The new trend of medical plant research in Japan

-Immunochemical strategy using monoclonal antibody-

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With the rapid development of the molecular biosciences and their biotechnological applications, immunoassays using monoclonal antibodies (MAbs) against drugs and low molecular weight bioactive compounds have become an important tool, because of their specificity, for receptor binding analyses, enzyme assays, and quantitative and qualitative analytical techniques in both animals and plants. For a long time we have been involved in studying the formation of MAbs against naturally occurring bioactive compounds and developing their applications.

1. Enzyme-linked immunosorbent assay (ELISA) for glycyrrhizin (GC) using anti-GC MAb

1) Direct Determination of Hapten-Carrier Protein Conjugate by MALDI-TOF Mass Spectorometry.

It is known that the hapten number in an antigen conjugate is important for immunization against small molecular compounds. We have previously described an analysis of hapten-carrier protein conjugates by MALDI-TOF mass spectrometry that can describe the suitability of hapten number for immunization. A broad peak coinciding with the conjugate of GC and BSA appeared around m/z 70,021. Using a molecular weight of 66 433 for BSA, the calculated value of the GC component (MW 823) is 3588, indicating at least four molecule of GC conjugated with the BSA. This hapten number is stimulated to be enough for immunization.

2) Production and characteristics of MAbs against GC.

The hyper-immunized BALB/c mice used to derive the cell clones described yielded splenocytes that were fused with P3-X63-Ag8-653 myeloma cell by the established procedure in our laboratory. Four hybridomas producing MAbs reactive to GC were obtained. The reactivities of the MAts to GC were tested by direct ELISA.

3) Assay sensitivity and assay specificity.

A competitive binding assay was established in which the MAb binds either free GC or GC-HSA conjugate adsorbed onto a polystyrene microtiter plate. Under these conditions, the full measuring range of the assay extended from 20 to 200 ng/ml (Fig.1). Since cross-reactivity is the most important factor in determining the value of an antibody and dominates the specificity of an immunoassay, the assay specificity was tested by determining the cross-reactivities of the MAbs with various related compounds. MAb 5A8 had 4.6%. cross-reactivity with glycyrrhetic acid 3-O-glucuronide and 2.1% with glycyrrhetic acid. There was no detectable cross-reaction with other steroidal compounds(Table 1).

Application of the competitive ELISA method was validated by determining the GC contents in three kinds of *Glycyrrhiza* roots stipulated by the Chinese Pharmacopoeia and the Japanese Pharmacopoeia as medicinal resources and present in various traditional Chinese prescriptions. GC

was not detectable in Danggui Shanyan San and Da Chaihu Tang, which contain no licorice.

Fig.1 Calibration curve of GC

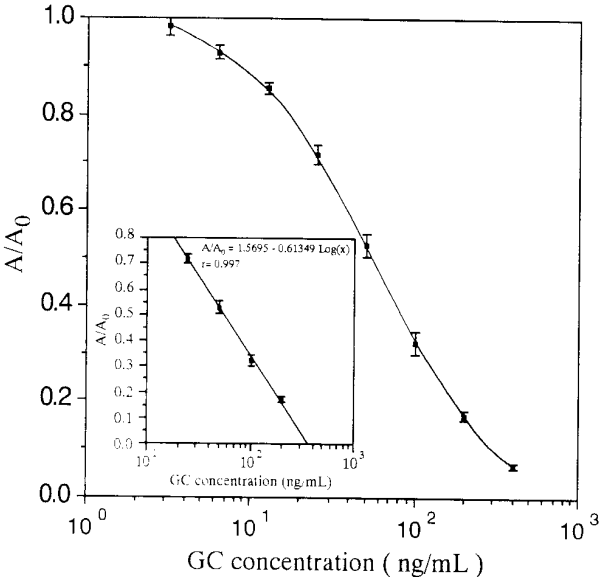


Table 1 Cross-reactivities

Table 1. Cross-Reactivities (%) of Anti-GC MAbs[a]

compound	5A8	5A5	4G6	5B4
glycyrrhizin	100	100	100	100
glycyrrhetic acid 3-*O*-glucuronide	4.36	46.30	73.50	81.03
glycyrrhetic acid	2.13	18.72	18.35	20.04
11-deoxo-18β-glycyrrhetic acid	2.32	5.45	11.37	1.17
18α-liquiritic acid	<0.02	<0.02	<0.02	<0.02
18β-liquiritic acid	<0.02	<0.02	<0.02	<0.02
11-deoxo-18β-liquiritic acid	<0.02	<0.02	<0.02	<0.02
deoxycholic acid	0.34	0.09	0.16	0.16
ursolic acid	<0.02	<0.02	<0.02	<0.02
oleanolic acid	<0.02	<0.02	<0.02	<0.02
hederagenin	<0.02	<0.02	<0.02	<0.02
betulin	<0.02	<0.02	<0.02	<0.02
lupeol	<0.02	<0.02	<0.02	<0.02
cholic acid	<0.02	<0.02	<0.02	<0.02
cholesterol	<0.02	<0.02	<0.02	<0.02
ginsenoside Rb1	<0.02	<0.02	<0.02	<0.02
saikosaponin a	<0.02	<0.02	<0.02	<0.02
solamargine	<0.02	<0.02	<0.02	<0.02
solasonine	<0.02	<0.02	<0.02	<0.02
digitonin	<0.02	<0.02	<0.02	<0.02
ergosterol	<0.02	<0.02	<0.02	<0.02
β-sitosterol	<0.02	<0.02	<0.02	<0.02

[a] The cross-reactivities were determined according to Weiler's equation (Weiler et al. 1976).

Correlation of GC contents in licorice samples measured by ELISA and HPLC. The contents determined by ELISA agreed well with those determined by HPLC.

2. Immunostaining of ginsenosides by eastern blotting using MAbs

Ginseng, the crude drug of *P. ginseng* is one of the most important Chinese medicine. Major active components are the ginsenosides, which consist of protopanaxatriol or

protopanaxadiol possessing a dammarane skelton in their molecules. It is well known that the concentration of ginsenosides are varying in the ginseng root or the root extracts depending on the method of extraction, subsequent treatment, or even the season of its collection. Therefore, standardization of quality is required. For this purpose we prepared anti-ginsenoside Rb1(GRb1) and Rg1 MAbs and set up an ELISA, a new eastern blotting and an immunoaffinity concentration method for one-step isolation of GRb1. In this lecture a new double staining for ginsenosides by eastern blotting using anti-GRb1 and GRg1 MAbs will be presented.

Ginsenosides were applied to TLC plates and developed with *n*-BuOH-EtOAc-H_2O. One TLC plate developed was dried and sprayed with H_2SO_4. Another TLC plate was dried and then sprayed with blotting solution mixture of isopropanol-methanol-H_2O. It was placed on a stainless steel plate, then covered with a PVDF membrane sheet. After covering with a glass microfiber filter sheet, the whole was pressed evenly 50 s with a 120 ºC hot plate. The PVDF membrane was separated from the plate and dried. The PVDF membrane blotted was dipped in water containing $NaIO_4$ under stirring at room temperature. After washing with water, carbonate buffer solution containing BSA was added, and stirred. The PVDF membrane was washed with TPBS for 5 min twice, and then washed with water. The PVDF membrane was immersed in anti-G-Rb1 MAb, stirred at room temperature for 1 h. After washing PVDF membrane twice with TPBS and water, diluted peroxidase-labeled goat anti-mouse IgG in GPBS was added and stirred at room temperature. The PVDF membrane was washed, then exposed to 4-chloro-1-naphtol-H_2O_2 in PBS.

For the staining by anti-Rg1 MAb, the PVDF membrane blotted was treated by the same way of anti-Rb1 MAb except being exposed to 3-amino-9-ethylcarbazole-H_2O_2 in acetate buffer.

The PVDF membrane blotted was treated with $NaIO_4$ solution. This reaction enhanced the fixing of ginsenosides via ginsenoside-BSA conjugates on PVDF membrane. The PVDF membrane incubated in the absence of $NaIO_4$ were essentially free of staining for ginsenosides.

When the mixture of anti-GRg1 and -GRb1 MAbs, and the pair of substrates were tested for staining for ginsenosides, all ginsenosides, GRg1, -Re, -Rd, -Rc and -Rb1 were stained as a blue color as shown in Fig.xx, although the purple color staining for GRg1 was expected because 3-amino-9-ethylcarbazole was used as a substrate. It is easily suggested that the sensitivities of substrate between 3-amino-9-ethylcarbazole and 4-chloro-1-naphtol might be different. Therefore, we performed successive staining of the membrane using anti-GRg1 and then anti-GRb1. Finally we succeeded the double staining of ginsenosides indicating that GRg1 and GRe were stained as purple color and the other did blue separately. From this result both antibodies can distinguish individual aglycons, protopanaxatriol and protopanaxadiol. For this application the crude extracts of various *Panax* species were analyzed by the newly developed double staining system. Major ginsenosides can be determined clearly by the double staining method.

This system enhanced to separately stain ginsenosides having protopanaxatriol or

protopanaxadiol in a molecule as an aglycon. On the other hand the Rf value of ginsenosides roughly suggest the number of sugar attached to the aglycon. Therefore, the both analyses make possible to suggest which aglycon attach and how many sugars combine leading to the structure of ginsenosides.

Fig.2 Double staining of ginsenosides by eastern blotting

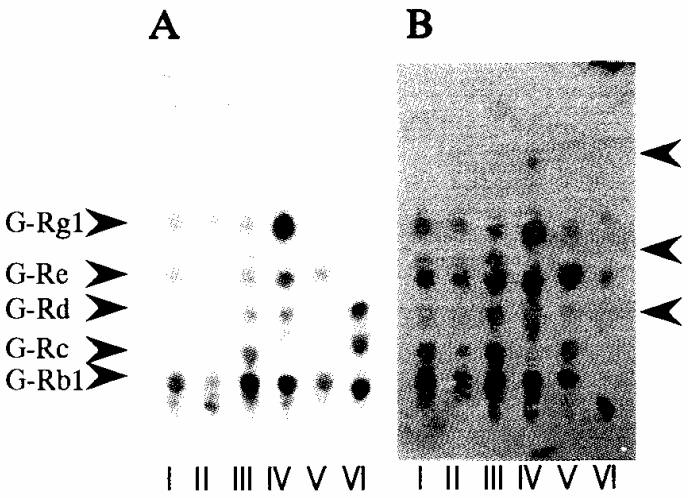


Fig. 4. Double Staining of Various *Panax* Samples

A, H_2SO_4 staining; B, Western blotting. Lanes I, II, III, IV, V, and VI indicate white ginseng, red ginseng, fibrous ginseng, *P. notoginseng*, *P. quinquefolium*, and *P. japonicus* (60 μg), respectively.

3. A one-step immunochromatographic assay for detecting ginsenosides Rb1 and Rg1

Quantitative and qualitative analysis of ginsenosides have been performed by HPLC, TLC and GC. Since rapid and simple assay is required to analyze large numbers of samples and small quantities of test material, immunoassays that use MAbs are very specific, and they are useful for quantitative and qualitative analysis. An immunochromatographic assay is based on a competitive immunoassay that utilizes antigen-antibody binding properties and provides a rapid and sensitive detection of analyte. In this lecture an immunochromatographic strip test for detecting GRb1 and GRg1 using anti-GRb1 and anti-GRg1 MAbs will be presented.

We selected GRb1 and GRg1 as quality control makers for *Panax* species. We previously reported that anti-GRb1 MAb (9G7) had weak cross-reactivity with GRc, GRd, GRe and GRg1 (0.02%, 0.02%, <0.01% and <0.01%, respectively). Furthermore, anti-GRg1 cross-reacted weakly with GRe, GRc, GRd and GRb1 (3.3%, 0.93%, 0.93% and 0.93%, respectively). Since MAbs do not cross-react with the other steroidal compounds, an

immunochromatographic strip test based on an immunoassay system with MAbs might detect GRb1 and GRg1 with a high degree of sensitivity and specificity.

The immunochromatographic strip test was based on a competitive immunoassay using anti-GRb1 and anti-GRg1 MAbs as detector antibodies. A sample solution was applied to the sample pad, and GRb1 and GRg1 in the sample were bound by the detector reagent in the conjugate pad. This pad contained two detector reagents, anti-GRb1 MAb and anti-GRg1 MAb colloidal gold conjugates. The GRb1 and GRg1 were bound to the detector reagents, and free GRb1, GRg1, and detector reagent migrated up the strip with the sample.

We used 1% BSA in PBS as a blocking solution to reduce non-specific adsorption and to immobilize the capture reagents on the nitrocellulose membrane. Any blocking agent that was not adsorbed was removed by washing the membrane with T-PBS washing buffer, which contained 0.05% Tween 20 to promote uniform rewetting of the membrane. BSA was used as a stabilizer for the anti-GRb1 and anti-GRg1 MAbs colloidal gold conjugates. Sucrose (2%) was added to increase solubility of the detector reagents on the conjugate pad. The addition of 0.04% Tween 20 in the detector reagent promoted solubilization, as did pretreatment of the sample pad by T-PBS.

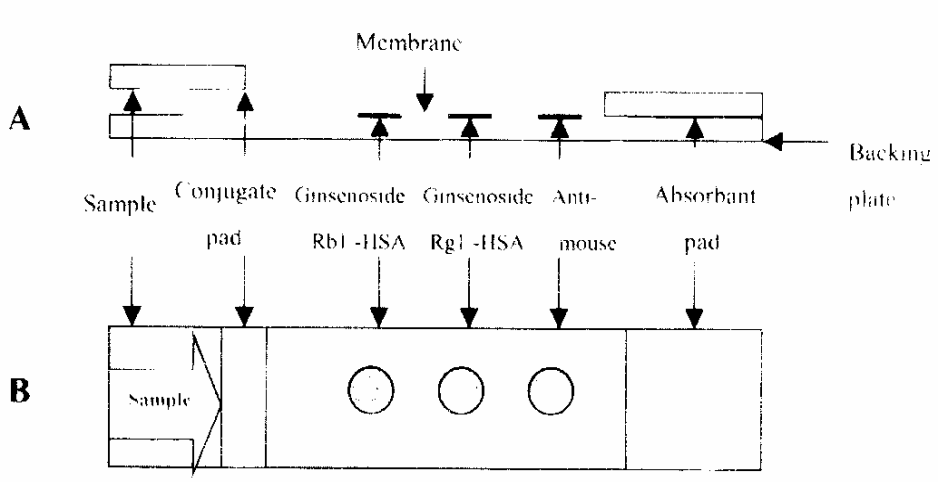
When the sample solution passed over the capture reagent (GRb1-HSA and GRg1-HSA), the detector reagent that was free of analyte bound the capture reagent at capture spots, whereas the control capture reagent (anti-mouse IgG) bound to the GRb1 and GRg1 MAbs held to the detector reagent at the control spot. When GRb1 and GRg1 were present in the sample, they competed with the immobilized GRb1 and GRg1 conjugate with HSA on the membrane for the limited amount of antibodies of the detector reagent. Thus, the immobilized capture reagent was prevented from binding with detector reagent on the membrane when sufficient amounts of GRb1 and GRg1 were present in the sample. Therefore, a positive sample produced no visible test spot in the test capture zone and the control test spot was always visible.

Color appeared at both capture spots if the sample contained no GRb1 and GRg1. On the other hand, no color developed on the two capture spots when the sample contained GRb1 and GRg1. The capture spot for GRg1 developed color when the sample contained only GRb1. In contrast, only the capture spot for GRb1 was colored if the sample contained GRg1 but no GRb1. Detection limits for both GRb1 and GRg1 using the strip test were 2 μg/ml. The appropriate sample volume was 200 μl, and the assay can be performed in about 10 min.

Panax species plants were analyzed and GRb1 and GRg1 were detected by the immunochromatographic strip test. *P. notoginseng, P. ginseng* and *P. quinquefolium* contained GRb1 and GRg1, and both were present at high levels. Only GRb1 was detected in *P. pseudoginseng* 1. On the other hand, GRb1 and GRg1 were below the detection limits in *P. pseudoginseng* 2 and 3. These results were confirmed by ELISA using anti-GRb1 and anti-GRg1 Mabs. For quantitative analysis of G-Rb1 and GRg1, a competitive ELISA method was used. The linear range of the assay was 20 to 400 ng/ml for GRb1 and 0.3 to 10 μg/ml for GRg1, respectively. *P. notoginseng , P. ginseng* and *P. quinquefolium* contained high levels of GRb1 and GRg1. *P. pseudoginseng* contained only GRb1. *P. pseudoginseng* 1 had the highest level of GRb1, as compared to *P. pseudoginseng* 2 and 3. These results were in

agreement with immunochromatographic strip test. Immunochromatographic assay accelerated the analytical procedure and did not require handling reagents. In addition, the assay can be conducted onsite where samples are collected. Therefore, the immunochromatographic strip assay was suitable as a rapid and simple procedure for screening GRb1 and GRg1 concentrations in plants and product preparations. In conclusion, both immunochromatographic assay described in this lecture and ELISA were useful methods for the qualitative and quantitative analysis.

Fig.3 Schematic diagram of immunochromatographic test strip (A) cross-section; (B) top view.



4. Immunoaffinity column conjugated with anti-GRb1 MAb

We describe an immunoaffinity column chromatography procedure for ginsenoside Rb 1 and its application in a single-step isolation of this component from a crude extract of ginseng root.

Because ginseng root contains a number of dammarane-type ginsenosides together with oleanane-type saponins, the isolation of individual saponins is quite tedious, requiring repeated Si

gel column chromatography or preparative HPLC. To avoid this, we have established a simple and reproducible purification method for ginsenoside Rb1 using an immunoaffinity column conjugated with an anti-ginsenoside Rbl MAb.

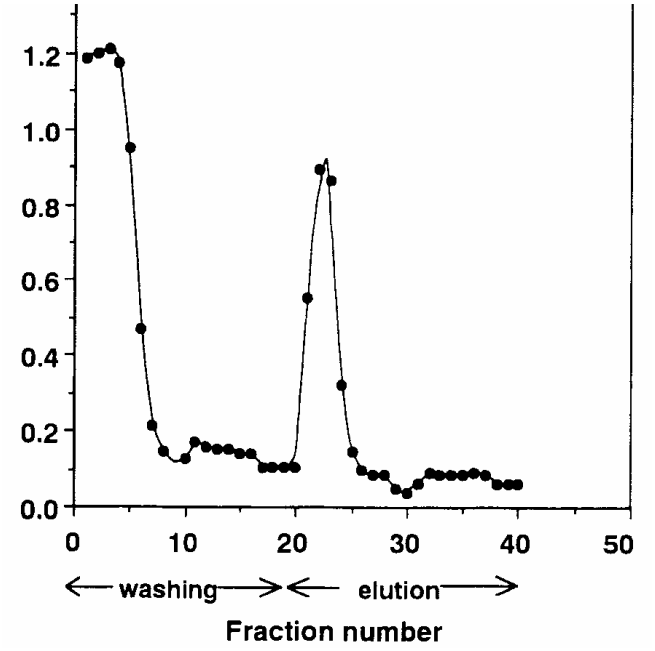
The recovery of 400 μg of ginsenoside Rb 1 was determined by an ELISA using various buffer solutions. The ginsenoside Rb1 concentration increased somewhat by eluting with a 20 mM phosphate buffer containing 0.5 M KSCN and 10% MeOH. When the 20 mM phosphate buffer was changed to 100 mM HOAc buffer, the elution ability reached the optimal level.

Although 20% CH3OH enhanced the elution of GRb1, higher concentrations were ineffective in this regard. From these results, 100 mM HOAc buffer containing 0.5 M KSCN and 20% CH3OH could be used routinely as an elution buffer solution.

A crude extract of *P. ginseng* roots was loaded onto the immunoaffinity column and washed with the washing solvent. The fractions 1-8 contained overcharged GRb1, which was determined by ELISA. GRc, Rd, Re and, Rgl were also detected in these fractions by the eastern blotting procedure. A sharp peak appeared around fractions 20-24, which contained GRb1. However, GRb1 purified by the immunoaffinity column was still contaminated by a small amount of malonyl GRb1 as detected by eastern blotting. This compound has almost the same cross-reactivity with GRb1. Therefore, the mixture was treated with a mild alkaline solution at room temperature to give pure GRbl. Overcharged GRb1, eluted with washing solution, was repeatedly loaded and finally isolated in pure form. The antibody was stable when exposed to the eluent, and the immunoaffinity column showed almost no decrease in capacity after repeated use more than 10 times under the same conditions.

This methodology is effective for the rapid and simple purification of GRbl and may open up a wide field of comparable studies with other families of saponins for which an acceptable method for single-step separation has not been developed.

Fig.4 Elution profile of crude extract of ginseng



5. Anti-solasodine glycoside single-chain Fv antibody stimulates biosynthesis of solasodine glycoside in plants

Recombinant antibody fragment, single-chain fragment-variable (scFv) antibodies have many biotechnological and biomedical applications because they have the same monovalent binding specificity and affinity as parent antibodies. The utility of scFv engineering as a diagnostic tool in plant pathology has recently been investigated. Other advances in antibody construction offer various means of conferring novel properties upon plants. For example, scFv has been expressed in plants to confer protection against virus attack. Furthermore, scFv can be used to study plant growth and to interfere with the biological activity of antigens.

We determined solasodine glycosides using a MAb against solamargine in an ELISA, a simple staining method called Eastern blotting and rapid immunoaffinity column separation. We also developed micropropagation systems to improve the quality control of medicinal plants. During ongoing efforts to breed a variety of *S. khasianum* that can yield larger quantities of solasodine glycosides, we genetically engineered an As-scFv. We present here, evidence that a functional As-scFv antibody produced in transgenic *S. khasianum* can increase the concentration of expressed solasodine glycosides.

The As-scFv was constructed from cDNAs encoding the VH and VL variable regions of As-MAb (SM-BD9) as follows. The cDNAs of the VH and VL chain were assembled using a DNA linker fragment encoding the amino acid sequence $(Gly_4Ser)_3$ and ligated into the pCANTAB5E phagemid. The DNA coding As-scFv fused with E-tag and an amber stop codon was amplified from the template pCANTAB5E. The gene was subcloned into the pET-28a(+) expression vector containing His- and T7-tags at the N-terminus as well as E-tag at the C-terminus.

After induction with IPTG, a large amount of recombinant protein was produced in the bacterial cytoplasm as inclusion bodies. The scFv protein refolded by rapid dilution was purified using a metal chelate affinity column resulting in a yield of 12.5 mg/100 ml of culture medium. MALDI mass spectrometry confirmed the purity of scFv and the amino acid sequence.

We investigated the cross reactivity of As-scFv and MAb against various steroidal compounds were almost identical to those for the MAb.

The As-scFv protein was directed to the endoplasmic reticulum (ER) by N-terminal yeast signal peptide (SUC2) and ER was retained using the C-terminal KDEL sequence. Specific expression and retention of scFv protein in the ER were essential to stabilize accumulation in plants. Adding the KDEL sequence to the C-terminus tripled increase scFv protein accumulation compared with expression without the KDEL sequence, indicating that the C-terminal KDEL sequence is essential to stabilize As-scFv protein. Kanamycin-resistant hairy roots were produced after infection with *A. rhizogenes* to yield 37 clones of transgenic hairy roots. We analyzed As-scFv protein expression and solasodine glycoside concentrations in the cloned roots.

To assess the ability of plants to produce a functional As- scFv protein, each transgenic hairy root was quantified by comparison with purified mouse Fab using an ELISA. Up to 220 ng of soluble scFv was expressed per mg of total soluble protein (TSP) extracted from the hairy roots.

After purification on an immobilized metal chelate affinity column, we analyzed the binding kinetics of the extract from transgenic hairy roots with scFv protein from transgenic hairy roots, original anti-solamargine MAb and scFv expressed in *E. coli* using a competitive ELISA. The average values of the dissociation constant (KD) of the MAb (SM-BD9), scFv in *E. coli* and scFv in hairy root cultures were 4.44 ± 0.41x10-9, 2.86 ± 0.25x10-9 and 2.43 ± 0.23x10-9 M, respectively. These results suggest that the functional affinity constant of the scFv protein derived from hairy roots is similar to that of the scFv expressed in *E. coli* and the original MAb. Moreover, dot blots showed that the specific binding activities of the two scFv proteins and the original MAb were the same binding (data not shown). The concentration of solasodine glycosides in all clones of As-scFv transgenic hairy roots ranged from 62-100 to 100.13±3.10 μg/g dry wt., whereas that of the wild type was 43 - 45 μg/g dry wt. (mean, 43.51±1.05 μg/g dry wt.), which was 2.3-fold higher than that of the wild type hairy root. Among 37 cloned transgenic hairy roots, 7 produced double the amount. Therefore, the expression level of As-scFv seems to be important for the increase in the solasodine glycoside concentration. The solasodine glycoside concentration was over double in the transgenic hairy root, indicating that As-scFv protein combines directly with solasodine glycosides and enhances their biosynthetic pathways in transgenic hairy roots. This evidence suggests that transgenic hairy roots expressing As-scFv produced the antigen-antibody complex that accumulated in plant cells. To confirm this notion, we regenerated plantlets from hairy roots. The fruits of transgenic *S. khasianum* contained 126.95±6.03 μg of solasodine glycosides per mg dry weight, which was over double that produced by the *S. khasianum* transformed by wild *A. rhizogenes* (53.43 ± 3.90 μg/mg dry wt.).

Fig.5 Structures of solasodine glycoside

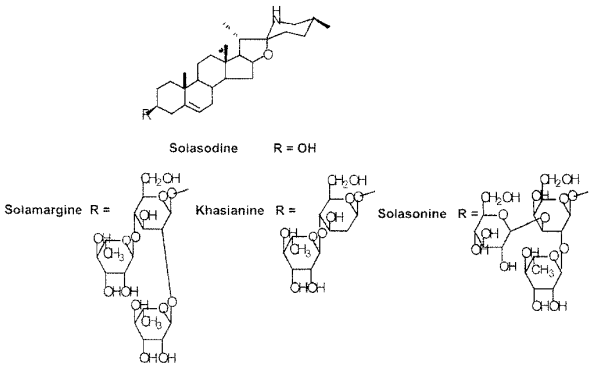


Fig.6 Comparison of solasodien glycoside concentrationfrom hairy root clones and scFv expression levels