Enzyme Immunoassay for a Characteristic Protein in the Animal Crude Drug Lumbricus

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A new method was developed to measure the content of a Lumbricus component in a traditional Chinese medicine (TCM). An antisem specific to Lumbricus was elicited in a rabbit following immunization with a suspension of Lumbricus fragments. A characteristic antigen protein, 70 kDa, was found in Lumbricus and was purified almost to singleness using a column chromatography series of gel filtration and DEAE-Sepharose. A selected antibody enzyme immunoassay (SAEIA) was developed using the antisem and the purified 70 kDa protein as a solid-phase antigen. The SAEIA was specific to Lumbricus species, and showed no cross-reaction with any crude drugs other than Lumbricus. This SAEIA detected 70 kDa protein in the amount of 10 ng/ml with excellent reproducibility (coefficient of variation = 3.0%) and an EC50 of 0.24 μg/ml. Using this assay, Lumbricus levels were easily determined in a Lumbricus-based TCM Kazecoll, but not in the control Kazecoll (Kakkonto) prepared without Lumbricus. The SAEIA for 70 kDa protein was simple, accurate, reproducible and may provide a general analytical method for the quality control of Lumbricus-based TCMS.

Key words Lumbricus; 70 kDa protein; selected antibody enzyme immunoassay; traditional Chinese medicine

Traditional Chinese medicines (TCMs) are undefined extracts from a mixture of many crude drugs from plants, animals and minerals, the prescription of which is defined for each TCM. Included among these crude drugs are many whose effective compounds have not yet been identified. This has made it difficult to control the quality of TCMs. In addition to assaying known effective compounds, namely Glycyrrhizin1,2 and Ginsenosides3,4 in Glycyrrhiza Radix and Panax Ginseng, respectively, an alternative method for finding and determining the characteristic compounds in crude drugs may be an effective way to achieve quality control.

We recently developed selected antibody enzyme immunoassays (SAEIA) for use in the quality control of TCMs containing plant crude drugs, Pinellia Tubers,5 Hoelen,6 Glycyrrhiza Radix,7-10 Trichosanthes Root11 and Panax Ginseng.12 In the development of SAEIA: 1) an antisem specific to each crude drug was produced, 2) a characteristic antigen component was separated from the crude drugs following immunoreactivity with the antisem, and 3) the assay method using the antisem and the separated antigen coated on solid-phase microtiter wells was shown to detect the component specifically and quantitatively. Success in these studies for plant crude drugs prompted us to develop SAEIA for an animal crude drug, Lumbricus, which has been used as a medicine to treat fever, bronchodilatation and hypotension.

We here present the first report of SAEIA for a Lumbricus component of a 70 kDa protein using anti-Lumbricus serum and the purified 70 kDa protein as a solid-phase. Optimal assay conditions for the SAEIA were established, and the method was successfully used to measure the levels of 70 kDa protein contained in the Lumbricus-based TCM Kazecoll.

MATERIALS AND METHODS

Materials A microtiter plate (Immunoplate II) was purchased from A/S Nunc, Roskilde, Denmark and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG/Fab' was purchased from MBL Co., Nagoya, Japan. Phenol reagent was obtained from Wako Pure Chemical Ind., Ltd., Osaka. Lumbricus kwangtungensis was purchased from the Dalian market of crude drugs, China. Lumbricus nativus and Kakkonto were from Kotoro Co., Ltd., Takastuki. Kazecoll was obtained from a commercial source from Taiso Pharmaceutical Co., Yokosuka. Other chemicals used were of reagent grade. The measurement of glycine was made by the phenol–sulfuric acid method12 using glucose as a standard, and that of protein by the Lowry method13 using bovine serum albumin as a standard as previously described.

Immunization Lumbricus was ground with a mortar and pestle, then filtered with a bolter (mesh No. 48). The Lumbricus powder (1 mg/ml) was suspended in 50 mM phosphate-buffered saline (PBS), pH 7.0, and homogenized by a sonic cell disruptor at 60 W for 1 min in an ice-water bath, then the crude drug fragment was used as immunogens. A white rabbit was primed with crude drug fragment (1 mg) suspended in 1 ml PBS plus an equal volume of Freund's complete adjuvant. Four booster injections of 0.5 mg of the crude drug fragment mixed with Freund's incomplete adjuvant given at biweekly intervals. The rabbit was bled from the ear vein at suitable periods after the priming, and the separated serum was stored at −30 °C.

SAEIA Method The wells in microtiter plates were coated with 100 μl of 70 kDa protein (which is described below) (1.0 μg/ml in 10 mM tris–HCl buffer, pH 8.5, containing 0.1 M NaCl and 10 mM NaN3) 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 A), and was blocked with 200 μl of the same buffer at 25 °C overnight for non-specific binding. As a control, 100 μl of either sample specimen or buffer A was added to the wells, in addition to 100 μl of anti-Lumbricus serum diluted 10000-fold in buffer A, and they were incubated at 25 °C for 3 h. After being rinsed four times with 10 mM phosphate buffer, pH 7.0, containing 0.1 M NaCl and 0.1% Tween 20 (PBST), the wells were incubated with 100 μl of HRP-labeled goat anti-rabbit

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IgG/Fab' diluted 2000 times with PBST at 25°C for 2 h. Following the final rinse, the amount of conjugated enzyme bound to each well was then measured using 100 μl of o-phenylenediamine (0.5 mg/ml) as a substrate; it contained 0.012% H₂O₂ in 0.1 M citrate-phosphate buffer, pH 5.2. The reaction was carried out for 10 min and then stopped by the addition of 100 μl of 2 N H₂SO₄. The absorbance at 492 nm was read with an ELISA analyzer (SLT-Labinstruments, Salzburg, Austria).

**Western Blot** Western blotting was carried out according to the reported method with a slight modification. Antigen components were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) run on 10% polyacrylamide gel, and then electroblotted onto an Immobilon PVDF membrane (Japan Millipore, Ltd., Tokyo) which was then incubated with 1000-fold diluted antiserum in buffer A at 4°C overnight. The filter received four washes with PBST, and was then incubated with 1000-fold diluted HRP-labeled goat anti-rabbit IgG/Fab' at 25°C for 1 h. After being rinsed with PBST, the HRP-labeled second antibody bound on the filter was then developed to brown bands with diaminobenzidine according to the method of Graham and Karnovsky.

**RESULTS**

**Antibody Response** An antibody specific to Lumbricular was produced in a rabbit which had been immunized with a suspension of Lumbricular fragments. The antibody titers in the serum samples were determined by the enzyme-linked immunosorbent assay (ELISA) using the antiserum, microtiter wells coated with the Lumbricular fragment suspension, and HRP-labelled goat anti-mouse IgG/Fab' as a second antibody. The titers, defined as the antibody binding to the solid-phase antigen, were found to peak 10 weeks after the first priming (data not shown). Pre-immunization serum showed no binding to the solid-phase antigens.

**Separation of Antigens** The Lumbricular powder (5 g) was extracted with 100 ml of boiling water for 2 h. After filtration with a cotton cloth, the filtrate was lyophilized to yield 743 mg of amorphous Lumbricular extract. This was then dialyzed against deionized water overnight with 5 changes of the outer solution (each 500 ml). The dialysate was applied to a column (25x30 cm) of Sephadex G-50 equilibrated with 20 mM phosphate buffer, pH 7.5, and it was found that almost all immunoreactive compounds appeared immediately after a void volume from the column, separating non-immunoreactive small molecular compounds (data not shown). The immunoreactive compounds (167 mg) were then chromatographed using a Sepharose 4B column (2x40 cm) as above, and the fractioned eluates were assayed for immunoreactivity and glycan amounts, as shown in Fig. 1.

Fraction Nos. 12 to 17, 18 to 32, and 33 to 42 were combined, dialyzed against deionized water and lyophilized to give 6.6 mg (AE1), 25.1 mg (AE2), and 39.6 mg (AE3), respectively. Among these, fraction AE3, with the highest immunoreactivity, was again chromatographed on a DEAE-Sepharose CL-6B column (1.5x5 cm) equilibrated with 20 mM phosphate buffer, pH 8.0 (Fig. 2), resulting in separation into two fractions, one passed through the column (AE3a), and the other adsorbed, which was eluted with 0.3 M NaCl in 20 mM phosphate buffer, pH 8.0 (AE3b). Fractions AE1, AE2, AE3a and AE3b were finally gel filtered on a Sepharose 6B column (2x63 cm) with an eluate of 20 mM phosphate buffer, pH 7.5. The purification process is summarized in Table 1. The AE3b, which was obtained at 0.85% of the final recovery and consists of 68.5% protein and 25.3% glycan components, showed about 30-times higher immunoreactivity compared to Lumbricular extract in terms of EC₅₀ value. AE3a had moderate immunoreactivity (EC₅₀=0.7 μg/ml). On the other hand, the lowest immunoreactivity occurred with AE1 and AE2, both of which consisted almost entirely of glycan components (Table 1).

Western blot analysis was performed using anti-Lumbricular serum, revealing that AE3b showed a single band at 70 kDa, although many protein bands were found in the Lumbricular extract (Fig. 3), indicating that a number of antibodies were raised against different protein components contained in Lumbricular. Also, by SDS-PAGE, AE3b showed a nearly single band. Thus, in the development of SAEIA for a 70 kDa protein, AE3b was used as a solid-phase antigen and a standard of 70 kDa protein.

![Fig. 1. Elution Profile of Lumbricular Antigens from a Sepharose 4B Column](image-url)

The dialysate (167 mg) was applied to a column (2x40 cm) of Sepharose 4B. Aliquots of each fraction were assayed for immunoreactivity by the SAEIA method using the antiserum and a solid-phase coated with a suspension of Lumbricular fragments (10 μg/ml), and for glycan levels by the phenol-sulphate method. Protein levels were monitored at 280 nm.
Fig. 2. Elution Profile of Lumbricus Antigens from DEAE-Sepharose CL-6B

AE3 (39.6 mg) was dialyzed against 20 mM phosphate buffer, pH 8.0, and the dialysate (3.0 ml) was applied to a column (0.5×2 cm) of DEAE-Sepharose CL-6B equilibrated with the same buffer. Fractions (3 ml each) were collected and the immunoreactivity (●), glycans (○), and protein (●) amounts were measured as in the Fig. 1. The arrow indicates the point at which the elution buffer was changed to 0.3 M NaCl in 20 mM phosphate buffer, pH 8.0.

Table 1. Purification of Lumbricus Antigen from Lumbricus Extract

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Amount (mg)</th>
<th>Recovery (%)</th>
<th>EC₅₀ (µg/ml)</th>
<th>Purification ratio</th>
<th>Glycan (%)</th>
<th>Protein (%)</th>
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<tbody>
<tr>
<td>Lumbricus extract</td>
<td>743</td>
<td>100</td>
<td>6.0</td>
<td>1.0</td>
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<tr>
<td>Sephadex G-50</td>
<td>167</td>
<td>22.5</td>
<td>1.8</td>
<td>3.3</td>
<td></td>
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</tr>
<tr>
<td>Sepharose 4B</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AE1</td>
<td>6.6</td>
<td>0.89</td>
<td>2.5</td>
<td>2.4</td>
<td>87.5</td>
<td>7.5</td>
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<tr>
<td>AE2</td>
<td>25.1</td>
<td>3.4</td>
<td>1.0</td>
<td>6.0</td>
<td>94.1</td>
<td>5.0</td>
</tr>
<tr>
<td>AE3</td>
<td>39.6</td>
<td>5.3</td>
<td>0.6</td>
<td>10.0</td>
<td>31.2</td>
<td>58.7</td>
</tr>
<tr>
<td>DEAE Sepharose CL-6B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AE3a</td>
<td>25.8</td>
<td>3.5</td>
<td>0.7</td>
<td>8.6</td>
<td>36.8</td>
<td>51.2</td>
</tr>
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<td>AE3b</td>
<td>6.3</td>
<td>0.85</td>
<td>0.2</td>
<td>30.0</td>
<td>25.3</td>
<td>68.5</td>
</tr>
</tbody>
</table>

Samples on each process were assayed by the SAIEA using anti-Lumbricus serum (1:10000) and a solid-phase coated with a suspension of Lumbricus fragments (10 µg/ml). a) The concentration required for 50% inhibition of antibody binding. b) Calculated as a ratio of EC₅₀ value to 6.0 of Lumbricus extract.

Fig. 3. SDS-PAGE and Western-Blot Analyses of Lumbricus Extract and AE3a with Anti-Lumbricus Serum

Lane 1, Lumbricus extract (0.1 mg/well); lane 2, AE3a (2 μg/well). SDS-PAGE: separated proteins were directly stained with Coomassie Brilliant blue R-250 (CBB). Western blot analysis: proteins separated by SDS-PAGE were transferred to the Immobilon PVDF membrane and incubated with anti-Lumbricus serum. The bound rabbit IgG was allowed to react with HRP-labeled goat anti-rabbit IgG/Fab′, followed by the reaction with 3,3′-diaminobenzidine to develop brown protein bands. Reference proteins, β-lactalbumin (14400), soybean trypsin inhibitor (20100), carbonyl reductase (30000), ovalbumin (43000), bovine serum albumin (67000) and phosphorylase b (94000) were used, and the molecular weights are shown by the arrows.

The SAIEA Method for 70 kDa Protein

The assay principle is based on competition between an analyte and 70 kDa protein for an antibody to 70 kDa protein in the antiserum. Optimal quantities and optimal incubation time for the first and second immunoreaction were established (see the SAIEA method in the Materials and Methods section). A standard calibration curve of 70 kDa protein quantification is presented in Fig. 4. For the 70 kDa protein assay, the EC₅₀ obtained was 0.24±0.01 µg/ml (n=5) of the protein when calculated from nonlinear regression analysis. The lower limit of detection by the assay was at 10 ng of 70 kDa protein per ml, and the working range was shown to be from 20 ng to 20 µg per ml. Quality control data of intraassays and interassays were determined, revealing this newly developed SAIEA for 70 kDa protein to be a reproducible technique. The coefficients of variation (C.V.) for intra- and interassays between 70 kDa protein concentrations of 20 ng to 20 µg per ml at five different levels each were 1.7 to 6.4% and 6.5 to 15.3%, respectively.

In recovery experiments, Kazecoll prepared without Lumbricus was used as a model control TCM, namely Kakkonto (Table 2). Samples containing 100 µg of the control Kazecoll were assessed with added 70 kDa protein (25, 50, 100 ng), and the recovery was calculated from the standard curve of the SAIEA. As shown in Table 2, analytical recovery ranged from 96.4 to 118.4%.

Antibody specificity was also examined by the SAIEA system using ten frequently prescribed animal crude drugs (Cow Bezor, Musk, Scorpio, Scolopendra, Hirudo, Bungarus Minimus, Squama Manus, Hairy Antler, Colla Corii Asini, and Red Ant) other than Lumbricus. It was found that none
of the crude drugs had any effect on antibody binding to the solid-phase (data not shown), indicating that the SAEIA was specific to the Lumbricus species.

Applications of SAEIA  The SAEIA for 70 kDa protein was used to determine 70 kDa protein levels in seven Lumbricus crude drugs, botanically identified as two of Lumbricus kwangtungensis and five of Lumbricus nativus (Table 3). Various amounts of the Lumbricus samples (1 to 100 μg/ml) gave SAEIA titration curves similar in shape to that of standard 70 kDa protein (data not shown). As shown in Table 3, 70-kD protein levels ranged from 37.5 to 60.1 μg per mg specimens with a mean of 47.0 μg. The range of C.V. was from 1.0 to 6.3% (n = 5).

The 70 kDa protein levels in the Lumbricus extract and a commercially available Lumbricus-based TCM, Kazecoll, were measured by the SAEIA. On the basis of the SAEIA titration curves shown in Fig. 4, the concentration of 70 kDa protein was determined to be 71 μg/mg of Lumbricus extract and 2.1 μg/mg of Kazecoll, with C.V. values ranging from 3.6 to 5.4% (n = 5). Almost no bound enzyme activity was observed in the control Kazecoll (Kakkonto) samples, even when a high concentration (10 mg/well) was used (Fig. 4).

The heat stability of 70 kDa protein was also examined by the SAEIA, since Lumbricus is extracted for use in TCMs. Thus, aqueous samples of 70 kDa protein were heated at 100°C, and 70 kDa protein immunoreactivity was measured occasionally by the SAEIA. A level of antigenicity of more than 90% remained, even when the 70 kDa protein was heated for 4 h (data not shown).

### Table 2. Analytical Recovery of 70 kDa Protein

<table>
<thead>
<tr>
<th>Added (ng)</th>
<th>Mean ± S.D. (%)</th>
<th>C.V. (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.0</td>
<td>29.6 ± 1.0</td>
<td>3.4</td>
<td>118.4</td>
</tr>
<tr>
<td>50.0</td>
<td>54.2 ± 1.6</td>
<td>2.9</td>
<td>108.4</td>
</tr>
<tr>
<td>100.0</td>
<td>96.4 ± 1.5</td>
<td>1.6</td>
<td>96.4</td>
</tr>
</tbody>
</table>

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

### Table 3. The Contents of 70 kDa Protein in Ten Lumbricus Determined by SAEIA Method

<table>
<thead>
<tr>
<th>Lumbricus sample</th>
<th>Place</th>
<th>Mean ± S.D. (μg)</th>
<th>C.V. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lumbricus kwangtungensis</td>
<td>C-1</td>
<td>60.1 ± 3.8</td>
<td>6.3</td>
</tr>
<tr>
<td>Lumbricus nativus</td>
<td>C-2</td>
<td>41.7 ± 1.2</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>C-1</td>
<td>54.5 ± 2.6</td>
<td>4.8</td>
</tr>
<tr>
<td></td>
<td>C-2</td>
<td>37.5 ± 0.6</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>C-3</td>
<td>46.2 ± 0.9</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>J-4</td>
<td>48.4 ± 0.5</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td>J-5</td>
<td>40.3 ± 1.0</td>
<td>2.5</td>
</tr>
</tbody>
</table>

An aqueous solution of Lumbricus powder (10 mg/ml) was heated in a boiling water bath for 2 h, and aliquots of the supernatant were assayed by SAEIA. a) Name of place where Lumbricus was produced. b) Expressed as μg of 70 kDa protein per mg of Lumbricus. c) Standard deviation of five replicated experiments. d) Coefficient of variation.

### DISCUSSION

Lumbricus contained in TCMs is used as an antispasmodic, diuretic, antidote and antipyretic. The present study was undertaken to develop an immunoassay method to find and determine specific antigen components in Lumbricus.

An anti-Lumbricus serum was obtained following the immunization of rabbit with a suspension of sonicated Lumbricus fragments 10 weeks after the first priming. Using the antisera, the Lumbricus extract was studied for a protein that could be used as a marker of Lumbricus. After the Lumbricus extract was chromatographed with a series of column gel filtration and DEAE-Sephrose, the four Lumbricus components, AE1, AE2, AE3a and AE3b, were separated (Table 1). Among these, AE3b, with the highest immunoreactivity, was found to show a single band at 70 kDa, as judged by Western blot analysis using the anti-Lumbricus serum (Fig. 3). Thus, AE3b was used as a solid-phase antigen, together with the anti-Lumbricus serum and HRP-labeled anti-rabbit IgG/Fab’ as primary and secondary antibodies, respectively, to develop a SAEIA for 70 kDa protein which proved sensitive (protein concentrations smaller than 10 ng/ml can be measured) and...
reproducible (3.0% variation intra assay as an average) (Fig.
4). This SAEIA was quite specific to the Lumbricus species
of both kwangtungensis and nativus, in which an almost fixed
amount of the protein (37.5—60.1 μg/mg) was detected,
though the seven Lumbricus used here were produced in dif-
ferent places (Table 3). However, this SAEIA showed no
cross-reactivity with ten animal crude drugs other than Lum-
bricus. This suggests that 70 kDa protein is a specific protein
component present only in Lumbricus, and not in the other
crude drugs tested. This assay yielded nearly quantitative
amounts of 70 kDa protein added to the SAEIA system
(Table 2). SAEIA also showed that the site of 70 kDa protein
recognized by the antiserum was stable when the 70 kDa pro-
tein was heated, indicating that the protein structure remains
stable under the extraction condition used for the preparation
of Lumbricus-based TCMs.

The utility of the SAEIA was demonstrated by measuring
levels of 70 kDa protein in Lumbricus extract and a TCM,
Kazecoll, which contains Lumbricus as a component of the
prescriptions.

In the present study we found a specific 70 kDa protein oc-
curring only in Lumbricus, and developed a SAEIA for
70 kDa protein using the anti-Lumbricus serum and purified
70 kDa protein. This assay should be useful for the quality
control of TCMs, measuring the protein as a marker of Lum-
bricus contained therein.

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