Development and Application of a New Method for Assay of the Content of Hoelen Extract in Chinese Traditional Medicines

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A newly selected antibody enzyme immunoassay (SAEIA) for aqueous hoelen extract was developed. Hoelen fragments were immunized and an antiserum specific for hoelen was elicited in rabbits. An aqueous extract of hoelen was converted to a solid-phase antigen using microtiter plates. The efficiency for immobilization of hoelen extract to three types of microtiter plates was compared and carbo-plate was chosen as the solid-phase antigen support. A new assay method for a hoelen extract, with a working range between 3 ng and 30 μg, was developed applying the novel assay principle of SAEIA. The selective binding of the antibodies specific for hoelen extract, contained in the anti-hoelen serum, to the solid-phase antigen, was detected using β-D-galactosidase labeled anti-rabbit immunoglobulin G as a tracer. Specificity of the SAEIA to the hoelen extract was also demonstrated. The contents of hoelen extract in three Chinese traditional medicines were successfully analyzed applying the SAEIA method.

Keywords hoelen; selected antibody enzyme immunoassay; SAEIA; Chinese traditional medicine; extract content

Introduction

Chinese traditional medicines consist of the extracts of a mixture of several Chinese crude drugs. It has been difficult to determine the content of the extract of any specific Chinese crude drug contained in a Chinese traditional medicine. Hoelen, a sclerotium of a basidiomycete fungus, *Poria cocos* (SCHW.) WOLF (*syn. Pachyma Hoelen* RUMPH.), grown on decayed pine tree roots under the ground, has been one of the principal medicaments in Chinese traditional medicines.[1-3] It was found that 90% of the weight of hoelen consists of a glucan named pachman.[4] Since the finding of Chihara *et al.* that chymaran, a modified product of pachman, possesses immune stimulant activity,[5] that activity was observed with hoelen itself[6-8] and its related glucans.[9,10] Although a number of Chinese traditional medicines contain hoelen, and several components of hoelen, such as the glucans described above and some terpenoids[11,12] reported, little is known about the content of hoelen extract and its exact role in Chinese traditional medicine.

An assay method which is capable of measuring the content of hoelen extract in Chinese traditional medicine would be of value for phamacological studies of many Chinese traditional medicines containing hoelen extract. A newly selected antibody enzyme immunoassay (SAEIA) for hoelen extract with high specificity and accuracy was developed. In the present paper, we also report on the application of SAEIA to measure the contents of hoelen extract in three kinds of Chinese traditional medicines.

Materials and Methods

Materials A microtiter plate with 96 wells (Immunoplate II) was bought from A/S Nunc, Denmark. Amino- and carbo-type microtiter plates were purchased from Sumitomo Bakelite Co., Ltd., Tokyo, Japan. The compositions of Chinese crude drugs for the preparation of three kinds of Chinese traditional medicines, Ninjin-yoei-to, Keishi-bukuryo-gan and Gorei-san, are summarized below (Table IV). The three medicines and their analogous pseudo-Chinese traditional medicines from which a component of either hoelen and/or chuling were omitted from the constituting Chinese crude drugs of the three medicines as well as five extracts of Chinese crude drugs, hoelen, chuling, ginseng, pinellia tuber, and glycyrrhiza, were prepared according to the manuals of Kotaroh Pharmaceutical Co., Ltd., Osaka, Japan. β-D-galactosidase (GAL)-labeled goat anti-rabbit immunoglobulin G (IgG) antibody was prepared according to the previous method.[13] Other chemicals were of reagent grade.

Media PBS (0.01 M phosphate buffer, pH 7.0, containing 0.1 M NaCl); coupling buffer (10 mM Tris–HCl buffer, pH 8.5, containing 10 mM NaCl and 10 mM NaN_3); buffer A (0.02 M sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% (w/v) bovine serum albumin (BSA) and 0.1% (NaN_3); buffer B (0.06 M sodium phosphate buffer, pH 7.4, containing 1 mM ethylenediamine tetraacetate) was used as the dilution medium except as otherwise stated; washing buffer (buffer A containing 0.05% Tween 20).

Equipment The autoanalyzer used for SAEIA was Easy reader (EAR 400AT), SLT-Labinstruments, Austria.

Preparation of Immunogen Hoelen was ground with a mortar and pestle and then filtrated with a bolter (mesh No. 48, 297 μm). The suspension of the powdered hoelen was homogenized by a cell disrupter (Branson Sonifier, model W 185E, Branson Sonic Power Co., U.S.A.) at 60 W for 3 min in an ice–water bath. A saline suspension of the disrupted hoelen was used as the immunogen.

Immunization One ml of a saline suspension of 1 mg of hoelen powder was emulsified with an equal volume of complete Freund's adjuvant. Two white female rabbits were given multiple subcutaneous and intramuscular injections along both sides of their backs. A booster injection was given at monthly intervals with half the dose of the first except that Freund's adjuvant of the incomplete type, instead of the complete type, was used. The rabbits were bled from the ear vein at biweekly intervals and then two weeks after the booster. Sera (anti-hoelen) was stored at −30 °C until use.

Preparation of the Extract of Hoelen The powdered hoelen was extracted with four times its volume of water at 100 °C, and then the mixture was filtrated with cotton cloth, and the filtrate was lyophilized.

Preparation of the Solid-Phase Antigen by Physical Absorption The wells in the microtiter plate were coated by loading 200 μl of either a solution of a hoelen extract (50 μg/ml) or a sonic disintegrated suspension of hoelen powder (50 μg/ml) in coupling buffer and being left for 1 h at 25 °C. After being washed with buffer B, each well was incubated with 300 μl of buffer B for 1 h at 25 °C in order to avoid non-specific adsorption. The microtiter plate was stored at 4 °C until use.

Preparation of Solid-Phase Antigen Using Chemical Absorption Type Microtiter Plate The wells of amino-plates were activated with 200 μl of 2% glutaraldehyde for 2 h at 25 °C. The wells of carbo-plates were activated with 200 μl of 0.1% 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (water soluble carbodiimide) for 2 h at 50 °C. After being washed with distilled water, both amino- and carbo-type microtiter plates were incubated with 200 μl of hoelen extract in PBS for 3 h at 25 °C. Each well was washed with PBS and then incubated with 300 μl of 1% ovalbumin for 1 h at 25 °C in order to block non-specific adsorption. The microtiter plates were stored at 4 °C until use.

Immunoassay Procedure by SAEIA Method The wells coated with hoelen extract were incubated with 100 μl of a solution of hoelen extract and 100 μl of a 10000 times diluted solution of anti-hoelen antiserum at 25 °C overnight. After three washes with 200 μl of the washing buffer, each well was incubated with 200 μl of GAL-labeled goat anti-rabbit IgG antibody for 3 h at 25 °C. The wells were washed three times with 200 μl

of buffer A, and the GAL activity bound to the well was assayed.

Measurement of GAL Activity The amount of the bound enzyme conjugate to each well was measured by the modification of a published method.[14] Each well was incubated at 37 °C for a suitable period of time with 0.2 ml of 0.1% *o*-nitrophenyl-*β*-D-galactoside in buffer A as the substrate. The reaction was stopped by adding 25 *μ*l of 1 M glycine–NaOH buffer, pH 10.6, and the resulting color intensity was measured at 414 nm using an enzyme-linked immunosorbent assay reader (EAR 400AT).

Results

Antibody Response An antiserum against hoelen (anti-hoelen) was produced in each of two rabbits immunized with a suspension of hoelen fragments. The serum from one of the two rabbits collected eight weeks after the immunization was found to possess a sufficient titer. The specific binding of the serum was detected by reacting variously diluted anti-hoelen antiserum with a solid-phase antigen, the immobilized hoelen fragments on the surface of the wells of a microtiter plate, using the GAL-labeled antibody to anti-rabbit IgG as the tracer. Typical binding curves of anti-hoelen serum from bleeding of one of the two rabbits are shown in Fig. 1. The antibody titer increased gradually and reached a maximum two weeks after the booster. Judging from the binding value, a 8000 times diluted solution of the highest titered serum was chosen for SAEIA. Use of preimmunization serum showed little non-specific binding to the solid-phase hoelen.

The Optimal Condition of Solid-Phase Antigen The optimal concentration of a hoelen extract in PBS for preparation of solid-phase antigens was first studied. Six different concentrations of hoelen extract were used for preparation of solid-phase antigens. Every binding of the antibody in anti-hoelen antiserum was competed by the presence of a known amount of hoelen extract ranging between 1 ng to 100 *μ*g and the results are summarized in Table I. The bound enzyme activity with competition of a known concentration of hoelen extract (B) was expressed as percent ratio of that without a competition of the extract (B_0). The lowest value of 9.5 was obtained at the concentration of 10 *μ*g/well for preparation of the solid-phase antigen and at the competition with 100 *μ*g of the extract. Thus, the concentration of 10 *μ*g/well was chosen for preparing the solid-phase antigen.

Similar experiments were performed to determine the optimal sonication time of the extract of hoelen used for the preparation of the solid-phase antigen. The result is shown in Table II. A time of 3 min was chosen.

Table III summarized the experiment to determine the optimal strength of sonic power for preparation of the solid-phase antigen. The strength of 60 W was chosen to obtain the steeper standard curve and the lower value of the negative control.

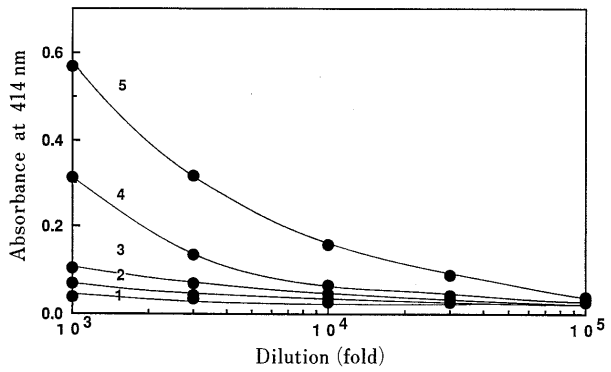

Fig. 1. Quantitative Estimation of Titer of Anti-hoelen Antiserum Samples

Sample 1 is bled before immunization. Samples 2, 3 and 4 are collected 2, 4 and 8 weeks after priming. Sample 5 is collected two weeks after the booster.

TABLE I. Effect of the Concentration of Extract of Hoelen Used for Immobilization as the Solid-Phase Antigen

Hoelen (*μ*g/well)	Bound enzyme activity (B/B_0%) Concentration of solid-phase antigen (*μ*g/well) 1	3	5	10	15	20
10^{-3}	73.3	95.6	90.3	95.6	95.6	98.4
10^{-2}	66.7	86.7	81.7	83.5	96.7	98.4
10^{-1}	60.0	80.0	75.6	76.4	96.7	96.0
1	43.3	66.7	59.9	65.4	79.5	78.2
10	40.0	42.2	35.2	32.0	37.0	40.3
10^2	26.7	20.0	16.9	9.5	12.7	18.5

TABLE II. The Optimal Time of Sonic Disintegration for Preparing Solid-Phase Antigen by Using Cell Disrupter

Hoelen (*μ*g/well)	Bound enzyme activity (B/B_0%) Time (min) 0	1	3	5	10
10^{-3}	93.5	97.1	92.6	99.3	101.8
10^{-2}	93.7	93.0	87.9	97.2	106.3
10^{-1}	86.9	88.7	84.6	95.1	96.4
1	63.9	67.6	66.4	80.4	82.1
10	32.8	33.9	36.9	42.0	47.3
10^2	18.0	10.6	9.4	11.4	14.3

TABLE III. Efficiency of the Strength of Sonic Power by Using Cell Disrupter

Hoelen (*μ*g/well)	Bound enzyme activity (B/B_0%) Sonic strength (W) 20	30	40	50	60
10^{-3}	93.3	98.4	96.0	100	96.2
10^{-2}	86.7	91.1	95.5	99.4	92.2
10^{-1}	86.1	86.4	93.6	94.9	91.6
1	64.2	74.3	78.3	70.0	63.8
10	39.4	42.9	41.4	41.4	37.3
10^2	13.3	12.6	13.4	14.9	11.0

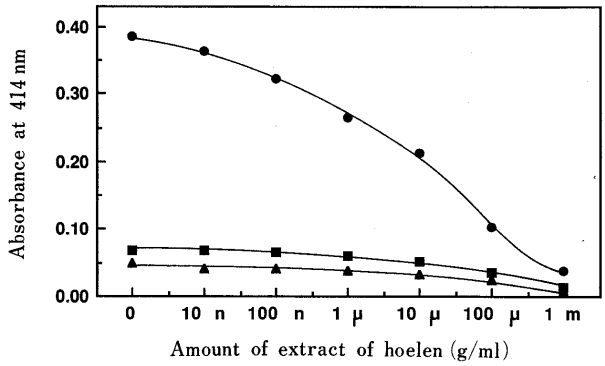

Fig. 2. Typical Dose–Response Curves of SAEIA for Hoelen Extract Using Carbo-Plate (●), Nunc Immunoplate II (▲) and Amino-Plate (■) as Solid-phase Supports of Hoelen Extract

Bound enzyme activity (A_{414}) plotted against log dose of the hoelen extract.

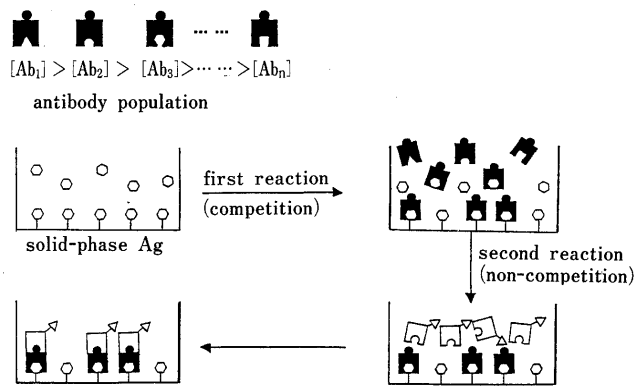

Fig. 3. The Illustration of the Principle of SAEIA Method

Free and solid-phase antigens were competed to the selected antibody (Ab_3). The bound antibody was reacted with GAL-labeled second antibody. Amount of the enzyme label bound to the solid-phase antigens was then measured.

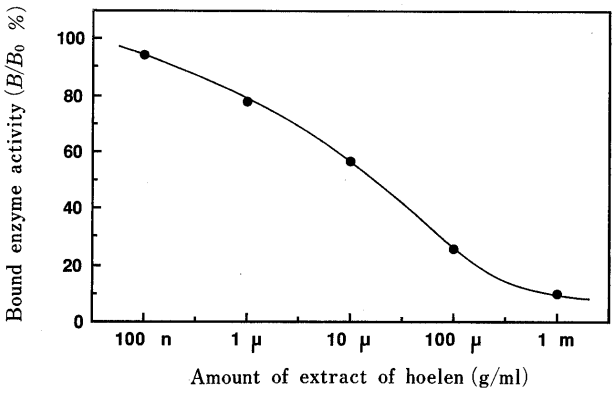

Fig. 4. Typical Dose–Response Curve of SAEIA for Hoelen Extract Using the Extract of Hoelen-Loaded Carbo-Plate

Bound enzyme activity (A_{414}) plotted against log dose of the extract of hoelen.

Comparison of Three Types of Microtiter Plate The efficiency of immobilization of hoelen extract on amino-plate, carbo-plate and Nunc Immunoplate II was compared. The extract of hoelen was converted to solid-phase antigens using the optimal conditions described above. The binding of antibody to each of the plates was measured at the competition with various amounts of the hoelen extract. Dose–response curves of SAEIA for the extract of hoelen using amino-plate, carbo-plate and Nunc Immunoplate II are shown in Fig. 2. The carbo-plate showed the highest binding activity with a steeper curve and it was chosen as the solid support.

SAEIA for the Extract of Hoelen The diagram of SAEIA is shown in Fig. 3. A typical dose–response curve of the SAEIA for a hoelen extract was determined using the solid-phase antigen, prepared under the optimal conditions described above, and 8000 times diluted anti-hoelen (Fig. 4). The working range of the SAEIA for the extract of hoelen was 10 ng to 100 μg/100 μl.

Specificity of the SAEIA The specificity of the SAEIA was examined by measuring the extract of several Chinese crude drugs, glycyrrhiza, ginseng and pinellia tuber. Fine powder of hoelen and the extract of chuling, a member of the same family as hoelen, were also measured for comparison. Cross-reaction values were calculated from their dose response curves according to the method of Abraham[15] (Fig. 5). The extracts of the crude drugs, glycyrrhiza, ginseng and pinellia tuber showed extremely low cross-reactivities. The cross-reactivity of the extract of chuling was also low (0.63%) and whole hoelen showed a cross-reactivity value of 7.9%.

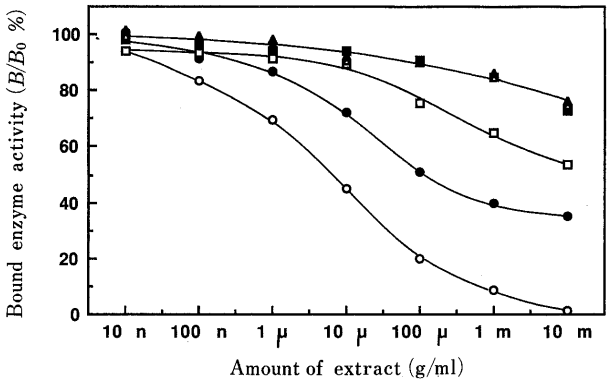

Fig. 5. The Dose–Response Curves for the Extract of Ginseng (■), Glycyrrhiza (△), Pinellia Tuber (▲), Chuling (□) and Hoelen (●) by the SAEIA Method (○).

Percentage of the bound enzyme activities with (B) and without (B_0) competition of the antigen is plotted against logarithmic dose.

TABLE IV. Compositions(g) of Chinese Crude Drugs in Three Kinds of Chinese Traditional Medicines before Extraction

Ninjin-yoei-to		Keishi-bukuryo-gan	
Ginseng	3.0	Cinnamon bark	4.0
Japanese angelica root	4.0	Hoelen	4.0
Peony root	2.0	Moutan bark	4.0
Rehmannia root	4.0	Peach kernel	4.0
Atractylodis rhizome	4.0	Peony root	4.0
Hoelen	4.0		
Cinnamon bark	2.5	Gorei-san	
Astragalus root	1.5	Alisma rhizome	6.0
Citrus unshiu peel	2.0	Chuling	4.5
Polygala root	2.0	Hoelen	4.5
Schisandra fruit	1.0	Atractylodis rhizome	4.5
Glycyrrhiza	1.0	Cinnamon bark	2.5

TABLE V. Measurement of the Extract of Hoelen in Seven Kinds of Normal- and Pseudo-Chinese Traditional Medicines

C.T.M.[a]	Measured amount of the extracted H[b] contained in either 100 μg or 10 μg of C.T.M. (μg/100 μg)	(μg/10 μg)
Keishi-bukuryo-gan[c]	1.30	0.125
without hoelen	N.D.	N.D.
Ninjin-yoei-to	0.94	0.088
without hoelen	N.D.	N.D.
Gorei-san	1.20	0.10
without hoelen	N.D.	N.D.
without chuling	1.55	0.11
without hoelen and chuling	N.D.	N.D.

a) Chinese traditional medicines. *b*) Hoelen. *c*) This was the extract of pseudo-Keishi-bukuryo-gan without Hoelen. N.D.: not detected.

Contents of Hoelen Extract in Three Chinese Traditional Medicines The compositions of Chinese crude drugs which were extracted for preparation of three Chinese traditional medicines, Keishi-bukuryo-gan, Ninjin-yoei-to and Gorei-san, are summarized in Table IV. The contents of hoelen extract in either 100 or 10 μg aliquot of the three

medicines were measured by SAEIA. For comparison, three pseudo-Chinese traditional medicines from which a component(s) of a Chinese crude drug, either hoelen or chuling, or both of them were omitted from their constituting Chinese crude drugs, were also prepared. The SAEIA was also applied to them (Table V). The content of hoelen extract in two different amounts of three Chinese traditional medicines were successfully determined. While all pseudo-medicines prepared by omitting hoelen were negative to this SAEIA (Table V).

Discussion

Immunoassay methods have been useful tools for various biological and immunological studies. Application of the methods, however, has been limited to water-soluble antigens and antibodies.[16-19] Most Chinese crude drugs are water-insoluble and most insoluble materials have been outside the scope of immunoassay. Chinese traditional medicines consist of the extract of a mixture of Chinese crude drugs and measuring the content of the extract of any specific Chinese crude drug has been unsuccessful.

In order to overcome this situation, we have been developing a new enzyme immunoassay named SAEIA[14] applicable to insoluble antigens.[20-22] A new method for assay of the extract of hoelen was studied. An antiserum specific for hoelen was elicited in a rabbit using disintegrated hoelen tissue as an immunogen. Although anti-hoelen serum contains an unknown population of antibodies specific for unidentified epitopes contained in hoelen tissue, a SAEIA for measuring the extract of hoelen has been developed using two immunological reagents, rabbit anti-hoelen antiserum and the hoelen extract-coated immunoplates as the solid-phase antigens, and GAL-labeled goat anti-rabbit IgG as a tracer. The assay principle of the SAEIA is as follows: rabbit anti-hoelen antiserum should contain a population of various antibodies specific for the corresponding epitopes of hoelen. When the extract of hoelen was used as the solid-phase antigen, only the selected antibodies specific for the epitopes contained in the hoelen extract should bind to the solid-phase antigen, but other populations of antibodies specific for other epitopes of hoelen would not bind. Competitions between the free and the solid-phase hoelen extracts against the selected antibodies were performed. The measurement of the amount of bound rabbit antibodies to the solid-phase antigen using GAL-labeled goat anti-rabbit IgG as a tracer was expected to assess the content of hoelen extract (Fig. 3).

Detailed conditions to develop a SAEIA for hoelen extract was studied, since the SAEIA method is a new immunoassay system and detailed conditions to set up the assay have not been reported. Comparison of three types of microtiter plates as the solid-phase support of the hoelen extract was first studied (Fig. 3). It was concluded that carbo-plate was the choice for the microtiter plate. The optimal conditions for immobilizing the extract of hoelen to the carboxyl groups of carbo-plate were established (Tables I, II and III).

Using the optimal conditions, a highly sensitive SAEIA for the hoelen extract has been developed. Its working range was between 100 ng and 100 μg (Fig. 4). The specificity of SAEIA was excellent as shown by the cross-reactions of the extract of either ginseng or chuling, the latter of which is in the same family as hoelen (Fig. 5).

TABLE VI. Recovery Test of 10 μg of Hoelen Extract Added to Aliquot of Keishi-bukuryo-gan, a Chinese Traditional Medicine

C.T.M.[a]		Measured amount of the H.E.[b] added to either 100 μg or 10 μg of C.T.M. (μg/100 μg)	(μg/10 μg)
Keishi-bukuryo-gan	(A)	1.23±0.46[c]	0.11±0.02[c]
(with 10 μg or H.E.)	(B)	11.0±2.3[c]	9.9 ±2.9[c]
B−A		9.8	9.8
Recovery (%)		98	98
Number of assays		3	3

a) Chinese traditional medicines. *b*) Hoelen extract. *c*) Mean±S.D.

The contents of the hoelen extract in three Chinese traditional medicines, Keishi-bukuryo-gan, Ninjin-yoei-to and Gorei-san were measured by the SAEIA method. The obtained results show that both values for different amounts of the medicines were parallel in each medicine (Table V). Five analogs of these Chinese traditional medicines, pseudo-medicines, were prepared from the extract of the mixture of all their constituting Chinese crude drugs in the same compositions except hoelen or/and chuling was omitted. All of them omitting hoelen were negative to the SAEIA (Fig. 6 and Table V). Neither the presence nor the absence of chuling in Gorei-san disturbed the assay method. To confirm the accuracy of the assay results, a recovery test of Keisi-bukuryo-gan was performed: the contents of hoelen extract in 10 and 100 μg of the medicine was assessed with or without the addition of 10 μg of the extract. The results are shown in Table VI. Accuracy of the assay results was confirmed by the good recovery percentages of 98% of the added extract obtained for both the doses of the medicine measured.

Although it has not been clarified which epitopes were determined to get a quantitative measurement of hoelen extract in Chinese traditional medicine by the SAEIA, the evidence clearly indicates that the SAEIA is able to measure the content of hoelen extract which had previously been impossible.

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