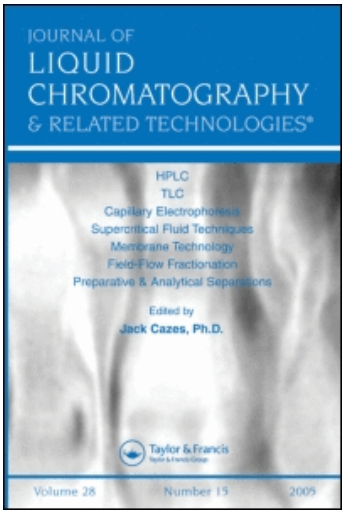

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IMMUNOAFFINITY COLUMN FOR ISOLATION OF BIOACTIVE COMPOUNDS USING MONOCLONAL ANTIBODIES

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ABSTRACT

Anti-solamargine and anti-ginsenoside Rb1 monoclonal antibodies were used for preparation of an immunoaffinity column. Total solasodine glycosides were separated directly from the crude extract of *Solanum khasianum* fruit, by the established immunoaffinity column. This method was specific for solasodine glycosides, which was detected by thin layer chromatography and eastern blotting. An immunoaffinity column, using anti-ginsenoside Rb1 monoclonal antibody, has made possible a single-step separation of ginsenoside Rb1 from a crude extract of ginseng roots (*Panax ginseng*).

Key Words: Immunoaffinity column; Monoclonal antibodies; ELISA

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INTRODUCTION

Monoclonal antibodies (MAbs) have many potential uses in addition to immunological methods to plant sciences. The immunoassay using MAbs against naturally occurring biologically active compounds having low molecular weights, have become an important tool[1] for the studies on receptor binding analysis, enzyme assay, and quantitative and/or qualitative analysis techniques in plants, owing to its specific affinity. In order to select the strain of higher yields of active compounds in plants, rapid and simple assay systems are required for a small amount of samples.

In our current investigation on the formation of MAbs against low molecular weights from natural products, we have reported previously the preparation of MAbs against ginsenoside Rb1, solamargine, their characterization and eastern blotting procedure using anti-solamargine, and ginsenoside Rb1 Mabs.[2–5] In extension of this approach, we describe, herein, the preparation of an imunoaffinity column using anti-solamargine and ginsenosside Rb1 MAbs for one-step-isolation of bioactive compounds from crude extracts of plants.

EXPERIMENTAL

Chemicals, Immunochemicals, and Plant Materials

Ginsenosides Rb1, Rc, Rd, Re, and Rg1 were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA) and human serum albumin (HSA) were obtained from Pierce (Rockford, IL, USA). Peroxidase labeled anti-mouse IgG was provided by Organon Teknika Cappel Products (West Chester, PA, USA). Polyvinylidene difluoride (PVDF) membranes (Immobilon-N) were purchased from Milipore Corporation (Bedford, MA). Glass microfilter sheets (GF/A) were purchased from Whatman International Ltd. (Maidstone, UK). All other chemicals were standard commercial products of analytical grade.

Fruit of *Solanum khasianum* was obtained from Faculty of Pharmaceutical Sciences, Nagasaki University, Japan. Solamargine and solasonine were isolated from fresh fruits of *S. khasianum* as previously described.[6] The roots of *Panax ginseng* were obtained from Tochimototennkaido Corporation (Osaka, Japan).

Purification of Monoclonal Antibodies

Monoclonal antibodies were purified using a Protein G FF column (0.46 × 11 cm, Pharmacia Biotech, Uppsala, Sweden) as previously reported.[5] The

cultured medium (500 mL) containing the IgG was filtered by a Millex-HV filter (0.45 μm filter unit, Millipore, Bedford, MA), subjected to the column, and the absorbed IgG was eluted with 100 mM citrate buffer (pH 3.0). The eluted IgG solution was neutralized with 1 M *tris*-HCl solution (pH 9.0), then dialyzed against H_2O, and finally lyophilized to give IgG.

Confirmation of the Purity of the MAb by Matrix-Assisted Laser Desorption Ionization-Time-of-Flight (MALDI-TOF) Mass Spectrometry

A small amount of the purified MAb was mixed with a 10^3-fold molar excess of sinapic acid in an aqueous solution containing 10% (w/v) trifluoroacetic acid. The mixture was inserted into a JMS-LDI 1700 time-of-flight mass monitor (JEOL, Japan) and irradiated with a N_2-laser (337 nm, 3 ns pulse). The ions formed by each pulse were accelerated by a 30 kv potential in a 1.7 m evacuated tube. The data were analyzed using a compatible computer.

Competitive ELISA

The individual fractions were determined by ELISA with modification, as reported previously.[2] A 96 well-immunoplate, which had been adsorbed by 100 μL of 2 μg/mL solamargine-HAS, was treated with 300 μL phosphate buffer saline (PBS) containing 5% skim milk. The plate was washed three times with PBS containing 0.05% Tween 20 (TPBS). Fifty micro liter samples diluted with 20% methanol were added to the above well. Fifty micro liter of MAb diluted with TPBS was further added to the well and then incubated for 1 h. The plate was mixed for 1 min. The plate was washed three times with TPBS. The plate was incubated with 100 μL of 1000 times-diluted peroxidase anti-mouse IgG for 1 h. After washing the plate three times with TPBS, 100 μL of substrate solution, 0.1 M citrate buffer (pH 4.0) containing 0.006% hydrogen peroxide and 0.3 mg/mL ABTS, were added to each well and incubated for 15 min. Absorbance at 405 nm was measured with a FAR 400 Electrophotometer (SLT-LABINTRUMENTS, Salzburg, Austria). All the reactions were carried out at 37°C. Competitive ELISA of ginsenoside Rb1 was also performed in the same manner.

Eastern Blotting

The fractions were applied to a TLC plate and developed with $CHCl_3$/MeOH/NH_3 (6 : 3 : 1) for solasodine glycosides and *n*-BuOH–EtOAc–H_2O

(15 : 1 : 4) for ginsenoside Rb1. The developed TLC plate was dried and then sprayed with a blotting solution mixture of *i*-PrOH/MeOH/H_2O (1 : 4 : 8, by vol.). It was placed on a stainless-steel plate, and then covered with a PVDF membrane sheet. After covering with a glass micro fiber filter sheet, the whole was pressed evenly 45 s with a 120°C hot plate as previously described[4,7] with modification. The PVDF membrane was separated from the plate and dried.

The blotted PVDF was dipped in water containing $NaIO_4$ (10 mg/mL) at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added, and stirred at room temperature for 3 h. The PVDF membrane was washed twice with TPBS for 5 min, and then washed with water. The PVDF was immersed in anti-solamargine MAb, stirred at room temperature for 1 h. After washing the PVDF membrane twice with TPBS and then water, 1000 times dilution of peroxidase-labeled goat anti-mouse IgG in GPBS was added and stirred for 1 h. The PVDF membrane was washed twice with TPBS and then exposed to 1 mg/mL 4-chloro-1-naphol 0.03% hydrogen peroxide in PBS solution, which was freshly prepared before use for 10 min at room temperature, and the reaction was stopped by washing with water. The immunostained PVDF membrane was allowed to dry. Eastern blotting of ginsenoside Rb1 was performed in the same manner.

Preparation of Immunoaffinity Column

Purified MAbs (2 mg for anti-solamargine MAb and 10 mg for anti-ginsenoside Rb1 MAb) was dialyzed against coupling buffer, pH 5.5, overnight at 4°C and after that MAb was oxidized with $NaIO_4$. The MAb oxidation was performed in a container covered with aluminium foil, and then mixed gently for 1 h at room temperature. Immediately after oxidation, glycerol was added at a final concentration of 20 mM, mixed for 10 min, and then dialyzed against a coupling buffer, pH 5.5 at 4°C. Oxidized MAb was added to a slurry of Affi-Gel Hz hydrazide gel (2 mL gel volume, Bio-Rad) in a coupling buffer and coupled by stirring at room temperature for 24 h. The eluant and washing solution (20 mM phosphate buffer, 0.5 M NaCl, pH 7.0) were combined, and the unbound protein was measured at 280 nm for determining coupling efficiency. The immunoaffinity gel was washed with PBS and packed in plastic mini-column. The gel was equilibrated with PBS contained 0.02% sodium azide and stored at 4°C. Preparation of an immunoaffinity column using anti-ginsenoside Rb1 was also performed in the same manner.

Immunoaffinity Column Chromatography for Isolation of Solasodine Glycosides

Protocol for Immunoaffinity Column Chromatography

The procedure was carried out at room temperature, except incubation. The immunoaffinity column was washed with PBS before use. A sample dissolved in PBS was loaded onto the immunoaffinity column. The loaded column was incubated at 4°C for 2 h with the flow stopped, and then washed with PBS. The column was eluted with 40% MeOH in PBS (15 mL). After elution of solasodine glycosides, the immunoaffinity column was washed with PBS, equilibrated with PBS containing 0.02% of sodium azide, and then stored at 4°C until subsequent use.

Determination of Absorption and Elution for Immunoaffinity Column

The capacity of an immunoaffinity column was determined. The immunoaffinity column (2 mL of gel) was loaded with solamargine (40 μg), solasonine (40 μg) and solasodine (20 μg) in PBS, which was added separately. The column was incubated at 4°C for 2 h, washed with PBS, and then monitored by ELISA until the substance disappeared. The loaded immunoaffinity column was eluted with 40% MeOH in PBS, and the concentration of each fraction (1 mL) was analyzed by ELISA.

Purification of Steroidal Alkaloid Glycosides by Immunoaffinity Column

The dried powder of *S. khasianum* fruit (20 mg) was extracted with MeOH (0.5 mL) five times, using an ultrasonic bath for 15 min. After filtering with a 0.45 μm filter, the filtrate was dried under N_2 stream and a vacuum drying oven. The residue was redissolved with MeOH and diluted with PBS. The solution was loaded on the immunoaffinity column and stood at 4°C for 2 h. The column was washed with the washing buffer solution. After the solasodine glycosides had disappeared, the column was eluted with 40% MeOH in PBS at 0.1 mL/min. The total solasodine glycosides concentration was assayed by ELISA. Solasodine glycosides were developed by TLC, and then sprayed with H_2SO_4 compared with the western blotting on PVDF membrane.

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Immunoaffinity Column Chromatography for Isolation of Ginsenoside Rb1

Protocol for Immunoaffinity Column Chromatography

The procedure was carried out at room temperature, except for incubation. The immunoaffinity column was washed with PBS before use. A sample dissolved in PBS was loaded onto the immunoaffinity column. The loaded column was incubated at 4°C for 2 h with the flow stopped, and then washed with PBS. The column was eluted with 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0). After elution of ginsenoside Rb1, the immunoaffinity column was washed with PBS, equilibrated with PBS containing 0.02% of sodium azide, and then stored at 4°C until subsequent use.

Determination of Adsorption, Elution Conditions, and Capacity for Immunoaffinity Column

Ginsenoside Rb1 (400 μg) was dissolved in PBS (3.5 mL) and loaded. After the column was incubated at 4°C over night, it was washed with PBS until the ginsenoside Rb1 disappeared by analysis with ELISA. The immunoaffinity column loaded with ginsenoside Rb1 was eluted with various solvent systems, and ginsenoside Rb1 contents in individual fractions (10 mL each) were determined by ELISA and eastern blotting.

The ginsenoside Rb1 loaded immunoaffinity column was eluted with 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0), and the total ginsenoside Rb1 content was analyzed by ELISA to determine its capacity.

Purification of Ginsenoside Rb1 from the Crude Extractives of Roots of *P. ginseng* by Immunoaffinity Column

The extracts of ginseng root was redissolved in PBS and then filtered by a Millex-HV filter (0.45 μm, Millipore) to remove insoluble portions. The filtrate was loaded on the immunoaffinity column and allowed to stand overnight at 4°C. The column was washed with the washing buffer solution (40 mL), and then eluted with 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH (pH 4.0, 20 mL). The ginsenoside Rb1 containing fraction was concentrated and surveyed by TLC developed with *n*-BuOH–AcOEt–H_2O (15 : 1 : 4), followed by eastern blotting. Ginsenoside Rb1 contaminated with malonyl ginsenoside Rb1 was treated with 0.1% of KOH in MeOH, at room temperature for 1 h, to give pure ginsenoside Rb1.

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RESULTS AND DISCUSSION

Immunoaffinity Purification of Solasodine Glycosides Using Wide-Cross Reactive Anti-Solamargine Mab

After MAb from the original clone (SMG-BD9) was purified by Protein G Sepharose 4FF column, the MALDI-TOF mass spectrometry was measured to confirm the purity of the MAb (SMG-BD9) as previously reported.[8] The molecular weight was 148,700, which is in good agreement with that of human IgG1 being determined as 146,000.[9] It became evident that the purified Mab, using a protein G column, can be used for the affinity column.

The purified MAb was coupled to Affi-Gel Hz hydrazide gel to give an immunoaffinity gel (7.54 μmol of MAb/2 mL gel). The elution system for the immunoaffinity column was investigated by using various elution buffers (data not shown). It appeared that PBS containing 40% MeOH was most effective and suitable for elution of solasodine glycosides.

To assess the capacities and the recoveries of solamargine, solasonine, and solasodine from the immunoaffinity column, each substance was added separately and run through the column. The column was washed with PBS, and then eluted with PBS containing 40% MeOH. The content of individual fractions were determined by ELISA. The established elution buffer system resulted in 95.31%, 97.20%, and 95.80% recovery of solamargine, solasonine, and solasodine, respectively. The capacity of the immunoaffinity column was determined to be 6.19, 12.92, 3.92 μg of solamargine, solasonine, and solasodine, respectively, per mL of immunoaffinity gel.

The crude extract of *S. khasianum* fruit was loaded on the immunoaffinity column, washed with PBS, and eluted with 40% MeOH in PBS. Figure 1 shows a chromatogram detected by ELISA. Fraction 1–8 contained over loaded solasodine glycosides like solamargine, solasonine, *L*-rhamnosyl-(1 → 4)-*O*-3-*β*-*D*-glucopyranosyl solasodine, and the other non-related unknown compounds, which were detected by TLC stained with H_2SO_4, as indicated in Fig. 2(B). The peak of fractions 22–29 shows the elution of total solasodine glycosides eluted with 40% MeOH in PBS. The fractions contained only solamargine, solasonine, and *L*-rhamnosyl-(1 → 4)-*O*-3-*β*-*D*-glucopyranosyl solasodine, which were determined by TLC stained with H_2SO_4 and the western blotting (Fig. 2-A,B). The above over loaded solasodine glycosides can be separated completely from the non-related unknown compounds by repeated column chromatography (data not shown). This result indicates, that solasodine-type steroidal alkaloids can be separated by the established immunoaffinity column.

In this investigation, PBS solution containing 40% MeOH was used for elution buffer, which proves most suitable for separation. The stability of antibody against PBS containing 40% MeOH is high, since the immunoaffinity column can

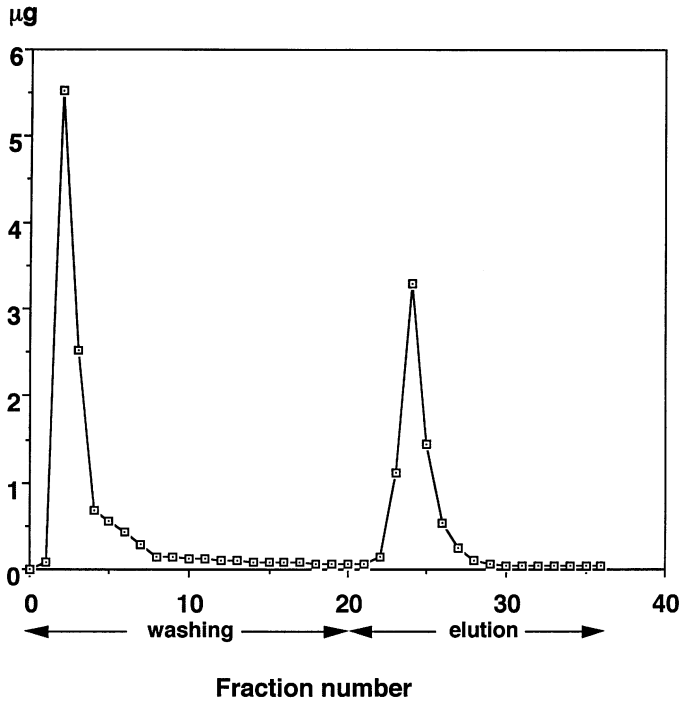

Figure 1. Elution profile of solasodine glycosides using an immunoaffinity column from the crude extract of *S. khasianum* fruit.

be used over 10 times or more under the same conditions, without any substantial loss of capacity. The capacity of the column for solasodine was determined to be 3.92 μg lower, compared to solamargine (6.19 μg) and solasonine (12.92 μg), due to smaller cross-reactivity (44%) with anti-solamargine Mab.[2] Since all solasodine glycosides can be cross-reacted with anti-solamargine Mab,[12,13] it is pointed out that the mixture of total solasodine glycosides can be separated by the immunoaffinity column. Therefore, this method can be available for the rapid and simple separation of total solasodine glycosides.

To expand this method, the crude extract of *S. khasianum* fruit was subjected onto the affinity column. In order to confirm the evidence of solasodine glycosides elution, TLC stained with H_2SO_4 and the western blotting were carried out to indicate the clear separation profile. It becomes clear that a single immunoaffinity column can separate only solasodine glycosides, which is as important as the starting materials of steroidal hormones from the crude extract of *S. khasianum* fruit.

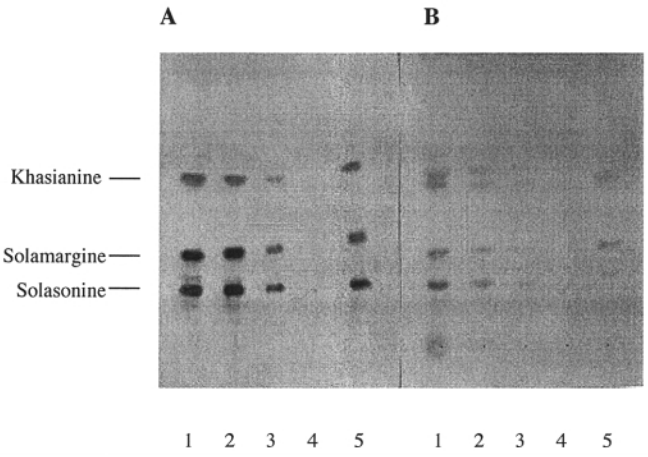

Figure 2. The profile of eastern blotting (A) and TLC stained with H_2SO_4 (B) separated by the affinity column as indicates in Fig. 1. Lane 1, Crude extract of *S. khasianum* fruit; Lane 2–4, washing fractions with PBS; Lane 5, eluting fraction with PBS containing 40% MeOH.

Single Step Isolation of Ginsenoside Rb1 from Crude Extract of Ginseng by Immunoaffinity Column Using an Anti-Ginsenodside Rb1 Mab

Since the ginseng root contains a number of dammarane-type saponins, ginsenosides together with oleanane-type saponins, the isolation of a saponin is very troublesome, requiring the repeated silica gel column chromatography. For this evidence, we established a simple and reproducible purification method for ginsenoside Rb1, using an immunoaffinity column conjugated with a purified anti-ginsenoside Rb1 MAb.

The concentration of ginsenoside Rb1 increased little by elution with the 20 mM phosphate buffer containing 0.5 M KSCN and 10% CH_3OH. If the 20 mM phosphate buffer was changed to the 100 mM AcOH buffer (pH 4.0), the elution ability reaches the optimum. Although, 20% MeOH enhanced the elution of ginsenoside Rb1, higher concentrations than 20% of MeOH did not affect the sample. From these results, 100 mM AcOH buffer containing 0.5 M KSCN and 20% MeOH can be routinely used as an elution buffer solution. The antibody was stable when exposed to the eluent, and the immunoaffinity column showed almost no decrease in capacity (20 μg/mL gel) after repeated use, more than 10 times under the same conditions.

In a preliminary trial, a ginsenosides mixture, ginsenoside-Rg1, -Re, -Rd, -Rc, and -Rb1, and chikusetsusaponin IV (an oleanane type saponin), were

separated by the newly prepared affinity column, using the separation solvent system. Figure 3 shows TLC and the eastern blotting profiles of these ginsenosides and chikusetsusaponin IV separated, using this method. When the column was washed with the washing solvent, ginsenoside-Rg1, -Re, -Rd, -Rc, chikusetsusaponin IV, and over-charged ginsenoside Rb1 appeared. After these compounds were washed out, the combined ginsenoside Rb1 was eluted using the elution solvent.

The crude extracts of *P. ginseng* root were loaded on the immunoaffinity column, and washed with 20 mM phosphate buffer containing 0.5 M NaCl (40 mL). Figure 4 shows the fractions 1 to 14 containing over-charged ginsenoside-Rb1, which was determined by ELISA. Ginsenoside Rc, ginsenoside Rd, ginsenoside Re, and ginsenoside Rg1 were detected by eastern blotting. A sharp peak appeared around fractions 20 to 24; they contained ginsenoside Rb1 determined by ELISA. However, this ginsenoside Rb1, purified by the immunoaffinity column, was still contaminated by a small amount of malonyl ginsenoside Rb1, as detected by eastern blotting. This compound has almost the same cross-reactivity with ginsenoside Rb1 as indicated previously.[3] Therefore, the mixture was treated with a mild alkaline solution at room temperature for 1 h, as previously reported,[10] to give pure ginsenoside Rb1. Over charged ginsenoside Rb1, eluted with washing solution, was repeatedly loaded and

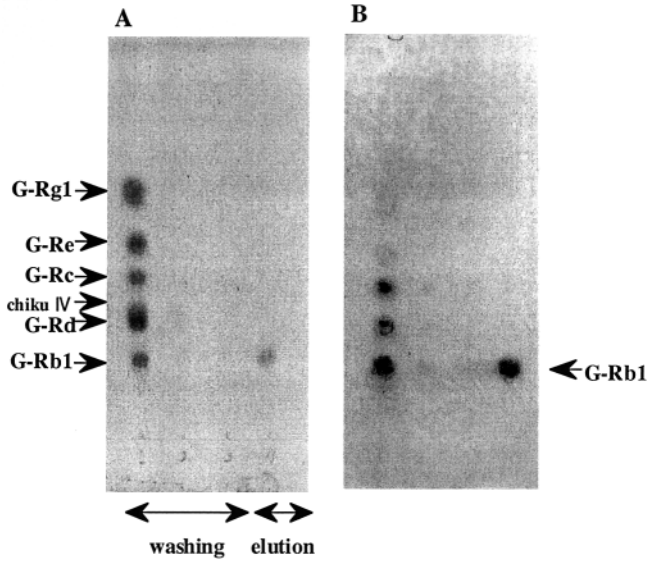

Figure 3. TLC stained with H_2SO_4 (A) and eastern blotting (B) profile of a standardized ginsenosides mixture (chikusetsusaponin IV = chiku IV).

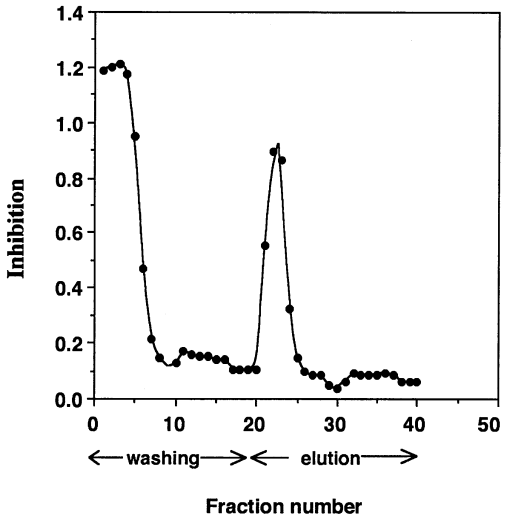

Figure 4. Elution profile of a crude extract of *P. ginseng* using an immunoaffinity column.

finally isolated. Furthermore, it became evident that the repeated separation of ginsenosides by the immunoaffinity column did make possible separation of ginsenoside Rc and Rd, although their cross-reactivities were very low (data not shown). A combination of immunoaffinity column chromatography, eastern blotting, and ELISA could be used for the survey of lower concentrations of ginsenoside Rb1 from plant origins, and the concentration of ginsenoside Rb1 in body fluid. We have succeeded in the isolation of ginsenoside Rb1 from a different plant, *Kalopanax pictus* Nakai, which was not known previously to contain ginsenosides, using this combination of methods (data not shown). Moreover, this combination system has also been used in a breeding program of *P. ginseng* using a tissue culture technique,[11–12] resulting in plants yielding higher ginsenoside Rb1 concentrations.

In conclusion, the wide cross-reaction to all solasodine glycosides of anti-solamargine MAb is the major advantage, because it is pointed out that the mixture of total solasodine glycosides from crude extracts can be separated by a single immunoaffinity column. In other words, if an aglycone is needed, like solasodine glycosides, we design MAb having a wide cross-reactivity. On the other hand, for immunoaffinity columns using anti-ginsenoside Rb1, this methodology is useful for the rapid and simple purification of ginsenoside Rb1, and may open up a wide field of comparable studies with other families of saponins that leads to an acceptable method for single step separation.

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