Regular Article

Development of Highly Sensitive Chemiluminescence Enzyme Immunostaining Assay to Determine Glycyrrhizin Content Using Anti-glycyrrhizin Monoclonal Antibody

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Licorice, the root of *Glycyrrhiza* spp., is used in a large number of herbal medicines, such as traditional Chinese medicines, Japanese Kampo medicines, and therapeutic drugs. Since glycyrrhizin (GL) is among the main components in licorice and exhibits numerous beneficial pharmacological activities, the content of GL directly affects biological activity. The quality control based on GL content is an important factor in ensuring biological activity; however, the content of GL in licorice varies depending on plant cultivation environment, genetic factors, and species type. Previously, we prepared an anti-GL monoclonal antibody (anti-GL mAb) and employed it in various immunochemical assays for quality control of licorice and licorice-based products. In this study, we employed the anti-GL mAb in chemiluminescence enzyme immunostaining (CLEIS) to develop a very simple, rapid, specific, and sensitive quality control assay for licorice products, with a limit of detection of 3.9 ng. Furthermore, the CLEIS assay enabled semiquantitative analysis of GL in Kampo medicines. Our results showed that multiple samples can be simultaneously analyzed using CLEIS, and it is a useful tool for determining GL content, as well as ensuring chemical quality control of licorice-containing products and herbal medicines.

Key words glycyrrhizin; monoclonal antibody; immunoassay; immunostaining; chemiluminescence; eastern blotting

Introduction

Glycyrrhiza spp. (licorice), a member of the Leguminosae family, is native to the drylands of China and Europe.[1)] The root of *Glycyrrhiza* spp., especially *G. uralensis*, *G. glabra*, and *G. inflata*, is among the most important crude drug and medicinal resources used in traditional, herbal, and Kampo medicines.[2,3)] Licorice is also consumed as a natural sweetener in snacks, drinks, and seasoning agents worldwide; thus, the global demand for licorice is high. In the food-manufacturing industry, licorice is used to mask bitter tastes because its sweetness has a slower onset than sugar and is long-lasting.[2,4)] To date, more than 500 compounds have been isolated from licorice. This includes more than 20 triterpenoids, such as glycyrrhizin (GL; Fig. 1), classified as an oleanane type triterpenoid. GL is the main constituent contributing to the sweet taste of licorice; it is 150 to 170-times sweeter than sucrose.[4,5)] The steric structure of the C- and E-rings of triterpenoids and the functional group at position C-30 are responsible for the taste of triterpenoids in licorice.[6)] GL is an important pharmacological compound because it exhibits antiviral,[7,8)] antioxidant,[9,10)] anti-atopic dermatitis,[11)] anti-inflammatory,[12)] and immunomodulatory activity[13)]; prevents cancer metastasis and chemoresistance[14)]; and protects against glucocorticoid-induced osteoporosis.[15)] From the perspective of pharmacokinetics, the bioavailability of GL is low[16)] because it is metabolized to its corresponding aglycone with or without chemical modification by several enterobacteria resulting in metabolites with various actions.[17–19)] However, since licorice is found in more than 70% of Kampo medicines in Japan, GL has been considered the main pharmacological compound and its content has a direct effect on pharmacological activity; Japanese pharmacopeia stipulates that the GL content in dried licorice is >2% (w/w). However, the content of GL in licorice varies depending on plant cultivation environment, species type, and harvesting season.[20)] Previously, we reported large variations in GL content in licorice extracted from plant roots grown on the same farm, suggesting that GL biosynthesis is genetically regulated.[21)] Therefore, quality control of licorice is crucial to ensure the pharmacological activities of licorice and licorice-based products. Based on these findings, accurate, sensitive, and rapid quantitative and/or qualitative analytical methodol-

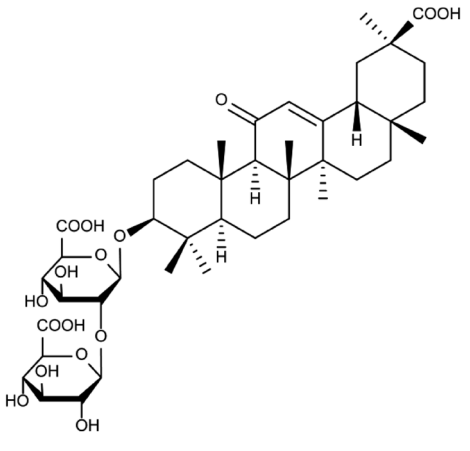

Fig. 1. Chemical Structure of GL

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ogy is required to ensure quality control.

We previously generated a specific monoclonal antibody (mAb) against GL (anti-GL mAb) and applied it to various immunoassays, including an indirect competitive enzyme-linked immunosorbent assay (icELISA).[22,23] Furthermore, we developed an immunostaining methodology using a glycoside as a motif, namely eastern blotting, for quality control analysis of licorice.[24] Eastern blotting is an effective high-throughput first screening tool for visual detection of GL. However, eastern blotting is time-consuming because it involves numerous steps and exhibits relatively low sensitivity.

In this study, we developed a chemiluminescence enzyme immunostaining (CLEIS) assay based on the dot blot technique and employed the concept of eastern blotting using the anti-GL mAb for the detection of GL. This simpler assay enables more rapid detection of GL with higher sensitivity, 120-times greater than that of typical immunostaining, such as eastern blotting. Finally, the developed CLEIS assay was applied to a semiquantitative analysis of GL content in Kampo medicines.

Experimental

Chemicals and Reagents GL (crude drug test grade), bovine serum albumin (BSA), sodium periodate, skimmed milk (powder form), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and 4-chloro-1-naphthol were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Horseradish peroxidase (HRP)-labeled anti-mouse immunoglobulin G (IgG) goat serum (Fc specific and whole molecule) was provided by Merck KGaA (Darmstadt, Germany). Polyethersulfone (PES) membrane (Mustang E) was supplied by the Pall Corporation (East Hills, NY, U.S.A.). ECL™ Prime detection reagent and a Luminescent Image Analyzer (LAS 4000 mini) were purchased from Cytiva (Tokyo, Japan). The Kampo medicines were manufactured by Tsumura & Co. (Tokyo, Japan). All other chemicals were standard commercial products of analytical grade.

Preparation of Standard and Sample Solutions GL was accurately weighed and dissolved in authentic methanol in a volumetric flask to prepare a 2 mg/mL stock solution. The stock solution was then diluted with methanol to prepare standard solutions. Each dried and homogenized Kampo medicine sample powder (100 mg each) was suspended in 1.2 mL of methanol containing 0.1% (v/v) ammonia and then sonicated for 20 min. After centrifugation at $9000 \times \boldsymbol{g}$, the supernatant was collected and the extraction steps were repeated five times, combining the resultant supernatant each time, and then evaporating the samples to yield a residue that was dissolved in 1 mL of methanol. The prepared standard and sample solutions were stored at 4 °C until used in the CLEIS assay and icELISA.

Generation and Characterization of Anti-GL mAb Previously, we established a hybridoma cell line secreting anti-GL mAb classified as IgG1 and having a λ chain and high-specificity for GL.[23] This cell line was obtained using a hybridoma technology by fusing immune spleen cells and mouse myeloma cells (SP 2/0) using polyethylene glycol. The obtained hybridoma cells were cultured in enriched RPMI1640-Dulbecco's-Ham's F12 (E-RDF) medium (Kyokuto Pharmaceutical Industrial AC., Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Finally, the cells were maintained in an FBS-free E-RDF medium, and after filtration, the collected culture medium was used for the experiments as the anti-GL mAb solution.

CLEIS for GL For the CLEIS assay, various concentrations of GL standards and sample solutions were applied to a PES membrane, which was then incubated in a solution of sodium periodate (10 mg/mL) for 30 min. After gently rinsing the membrane with water, 1% BSA in 50 mM carbonate buffer (pH 9.6) was added, and the membrane was gently shaken for 3 h. After washing with 0.1% Tween 20 in Tris-buffered saline (TBST), the membrane was incubated in TBST containing 2% skimmed milk for 1 h to reduce the non-specific reaction. Following three washes with TBST, the membrane was immersed in the anti-GL mAb solution for 3 h. Following washing with TBST, an HRP-labeled anti-mouse IgG (whole molecule) secondary antibody solution, diluted 1000-fold with TBST and containing 2% skimmed milk, was added and the membrane was shaken for 1 h. After washing three times with TBST and once with TBS, the membrane was exposed to the detection reagent (ECL™ Prime) for 30 s and the resulting chemiluminescence was measured using a Luminescent Image Analyzer. The amount of chemiluminescence was quantified using Image Quant TL software (Cytiva, Tokyo, Japan). In addition, typical immunostaining using 4-chloro-1-naphthol as a detection reagent was carried out and the same protocol was used until before the detection step. In this case, the PES membrane was incubated for 20 min in a phosphate-buffered saline (PBS) solution containing 4-chloro-1-naphthol (1 mg/mL) and hydrogen peroxide (0.03%). The membrane was then transferred to water to stop the color development reaction. All the GL-detection procedures were conducted at 25 ± 2 °C.

icELISA for GL Quantitative analysis of GL using icELISA using the anti-GL mAb was conducted as previously reported.[25] Briefly, 100 μL of GL-human serum albumin conjugate (1 μg/mL) in 50 mM carbonate buffer (pH 9.6) was immobilized in a 96-well immunoplate (Thermo Fisher Scientific). To prevent non-specific adsorption, each well was blocked with 300 μL of 5% skimmed milk in PBS before adding 50 μL of various concentrations of GL and sample solutions and incubating with anti-GL mAb. The antigen-antibody complexes in each well were reacted with HRP-labeled anti-mouse IgG (Fc specific) secondary antibody (100 μL). Subsequently, 100 μL of substrate solution (100 mM citrate buffer, pH 4.0, containing 0.003% [v/v] hydrogen peroxide and 0.3 mg/mL ABTS) was added and incubation continued for 20 min at 37 °C to allow for color development. Finally, the absorbance was measured at 405 nm using a microplate reader (Thermo Fisher Scientific). All reactions were carried out at 37 °C for 1 h unless stated otherwise. In addition, the immunoplate was washed three times between each step using 0.1% Tween 20 in PBS.

Results and Discussion

We developed the CLEIS technique to improve the sensitive detection of GL based on our previously described eastern blotting technique. A schematic of the CLEIS assay is shown in Fig. 2. In the first step, the sample solution was applied to a PES membrane (Fig. 2a). To ensure the fixation of low molecular-weight compounds (*e.g.*, glycosides), sodium periodate was added to oxidize the glycosides and generate an aldehyde

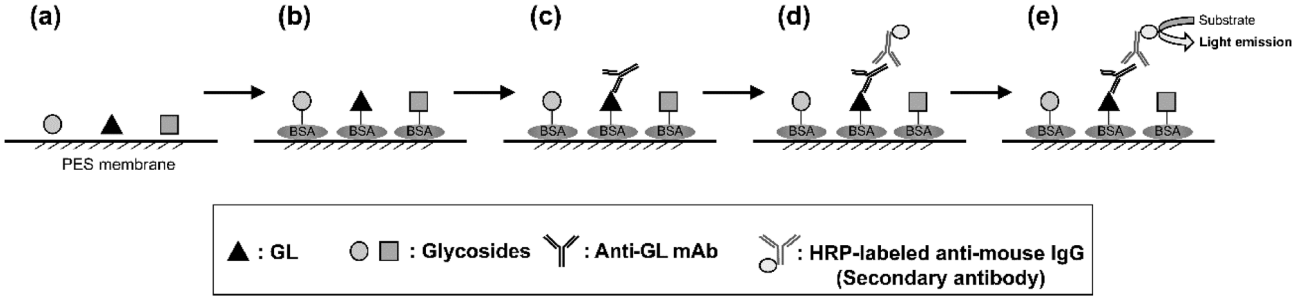

Fig. 2. CLEIS Assay Scheme

The CLEIS assay comprises five steps: (a) Application of sample or GL solution to the PES membrane. (b) Treatment with sodium periodate to fix GL or other glycosides to the PES membrane using BSA. (c) Addition of anti-GL mAb and binding to GL-BSA conjugates. (d) Addition of HRP-labeled secondary antibody. (e) Chemiluminescence detection.

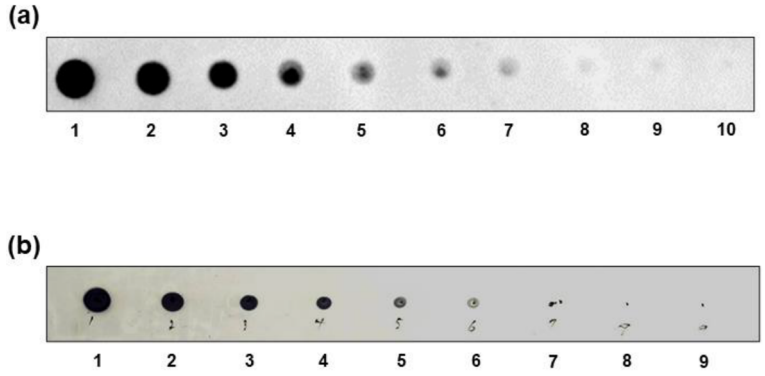

Fig. 3. Comparison of CLEIS and Typical Immunostaining Detection Limits for GL

The detection limit using (a) CLEIS and (b) typical immunostaining was 3.9 ng and 500 ng, respectively. The CLEIS assay was tested using ten concentrations of GL (lanes 1–10: 2000, 1000, 500, 250, 125, 62.5, 31.2, 15.6, 7.8, and 3.9 ng), and typical immunostaining was conducted using nine concentrations of GL (lanes 1–9: 16000, 8000, 4000, 2000, 1000, 500, 250, 125, and 62.5 ng).

group. Under alkaline conditions (pH = 9.6), the aldehyde group forms a Schiff-base complex with the lysine residue in BSA on the PES membrane, thus fixing the glycosides to the membrane *via* BSA (Fig. 2b). Subsequently, GL was recognized by the anti-GL mAb (Fig. 2c). After binding the secondary antibody to the antigen-antibody complexes (Fig. 2d), GL was detected using the chemiluminescent substrate (Fig. 2e).

The detection of various concentrations of GL using CLEIS and typical immunostaining is compared in Fig. 3. The limit of detection using CLEIS and typical immunostaining was 3.9 and 500 ng GL, respectively. Although the limit of detection using typical immunostaining was almost the same as in our previous study using the eastern blotting technique,[24] the new CLEIS technique could detect GL at a level 120-times lower than that of immunostaining. Therefore, the newly developed CLEIS technique enables a high sensitivity detection system for GL.

We also observed that the spot diameter and intensity detected using CLEIS corresponded to the concentration of GL (Fig. 3a). Therefore, the chemiluminescence signal was quantified using image analysis software to generate a calibration curve for GL initially ranging from 7.8 to 2000 ng. When the calibration curve was examined over a range of 15.6–1000 ng GL, a high correlation coefficient ($R^2 = 0.9994$) was observed (Fig. 4). Previously, Morinaga *et al.* applied eastern blotting to quantitatively analyze the PES membrane-bound GL.[26] The lower limit of their calibration curve was 1.0 μg; thus, our CLEIS assay is at least 64-times more sensitive than their

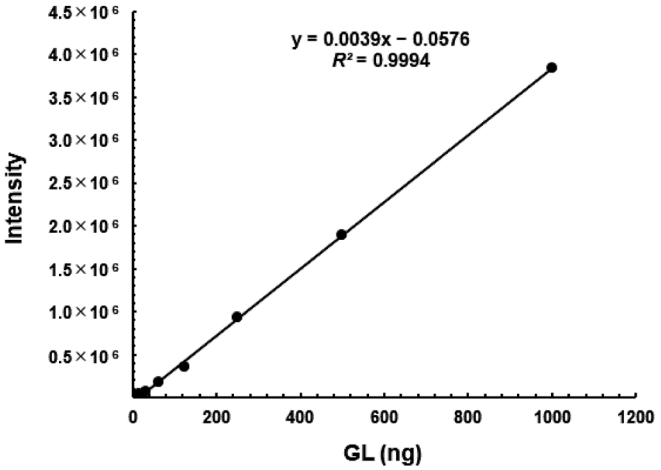

Fig. 4. Calibration Curve for GL Obtained Using CLEIS Assay

The calibration curve obtained using a GL concentration range of 15.6 to 1000 ng exhibited a high correlation coefficient ($R^2 = 0.9994$).

assay. Furthermore, since our new approach does not require an organic solvent or additional time for the development of components and transfer to the membrane, it is eco-friendly and rapid.

The content of GL in nine Kampo medicine samples containing licorice (samples 1–9) and one without licorice (sample 10) was determined using icELISA (Fig. 5a) and CLEIS (Fig. 5b) using the anti-GL mAb. GL was detected on

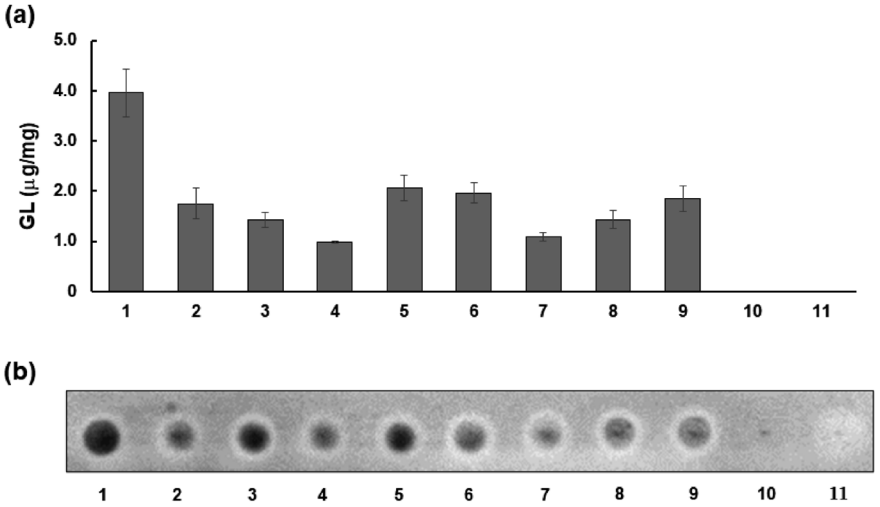

Fig. 5. Detection of GL in Various Kampo Medicines Using CLEIS

Samples 1–9; Kakkonto, Anchusan, Kamishoyosan, Bakumondoto, Juzendaihoto, Junchoto, Yokuininto, Heiisan, and Saikoseikanto. Sample 10; Hangekobokuto, which did not contain licorice. Sample 11; BSA, which was used to fix GL to the PES membrane. GL content was quantitated by icELISA (a) and the results were compared with the spot diameter and intensity results obtained using CLEIS (b).

all nine Kampo medicines (samples 1–9) containing licorice. In contrast, GL was not detected using CLEIS in sample 10 (Hangekobokuto) which does not contain licorice, or in the BSA negative control sample 11, which was used as a carrier protein to fix GL to the PES membrane. Based on these results, we conclude that the use of CLEIS with the anti-GL mAb can detect GL without being affected by various analogs in the sample solution and carrier protein used in the fixation of samples to the membrane. Our previous eastern blotting using anti-GL mAb ensured the high specificity detection for GL without the cross-reactivity against GL analogs and constituents in licorice.[24] Therefore, the developed CLEIS is possible for specific detection of GL. Furthermore, the sensitivity of CLEIS was satisfactory for detecting GL in crude extracts. A good correlation was found between the spot diameter and intensity using CLEIS and quantitative values of icELISA (Fig. 5). Subsequently, the detected spot on the PES membrane was quantified using an image analysis software to determine the content of GL detected by CLEIS. The quantitative values of GL in the samples were determined by both CLEIS assay and icELISA, and exhibited a good correlation (Table 1). Previously, we reported on using icELISA in conjunction with the specific anti-GL mAb for quality control and breeding of licorice.[21] Quantitative analysis of GL using icELISA indicated a good correlation with that of HPLC analysis. Quantitative analyses of compounds on membranes have been reported,[26,27] however, these methodologies have a drawback in terms of detection sensitivity and analysis time, as well as the difficulty in simultaneously analyzing many samples owing to the need for developing components in the sample. The advantages of CLEIS are that it is highly sensitive and simultaneous analysis of multiple samples can be performed because the methodology is based on the dot blot technique. Although HPLC is the most commonly used analytical method, CLEIS, as developed in this paper, has several significant advantages: first, the initial cost is small because CLEIS does not require an expensive device like HPLC, and the running cost of separation columns, eluents, *etc.* is small, which means that it can be easily introduced in a laboratory. Second, CLEIS can be used for simultaneous analysis of multiple samples in a single assay because the reaction is completed on a single membrane. Third, although HPLC is needed for some complicated pretreatments, CLEIS can directly assay using crude sample extract without pretreatment because it is ensured by the specificity of anti-GL mAb. Furthermore, the high sensitivity of CLEIS also means that even small sample volumes can be analyzed. Taken together, the newly developed CLEIS assay is a potentially useful analytical tool for the detection of GL in terms of simplicity, rapidity, and sensitivity.

Table 1. Content of GL in Various Kampo Medicines Determined Using CLEIS and icELISA with Anti-GL mAb

(No.) Samples	GL (μg/mg dry weight)	
	CLEIS	icELISA
(1) Kakkonto	3.87 ± 0.15	3.96 ± 0.47
(2) Anchusan	1.82 ± 0.27	1.75 ± 0.31
(3) Kamishoyosan	1.85 ± 0.67	1.42 ± 0.15
(4) Bakumondoto	0.92 ± 0.16	0.98 ± 0.02
(5) Juzendaihoto	2.09 ± 0.31	2.06 ± 0.26
(6) Junchoto	1.06 ± 0.26	1.96 ± 0.20
(7) Yokuininto	1.19 ± 0.27	1.09 ± 0.08
(8) Heiisan	1.93 ± 0.44	1.43 ± 0.18
(9) Saikoseikanto	1.63 ± 0.03	1.85 ± 0.26
(10) Hangekobokuto	N.D.	N.D.

Data are presented as mean ± standard deviation ($n = 3$). N.D., not detected.

Conclusion

In this study, anti-GL mAb was applied to the CLEIS technique, which is based on the dot blot concept with eastern blotting, and we demonstrated high sensitivity and specificity in the identification of GL in Kampo medicines. Furthermore, CLEIS achieved semiquantitative analysis of GL in crude extracts. Our previous study demonstrated that the eastern blotting technique using the anti-GL mAb is a rapid assay, in particular for quantitative and/or qualitative analysis of numerous samples,[24] and this assay system can analyze directly using crude extract because it uses the high specific anti-GL

mAb. The CLEIS does not require blotting to the membrane and the development of the constituents which must require eastern blotting. Thus, compared with other typical immunostaining detection systems, CLEIS is a simple, rapid, sensitive, specific, and eco-friendly procedure. Furthermore, it is suitable for simultaneous analysis of numerous samples because it is based on the dot blot technique, and can be applied directly to crude extracts without any pretreatment. Currently, the global demand for licorice is high; thus, there is a need for the development of an analytical methodology for GL as a quality control marker for licorice to ensure pharmacological activity. Therefore, the developed CLEIS assay, which can be used for the detection of GL content, is a useful and high throughput tool for the first screening in quality control. Furthermore, in addition to CLEIS, incorporating icELISA, using the anti-GL mAb, is expected to take quality control analysis of licorice and licorice-based products to an even higher level.

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Conflict of Interest The authors declare no conflict of interest.

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