100% according to the method of Abraham.[9]

Extraction of Crude Karasurin A 1) After peeling, 1 g of fresh root tuber of TKMJK was sliced and homogenized with 5 ml of water. One ml of the homogenate was incubated with the same volume of either 2% aqueous ammonia (final concentration, 1%), 2% acetic acid or water overnight. After centrifugation at 600 × ***g*** for 5 min, the karasurin A content in each solution was measured by the SAEIA method. 2) A homogenate of 5 g of fresh root tuber of TKMJK received three successive extractions with 20 ml of 1% aqueous ammonia using centrifugation at 600 × ***g*** for 5 min, and the karasurin A content in each extract was assayed by SAEIA.

Western Blotting Western blotting was carried out by the method of Laemmli[10] with a slight modification. Sample proteins were separated by SDS-PAGE, and transferred electrophoretically to a Immobilon™ filter (IPVH000 10, Japan Millipore Ltd., Tokyo). The filter was blocked with Block Ace (Dainippon Pharmaceutical Industries, Suita) at 37 °C for 1 h, and then was incubated with 1000-fold diluted anti-karokon in dilution buffer at 4 °C overnight. The filter was washed 6 times with PBST, and was then incubated with 1000-fold diluted HRP-labeled Fab′ of goat anti-rabbit IgG at room temperature for 30 min. After 6 washes with PBST for 15 min, the HRP-labeled second antibody bound on the filter developed to brown bands with substrate solution B at 25 °C for 5 min.

Results

Antibody Response Both rabbits immunized with a suspension of karokon fragments elicited antibodies specific to karokon fragments. The binding of antibodies contained in diluted anti-karokon serum samples of one of the rabbits, collected at four different periods after the priming, to solid phase karokon fragments was measured using HRP-labeled Fab′ of goat anti-rabbit IgG as the second antibody (Fig. 1). Use of preimmunization serum (0 week) of the rabbit showed little non-specific binding to solid phase karokon. The highest titer serum of the rabbit bled at 10 weeks after the priming was used as anti-karokon serum. The highest titer serum collected from the second rabbit possessed a slightly lower titer than that of the rabbit whose serum was used (data not presented).

Enzyme immunoassay (EIA) Method When karasurin A was used as the solid phase antigen, only the selected antibody (or antibodies) specific to karasurin A contained in serial dilutions of anti-karokon serum bound to the solid phase antigen. It was also found that the binding of the selected antibody was exclusively inhibited by the presence of 10 μg/ml of free karasurin A (data not presented). Based on this finding, a highly sensitive selected antibody EIA for karasurin A with a detection limit of 1 ng/ml was developed (Fig. 2). The working range of karasurin A was arbitrarily set between 1 ng and 1 μg per ml based on the precision data for the SAEIA (Table I). The intraassay (same-run assays) and interassay (independent assays) coefficients of variation at three different levels were 7.0—10.6% and 9.9—14.0%, respectively.

Specificity The dose response curves of several antigens were assayed by SAEIA. CR values were calcu-

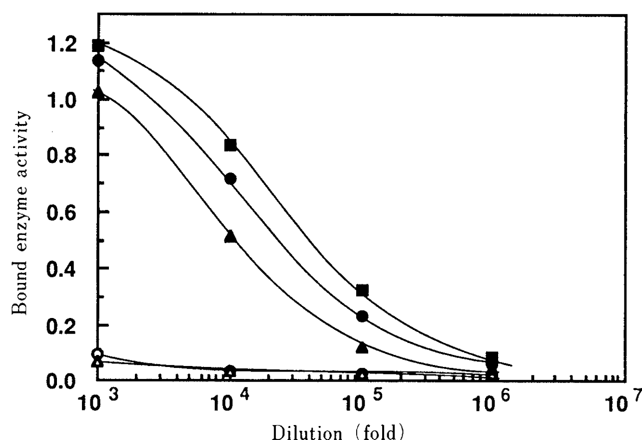

Fig. 1. Binding Activities of Antibodies Contained in a Series of 10-Fold Dilutions of Anti-karokon Serum Samples to Solid-Phase Karokon Fragments

The samples were collected at suitable periods following immunization, and preimmunization serum (0 week) of the rabbit was used as control. ○, 0 week; △, 2 weeks; ▲, 6 weeks; ●, 8 weeks; ■, 10 weeks.

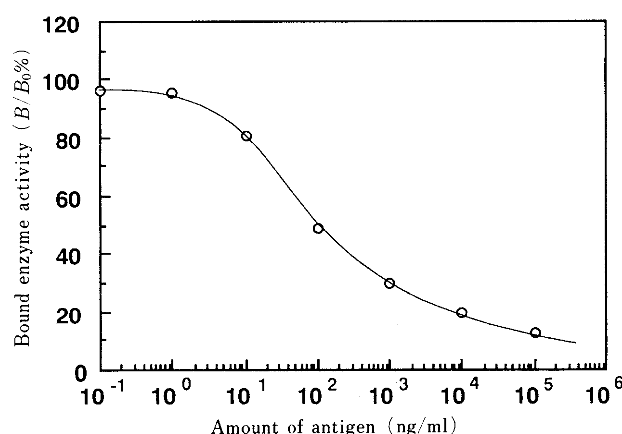

Fig. 2. Typical Dose Response Curve of SAEIA for Karasurin A

The curve shows the bound enzyme activities of various log doses of competing antigen (B) as a percent of bound enzyme without a competing antigen (B_0). Each data point is the average of five replicated experiments.

Table I. Precision of SAEIA for Karasurin A

	Content (ng/ml)	Estimated[a] (ng/ml)	CV[b] (%)
Intraassay	10	10.4 ± 1.3	10.4
	100	102.6 ± 7.2	7.0
	1000	933 ± 95.7	10.6
Interassay	10	10.7 ± 1.5	14.0
	100	108.5 ± 10.7	9.9
	1000	980 ± 112	11.4

a) Values represent the mean ± standard deviation of five experiments. b) Coefficient of variation.

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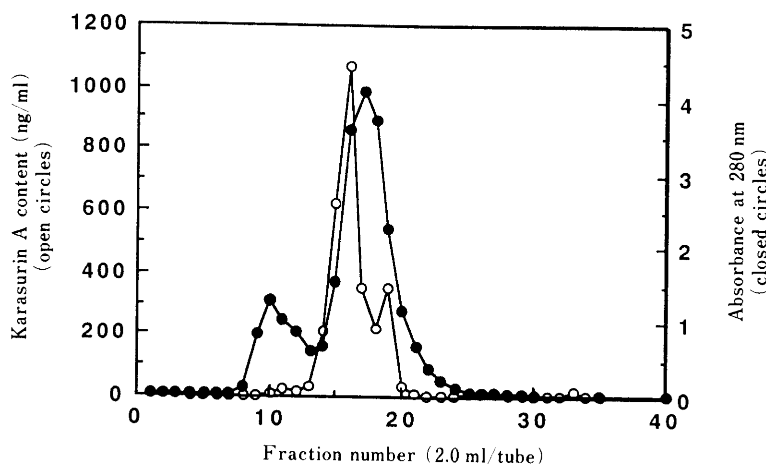


Fig. 3. Gel-Filtration Chromatography of Crude Karasurin A Monitored in Terms of the Absorbance at 280 nm, and SAEIA for Karasurin A

A crude extract (1 ml) containing 10.2 mg of karasurin A was put on a column of Sephacryl S-300 (1.5×30 cm), which was previously equilibrated and eluted with water: absorbance at 280 nm (closed circles; right vertical axis) was measured and karasurin A content (open circles, left vertical axis) was assayed by the SAEIA method using 100 μl/well of 1000-fold diluted solution of each fraction (2 ml/tube). Each karasurin A content data point is the mean of three replicated experiments.

TABLE II. CR Values of Five Kinds of Proteins and Karokon, a Chinese Medicinal Herb, Determined by the SAEIA Method Using Anti-karokon Antiserum and Solid-Phase Karasurin A

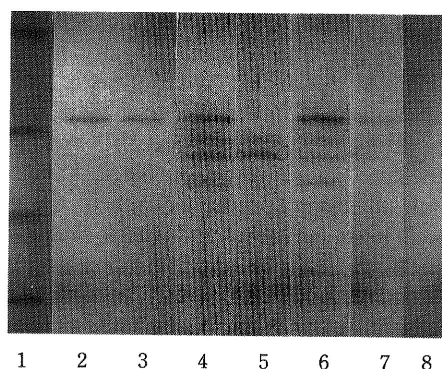
Sample	CR (%)
Karasurin A	100
Karasurin B	57.4
Karokon	0.15
Hemocyanin	<0.001
Human IgG	<0.001
Mouse IgG	<0.001
Horse albumin	<0.001

lated from B/B_0 50% values of their dose response curves.[9] SAEIA was specific to karasurin A. The CR value of karasurin B, the structure of which is quite similar to that of karasurin A[7c] was 57.4% and that of karokon fragments, the immunogen of anti-karokon, was 0.15%. B/B_0 values of 10 mg/ml of four other proteins were larger than 70%, indicating their CR values were less than 0.001% (Table II).

Karasurin A Content Three solutions were compared for efficiency of extraction of karasurin A from a one ml portion of a homogenate of root tuber of TKMJK using the SAEIA method. Although karasurin A is a basic protein and extraction has been performed by water,[7] the quantities of karasurin A in 1% aqueous ammonia, water, and 1% acetic acid were 657, 29 and 0.19 μg/ml, respectively.

A homogenate of 5 g of root tuber of TKMJK was subjected to three successive extractions with 20 ml of 1% aqueous ammonia, and the karasurin A content in each extract was assessed by SAEIA: in extract I the content was 22.4 mg (90.6%), in extract II it was 2.3 mg (9.0%) and in extract III only 0.1 mg (0.4%). Extract I give 202.4 mg of lyophilized residue containing 22.4 mg of karasurin A.

A 100 mg portion of the lyophilized residue of extract I was subjected to gel-filtration. Figure 4 shows the elution profiles of crude karasurin A on gel filtration with a Sephacryl S-300 column. The elution profile determined by the absorbance at 280 nm showed two broad peaks: a main peak at fractions no. 14—20 and a minor peak at fractions no. 7—12. The elution profile assayed by SAEIA for karasurin A, using 1000-fold diluted solutions, possessed a main sharp peak at fraction no. 16 containing



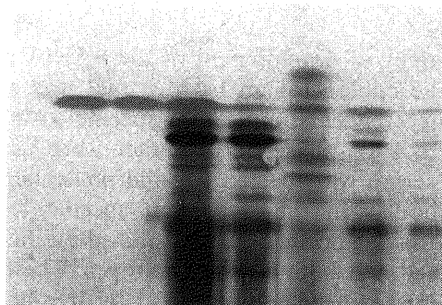


Fig. 4. SDS-PAGE and Western-Blot Analysis of Karasurin A, Karasurin B and Crude Karasurin A Extract and Its Chromatographic Fractions

Lane 1, standard proteins (ribonuclease A, chymotrypsinogen A, ovalbumin, bovine serum albumin); lane 2, karasurin A; lane 3, karasurin B; lane 4, crude karasurin A extract of 1% ammonia solution; lane 5, fraction no. 10; lane 6, fraction no. 16; lane 7, fraction no. 19; lane 8, fraction no. 25. Fig. 4A (upper figure), Coomassie Brilliant Blue R-250 staining. Fig. 4B (lower figure), Western blot analysis; proteins separated by SDS–PAGE were transferred to the IPVH-filter, incubated with anti-karokon serum. The rabbit IgG bound to proteins 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.

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1.1 μg/ml of karasurin A and a minor peak at no. 19. A low content of the protein at fractions no. 7—12 was observed.

Detection of Anti-karasurin A Antibody SDS-PAGE and Western-blot analysis were chosen as assay methods for the detection of anti-karasurin A antibody in anti-karokon serum. Purified karasurin A and karasurin B, and sample proteins contained in crude karasurin A extract in 1% aqueous ammonia (preceding section), and its three gel filtration fractions, no. 10, 16 and 25, obtained by chromatography on a Sephacryl S-300 column (Fig. 3) were separated using SDS-PAGE (15% gel) under non-reducing conditions. Ribonuclease A molecular weight (mw 13700), chymotrypsinogen A (mw 25000), ovalbumin (mw 43000) and bovine serum albumin (mw 67000) were used as reference proteins. Both staining methods, Coomassie Brilliant Blue R-250 staining and the Western blot analysis using an anti-karokon serum and HRP-labeled second antibody, were applied. The detection of protein bands was more sensitive by the Western blot analysis (Figs. 4A and B); purified karasurin A (lane 2) and karasurin B (lane 3) showed a single band indicating that anti-karokon serum contains anti-karasurin antibody. All protein bands developed by Coomassie Brilliant Blue R250 which appeared in lanes 2 to 8 were observed for the corresponding Western-blot assay but not the reference proteins in lane 1, showing that anti-karokon serum contained an antibody specific to each of the proteins in lanes 2 to 8. Karasurin A band was observed in crude karasurin extract (lane 4), and fractions no. 16 (lane 6) and no. 19 (lane 7) but not in no. 25 (lane 8) (Fig. 4A), though the band was positive for lanes 2 to 8 by sensitive Western-blot assay (Fig 4B).

Discussion

The strategy of development of the SAEIA method to measure the content of an herb extract was as follows: two immune reagents, antiserum specific to a medicinal herb and a solid-phase herb extract were used. When anti-herb serum was reacted to solid-phase herb extract, only antibodies in the antiserum specific to epitopes contained on the surface of the solid-phase herb extract were selected and bound to the epitopes. The binding of the antibodies can be competed for by free herb extract with a dose response relationship and the dose of free herb extract in specimens can be determined.

A medicinal herb extract consists of a mixture of many undefined components. Consequently, we were unable to identify what epitopes were used in the SAEIA assay of the extract of either herb Pinellia tuber or Hoelen. This would make it difficult to understand the principle of the SAEIA method for investigators in the field of pharmacognosy who are not familiar with immunoassay methods.

In a series of studies using SAEIA assay, this method was investigated for a purified non-enzymatic protein karasurin A, an effective component of a medicinal herb TKMJK. Highly titered antisera specific to karokon were elicited in two rabbits using karokon fragments as the immunogen, and the highest titer serum of one rabbit was used (Fig. 1). Antibody specific to karasurin A was identified in anti-karokon serum by the Western blotting method: after separation by SDS-PAGE, purified or crude karasurin A contained in extracted solutions and fractions of gel filtration chromatography were selectively bound to anti-karasurin A antibody contained in rabbit anti-karokon serum. The rabbit antibody bound on protein bands was detected by the reaction with HRP-labeled second antibody followed by color reaction with substrate solution of the HRP-label (Fig. 4). It is obvious that anti-karokon serum contains a number of antibodies specific to various protein components. In addition, antibodies specific to other components of the herb such as glucans should be present, though these could not be detected by the Western blotting method.

The SAEIA method is based on the selection of specific antibody (antibodies) in an antiserum by a solid-phase antigen. When a solid-phase karasurin A was incubated with anti-karokon serum, only the antibody specific to karasurin A bound to this antigen, though anti-karokon contains a number of antibodies of different specificities. That the binding was limited to the antibody specific to karasurin A was shown by evidence that the binding was competed for by presence of karasurin A (Fig. 2). Sensitive and specific measurement of the contents of karasurin A in several solutions extracted from its natural source and in fractions of its gel-filtration chromatography were made using the SAEIA method.

An immunoassay of a protein has been performed using an antiserum specific to the protein. In the present study, the immunoassay of karasurin A was achieved using an antiserum specific to an herb, a raw material of the protein, instead of an anti-karasurin A serum. The SAEIA method was shown to also be applicable to a single component of a medicinal herb, though anti-herb serum contains a number of antibodies of different specificities. In addition, it was shown that a sensitive and specific method for the quantitative analysis of a non-enzymatic protein can be developed by the SAEIA method when the protein has been isolated from an herb, if an antiserum specific to the herb has been prepared preceding the isolation, because all other immunological reagents and tools required for developing the SAEIA are commercially available. The SAEIA method will be a useful new tool for various studies of pharmacognosy.

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