Monoclonal antibodies against naturally occurring bioactive compounds

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Abstract

The ratio of hapten to bovine serum albumin (BSA) in an antigen conjugate was determined by matrix-assisted laser desorption/ionization (MALDI) tof mass spectrometry. A hybridoma secreting monoclonal antibody (MAb) was produced by fusing splenocytes immunized with an antigen-BSA conjugate with HAT-sensitive mouse myeloma cells. The cross-reaction of anti-forskolin antibodies with 7-deacetyl forskolin was 5.6%. A very small cross-reaction appeared with other derivatives. The full measuring range of the assay extends from 5 ng to 5 μ g/ml of forskolin. Immunoaffinity column chromatography using anti-forskolin MAbs appears to be far superior to previously published separation methods. The capacity of the immunoaffinity column as determined by ELISA is 9 μ g/ml. Forskolin has been isolated directly from the crude extracts of tuberous roots and the callus culture of *Coleus forskohlii*. A MAb against tetrahydrocannabinolic acid (THCA) was produced. The cross-reaction of anti-THCA antibody against other cannabinoids was very wide. Many cannabinoids and a spiro-compound were reactive, but did not react with other phenolics. It became evident that this ELISA was able to be applied to the biotransformation experiments of cannabinoids in plant tissue culture system. Anti-ginsenoside Rb1 MAbs were produced. New western blotting method of determination for ginsenosides was established. Ginsenosides separated by silica gel TLC were transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was treated with NaIO₄ solution followed by BSA, resulting in a ginsenoside-BSA conjugate. Immunostaining of ginsenosides was more sensitive compared to other staining. Immunostaining of ginsenosides in the fresh ginseng root was succeeded using anti-ginsenoside Rb1 (GRb1) MAb after blotting to PVDF membrane.

Introduction

Although the immunoassay system using monoclonal antibodies (MAbs) is now indispensable to various investigations, it is not so familiar for naturally occurring bioactive compounds having smaller molecular weight except against drugs like morphine. In the process of MAb production the confirmation of antigen conjugate is necessary for the immunization. The strategy for the determination of molecular weight of hapten-carrier protein conjugate by MALDI tof mass analysis provides us more accurate molecular ratio of hapten and carrier protein especially for those compounds having no specific uv absorbance where difficulties and ambiguities remain. In this review, three naturally occurring bioactive compounds, an

activator of adenylylase, forskolin, marihuana compound, tetrahydrocannabinolic acid (THCA) and a ginseng saponin, ginsenoside Rb₁ (GRb1) have been evaluated.

Formation of anti-forskolin MAb and its application

Forskolin (Figure 1), a labdane diterpenoid, was isolated from the tuberous roots of *Coleus forskohlii* Briq. (Figure 2) (Lamiaceae) (Bhat et al., 1977) which has used as an important folk medicine in India, and was found as a typical activator of adenylate cyclase (Metzger and Lindner, 1981), leading to an increase of c-AMP, which affects heart action, blood and in-

Figure 1. Structures of forskolin and related compounds. Left five compounds were isolated from *Coleus forskohlii*. Right three compounds were isolated from *Ptychanthus striatus*.

traocular pressure, resulting in an important drug in Germany, India and Japan. The production of forskolin is completely dependent on the commercial collection of wild and cultivated plants in India. However, it has been reported that the natural resources have been decreasing in India (Vishwakarma et al., 1988). This is the reason why the suspension culture (Mersinger et al., 1988), the root culture (Sen et al., 1992) and the transformed root culture (Kromholz et al., 1992) of *C. forskohlii* have been investigated. On the extension of these investigations, we are interested in the breeding of *C. forskohlii* containing higher amount of forskolin *in vitro*. Vishwakarma et al. (1988) surveyed and selected the *C. forskohlii* strain having higher amount of forskolin. They found the evidence that wide variation for forskolin content in the wild grown strains occurred. This suggests the possibility of breeding for the strain higher yielding forskolin. The rapid, simple, high sensitive and reproducible assay systems are needed for a large number of cultured plants and a limited small amount of sample *in vitro* in order to produce the strain of higher yielding forskolin. No evidence regarding MAb for forskolin has been reported yet, although Ho and Ho (1987) reported about the production of polyclonal antibodies against forskolin for understanding of the mechanism of adenylate cyclase activation by forskolin.

Table 1. List of monoclonal antibodies against forskolin

MAb	Isotype	Light chain
1A9	G1	κ
4G12	G2b	κ
9D12	M	κ
9E12	M	κ
11A9	M	κ
12F9	M	κ

Table 2. Cross-reactivities of MAb reactive to forskolin (1A9) against forskolin analogs

Analog	Cross-reactivity (%)
1-DF	0.63
1,9-DDF	1.30
6-A-DF	1.13
7-DF	5.57
PS-3	0
PS-4	0
PS-7	0

Direct determination of hapten-BSA conjugate by matrix-assisted laser desorption tof mass spectrometry (Shoyama et al., 1993)

The MALDI tof mass spectrum of BSA is shown in Figure 3. The molecular weight is 66,928 (average

Figure 2. Picture of *Coleus forskohlii*.

for triplicates, 0.09%) in agreement with a previous report (Reed et al., 1980) of which the calculated molecular weight of BSA was 66,267, a difference of 0.997%. Figure 3 also shows only the singly, doubly, and triply ionized molecular ions of the intact BSA. Fragmentation is not an artifact of the ionization technique. Figure 4 shows the co-mass spectra of the antigen conjugate of forskolin and BSA which is added as an internal standard. A sharp peak coinciding with the conjugate appeared at m/z 72,160 (average for triplicates, c.v.: 0.49%) resulting in the calculated molecular weight of 71,447. Using a molecular

weight of 66,928 for BSA, the calculated value of the 7-deacetyl forskolin 7-hemisuccinyl residue (MW 451) is 4,519 resulting in 10 molecules of forskolin conjugated with BSA. These results provide rapid, exact determination of molecular weight and direct combining information.

Production and characteristics of MAb against forskolin (Sakata et al., 1994; Yanagihara et al., 1996)

The hyperimmunized BALB/c mice used to derive the

Figure 3. Direct determination of BSA by matrix-assisted laser desorption/ionization mass spectrometry. $[M+H]^+$, $[M+2H]^{2+}$, $[M+3H]^{3+}$ are single, double and triple protonated molecule of BSA, respectively.

cell clone described in this study yielded 4×10^8 splenocytes which were fused with P3-X63-Ag8-U1 myeloma cells by the routinely established procedures in this laboratory (Sakata et al., 1994). Six hybridomas producing MAbs reactive to forskolin were obtained, and classified as indicated in Table 1. They consisted of IgG1 (1A9), IgG2b (4G12) and IgMs (9D12, 9E12, 11A9 and 12F9) which had *k* light chains. The reactivities of IgG type MAbs against to forskolin were investigated resulting in different affinities. 1A9 had higher reactivity than that of 4G12.

One of the original clone (1A9) secreted anti-forskolin antibody of which concentration was $4.0 \mu\text{g/ml}$ was purified by Protein A affinity column chromatography to give 75% of recovery. In order to confirm the purity of IgG the MALDI tof mass spectrometry of purified IgG and BSA added as the internal standard was measured. The results are indicated in Figure 5. The molecular weight was 148 600

in good agreement with that of human IgG1 being determined as 146 000 (Langone, 1982). Furthermore, since only minor molecular peaks were observed except those related to BSA and IgG, it became evident that the purification of IgG using protein A is almost satisfactory.

The cross-reactivities of related compounds as indicated in Table 2 are calculated by the method reported by Weiler and Zenk (1976). Since the cross-reactivity of 7-deacetyl forskolin was higher than the other, the detailed assay was done to determine 5.6%. Very small cross-reaction appeared in 1-deoxy, 9-deoxy and 1,9-dideoxy forskolin derivatives, however, 11-acetoxyl forskolin derivative did not show any cross-reaction. From these results, it is obvious that the hydroxyl group at C-1 and/or C-9 position is not strongly related to immunization. On the other hand, the carbonyl group at C-11 position may play the most important role in the structure of forskolin.

Figure 4. Direct determination of forskolin-BSA conjugate by matrix-assisted desorption/ionization mass spectrometry. Protonated molecules of BSA and forskolin-BSA conjugate are indicated in the same way as Figure 3.

Figure 5. Analysis of purified IgG reactive to forskolin by matrix-assisted laser desorption/ionization mass spectrometry. $[M+H]^+$, $[M+2H]^{2+}$ are single and double protonated molecule of IgG, respectively.

Figure 6. Standard curve for ELISA assay of forskolin.

Assay system by ELISA and rapid analysis of small samples containing forskolin using anti-forskolin MAb (Yanagihara et al., 1996)

The MAb is bound to polystyrene microtitration plates precoated with a forskolin-HSA. Under these conditions, the full measuring range of the assay extends from 5 ng/ml to 5 μ g/ml of forskolin as indicated in Figure 6. The ELISA system established in this study was more sensitive compared to TLC (Inamdar et al., 1984), GLC (Inamdar et al., 1980) or HPLC (Vishwakarma et al., 1988; Inamdar et al., 1984) analysis, furthermore no needs any pretreatment. This methodology can be utilized for the assay of forskolin contained in *in vitro* plantlets, therefore it makes possible to study for a large number of cultured plantlets, and a limited small amount of sample *in vitro*.

It is well known that the tuberous root of *C. forskohlii* contains a higher amount of forskolin than other organs. Due to a lack of detailed data, flowers, rachises, leaves, stems, tuberous root, and roots of a single one-year-old plant cultivated were separately

analyzed to determine the distribution of forskolin (Figure 7). Forskolin was not detected in flowers, but the rachises contained 0.11 μ g/mg dwt of forskolin. Leaves contained a small amount of forskolin, between 0.003 to 0.011 μ g/mg dwt. Young leaves contain higher amount of forskolin than older leaves. The concentration of forskolin in the stem base (4.04 μ g/mg dwt) exceeded that found in both the upper stem (1.58 μ g/mg dwt) and near the base stem was observed in individual branches, except for the lowest branch base which contained the highest amount of forskolin (5.97 μ g/mg dwt). The tuberous root base contained 3.83 μ g/mg dwt, which decreased to a minimum of 1.92 μ g/mg dwt in the top of the tuberous root. The concentration of forskolin in roots was constant, approximately 1.5 μ g/mg dwt.

From these results it becomes evident that the newly established ELISA can be utilized for the assay of wide range of forskolin contents suggesting that it is possible to study a large number of cultured plantlets containing a small amount of forskolin.

Figure 7. Distribution pattern of forskolin in different segments in *Coleus forskohlii*.

Production of immunoaffinity column chromatography using anti-forskolin MAb and direct purification of forskolin by its column (Yanagihara et al., 1996)

Due to the fact that it is difficult to purify and isolate forskolin by usual column chromatography because of the slight contents of forskolin and of the complex of

many related compounds (Gabetta et al., 1989), an effective methodology needs for the rapid and simple purification of forskolin.

Purified anti-forskolin MAb was conjugated with CNBr-activated Sepharose 4B as reported previously (Yanagihara et al., 1996). Since forskolin was almost insoluble in water, 6% of MeOH was used for ELISA system (Sakata et al., 1994). Therefore, in

Figure 9. Elution profile of forskolin in tuberous root of *Coleus forskolii* from the immunoaffinity column.

Figure 8. Elution profile of forskolin from the immunoaffinity column. Column washed with PBSM then eluted by PBS containing 45% methanol until forskolin disappeared. Individual fractions by ELISA.

order to determine the most suitable concentration of MeOH as an eluent, MeOH was been increasing in the elution buffer solution. Figure 8 shows the recovery

of forskolin determined by ELISA when the MeOH concentration increased. The forskolin contents increased rapidly from 20% of MeOH, and reached to the optimum at 45% of MeOH. From these results PBS containing 45% of MeOH can be routinely used as an elution buffer solution. In addition the capacity of immunoaffinity column chromatography was determined to be $9 \mu\text{g/ml}$ immunoaffinity gels. Therefore, application of this column for a large amount of forskolin contained in crude extractives needs more improvement.

The crude acetone extractives of tuberous root of *C. forskolii* were loaded on the affinity column chromatography, and washed with PBS containing 6% of MeOH 5 times. Figure 9 shows the fractions 2 to 8 contained over-charged forskolin which was determined by ELISA together with 1-deoxyforskolin, 1,9-dideoxyforskolin, 7-deacetylforskolin and 6-acetyl-7-deacetylforskolin which were detected by TLC (Figure 10). Forskolin eluted by washing solution was

Figure 10. TLC profiles of adsorption, washing and elution and structures of forskolin and related compounds.

Table 3. List of monoclonal antibodies against Δ^1 -THCA

MAB	IgG subclass	Light chain
2A6	2b	κ
3H11	1	κ
4A4	2b	κ
4F5	1	κ
5D11	1	κ
6C6	1	κ
7C4	1	κ
7H12	1	κ
8A11	2a	κ
10F6	1	κ

repeatedly loaded and finally isolated. However, forskolin purified by the immunoaffinity column chromatography was still contaminated by a small amount of 7-deacetyl forskolin because of this compound has a 5.5% of cross-reactivity as previously indicated (Sakata et al., 1994). Therefore, the mixture was treated with pyridine and acetic anhydride at 4 °C for 2 h to give pure forskolin.

The MeOH extractives of callus tissues were also purified by the same procedure with that of tuberous roots (data not shown).

The antibody was stable when exposed to the eluent and the immunoaffinity column showed any decrease of capacity after repeated use more than 10 times under the same condition.

This methodology can be available for the rapid and simple supplement of pure forskolin which is

cannabinoid **ligand**

Figure 11. Structures of cannabinoids and endogenous ligands.

important drug, and reagent for various kinds of biochemical experiments. Moreover, it makes possible that the combination of immunoaffinity column and ELISA can be used for the survey of forskolin from plant origins because it may be possible to concentrate the forskolin content by the immunoaffinity column. Breeding project of *C. forskohlii* yielding higher forskolin has been progressed now using the tissue culture technique. In this case ELISA needs 12-week-cultivation for the quantitation of forskolin contained in the regenerated plants treated by mutagens (Yanagihara et al., 1996). Moreover, this combination system may be available for the analysis of animal plasma samples in the drug developing program of forskolin since very low plasma forskolin concentrations will be expected.

Production of MAb against marihuana compounds (cannabinoids) and its application

Since the receptor for the most pharmacologically active cannabinoid, Δ¹-THC in brain (Matsuda et al., 1990), and an endogenous ligand, anandamide for its receptor were found (Devane et al., 1992), cannabinoids have attracted interest as resources of drugs. The structures of cannabinoids and ligands were indicated in Figure 11. In our ongoing studies on marihuana, we have examined the facilitating effects of Δ¹-THC on the rise in intrasynaptosomal Ca²⁺ concentration (Okada et al., 1992), the relationship between dopamine D1 receptors and Δ¹-THC (Miyamoto et al., 1996), the purifications of THCA synthase (Taura et

Figure 12. Calibration curve of Δ^1 -THCA. Various concentrations of Δ^1 -THCA were incubated with MAb in the precoated with THCA-BSA ($1 \mu\text{g/ml}$). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.

Table 4. Cross-reactivities of MAb reactive to Δ^1 -THCA (4A4) against phenolic compounds

Compound	Cross-reactivity (%)
<i>Alkaloid</i>	
morphine	0
<i>Curcuminoid</i>	
curcumin	0
<i>Anthraquinone</i>	
sennoside A	0
1,8-dihydroxyanthraquinone	0
<i>Flavonoid</i>	
baicalin	0
wogonin	0
wogonin 7-O- β -D-glucuronide	0
<i>Tannin</i>	
cinnamtannin B1	0
(+)-catechin	0

al., 1995), CBDA synthase (Taura et al., 1996) and CBCA synthase (Morimoto et al., 1997).

An immunological approach for assaying small quantities of THC and its isomers in body fluids and cell preparation have been investigated in many groups (Gross et al., 1974; Teale et al., 1974; Teale et al.,

Figure 13. Structures of cannabinoids, related compounds and metabolites. The cross-reactivities are given in parentheses.

Figure 14. Structure of cannabisirol.

Table 5. Hapten numbers of conjugates

Conjugate	Hapten number
Ginsenoside Rb ₁ -BSA	12
Ginsenoside Rb ₁ -HSA	5

1975; Cais et al., 1975). However, since no production of MAb against cannabinoid acids has been published yet, we wish to summarize the preparation and determination of the THCA-carrier protein conjugate by MALDI tof mass spectrometry (Goto et al., 1994), the formation of MAb against cannabinoids and an immunoassay system for the detection of THCA using a MAb (Tanaka et al., 1996), and an application for biotransformation of CBN, CBD and CBDA using plant tissue culture (Tanaka et al., 1996).

Production and characteristics of MAb against THCA

Ten hybridomas producing MAbs reactive to THCA were obtained, and classified as indicated in Table 3. They consisted of IgG1 (3H11, 4F5, 5D11, 6C6, 7C4, 7H12 and 10F6), IgG2a (8A11) and IgG2b (2A6 and 4A4) which had *k* light chains. The reactivities of IgG type MAbs against to THCA were investigated by performing a straight dilution curve resulting in different affinities. Since 4A4 had a higher reactivity than the others, the original clone 4A4 secreted an anti-THCA antibody was purified by a Protein A affinity column chromatography. The purity of IgG was confirmed by the co-MALDI-tof-mass spectrometry using BSA as an internal standard as previously described (Sakata et

al., 1994). The molecular weight was 148 600 in good agreement with that of human IgG1 being determined as 146 000 (Langone, 1982).

Competition ELISA and cross-reactivity against cannabinoids and related compounds

The free MAb following competition is bound to polystyrene microtitre plates precoated with a THCA- β -alanine-BSA. Figure 12 indicated the calibration curve for THCA indicating the concentrations between 10 and 500 μ g/ml of THCA is linear.

The cross-reactivities of related cannabinoids as indicated in Figure 13 are calculated using p mol of THCA yielding midrange and p mol of cannabinoids under investigation yielding midrange by the method reported by Weiler and Zenk (1976). Since CBGA, CBNA, CBDA, CBLA and methylolivetolate were all reactive to MAb, the terpene moiety was not immunized. CBGV and CBGO had different cross-reactivities. Therefore, alkyl moiety was related to immunization. As we reported, marihuana contains spiro-compounds (Figure 14) (Shoyama and Nishioka, 1978), acetyl cannabisirol, cannabisirol, cannabispiron and cannabispirenone which have the same basal skeleton with cannabinoids and are biosynthesized from the same precursor of cannabinoids (Shoyama et al., 1984). Therefore, one of them, cannabisirol was assayed. Same reactivity of cannabisirol with Δ^1 -THCA was observed. From these results this MAb immunized only olivetol moiety. This anti-THCA MAb, however, did not react with other naturally occurring phenolics such as flavonoids and anthraquinones (Table 4) only limited to cannabinoids including spiro-compounds. This wide cross-reactivity is the major advantage of this ELISA. The marihuana samples and the fresh *Cannabis* leaves can be detected and distinguished from other plant species by this ELISA without any analytical instruments such as GC and HPLC. Although we analyzed more than 40 plant leaves by the ELISA, nothing positive plants were detected except *Cannabis* leaves (data not shown). Namely, newly established ELISA might be important in the field of judgement of marihuana samples used illicitly.

Application of ELISA for metabolic experiments

In a previous paper, we reported the biotransformation of CBN into its glucoside and hydroxylated glucoside (Tanaka et al., 1993). Therefore, CBDA was incubated with *Pinellia ternata* tissue mass as described

Figure 15. Separation of converted products of CBDA by HPLC. CBDA was incubated with the tissue mass suspension of *Pinellia ternata* in MS medium supplemented with 2,4-D on a shaker (60 rpm) at 25 °C under a 16 h photoperiod. Tissue masses were dried, powdered and extracted with MeOH. The extractives were separated by HPLC. The HPLC was performed by a Cosmosil 5C18-MS column and a variable wave length detector (268 nm). The mobile phase was a gradient acetonitrile-H₂O mixture (20 to 100%) containing 5 mM phosphoric acid. The flow rate was 1.0 ml/min.

previously (Tanaka et al., 1996) for an application of this ELISA. The biotransformation products of CBDA were applied to HPLC to give several peaks. Individual peaks were assayed by ELISA. Figure 15 showed individual reactivities of Fr. 2, 5, 6, 7, 8 and 9 resulting in 32, 27, 45, 65, 63 and 49%, respectively compared to the control assay. The structures of constituents contained in Fr. 2 and 6 were elucidated to be CBDA-monoglucoside and hydroxy-CBDA-monoglucoside by their FAB MS and ¹³C NMR, respectively. Fr. 8 was identified with original

CBDA by the comparison with authentic sample by UV and FAB-MS spectra. Fr. 5 and 7 were difficult to identify because there were so few of them although they showed ELISA positive.

Many metabolic studies on cannabinoids have been carried out using animals, their organs and microsomal fractions. Nearly 70 metabolites of THC analogs have been found. However, since we have been having difficulties in obtaining such urine samples, only two major metabolites, 7-oxo- Δ^6 -THC and 7-hydroxy- Δ^6 -THC were determined (Burstain, 1973; Yamamoto

Figure 16. Calibration curve of GRb₁. Various concentrations of GRb₁ were incubated with MAb in the precoated with GRb₁-HSA (1 μ g/ml). After washing with TPBS, the wells were again incubated with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.

Table 6. Cross-reactivities of MAb (9G7) against ginsenosides

Compound	Cross-reactivities (%)
Ginsenoside Rb ₁	100
Ginsenoside Rc	0.024
Ginsenoside Rd	0.020
Ginsenoside Re	<0.005
Ginsenoside Rg ₁	<0.005
Glycyrrhizin	<0.005
Gigitonin	<0.005
Tigogenin	<0.005
Tigonin	<0.005
Gitogenin	<0.005
Digitonin	<0.005
Solamargine	<0.005
Solasonine	<0.005
Cholesterol	<0.005
Ergodterol	<0.005
Ulsolic acid	<0.005
β -Sitosterol	<0.005
Cholic acid	<0.005
Deoxycholic acid	<0.005

The cross-reactivities of ginsenosides were determined according to Weiler's equation.

Table 7. Ginsenoside Rb₁ contents of various Ginseng

Sample	Content (μ g/mg dry wt.)
Ginseng (<i>P. ginseng</i>)	6.20 $\pm$ 0.39
Red Ginseng	3.75 $\pm$ 0.36
Fibrous Ginseng	48.44 $\pm$ 4.70
San-chi Ginseng (<i>P. notoginseng</i>)	35.46 $\pm$ 1.38
American Ginseng (<i>P. quinquefolium</i>)	26.49 $\pm$ 2.32
Japanese Ginseng (<i>P. japonicus</i>)	2.01 $\pm$ 0.26

Figure 17. Western blotting of ginsenoside Rb₁ and related compounds (A). Ginsenoside Rb₁ (lane 1), ginsenoside Rc (lane 2), ginsenoside Rd (lane 3), ginsenoside Re (lane 4), ginsenoside Rg₁ (lane 5), chikusetsusaponin III (lane 6). B was stained by H₂SO₄.

et al., 1986). It became clear that two major metabolites and the biotransformed marihuana compounds having a free phenolic hydroxy group in a molecule showed clearly positive for ELISA although these sensitivities were different.

Furthermore, this ELISA can be available for the preliminary survey of cannabinoids, not limited to THCA against users of marihuana because the marihuana samples contain many kinds of original and artificial cannabinoids which consist of more than 70 which depend upon its storage condition and upon its strain such as THCA-, THCVA-, CBDA-and CBDVA-strain (Shoyama et al., 1977) of *C. sativa* resulting in the majority like pentyl-, propyl- and methyl-cannabinoids. For instance although the CBDA strain bred by us (Shoyama 1993) contains CBDA as a major

Ginsenosides

Figure 18. Schematic evidence for western blotting of ginsenoside Rb1 in PVDF membrane using anti-GRb1 MAb.

and CBCA as a minor marihuana compound, and none of THCA, this ELISA is available for the detection of such non-THCA *Cannabis* plant or marihuana too. Moreover, it is very difficult to detect cannabinoids of slight contents contained in old marihuana samples by usual methods such as GLC, HPLC and TLC because of almost all cannabinoids are fragile and decomposed with respect to light (Shoyama et al., 1970), auto-oxidation (Razdan et al., 1972), free radical oxidation (Miller et al., 1982) and/or high temperature (Lewis et al., 1978; Shoyama et al., 1970) during their storage. However, since spiro compounds which were stable compared to cannabinoids and showed an ELISA positive, this ELISA may be helpful for the determination of old marihuana samples.

Formation of anti-ginsenoside Rb1 MAb and its application (Tanaka et al., 1998)

Almost all *Panax* spp. (Araliaceae family) have been used in folk medicine. The most famous variety is *P. ginseng*, which was recorded in Chinese *Materia Medica* 2,000 years ago. Ginseng is one of the most important Chinese medicines used in the world in tonics to combat stresses and cancer, disturbances of the central nervous system, and hypothermia, and for radio-protection (Tanaka, 1973; Shibata, 1982). It contains many dammarane and oleanane saponins

(Besso et al., 1982; Koizumi et al., 1982), polyacetylene derivatives (Hansen and Boll, 1986) and polysaccharides (Tomoda et al., 1993) of which the biological activity has been studied widely. An immunological approach for assaying quantities of ginsenosides using a polyclonal antibody has been investigated by Sankawa et al. (1982). However, since no result of MAb related to ginsenosides has been reported yet, we will communicate the formation of MAb against a major ginseng component, ginsenoside Rb₁ having a sedative activity and a unique immunostaining of ginseng organs.

Direct determination of hapten-carrier protein conjugate by matrix-assisted laser desorption tof mass spectrometry

A broad peak coinciding with the conjugate of GRb₁ and BSA appeared at around m/z 79 469 in the MALDI tof mass spectrum. Using a molecular weight of 66 433 for BSA, the calculated values of GRb₁ component (MW1,109) is 13 036 resulting in at least 12 molecules of GRb₁ conjugated with BSA. The hapten number was estimated to be enough for immunization. The number of GRb₁ contained in the GRb₁-HSA conjugate was also determined to be around 5 molecules by its spectrum (Table 5).

Figure 19. Western blotting of ginsenosides in a sliced *P. ginseng* root.

Production and characteristics of MAb against GRb₁

The hyperimmunized BALB/c mice used to derive the cell clone described in this study yielded splenocytes which were fused with P3-X63-Ag8-653 myeloma cells by the routinely established procedure in this laboratory (Sakata et al., 1994). Hybridoma producing MAb reactive to GRb₁ was obtained, and classified into IgG2b which had *k* light chains.

The reactivity of IgG type MAb, 9G7 was tested by varying antibody concentration and by performing a dilution curve. The antibody concentration (0.418 μ g/ml) at which the OD was about 0.8 was selected for competitive ELISA.

The free MAb following competition is bound to polystyrene microtitre plates precoated with GRb₁-HSA. Under these conditions, the full measuring range of the assay extends from 20 to 400 ng/ml as indicated in Figure 16.

Cross-reactivity is the most important factor in determining the value of antibody. Since the ELISA for GRb₁ was established for phytochemical investigations involving crude plant extracts, the assay specificity was checked by determining the cross reactivities of the MAb with various related compounds. The cross-reactivities of MAb obtained was examined by competitive ELISA and calculated using p mole of GRb₁ yielding midrange and p mole of derivatives of GRb₁ under investigation yielding midrange by the method reported by Weiler and Zenk (1976). The cross-reactivity of GRc and GRd which possess a diglucose moiety attached to C-3 hydroxy group are weak comparing with GRb₁. Moreover, GRE and

GRg₁ show no cross-reactivity. From these results a diglucose moiety at C-3 position was necessary. On the other hand a sugar moiety at C-20 position is not related for the reactivity. The effect of the sugar moiety conjugated at C-6 is not clear because such derivatives are not available from natural resources. It becomes evident that the MAb reacted only with small number of structurally related GRb₁ very weakly, and did not react with other steroidal compounds as shown in Table 6 resulting in that the MAb against GRb₁ exhibited high specificity. Therefore, the newly established MAb against GRb₁ can be routinely used for the phytochemical investigations involving crude plant extracts without any pretreatment.

Application of ELISA

The ELISA was utilized to measure the contents of GRb₁ in various ginsengs (Table 7). Kitagawa et al. analyzed the ginsenoside contents in various crude ginsengs by HPLC. More recently Samukawa et al. (1995) reported the comparative contents of ginsenosides in the various commercial ginseng radices analyzed by HPLC. Our results indicate that the ELISA and HPLC methods closely correlate over the range of concentrations found in the plant materials. The newly established ELISA was more sensitive than the TLC (Tani et al., 1981) or HPLC methods (Sticher et al., 1979; Soldati et al., 1980). Furthermore no pretreatment of crude extracts was necessary. This methodology can be utilized for the assay of GRb₁, therefore it is possible to study a large number of plantlets cultured in this laboratory (Shoyama et al., 1995; Shoyama et al., 1997), and a small sample size *in vitro* for the breeding of *Panax* species to yield high concentration of GRb₁.

Western blotting and immunostaining of ginseng root using anti-ginsenoside Rb₁ MAb

Although the western blotting is one of the most common methodology in the fields of higher molecular substances, small molecular compounds have been ruled out. It is well known that the direct immunostaining of small molecular compounds on TLC plate is impossible. Therefore, a new surveying method bearing an ability to react with small molecular compounds like ginsenosides is required for the finding of naturally occurring bioactive compounds. Moreover, if they can be blotted to a membrane, the fixing on membrane needs some new methodology. Since we synthesized the hapten-BSA conjugate as indicated above, it seems

to be that this reaction has been induced for the fixing on membrane.

Ginsenosides were applied to TLC plate and developed with *n*-BuOH-EtOAc-H₂O(15:1:4). One TLC plate developed was sprayed and colored with H₂SO₄. Other TLC plate developed was blotted on the PVDF membrane by heating as previously described with modification. The PVDF membrane blotted was dipped in water containing NaIO₄ for 1 h. After washing with water, 50 mM carbonate buffer solution containing BSA was added, and stirred for 3 h. The PVDF membrane was stained by standard protocols of western blotting using MAb 9G7.

Figure 17 shows the western blotting (A) and H₂SO₄ staining (B) of ginsenosides. Lanes 1 to 5 show the bands of ginsenosides, G-Rb1, -Rc, -Rd, -Re and -Rg1, respectively. Although the H₂SO₄ staining detected many spots, the western blotting did only ginsenosides bearing panaxatriol resulting that chikusetsusaponin III which is an oleanane-type saponin contained in *P. japonicus* can not be detected by the western blotting (Figure 17, lane 6). When compared the sensitivities of staining by two methods, the western blotting was the higher followed to the H₂SO₄ staining. Interestingly, an aglycone of ginsenosides, panaxatriol (arrow shows) was not detected by the western blotting evidently indicating that the newly established western blotting needed sugar moieties. Namely this reaction enhanced the fixing of ginsenosides via ginsenoside-BSA conjugates on PVDF membrane. From the evidence that the PVDF membrane incubated in the absence of NaIO₄ were essentially free of staining for ginsenosides (data not shown) the above hypothesis became clear as indicated in Figure 18. Moreover, the antigen synthesized by NaIO₄ cleavage via the conjugation with carrier protein is necessary. Because when the different type of hapten-carrier protein conjugate is used, the western blotting can not be detectable.

The different sensitivities between ginsenosides were observed, and the sensitivity of the GRb1 was apparently higher rather than that of other ginsenosides. Detectable limitation was 400 ng of G-Rb1. Relations between the binding site of sugar moiety and the staining phenomenon were ruled out that the sugar moiety at C-3 position was necessary for staining, but that at C-6 position inhibited staining. Differences of sensitivity on ginsenosides may depend upon their structures and cross-reactivities (Figure 17). The bands of G-Re and -g₁ were stained instead of almost no cross-reactivity against MAb 9G7, but disappeared after

few hours (Figures 17, arrow-1 and arrow-2). This phenomenon suggested that the frame of aglycone, panaxatriol and a part of sugars might be immunized, and functioned as epitopes in the structure of ginsenosides. In addition it is suggested that the specific reactivity of sugar moiety in the ginsenoside molecule against MAb may loosen by the NaIO₄ treatment of ginsenosides on the membrane resulting that G-Re and -g₁ become detectable by the newly established western blotting.

As an application of this methodology the immunocytolocalization of ginsenosides in ginseng organs has been investigated. Figure 19 indicates the immunocytolocalization of ginsenosides in the fresh ginseng root. Endodermis cells (or the pericycle) (B) contained the highest contents of ginsenosides, and decreasing to that of exodermis tissues (A) and then that of radial vascular bundle (C).

This is possibly the first report when the western blotting for ginsenosides and its application is described. This assay method can be routinely used for survey of natural resources of ginsenosides as a simple and rapid analysis. Moreover, this methodology may be available for the assay *in vitro* plantlets of *P. ginseng*, therefore it makes it possible to study a large number of cultured plantlets, and a limited small amount of sample *in vitro* for the breeding of *Panax* species containing a higher amount of ginsenosides in the continuation of our medicinal plant breeding studies. Furthermore, the immunocytolocalization of ginsenosides may be available for the analysis of animal tissue samples of ginsenosides not limited to ginsenosides distributions in plant organs or tissues since very low concentrations are expected. Since it was difficult to detect a small molecular compound by the western blotting and/or immunocytolocalization, the approach described here will open a wide field of comparable situations as a new attractive methodology. This methodology can be theoretically expanded for all sugar conjugates such as saponins and various kind of glycosides, moreover glucuronides and aminosugar conjugates if individual MAbs are available.

Conclusion

The molecular ratio of hapten and carrier protein is important for immunization. In our laboratory MALDI tof mass spectrometry had been inducing for the determination of hapten number in hapten-carrier protein

conjugate resulting in the rapid and exact determination of hapten molecule number.

Formations of MAb against three kinds of naturally occurring bioactive compounds, forskolin, THCA and ginsenoside Rb1 have been set up. Anti-forskolin MAb can be used as a higher sensitive and reproducible assay system between 5 ng/ml to 5 µg/ml of forskolin. An affinity column chromatography using anti-forskolin MAb was prepared to apply for the direct purification of forskolin from the crude extract of *C. forskohlii* root. The possibility to resolve a synthesized racemic forskolin by an affinity column chromatography using anti-forskolin MAb has been opened.

On the other hand, anti-THCA MAb showed an unique characteristics having a wide cross-reactivities limited to various cannabinoids, but no cross-reaction for other phenolic compounds. Therefore, we apply this MAb for the detection of metabolites of various cannabinoids. These results expand to make it possible to detect many cannabinoid metabolites suggesting that the ELISA can be routinely used for the first screening for the marihuana users.

In the final investigation, we succeeded the western blotting of ginsenosides developed on TLC using anti-GRb1 MAb after transferred to membrane. This new western blotting needs the oxidation cleavage step of sugar moiety by NaIO₄ because ginsenosides are washed out by buffer or water solution. The newly established methodology opens a wide application for sugar containing compound like glucosides, glucuronides and/or glucosamine derivatives.

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