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# Isolation of chromosaponin I-specific antibody by affinity chromatography

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**Abstract**

Chromosaponin I (CSI), a γ-pyronyl-triterpenoid saponin isolated from pea and other leguminous plants, modulates several developmental processes of plant roots and activates the sugar taste receptor cells in blowflies. CSI is a unique saponin for its reducing power and biological activities in both plants and insects. In the present paper, we described the method of preparation for CSI-specific antibody using CSI-affinity and soyasaponin I-affinity columns. The antibody's-specific binding activity to CSI was confirmed by a bioassay using *Arabidopsis* roots and a ligand–molecule interaction analysis using BIAcore 3000. Because of the lability of CSI, the CSI-affinity column was made only by a moderate reaction condition in which CSI was coupled to EAH Sepharose 4B in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC). The special control of the reaction temperature was essential to complete the coupling reaction; the reaction with EDC at 0 °C followed by a gradual increase in temperature.


*Keywords:* Affinity chromatography; Antibody; BIAcore; Chromosaponin I; Coupling reaction; EDC; Saponin

Saponins, i.e., sapogenin glycosides, are widely distributed in plants and a great number of saponins have been reported. Chromosaponin I (CSI), a γ-pyronyl-triterpenoid saponin, was isolated from pea [1] and other leguminous plants [2–4]. It is an amphipathic natural reductant [5] and shows antioxidative activity in several assays [6,7]. CSI stimulates the growth of roots in a variety of plants [8,9] and in *Arabidopsis* it modulates several root growth developmental processes including cell elongation, gravitropic response, and root hair development by interacting with auxin influx carrier protein AUX1 [10,11]. CSI also has been shown to stimulate the sugar taste receptor cells by activating a G protein-mediated transduction in the blowfly, *Phormia regina* [12,13]. All these findings along with high concentration (2–3 mM) of CSI in the actively growing tissue of pea seedlings prompted us to elucidate the role of CSI in plants. However, the intracellular localization of this compound is still unknown. To clarify the role of CSI as well as its localization in plants, we tried to isolate CSI-specific antibody from the polyclonal antibody raised against CSI. To this end CSI-affinity column and soyasaponin I (SI)-affinity column are required. SI is an analogue of CSI and has the same sugar and triterpenoid moieties as CSI but does not have the γ-pyronyl moiety (Fig. 1). CSI-specific antibody is expected to be obtained from the fraction which does not bind to SI-affinity column but binds to CSI-affinity column. Our idea is that antibodies having an affinity to the sugar moiety of CSI including rhamnose, galactose, and glucuronic acid can be removed by passing the SI-affinity column. However, we found difficulties in preparing the CSI-affinity column because of the labile property of CSI. CSI has been decomposed by many kinds of coupling reagents we have tested.

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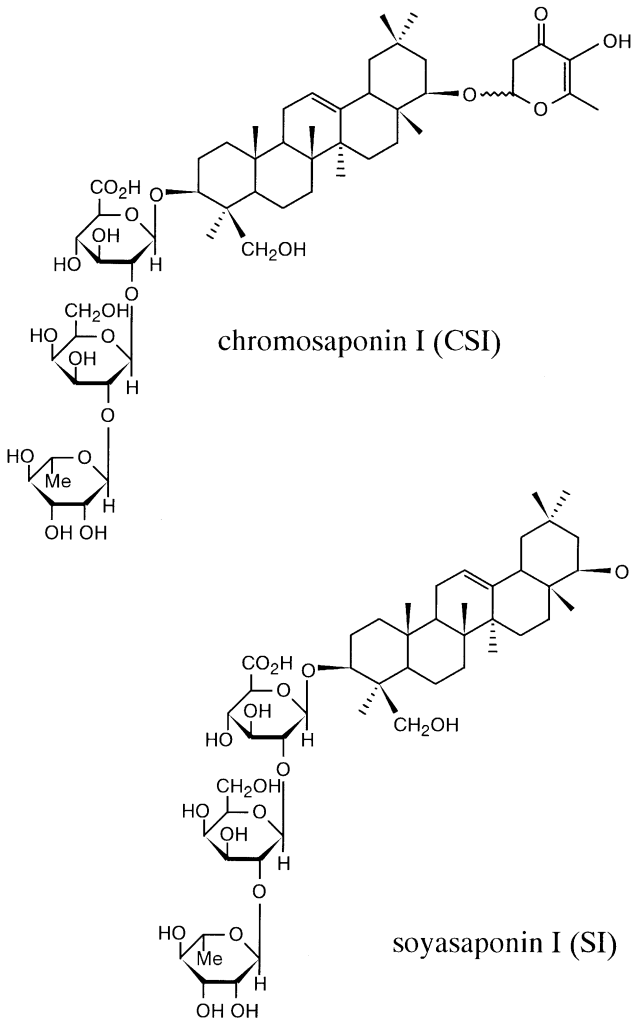


Fig. 1. Chemical structures of CSI and SI.

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) is the only coupling reagent which is compatible with CSI. Although the standard method for EDC to couple CSI with EAH Sepharose 4B was unsuccessful, we could complete the coupling reaction by controlling the reaction temperature. The problem of making affinity column for labile compounds would be overcome by this method.

## Materials and methods

*Chemicals*. CSI (Fig. 1) was isolated from pea and purified as described previously [1]. SI (Fig. 1) was originally isolated from soybean [14], but we prepared it by alkaline hydrolysis of CSI [1]. Protein A immobilized on Sepharose CL-4B, bovine serum albumin (BSA), glycyrrhizin, digitonin, tomatin, and Quillaja saponin were purchased from Sigma Chemical (St. Louis, MO, USA). EAH Sepharose 4B was from Amersham–Pharmacia Biotech AB (Uppsala, Sweden). Keyhole limpet hemocyanin (KLH) was from Calbiochem–Novabiochem (La Jolla, CA, USA). Freund's complete and incomplete adjuvants were from Difco Labs. (Detroit, MI, USA).

1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was from Peptide Institute (Osaka, Japan). Tea saponin, *Mycobacterium butyricum* Inactive, *Bordetella pertussis* Inactive, and tris(hydroxymethyl)aminomethane (Tris) were from Wako Pure Chemical Industries (Osaka, Japan).

3-(*N*-morpholino)-propanesulfonic acid (MOPS) and 2-(*N*-morpholino)-ethanesulfonic acid (MES) were from Dojindo Lab. (Kumamoto, Japan). Yucca saponin and Mukurozi saponin were the gifts from Maruzen Pharmaceuticals (Hiroshima, Japan).

*Immunization*. The immunogen was prepared by binding CSI to KLH. Two milligrams CSI was allowed to react with 2 mg KLH in the presence of EDC in 700 μl of 0.1 M MES buffer (pH 5). The resulting CSI–KLH conjugate was dialyzed against PBS (0.1 M sodium phosphate, 0.1 M NaCl, pH 7.2) overnight and washed 4 times with the buffer by repeated centrifugation at 10,000 rpm at 4 °C for 5 min. The washed immunogen was stored at −20 °C. A rabbit was immunized subcutaneously with 0.2 mg CSI–KLH conjugate in Freund's complete adjuvant including 5 mg of *M. butyricum* Inactive and further injected subcutaneously with 50 μl *B. pertussis* Inactive in 450 μl PBS. After 14 days, it was immunized subcutaneously with 0.2 mg CSI–KLH conjugate in Freund's incomplete adjuvant. Booster dose was given in every 2 week interval. Both non-immunized and immunized sera were stored at −80 °C.

*Measurement of antibody titer by ELISA*. Anti CSI–BSA titers in sera were determined by ELISA. Wells of microtiter plates (MaxiSorp, Nunc, Rockilde, Denmark) were coated with 50 μl CSI–BSA conjugate dissolved in PBS. CSI–BSA conjugate was prepared by the same method as CSI–KLH conjugate. The plates were incubated for 2 h at room temperature and then washed with 100 μl T-TBS (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Each well was added by 250 μl of 1% BSA as blocking agent, incubated for 2 h, and then washed with 150 μl T-TBS. Rabbit sera diluted with T-TBS were added (50 μl/well) to the CSI–BSA coated plate and left overnight at 4 °C. The plates were washed with 100 μl T-TBS. Then alkaline phosphatase-labeled goat anti-rabbit antibody (Cappel) diluted 1:2000 in T-TBS was added to each well and incubated for 1 h. After washing the plate twice with 100 μl T-TBS, 50 μl of a substrate solution (6.45% triethanolamine, 1 mM magnesium chloride, 0.1% *p*-nitrophenylphosphoric acid disodium salt, and 0.02% sodium azide) was added to each well. The plates were kept at 37 °C and color development was stopped with an addition of 0.1 N NaOH (50 μl/well). Absorbance at 405 nm was read using a microplate reader EIA-READER (Bio-Rad Lab. USA). Anti CSI–BSA titers in sera increased gradually 2 weeks after the first immunization and reached its peak in 18 weeks. We used the 18-week sera for the following experiments.

*Isolation of total antibody using a protein A column*. The pH of 18-week sera was adjusted to 8.0 by adding 1/10 volume of 1.0 M Tris (pH 8.0). The sera of 1 ml were applied to 1 ml of a protein A bead column (protein A immobilized on Sepharose CL-4B) equilibrated with 100 mM Tris (pH 8.0). The beads were then washed with 10 column volumes of 100 mM Tris (pH 8.0) followed by a wash of 10 column volumes of 10 mM Tris (pH 8.0). The total antibody (Total-IgG) was eluted with 100 mM glycine (pH 3.0). Each 500 μl eluate was collected in a 1.5 ml Eppendorf tube containing 50 μl of 1.0 M Tris (pH 8.0). Each tube was mixed gently to bring the pH back to neutral and kept at −80 °C. The protein content of Total-IgG obtained from 1 ml of sera was about 94 μg. Protein content was determined by the method of Bradford [15].

*Preparation of CSI-affinity column*. One milliliter of EAH Sepharose 4B pre-swollen gel was washed with 20 ml of 0.5 M NaCl. Eight milligrams of CSI was dissolved in 2 ml of deionized water by adding minimal amount of KOH. The pH of the solution was kept lower than 8 because CSI is destroyed in alkaline pH higher than 9. The CSI solution was mixed with the washed gel by stirring slowly at 0 °C. EDC 19.2 mg in powder was added three times with at least 1 h interval between each addition to make 0.1 M finally while keeping the temperature at 0 °C. The solution was further stirred on ice for at least 6 h. And then the temperature of solution was increased gradually to room temperature (line c in Fig. 2A). The total time from the beginning until the end of the reaction was 24 h. The gel was washed with deionized

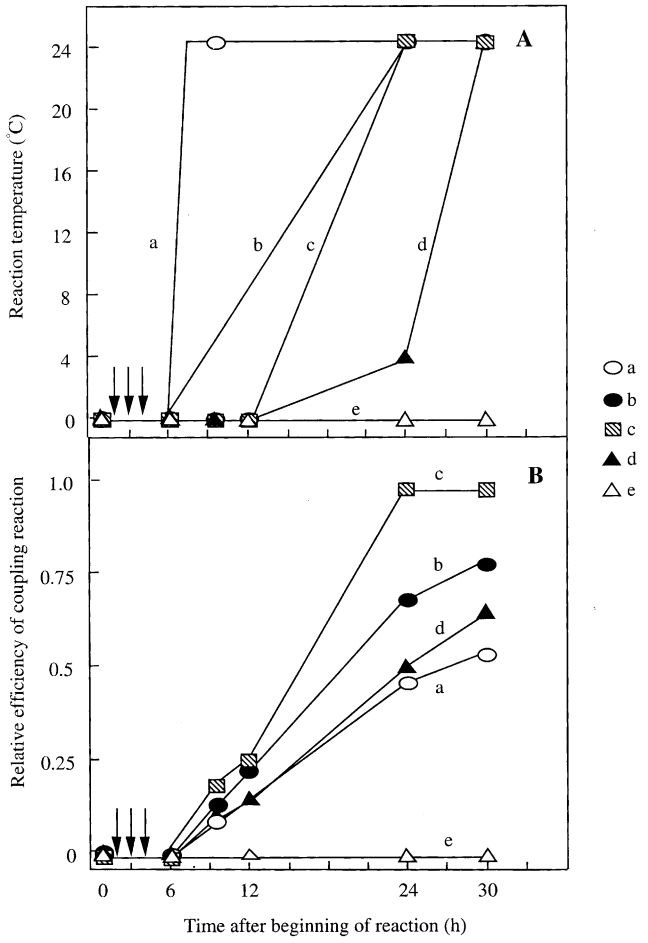


Fig. 2. Temperature schedule for the coupling reaction of CSI and EAH Sepharose 4B with EDC (A) and the relative efficiency of the coupling reaction (B). The schedule c resulted in the highest efficiency. Arrows indicate the addition of EDC three times.

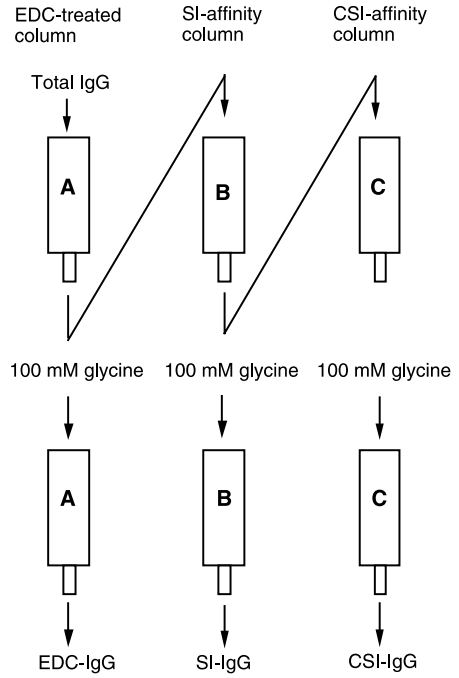


Fig. 3. A schematic diagram showing the method for isolating CSI-specific antibody. Total antibody (Total-IgG) was passed serially through EDC-treated column (A), SI-affinity column (B), and CSI-affinity column (C). To ensure complete binding to columns, each eluate was further passed through the same column twice. Antibodies bound to each column were eluted with 100 mM glycine (pH 3.0) separately from the three columns after washing the columns with 10 mM Tris buffer (pH 8.0) and 10 mM Tris including 0.5 M NaCl (pH 8.0).

water several times and then packed into a column (disposable polystyrene columns, 2 ml; Pierce, USA). Since CSI has an absorption peak at 295 nm (log $\varepsilon$, 3.89 in water), the efficiency of the coupling reaction was determined by measuring the absorbance of gel suspension at 295 nm after washing the gel with 80% methanol. SI-affinity column was prepared in the same way using 8 mg SI. As a control, we also prepared EDC-treated column by treating the gel with EDC in the absence of saponin.

*Purification of CSI-specific antibody*. CSI- and SI-affinity columns and EDC-treated column were washed with 10 bed volumes of 10 mM Tris (pH 8.0). Then they were washed with 10 bed-volumes of 100 mM glycine (pH 3.0), followed by 10 bed-volumes of 10 mM Tris (pH 8.0). The total antibody solution was passed serially through these three types of columns (Fig. 3, first the EDC-treated column, second the SI-affinity column, and third the CSI-affinity column). To ensure complete binding to columns, each eluate was further passed through the same column twice. The columns were washed serially with 20 bed-volumes of 10 mM Tris (pH 8.0) and 20 bed-volume of 10 mM Tris containing 0.5 M NaCl (pH 8.0). The antibody bound to the columns was then eluted by acid-sensitive interaction by passing 10 bed-volumes of 100 mM glycine (pH 3.0). The eluate was collected in a tube containing 1 bed volume of 1 M Tris (pH 8.0). EDC-IgG, SI-IgG, and CSI-IgG fractions were the eluates from EDC-treated, SI-affinity, and CSI-affinity columns, respectively. The volume of antibody solution was reduced to 1/10 using protein A–Sepharose bead column. Protein content of each fraction obtained from 3 ml of sera was 10 μg (EDC-IgG), 4.2 μg (SI-IgG), and 6.25 μg (CSI-IgG), respectively.

*Root growth bioassay*. Wild type of *Arabidopsis thaliana* (L) Heynh., ecotype Landsberg erecta was used as plant material to see the effect of CSI-specific antibody on CSI action. Methods for the bioassay were described earlier [9]. In brief, *Arabidopsis* seeds were germinated in 2.6 cm Petri dish on filter paper (advantec no. 2; Toyo Roshi Kaisha, Tokyo, Japan) wetted with 300 μl of 20 mM MOPS buffer with salts (pH 6.6), and the seedlings were allowed to grow in the presence and absence of 300 μM CSI with or without antibody in the light at 23 °C for 3 days. The length of roots was measured under a microscope and the means (±SE) for 10–15 seedlings was calculated. Each assay was repeated at least 6 times.

*Ligand molecular interaction analysis with BIAcore 3000*. BIAcore 3000 system, BIAevaluation software 3.0, Sensor Chip CM5 (coated with carboxyl methyl dextran matrix, research grade), and amine coupling kit were purchased from BIAcore AB (Uppsala, Sweden). The carboxyl groups on the surface of sensor CM5 were activated with 0.2 M EDC and 0.05 M *N*-hydroxysuccinimide (NHS). The specific surface was obtained by injecting CSI-IgG (25 μg/ml) in 10 mM acetate buffer (pH 5.0). The immobilization procedure was completed by an injection of 1 M ethanolamine hydrochloride to block the remaining ester groups. Association with saponins was measured in concentrations ranging from 10 to 100 μM in 50 mM MOPS (pH 7.2) for 3 min using a flow rate of 40 μl/min. Dissociation of bound saponins was measured with 300 mM glycine buffer (pH 3.0). Saponin solutions were allowed to flow into two pathways; one is CSI-IgG coated dextran and the other is a plain dextran matrix (control). The response shown in Fig. 5A is the difference between the two measurements. $K_D$ was determined from the steady state affinity plot using BIAevaluation 3.0 software.

## Results and discussion

### *Preparation of CSI-affinity column*

To purify the CSI-specific antibody from total antibody, we used EDC-treated column, SI- and CSI-affinity columns. To prepare the CSI-affinity column we tested several linkers, but most of them destroyed the CSI molecule. EDC was the only safe linker for CSI. However, the standard method using EDC was not successful to couple the CSI molecule with EAH Sepharose 4B. To complete the coupling reaction, special care on the reaction temperature was required. Fig. 2 shows the typical results of reaction temperature on the coupling reaction. The temperature of reaction solution should be 0 °C before starting the reaction and kept at 0 °C while EDC reacts with CSI. The gradual increase of temperature from 0 °C to room temperature accelerated the coupling reaction. Continuous treatment at 0 °C or room temperature was not successful for the coupling. The highest coupling efficiency was obtained with the temperature schedule shown in line c of Fig. 2A. We suspect that the low temperature reduces the steric hindrance between EDC-activated CSI molecule and EAH Sepharose 4B and permits the close association of the two molecules, and then the gradual increase of temperature stimulates the coupling reaction. A rapid increase of reaction temperature (line a in Fig. 2A) or a slower increase of it (line d in Fig. 2A) resulted in less efficiency of the coupling compared with the line c. The EDC-treatment at 0 °C and a gradual increase of reaction temperature were found to be the essential steps to complete the coupling reaction. This method would resolve the problem of making affinity column for labile compounds.

### *Purification of CSI-specific antibody*

SI is an analog of CSI (Fig. 1). The sugar moiety and the triterpenoid moiety of SI are the same as those of CSI but the former does not have the γ-pyronyl moiety. The CSI-specific antibody is expected to be obtained from the fraction which does not bind to both EDC-treated and SI-affinity columns but binds to CSI-affinity column. The total antibody was passed serially through these three types of columns as shown in Fig. 3. The antibody fractions EDC-IgG, SI-IgG, and CSI-IgG are the eluates with 100 mM glycine (pH 3.0) from EDC-treated column, SI-affinity column, and CSI-affinity column, respectively.

### *Root growth bioassay*

To examine the activity of antibody obtained above, we performed bioassay using *Arabidopsis* seedlings. CSI stimulates the growth of *Arabidopsis* roots by accelerating cell division and cell elongation [9] as shown in control of Fig. 4. The content of each antibody in a test solution was adjusted to the amount equivalent to 1/100 of 3 ml of serum. Antibody itself did not show any effect on the root growth (data not shown). In the presence of CSI, the CSI-IgG fraction counteracted the stimulatory effects of CSI, whereas SI-IgG, EDC-IgG, and buffer alone did not show any effect.

### *Ligand–molecule interaction analysis of CSI-specific antibody with saponins*

Having demonstrated that CSI and the purified antibody interact directly by affinity chromatography, we determined the $K_D$ for their interaction using a surface plasmon resonance detection machine BIAcore 3000. This method and technology has been widely accepted to investigate the ligand–molecule interaction [16]. The BIAcore 3000 has an advantage of analyzing the binding of low molecular weight compound such as CSI (mw 1068). The CSI-IgG was immobilized to the surface with a dextran matrix CM5. Our initial investigation into the interaction between CSI and the purified antibody on the BIAcore found that the on and off rates for the interaction were too fast to be amenable to a kinetic analysis. Therefore, we utilized an equilibrium approach on the BIAcore to determine the dissociation constant [16]. Various concentrations of CSI solutions were passed over the antibody covalently attached to a CM5 sensor chip. The resulting sensograms were subtracted from the data as background. Fig. 5A shows an overlay plot of sensograms representing CSI injections of varying concentrations. It shows the typical response curves when CSI or SI was flowed in the concentration range from 10 to 60 μM and the following dissociation curves

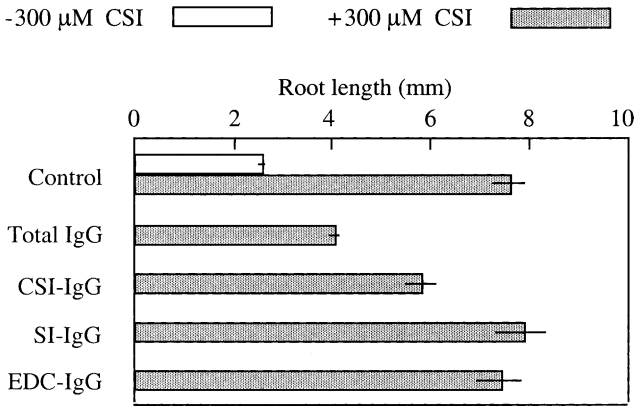


Fig. 4. The effect of antibodies on CSI action in the root growth assay. *Arabidopsis* seedlings were grown on filter paper wetted with 20 mM MOPS buffer (pH 6.7) in the presence (shaded bars) and absence (blank bars) of 300 μM CSI without (control) or with antibodies in the light for 3 days. The amount of each antibody in a test solution was adjusted to equivalent to 1/100 of 3 ml of serum. Control included the same buffer as Total-IgG, EDC-IgG, SI-IgG, and CSI-IgG. Data are the averages from 10 to 15 seedlings (±SE).

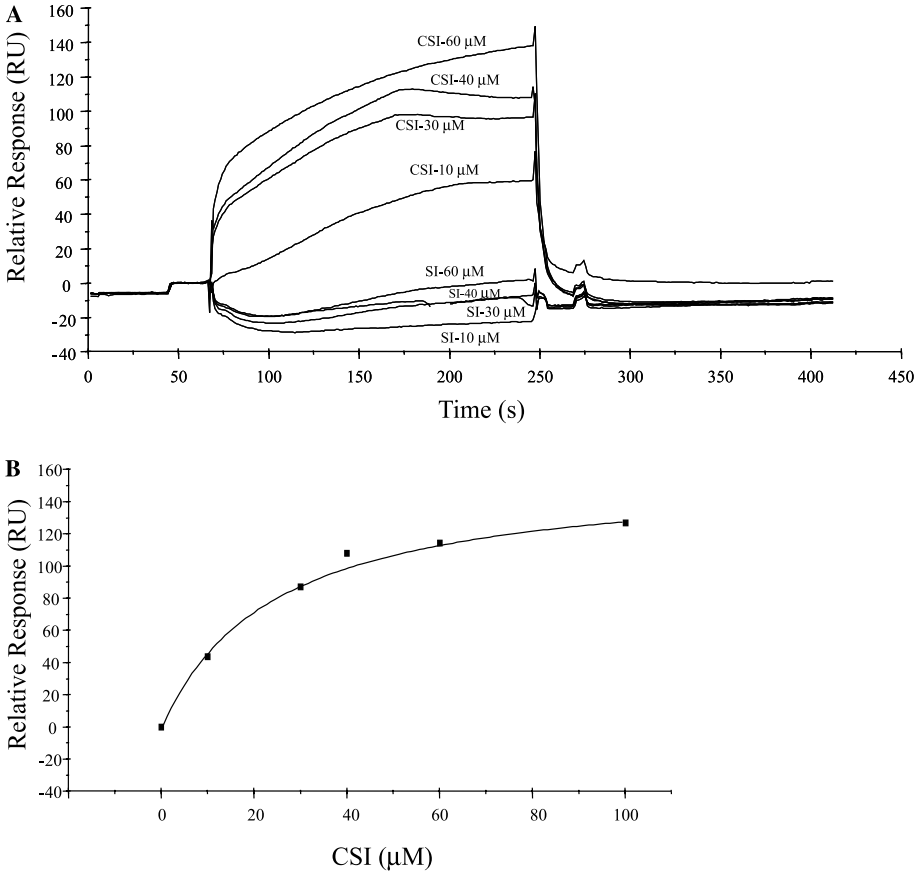


Fig. 5. Determination of $K_D$ for the interaction of CSI with CSI-specific antibody. (A) Response curves showing the association of saponins with CSI-specific antibody (CSI-IgG) immobilized on the sensor of BIAcore 3000. Association with CSI or SI was measured in concentrations ranging from 10 to 60 μM in 50 mM MOPS (pH 7.2) for 3 min using a flow rate of 40 μl/min. Dissociation of bound saponins was measured with 300 mM glycine buffer (pH 3.0). Saponin solutions were allowed to flow into two pathways; one is CSI-IgG coated dextran and the other is a plain dextran matrix (control). The response is the difference between the two measurements. (B) The $R_{max}$ from response curve was plotted against various CSI concentration. Data are the averages from 5 experiments. The $K_D$ was obtained from fitting the data to a steady state affinity model and determined to be $2.4 \times 10^{-5}$ M.

when changed to glycine buffer. The response to CSI increased depending on the concentration of CSI, whereas SI did not show any clear binding response. The $R_{max}$ from sensogram was plotted as a function of the CSI concentration (Fig. 5B) and was fitted to the model of steady state affinity $(RU_{bound} = [CSI](RU_{max})/([CSI] + K_D))$. From this model, the dissociation constant $K_D$ for CSI was calculated as $2.4 \times 10^{-5}$ M. We also examined other saponins, Yukka saponin, Quillaja saponin, tea saponin, glycyrrhizin, digitonin, tomatin, and Mukurozi saponin, but could not find any binding response for the antibody (data not shown). Since the concentration of CSI in the hook or root tip of pea seedlings is 2–3 mM, the $K_D$ value of this antibody is enough to detect CSI in vivo. In the present paper, we directly demonstrated by affinity column chromatography and surface plasmon resonance that only CSI but no other saponin forms a tight complex with the purified CSI-specific antibody. We also described the method to solve the problem of making affinity column for labile compounds.

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