Sensitive Detection of Glycyrrhizin and Evaluation of the Affinity Constants by a Surface Plasmon Resonance-based Immunosensor

Takatoshi SAKAI,* Katsura SHINAHARA,* Akira TORIMARU,* Hiroyuki TANAKA,**
Yukihiro SHOYAMA,** and Kiyoshi MATSUMOTO*†

*Department of Bioscience and Biotechnology, Faculty of Agriculture, Kyushu University,
Fukuoka 812-8581, Japan

**Department of Chemo-Pharmaceutical Sciences, Faculty of Pharmaceutical Sciences, Kyushu University,
Fukuoka 812-8582, Japan

A sensitive and selective determination of glycyrrhizin (GC) based on surface plasmon resonance (SPR) was performed by using an anti-GC monoclonal antibody (GC-MAb) and GC-bovine serum albumin (GC-BSA) conjugate (antigen). GC-BSA was immobilized on an Au thin film of the SPR sensor chip by physical adsorption, and GC determinations were performed by an indirect competitive method. The addition of GC into the GC-MAb solution (5 µg/ml) was found to decrease the incident-angle shift sharply because of an inhibition effect of GC. The RSDs ($n = 3$) of each point were less than 4%. The lowest detection limit for GC by SPR was almost the same as that by ELISA, 60–75 ng/ml. An evaluation of the affinity constant between GC-MAb and GC using the data from ELISA and those from SPR measurements was performed. The values of the association constant (K_A) from three different analyses of ELISA data and from SPR measurements are discussed in detail. As a whole, the affinity constant (K_A) between GC-MAb and GC was on the order of 10^7 M^{-1} .

(Received September 8, 2003; Accepted December 8, 2003)

Introduction

Licorice (*Glycyrrhiza* spp.) root is one of the world's most common herbal medicines, used in European, Mideastern, Asian, and Chinese traditions. Glycyrrhizin (GC) is an extract from licorice root, and amounts to about 15% of the whole fresh root extract. The pharmaceutical properties of GC are, so far, reported as having protein kinase inhibitory activity,¹ anti-ulcer² and anti-viral³ activity, immunomodulatory activity against HIV,⁴ and therapeutic effects on liver disease.⁵ The rapid and selective quantitation of low-molecular-weight bioactive compounds is very important for investigating the distribution of the compounds in plants or animals and for elucidating the metabolism of such compounds. Measurements of GC using HPLC⁶ and capillary electrophoresis⁷ have been reported. These methods, however, require a pretreatment in order to measure GC in plant extracts or serum samples.

Biochemical techniques using monoclonal antibodies (MAbs) are powerful tools for the detection of drugs and low-molecular-weight bioactive compounds. Recently, Shan *et al.* developed a sensitive method for determining GC by using an enzyme linked immunosorbent assay (ELISA).⁸ Though it is sensitive and selective for measuring GC, it is still time consuming (in general, 3–4 h per assay).

An immunosensor based on surface plasmon resonance (SPR) has been receiving increasing attention in recent years, due to its potential as a label-free, real-time, rapid, high-selectivity

immunoassay technique.^{9,10} These methods are able to perform an assay in 5–30 min.

In the present study, we aimed to develop a simple and rapid method for quantifying GC by SPR. In addition, we evaluated the affinity constants (K_A) between antigens and antibodies (GC and GC-MAb) by assuming a Langmuir adsorption model for the immunoreaction.

Experimental

Chemicals and apparatus

GC and Tween 20 were purchased from Wako Pure Chemicals Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA), sodium periodate (NaIO_4), *p*-nitrophenyl phosphate disodium salt (*p*-NPP), and gelatin were from Nacalai Tesque, Inc. (Kyoto, Japan). Human immunoglobulin G (*hIgG*, reagent grade), anti-*hIgG* antibody (affinity purified) and alkaline phosphatase labelled anti-mouse IgG were provided by Sigma (St. Louis, MO, USA). All other chemicals were of analytical reagent grade. All buffer solutions were prepared using water purified with a Milli-Q (Millipore, Bedford, UK) system. Anti-GC MAb 5A8 (GC-MAb, 5A8) was supplied by Tanaka and Shoyama.⁸

SPR measurements were performed using a Spreeta™ evaluation module kit (Texas Instruments, Inc., Texas, USA) attached to a sensor chip, a flow-through cell ($2 \times 18 \times 0.2 \text{ mm}$) and a microtube pump (EYELA, SMP-23S, Tokyo, Japan). ELISA measurements were performed using 96-well immunoplates (NUNC, No. 446612, Roskilde, Denmark) and a microplate reader (Spectra 1, Wako, Osaka, Japan).

† To whom correspondence should be addressed.
E-mail: kmatsu@agr.kyushu-u.ac.jp

Fig. 1 Response transients of the GC-BSA immobilized sensor to the GC-MAb without antigen (θ_0) and with antigen (θ_1).

Synthesis of GC-BSA conjugate

GC-BSA conjugate was synthesized by a method reported by Shan *et al.*⁸ Briefly, a methanol solution (0.27 ml) of GC (5.2 mg) was added dropwise to a solution (0.4 ml) containing NaIO_4 (5 mg) and stirred at room temperature for 1 h. Carbonate buffer (50 mM, pH 9.6, 1.4 ml) containing BSA (5 mg) was added to the above-mentioned reaction mixture. The whole reaction mixture was adjusted to pH 9.0 with a 1 M Na_2CO_3 solution and stirred at room temperature for 6 h. The reaction mixture was dialyzed against five changes of H_2O at 4°C for 3 days, and then lyophilized to produce GC conjugate (GC-BSA) (4.8 mg).

Indirect competitive ELISA for GC

ELISA for GC was performed as described by Shan *et al.*⁸ with some modifications. Briefly, 96-well immunoplates were coated with 100 μl of GC-BSA conjugate (10 $\mu\text{g}/\text{ml}$) in 50 mM carbonate buffer (pH 9.8) overnight at room temperature. The following day, the plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) and treated with 1% gelatin for 1 h at room temperature. The plates were washed three times with PBST and reacted with 100 μl of equivalent mixtures of GC-MAb 5A8 (10 $\mu\text{g}/\text{ml}$) and serial diluted GC antigens for 1 h at room temperature. The plates were washed three times with PBST, and then reacted with 100 μl of alkaline phosphatase labelled anti-mouse IgG (1000 fold diluted with PBS) for 1 h at room temperature. After washing three times with PBST, the substrate solution (2 mg/ml *p*-NPP in 50 mM carbonate buffer, pH 9.8, containing 1 mM MgCl_2 and 0.1 mM ZnCl_2) was added to each well and incubated for 30 min at room temperature. The absorbance at 405 nm was measured using a microplate reader.

Indirect competitive SPR measurement for GC

For highly sensitive detection, we used an indirect competitive method, which was effective to detect chemical substances with low molecular weight.¹¹⁻¹³ The refractive index (or incident angle) of Au thin film modified with an antigen-protein conjugate depends on the concentration of the antigen; this small change can be detected using the SPR. A schematic diagram of the indirect inhibition SPR measurement is shown in Fig. 1. In this method, antigen proteins were immobilized on the sensor chip (Au thin film) by circulating the GC-BSA solution (1 mg/ml in PBS) for 1 h (physical adsorption). The

Fig. 2 Effect of the repetitive binding of anti-*h*IgG on the immobilized *h*IgG. Anti-*h*IgG injected was 20 $\mu\text{g}/\text{ml}$. The flow rate was constantly maintained at 300 $\mu\text{l}/\text{min}$.

sensor chip was washed by circulating the carrier buffer (PBS) for 5 min. A BSA solution (2 mg/ml in PBS) was circulated for 30 min to reduce any nonspecific adsorption of the antibody. After washing with the carrier buffer (State A in Fig. 1), the GC-MAb (5 $\mu\text{g}/\text{ml}$) solution was allowed to flow for 3 min, and changes in the incident-angle shift ($\Delta\theta_0$) caused by the association of the antibodies (GC-MAbs) to the immobilized antigens (GC-BSA) was measured (State B). After 3 min, the flowing solution was changed to the carrier buffer and allowed to flow for another 3 min. The sensor chip was then regenerated by the circulation of glycine-HCl buffer (10 mM, pH 2.2) for 1.7 min (State C). After the chip was washed with the carrier buffer and the signal base line was recovered (State D), 1 ml of an equivalent mixture of GC-MAb (10 $\mu\text{g}/\text{ml}$) and an appropriate amount of GC was allowed to flow to obtain a decreased incident-angle shift ($\Delta\theta_1$) with inhibition by GC (State E). This cycle was performed for mixtures of GC-MAb and serial-diluted GC. A quantitative determination of the antigen concentration can be made from the difference of $\Delta\theta_0$ and $\Delta\theta_1$. The flow rate was constantly maintained at 300 $\mu\text{l}/\text{min}$, and all of the procedures were conducted at room temperature.

Indirect competitive SPR measurement for hIgG

An *h*IgG measurement using SPR sensor was performed by almost the same procedures as for GC, except for the antigen immobilization (1 mg/ml *h*IgG) and anti-*h*IgG antibody (final concentration: 20 $\mu\text{g}/\text{ml}$).

Results and Discussion

Optimization of the hIgG assay by SPR

*h*IgG was widely used as a model antigen for evaluating the sensor based on the immunoreaction. In order to grasp the properties of the Spreeta™ sensor kit attached to the flow cell, we first tried to measure *h*IgG by an indirect competitive method, and evaluated the sensitivity and accuracy of the assay by using the SPR method. Figure 2 shows the effect of repetitive binding of anti-*h*IgG on the immobilized *h*IgG. As shown in Fig. 2, the amount of associated anti-*h*IgG antibody to *h*IgG was decreased by about 20% in the first few trials because of a slight denaturation of the *h*IgG protein by the regeneration buffer (glycine-HCl). The response, however, became nearly

Fig. 3 Response transient of the incident angle of the *hIgG* immobilized sensor to the anti-*hIgG* (a) and the dependence of the incident angle shift on the anti-*hIgG* concentration (b).

constant after five repetitions, indicating that constant results were achieved by this pre-conditioning. The thus-pre-conditioned *hIgG* immobilized chip was exposed to the flow of anti-*hIgG* antibody at various concentrations (2.0–60 µg/ml). The response transient of the incident angle of the *hIgG* immobilized chip to a flow of 20 µg/ml anti-*hIgG* is shown in Fig. 3(a). Upon exposure to the flow of antibodies, the incident angle increased rapidly due to the association of the anti-*hIgG* antibody on the sensor chip by antigen-antibody binding, and attained a steady state in about 3 min. The incident-angle shifts, measured by sequential injection of increased antibody concentration, were plotted against the concentration of the antibody. The plots are shown in Fig. 3(b). The incident-angle shift increased rapidly with increasing concentration of the anti-*hIgG* antibody up to approximately 40 µg/ml, and then leveled off. Considering the use of the indirect competitive method, we chose approximately 20 µg/ml anti-*hIgG* as the best antibody concentration.

Determination of *hIgG* by SPR

Anti-*hIgG* antibody solutions were mixed with *hIgG* to prepare mixtures containing the antibody with the final concentration of 20 µg/ml and *hIgG* of different concentrations (0.5–100 µg/ml). The mixtures were incubated for 5 min at room temperature prior to flowing over the *hIgG* immobilized chip. The percent inhibition, which was the decrease in the response relative to that of no *hIgG* antigen, $(\Delta\theta_0 - \Delta\theta_i)/\Delta\theta_0 \times 100$, was plotted against the concentration of *hIgG*, where $\Delta\theta_i$ stands for the incident-angle shift of the *i*-th mixture of anti-*hIgG* and *hIgG*; the plots are shown in Fig. 4. The percent inhibition was increased rapidly with an increase in the concentration of *hIgG* higher than 5 µg/ml. The relative standard deviations (RSDs, $n = 3$) of each point were 1.29, 3.46, 2.65, 3.00, 2.39, and 3.29% for 0.5, 1.0, 5.0, 10.0, 20.0, and 50.0 µg/ml, respectively. These results indicated that high-reproducible measurements can be obtained by using this SPR method. The lowest detection limit is defined as the concentration where the percent inhibition is increased by 15%, which is approximately

Fig. 4 Determination of *hIgG* by the indirect competitive SPR method. The error bars indicate $\pm$ standard deviations ($n = 3$). 20 µg/ml of anti-*hIgG* was used.

Fig. 5 Dependence of the incident angle shift of the GC-BSA immobilized SPR sensor on the GC-MAb concentration.

three SDs, from the mean incident angle shift observed in the absence of *hIgG*. The lowest detection limit for *hIgG* is approximately 5 µg/ml (corresponding to about 3.3×10^{-8} M).

Optimization of the GC assay by SPR

A GC-BSA immobilized chip was exposed to the flow of GC-MAb at various concentrations (2.5–20 µg/ml). The incident-angle shifts were plotted against the concentration of GC-MAb; the plots are shown in Fig. 5. The incident-angle shift again increased rapidly with increasing concentration of GC-MAb up to approximately 10 µg/ml, and then increased meagerly above this concentration. From this figure, the use of an approximately 5 µg/ml GC-MAb solution is considered to be the best for GC sensing experiments, considering the use of the indirect competitive method.

Detection of GC with SPR and a comparison with ELISA

GC-MAb solutions (10 µg/ml) were mixed with equal volumes of GC of various concentrations, and incubated for 5 min at room temperature prior to flowing over the GC-BSA immobilized sensor chip. The percent inhibitions were plotted against the concentration of GC in the same way as in the case of *hIgG*; the plots are shown in Fig. 6. In this case, pre-conditioning was not required for the association of GC-MAb to the GC-BSA immobilized chip. The RSDs ($n = 3$) of each point were less than 4.0%. The detection of GC was also done by indirect competitive ELISA; the results observed by both the SPR and ELISA methods are compared (Fig. 6). The lowest detection limit for GC by SPR was almost the same as that by

Fig. 6 Standard curve of inhibition by GC. ●, Indirect competitive SPR method; ○, indirect competitive ELISA.

ELISA, 60–75 ng/ml (corresponding to $7.2\text{--}9.1 \times 10^{-8}$ M). GC is contained in mg-quantity in plant extracts,^{7,8} whereas, to evaluate the pharmacokinetics and bioavailability of GC, μg/ml levels of sensitivity should be needed.^{14,15} The SPR method might give a simple and rapid detection of GC for wide applications from plant screening to pharmacokinetic studies.

Evaluation of affinity constants

To determine an affinity constant (K_A), or its reciprocal, the dissociation constant (K_D) is frequently useful in the study of antigen-antibody interactions. The evaluation of K_D , however, is an afflictive process because of the different values obtained by the method used for the analysis. The introduction of a convenient method for calculating K_D using indirect competitive ELISAs, based on the method by Friguet *et al.*,¹⁶ has been followed by its widespread application. Subsequently, a corrected method that takes into account the bivalent nature of IgG was suggested by Stevens.¹⁷ Seligman¹⁸ discussed the influence of the solid-phase antigen in indirect competitive ELISAs on the calculated antigen-antibody dissociation constants (K_D). We first tried to evaluate the values of K_D with our GC determination data obtained in indirect competitive ELISA by using Friguet's, Stevens' and Seligman's methods. The data points for these analyses were used from No. ③ to No. ⑦ in Fig. 7. Table 1 summarizes the results. The values of K_D ranged from 4.84×10^{-8} M to 19.7×10^{-8} M. The highest value was from Friguet's method, which did not incorporate any correction for bivalency. The value (19.7×10^{-8} M) from Friguet's method was almost four-fold higher compared with that (4.87×10^{-8} M) from Seligman's method, and the value (4.84×10^{-8} M) from Stevens' was almost the same as that from Seligman's method. We also estimate the K_D value from the ELISA data reported earlier by Shan *et al.*⁸ with the same antigen-antibody system by using Seligman's method. The data points for this analysis were used from No. ①' to No. ⑤' in Fig. 7. The evaluated value of K_D was 0.81×10^{-8} M ($K_A = 1.23 \times 10^8$ M⁻¹). The value of 0.81×10^{-8} M was approximately one-sixth lower than our results (4.87×10^{-8} M) and the difference was interpreted by the influence of the amount of solid-phase antigen. The amount of solid-phase antigen used by Shan *et al.* was 1 μg/ml, whereas it was 10 μg/ml by us. Hetherington¹⁹ investigated the influence of a solid-phase antigen on a competitive ELISA, and found that an eight-fold increase in the amount of solid-phase antigen applied to the microtiter plate caused an approximately five-fold increase in the calculated K_D . Seligman¹⁸ also obtained almost the same tendency of the eight-

Fig. 7 Plots of percent inhibition versus the GC concentration using indirect competitive ELISA. ●, our data; ○, data reported by Shan *et al.* The solid and dashed lines are theoretical inhibition curves from $K_A = 2.05 \times 10^7$ M⁻¹ and $K_A = 1.24 \times 10^8$ M⁻¹, respectively.

fold increase in the calculated K_D when the amount of solid-phase antigen increased from 6 pmol/well to 600 pmol/well. The lower amount of solid-phase antigen would lead a more reliable value of K_D . The solid and dashed lines in Fig. 7 show theoretical inhibition curves for indirect competitive antigen-antibody reactions using our calculated K_D -value (4.87×10^{-8} M) and that (0.81×10^{-8} M) of Shan *et al.*,⁸ respectively, indicating that each measured datum point closely falls on both lines.

Next, we tried to evaluate the affinity constants of the GC-MAb to immobilized GC-BSA and GC by using the SPR data. The analyses were followed by a method reported by Sakai *et al.*^{20,21} They evaluated the affinity constants relevant to an immunoassay system by assuming a Langmuir-type adsorption model for the immunoreaction.^{20,21} The present system involves two competitive immunoreactions, as follows:

Here, MAb stands for GC-MAb, and K_1 and K_2 are affinity constants. Three assumptions are required to correlate the incident-angle shift with each immunoreaction. First, the antigen-antibody reaction will proceed by monovalency. Second, a Langmuir-type adsorption model can be adopted. Third, the incident-angle shift is proportional to the coverage of the adsorption sites (immobilized GC-BSA) by GC-MAb. Based on these assumptions, we can obtain the following equation for the first immunoreaction:

$$[\text{MAb}]/\Delta\theta_0 = [\text{MAb}]/\Delta\theta_{0,\text{max}} + 1/\Delta\theta_{0,\text{max}}K_1 \quad (3)$$

Here, [MAb] is the molar concentration of antibody, $\Delta\theta_0$ is the equilibrium incident-angle shift to a given concentration of antibody, and $\Delta\theta_{0,\text{max}}$ is the maximum incident-angle shift at the full coverage. Assuming the latter immunoreaction also to be in equilibrium, the following equation can be derived:

$$1/\Delta\theta = 1/\Delta\theta_0 + K_2[\text{GC}]/\Delta\theta_{0,\text{max}}K_1[\text{MAb}]_0 \quad (4)$$

Here, $\Delta\theta$ and $\Delta\theta_0$ are the angle shifts in the presence and absence of GC, respectively. $[\text{MAb}]_0$ is the initial molar concentration of MAb (GC-MAb).

Table 1 Calculation of dissociation constants by various methods using data from competitive ELISA of GC

Method	Formula	K_D ($\times 10^{-8}$ M)	K_A ($= 1/K_D, \times 10^7$ M $^{-1}$)
Friguet <i>et al.</i>	$1/v = 1 + K_D/a_0$	19.7	0.51
Stevens correction	$1/f = 1 + K_D/a_0$	4.84	2.07
Seligman	$a_0/f = K_D/(1-f) + i_0$	4.87	2.05

Analyses were performed using formulae shown above and data points from ③ to ⑦ of Fig. 7.

$v = (A_0 - A_i)/A_0$ where A_0 is the absorbance without competing antigen and A_i is the absorbance with competing antigen; a_0 , initial concentration of competing antigen; $f = \sqrt{v}$; i_0 , initial antibody concentration.

From Eq. (3), $[Mab]/\Delta\theta_0$ should be linear to $[Mab]$, the slope and the intercept giving values of $\Delta\theta_{0,max}$ and K_1 , respectively. The data in Fig. 5 are replotted in this way in Fig. 8(a). A linear correlation was obtained, as indicated, which gave $\Delta\theta_{0,max} = 0.099$ deg. and $K_1 = 8.69 \times 10^7$ M $^{-1}$. From Eq. (4), the inverse $\Delta\theta$ should be linear to $[GC]$, K_2 being obtained from the slope and the known values of $\Delta\theta_{0,max}$, K_1 and $[Mab]_0$. The data of SPR in Fig. 6 are replotted in Fig. 8(b). The obtained linear correlation gave $K_2 = 4.64 \times 10^7$ M $^{-1}$. The evaluated K_2 by SPR is the affinity constant between GC-MAb and free GC, which corresponds to K_A evaluated from ELISA. The value of K_2 (4.64×10^7 M $^{-1}$) from SPR is approximately two-fold higher than that (2.05×10^7 M $^{-1}$) obtained from our ELISA data by using Seligman's method. The reason for this difference is not yet clear, but one of the reasons would be neglecting the bivalent nature of IgG from Eqs. (1) to (4). Seligman's method also has a vulnerable point, because the method does not take into account the interaction between GC-MAb and immobilized GC-BSA, which causes the difference of K_A due to the influence of the amount of solid-phase antigen. One of the advantages of the SPR evaluation method over the ELISA methods is to evaluate each affinity constant between the antibody and the solid-phase antigen (K_1) and between the antibody and the free antigen (K_2). As a whole, we can say that the affinity constant (K_A) between GC-MAb and GC is on the order of 10^7 M $^{-1}$.

References

1. K. Ohtsuki and N. Ishida, *Biochem. Biophys. Res. Commun.*, **1988**, 157, 597.
2. R. Doll, I. D. Hill, C. Hutton, and D. L. Underwood, *Lancet*, **1962**, 2, 793.
3. R. Pompei, O. Flore, M. A. Marccialis, A. Pani, and B. Loddo, *Nature*, **1979**, 281, 689.
4. T. Hattori, S. Ikematsu, A. Koito, S. Matsushita, Y. Maeda, M. Hada, M. Fujimaki, and K. Takatsuki, *Antiviral Research*, **1989**, 11, 255.
5. T. G. van Rossum, A. G. Vulto, R. A. de Man, J. J. Brouwer, and S. W. Schalm, *Alimentary Pharmacology & Therapeutics*, **1998**, 12, 199.
6. E. S. Ong and S. M. Len, *Anal. Chim. Acta*, **2003**, 482, 81.
7. H. R. Ren and S. J. Sheu, *J. Chromatogr. A*, **1993**, 653, 184.
8. S. Shan, H. Tanaka, and Y. Shoyama, *Anal. Chem.*, **2001**, 73, 5784.
9. F. Deckert and F. Legay, *Anal. Biochem.*, **1999**, 274, 81.
10. T. P. Vikinge, A. Askendal, B. Liedberg, T. Lindahl, and P. Tengvall, *Biosens. Bioelectron.*, **1998**, 13, 1257.
11. K. V. Gobi, S. J. Kim, R. Harada, and N. Miura, *Electrochem.*, **2003**, 71, 430.
12. N. Miura, M. Sakai, G. Sakai, and K. V. Gobi, *Chem. Lett.*, **2002**, 31, 342.
13. N. Miura, M. Sakai, K. V. Gobi, C. Kataoka, and Y. Shoyama, *Biosens. Bioelectron.*, **2003**, 18, 953.
14. S. Krahenbuhl, F. Hasler, and R. Krapf, *Steroids*, **1994**, 59, 121.
15. G. Cantelli-Forti, M. A. Raggi, F. Bugamelli, F. Maffei, A. Villari, and N. M. Trieff, *Pharmacol. Res.*, **1997**, 35, 463.
16. B. Friguet, A. F. Chaffotee, L. Djavadi-Ohanian, and M. E. Goldberg, *J. Immunol. Methods*, **1985**, 77, 305.
17. F. Stevens, *Mol. Immunol.*, **1987**, 24, 1055.
18. S. J. Seligman, *J. Immunol. Methods*, **1994**, 168, 101.
19. S. Hetherington, *J. Immunol. Methods*, **1990**, 131, 195.
20. G. Sakai, K. Ogata, T. Uda, N. Miura, and N. Yamazoe, *Sens. Actuators B*, **1998**, 49, 5.
21. G. Sakai, S. Nakata, T. Uda, N. Mira, and N. Yamazoe, *Electrochim. Acta*, **1999**, 44, 3849.

Fig. 8 Graphic analyses for evaluating K_1 and K_2 . (a) Relationship between $[Mab]$ and $[Mab]/\Delta\theta$; (b) relationship between $[GC]$ and $1/\Delta\theta$.