Chapter 2

Applications of Anti-natural Compound Immunoaffinity Purification on Quality Control

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Additional information is available at the end of the chapter

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1. Introduction

Worldwide demand of herbal medicines has increased in recent years owing to rising interest in the health benefits. Among with this, the quality control of plant extracts and plant-derived medicines is growing in importance to ensure their efficacy and safety. Effective quality control of the traditional Chinese medicines (TCM) and plant crude extracts requires the rapid and sensitive methods for separation and quantification of bioactive compounds. Various methods have been employed for the separation and quantification of certain constituents in medicinal plants or herbal medicines. However, the current methods in use are not necessarily optimal approaches. For example, separation and quantification of glycyrrhizin (GC), the main active constituent in licorice (*Glycyrrhiza* spp.), have been used gas chromatography, high performance liquid chromatography (HPLC) and micellar trokinetic chromatography and so on [1,2]. Commercial purification of GC typically progressed through several steps, including crystallization, column chromatography, and liquid partitioning. These current methods are not sufficiently approaches because of insufficient sensitivity and reproducibility, large consumption of organic solvent for extraction and analysis, and long analysis time.

Immunoassay systems using monoclonal antibody (MAb) against drugs and small molecular weight bioactive compounds have become an important tool for studies on receptor binding assays, enzyme assays, and quantitative and qualitative analytical techniques both *in vivo* and *in vitro* studies. Although immunoaffinity purification against higher molecule analyte such as peptides and proteins are widely used in the research and commercial ways, there are too few cases of immunoaffinity purification targeting a small molecule compound such as natural compounds. Our laboratory has prepared many kinds of MAbs against

naturally occurring bioactive compounds such as terpenoids [3-5], alkaloids [6,7], saponins [8-12], and phenolics [13-16], and developed several applications. One of the applications by using MAbs is immunoaffinity column conjugated with anti-natural compound-specific MAbs and work by specifically binding and removing the target compounds. We have been establishing several affinity columns against a kind of terpenoid, forskolin [17], solasodine glycosides [18], ginsenosides Rb_1 [19], and GC [20]. Application of an immunoaffinity column to isolate and concentrate a natural compound may decrease the amount of solvent consumption and the number of purification steps, shorten analysis time, and simplify sample analysis compared to traditional cleanup techniques.

In this chapter, we focus on the immunoaffinity purification to separate and concentrate the target bioactive compounds from the crude extract. Our approaches effectively succeeded one-step purification of target compounds by MAb-conjugated immunoaffinity column, which leads to high-sensitivity detection and isolation of target compounds. In addition, the immunoaffinity column can prepare the knockout (KO) extract which contains all components except an antigen molecule, and KO extract will be useful for the pharmacological investigation to reveal the real effects of bioactive compound in the crude extract.The information in this chapter may provide new insight into quality control of plant-derived medicines.

2. Preparation of anti-ginsenoside Rb_1 immunoaffinity column and its application

Ginseng, the root of *Panax ginseng*, has been an important component in traditional medicines for more than 1000 years in Eastern Asia. It is now one of the most extensively used alternative medicines all over the world and appears in the pharmacopoeias of several countries. The biological and pharmacological activities of ginseng have been reported to have anti-aging, anti-cancer, anti-inflammation, anti-diabetics, anti-stress, maintenance of homeostasis, and to affect on central nervous system and immune function [21]. The bioactive components responsible for ginseng actions are ginsenosides, which are triterpenes saponins that possess a dammarane skeleton with sugar moieties [22]. Up to now more than 60 kinds of ginsenosides have been isolated from *Panax* genus [23]. It is well-known that the concentrations of ginsenosides vary in the ginseng root or the root extracts depending on the method of extraction, subsequent treatment, or even the season of its collection [24,25]. Due to the importance of ginseng, a number of researches has been carried out to develop the methods for the identification, quantification and quality control of ginsenosides in raw plants materials, extracts and commercial products. Currently, analytical and preparative HPLC are commonly used to quantify and purify the individual ginsenosides from ginseng [26]. However, isolation of ginsenosides by HPLC requires the repeated purification steps, including cumbersome handling and lengthy analysis times, and may result in the decrease of the final yield.Thus, the developed approaches are required for quality control of ginseng in the field of TCM.

Ginsenoside Rb_1 (G-Rb_1) is one of the main ginsenosides responsible for many pharmaceutical actions of ginseng [27]. G-Rb_1 has various biological activities, including facilitating acquisition and retrieval of memory [28], scavenging free radicals [29], inhibition of calcium over-influx into neurons [30], and preserving the structural integrity of the neurons [31].

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In order to develop efficient quality control of ginseng, we have prepared anti-G-Rb_1 MAb, set up of enzyme-linked immunosorbent assay (ELISA), and a new immunostaining method named Eastern blotting [8,32]. Furthermore, we established an immunoaffinity column against G-Rb_1 and its application for one-step isolation from crude extract of ginseng root [19, 32]. Herein we describe the preparation of anti-G-Rb_1 immunoaffinity column and it applications for identification and concentration of G-Rb_1.

2.1. Preparation of MAb and immunoaffinity column against G-Rb_1

2.1.1. Analytical methodology for determination of hapten number in antigen, hapten-carrier protein conjugate

The first step for the MAb production is the synthesis of a hapten-carrier protein conjugate. Bovine serum albumin (BSA) conjugated with G-Rb_1 was produced for the preparation of specific MAb in mouse [8]. There had been no direct and appropriate methods for the determination of haptens conjugated carrier proteins without differential UV analysis, radiochemical or chemical methods. Therefore, immunization by the injection of hapten-carrier protein conjugate was unreliable. Wengatez *et al.* determined the hapten density of immuno-conjugates by matrix-assisted UV laser desorption/ionization (MALDI) mass spectrometry [33]. We also reported the direct analytical method of hapten and carrier protein conjugates by a MALDI tof mass spectrometry using internal standard [3-16]. Figure 1 shows the MALDI tof mass spectra of G-Rb_1-BSA conjugate. A broad peak coinciding with the conjugate of G-Rb_1 and BSA appeared from *m/z* 70,000 to 90,000 centering at around *m/z* 79,469. Using experimental results and a molecular weight of 66,433 for BSA, the calculated values of G-Rb_1component (MW1,109) are from 3,327 to 23,289 resulting in the range of 3 to 21 (12 in average) molecules of G-Rb_1 conjugated with BSA [8]. This method is suitable for characterization of conjugates between small molecule natural compound and carrier protein conjugates.

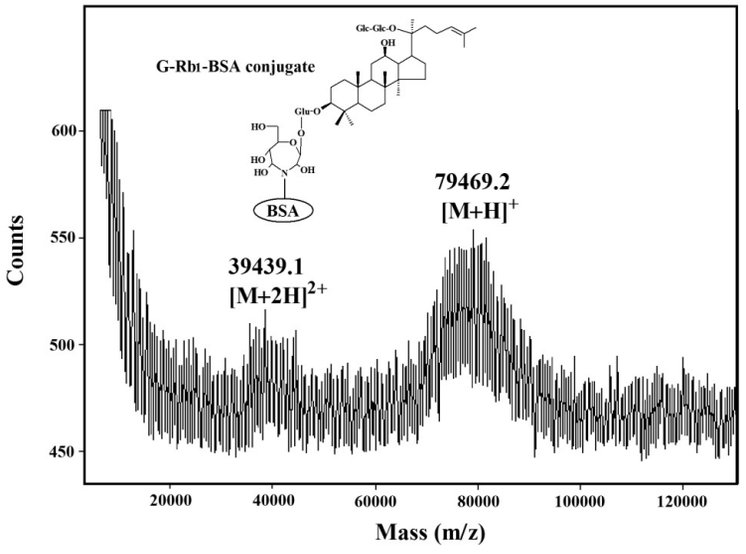

Figure 1. Direct detection of G-Rb_1-BSA conjugate by MALDI tof mass spectrometry. $[M+H]^+$, $[M+2H]^{2+}$ are single and double protonated molecules of G-Rb_1-BSA, respectively.

2.1.2. Preparation of anti-G-Rb_1 MAb and ELISA as an assay system

A hybridoma-producing MAb reactive to G-Rb_1 was obtained by general procedure and classified into IgG2b which had κ light chains [8]. The reactivity of IgG type MAb, 9G7 was tested by varying antibody concentration and by performing a dilution curve. The antibody concentration was selected for competitive ELISA. The free MAb following competition is bound to polystyrene microtiter plates precoated with G-Rb_1-human serum albumin (HSA). Under these conditions, the full measurement range of the assay extends from 20 to 400 ng/mL. The cross-reactivity against G-Rc and G-Rd, which possess a diglucose moiety attached to the C-3 hydroxy group, were weak compared with G-Rb_1 (0.024 and 0.020 %, respectively). G-Re and G-Rg_1 showed no cross-reactivity (less than 0.005 %). It is evident that the MAb reacted only with a small number of structurally related G-Rb_1 molecules, and very weakly and did not react with other steroidal compounds.

2.1.3. Preparation of anti-G-Rb_1 immunoaffinity column and appropriate buffer systems for separation of G-Rb_1

The purified IgG (10 mg) was treated by $NaIO_4$ to give dialdehyde group in sugar moiety which was coupled to Affi-Gel Hz hydrazide gel resulting in a hydrozone-type immunoaffinity gel [32]. The immunoaffinity gel was packed into plastic mini-column (Figure 2). Due to examine the optimal conditions of adsorption and elution, 400 μg of G-Rb_1 was dissolved in phosphate buffered saline (PBS) and loaded on anti-G-Rb_1 affinity column. After washing with washing buffer (20 mM PB containing 0.5 M NaCl), various buffer solutions for elution were loaded on the column, and then the recovery efficiency was determined by ELISA. The G-Rb_1 concentration was somewhat increased 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 AcOH buffer (pH 4), the elution ability reached the optimal level. Although 20 % MeOH could enhance the elution of G-Rb_1, higher MeOH concentration of over 20 % was ineffective. Thus, 100 mM AcOH buffer containing 0.5 M KSCN and 20 % MeOH could be used as an elution buffer in the immunoaffinity chromatography.

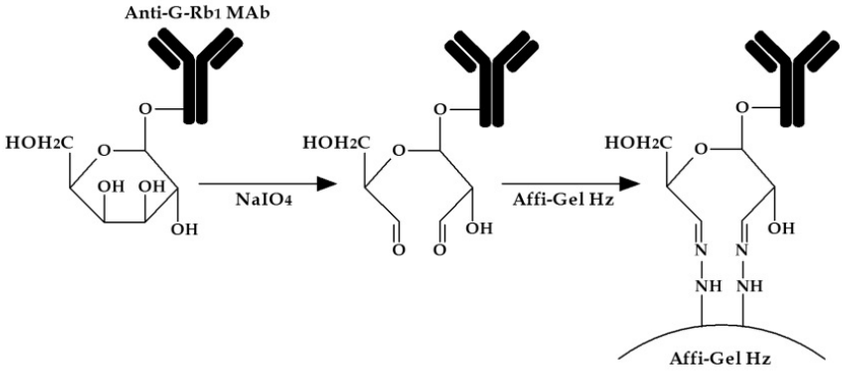

Figure 2. Preparation of anti-G-Rb_1 immunoaffinity column

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2.2. Purification of G-Rb_1 by immunoaffinity column

2.2.1. One-step purification of G-Rb_1 from crude extract of P. ginseng roots by anti-G-Rb_1 immunoaffinity column

A crude extract (3.8 mg) of *P. ginseng* roots was loaded onto anti-G-Rb_1 immunoaffinity column. The column was washed with the washing buffer (fractions 1-20), and then eluted with elution buffer (fractions 21-40). As shown in Figure 3, the fractions 1-8 contained the overloaded G-Rb_1, which determined by ELISA. The other ginsenosides such as G-Rg_1, Rc, Re and Rd were also detected in these fractions by Eastern blotting procedure. After washing, a sharp peak was observed around fractions 21-24 of elution buffer, which contained G-Rb_1. However, these eluted fractions were still contaminated by a small amount of malonyl-G-Rb_1 as detected by Eastern blotting. The malonyl-G-Rb_1 has almost the same cross-reactivity with G-Rb_1 [32]. Therefore, the eluted fractions were treated with a mild alkaline solution (0.1 % KOH in MeOH) at room temperature to give pure G-Rb_1 [19]. Overcharged G-Rb_1 in washing solution (fractions 1-8) was repeatedly loaded and finally isolated in pure form. The anti-G-Rb_1 MAb was stable during all procedures, and the immunoaffinity column showed almost no decrease in capacity (20 μg of G-Rb_1/ml gel) after repeated use more than 10 times under same conditions, as reported for a one-step purification of forskolin from a crude extract of *Coleus forslohlii* root [17].

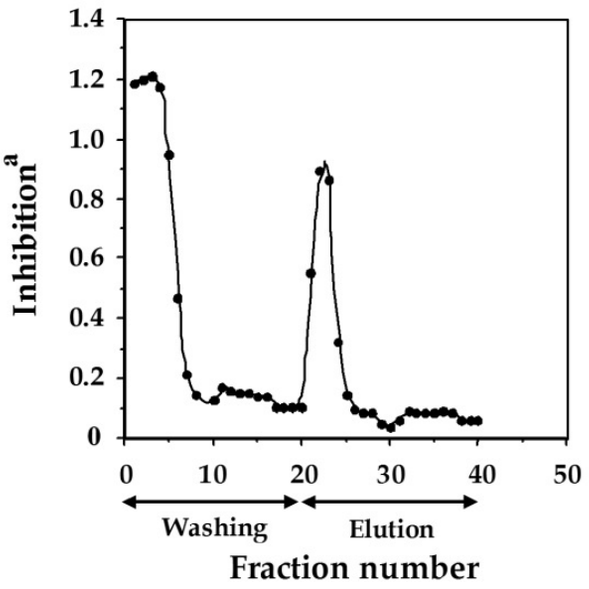

Figure 3. Elution profile of *P. ginseng* crude extract separated by anti-G-Rb_1 immunoaffinity column. The concentration of G-Rb_1 in each fraction was monitoring by ELISA using anti-G-Rb_1 MAb. Individual fraction (2 mL) were assayed by ELISA. [a]Inhibition = $(A_0\text{-}A)/A_0$;A_0 is the absorbance in the absence of the test compounds. A is the absence in the presence the test compounds.

This methodology is effective for the rapid and simple purification of G-Rb_1 and may open up a wide field of comparable studies with other families of saponins for which an acceptable method for one-step separation has not yet been developed. Furthermore, to separate the

total ginseng saponins, a wide cross-reactive MAb against ginsenoside, like anti G-Re MAb which showed wide cross-reactivity, could be designed [34]. A combination of immunoaffinity column, Eastern blotting and ELISA could be used to survey low concentrations of ginsenoside Rb_1 of plant origin and/or in experimental animals and human. In fact we have succeeded in the isolation of G-Rb_1 from a different plant, *Kalopanax pictus* Nakai, which was not known previously to contain ginsenosides, using this combination of methods [35].

2.2.2. Isolation and determination of unknown compounds related to G-Rb_1 by anti-G-Rb_1 immunoaffinity column

Several species of ginseng are known to exist and contain different amount and kinds of ginsenosides. *P. japonicus* is distributed in Japan and China and it is morphologically different from the other *Panax* species. Yahara *et al.* indicated that G-Rb_1 was not detected in *P. japonicus*, and isolated oleanane-type saponins called chikusetsusaponins and determined their structures [36]. Morita *et al*. reported the varieties of saponins in *P. japonicus* by chemical analysis. These results suggested that the concentration of G-Rb_1 might be trace level in *P. japonicas* [37]. We previously analyzed the G-Rb_1 concentration in several ginseng roots by ELISA using anti-G-Rb_1 MAb and HPLC after pre-treatment under mildly alkaline condition [32]. As shown in Table 1, G-Rb_1 concentrationsof *P. ginseng*, *P. notoginseng* and *P. quinquefolius* were correlated between ELISA and HPLC. However, the G-Rb_1 of *P. japonicus* was higher concentrations compared with HPLC and previous reports [37]. This data suggest that anti-G-Rb_1 MAb using ELISA has the cross-reactivity with some unknown compounds contained in *P. japonicus*.

		G-Rb_1 concentration (μg/mg dry weight powder)	
Sample		**ELISA**	**HPLC**
P. ginseng	**White ginseng**	**5.49 ±0.75**	**4.96 ± 0.05**
	Red ginseng	**3.57 ± 0.62**	**3.93 ± 0.34**
	Fibrous ginseng	**64.44 ± 3.64**	**69.75± 1.45**
P. notoginseng		**47.08 ± 3.34**	**42.39 ± 1.39**
P. quinquefolium		**48.51 ± 1.79**	**47.96 ± 1.04**
P. japonicus		**1.37 ± 0.34**	**0.63 ± 0.06**

Table 1. G-Rb_1 concentration in various ginseng samples

To clarify the unknown compounds bound to anti-G-Rb_1 MAb, the crude extract of *P. japonicus* was concentrated by immunoaffinity column using anti-G-Rb_1 MAb. The crude root extract was loaded on the column and washed with the washing Buffer, followed by the elution buffer as already indicated. Figure 4 shows the H_2SO_4 staining (A) and the Eastern blotting (B) profiles of the washing fractions 1-4 and eluted fraction 5. Fraction 1 is first elut-

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ed fraction by the washing buffer, and showed many spots, indicating chikusetsusaponins, similar to the original extract of *P. japonicus*. After washing, the column was eluted by elution buffer (fraction 5), and then one spot was detected. As shown in Figure 4B, Eastern blotting indicated two different spots in washing fraction (Compound 1) and eluted fraction (Compound 2). These compounds bound with anti-G-Rb_1 MAb have a dammarane saponin having protopanaxadiol as a framework.

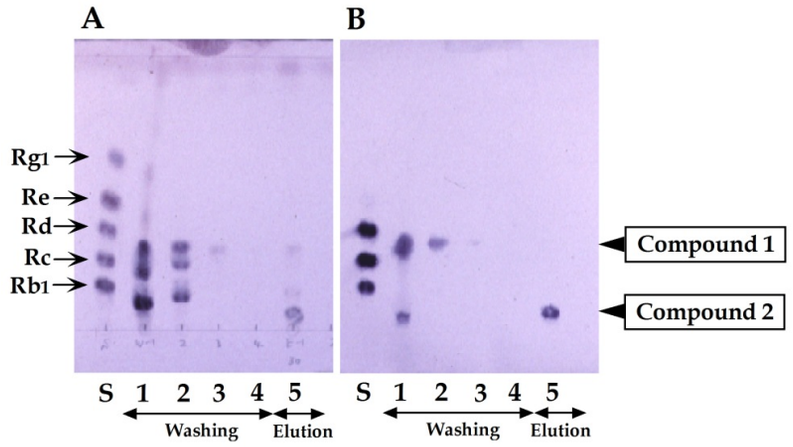
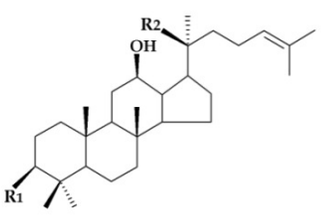


Figure 4. Purification and determination of ginsenosides of *P. japonicus* by the anti-G-Rb_1 immunoaffinity column. TLC (A) and Eastern blotting (B) profiles of the separated fractions from *P. japonicus* crude extract by the anti-G-Rb_1 immunoaffinity column. Lane S indicates the standard of ginsenosides (G-Rd, G-Rc, G-Rb_1, G-Rg_1, and G-Re). Lane 1-4 and Lane 5 were the washing fractions and the eluted fraction, respectively.



Compound	R1	R2
G-Rb1	-O-Glc-Glc (2)	-O-Glc-Glc (6)
Chikusetsusaponin III	-O-Glc-Xyl (6), Glc (2)	-OH
Chikusetsusaponin VI	-O-Glc-Xyl (6), Glc (2)	-O-Glc-Glc (6)

Figure 5. Chemical structures of G-Rb_1 and chikusetsusaponins purified from *P. japonicus* by the anti-G-Rb_1 immunoaffinity column.

Compound 1 has three sugar moieties in a molecule because that the R_F value closes to that of G-Rd, indicating that this compound is chikusetsusaponin III (Figure 5). Finally, we identified that this compound as chikusetsusaponin III in a direct comparison with authentic sample [32]. Another unknown spot, compound 2 appeared in fraction 5 of the eluted fraction. Thus, compound 2 has a similar molecular structure and high cross-reactivity with G-Rb_1, and seems to be related ginseng saponin having protopanaxadiol as an aglycone. Moreover, compound 2 might have the same sugar fragments and possess five sugar moiety compared with G-Rb_1, as indicated by their R_F value. From these evidences compound 2

might be chikusetsusaponin III-20-*O*-gentiobiose, chikusetsusaponin VI (Figure 5), which has 5 sugars in a molecule in good agreement with the R_F value previously reported [38] and we confirmed that compound 2 is chikusetsusaponin VI by the direct comparison with authentic sample.

These data suggested that the anti-G-Rb_1 immunoaffinity column could isolate some unknown structurally resemble compounds having cross-reactivity against anti-G-Rb_1MAb. Therefore, this purification system will be applied to survey new compounds related to target compound of MAb. In our previous studies, we demonstrated the immunoaffinity purification against all solasodine glycosides from crude extract by one-step purification. In this case, all solasodine glycoside have almost same cross-reactivity against anti-solamargine MAb [12].

2.2.3. Preparation of G-Rb_1 knockout extract by anti-G-Rb_1 immunoaffinity column

The capacity of this anti-G-Rb_1immunoaffinity column is 20 μg of G-Rb_1/ml gel [32]. By loading the samples not to exceed the binding capacity against G-Rb_1, this immunoaffinity column becomes possible to remove all G-Rb_1 from crude ginseng extract. Figure 6 showed H_2SO_4 staining of TLC of the purification steps by the immunoaffinity column. Lane 1 and 2 were spotted the standard of ginsenosides (G-Rd, G-Rc, G-Rb_1, G-Rg_1, and G-Re). Lane A, B, and C were the crude extract, the washing fraction, and the eluted fraction, respectively. In the crude extract (lane A), all spots of ginsenosides were clearly detected. On the other hand, the washing fraction (lane B) contained all of the ginsenosides in the crude extract except G-Rb_1. Furthermore, the spot of G-Rb_1 was detected in the eluted fractions (lane C). These data strongly indicated that G-Rb_1 molecule in the ginseng extract can be eliminated by an anti-G-Rb_1 immunoaffinity column and the washing fractions was knockout only by the antigen molecule, G-Rb_1. Thus, we named the washing fractions a knockout (KO) extract [39,40]. This KO extract may be useful for the determination of real pharmacologically active principle in the TCMs.

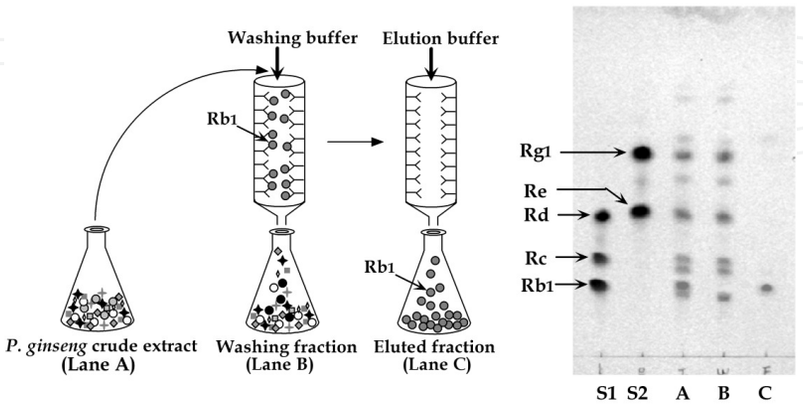


Figure 6. Preparation of G-Rb_1-KO extract from *P. ginseng* crude extract using anti-G-Rb_1 immunoaffinity column. Lane S1 and S2 indicate the standard of ginsenosides (G-Rd, G-Rc, G-Rb_1, G-Rg_1 and G-Re). Lane A, B, and C were the crude extract, the washing fraction, and the eluted fraction, respectively.

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3. Glycyrrhizin-knockout extract and its application for *in vitro* assay

Licorice (*Glycyrrhiza* spp.) is also important crude drug used in over 70 % of the TCMs and Japanese Kampo medicines. It is prescribed with other herbal medicines as a demulcent in the treatment of sore throats, an expectorant for coughs and bronchial catarrh, an antitussive, a taste-modifying agent for relieving pain, an anti-inflammatory agent for anti-allergic reactions, rheumatism and arthritis, a prophylactic for liver disease and tuberculosis and adrenocorticoid insufficiency [41-43]. Accumulated evidence indicated that GC, a main saponin component of licorice, is one of the biologically active compounds. It has been reported that GC exhibits numerous pharmacological effects such as anti-inflammation, anti-ulcer, anti-tumor, anti-allergy, and hepatoprotective activities [44,45]. Clinically, GC has been used to treat patients with chronic hepatitis [46,47]. Although GC is supposed to be a major active principle in licorice crude extract, a number of studies by HPLC profiles suggested that licorice has many other bioactive components, including flavonoids, isoflavonoids and chalcones [43,48]. Biological studies showed that various flavonoid glycosides and their aglycones of licorice exhibit anti-inflammatory, anti-oxidative, anti-microbial, superoxide scavenging, and anti-carcinogenic activities [43,48]. In order to confirm the role of GC in TCM, we previously purified GC from TCM using an immunoaffinity column conjugated with anti-GC MAb [20]. In this section, we describe the preparation of GC-KO extract and its application for functional analysis of GC in licorice crude extract.

3.1. Preparation of GC-KO extract by anti-GC immunoaffinity column and the characterization of GC-KO extract

Our previous study demonstrated the preparation of anti-GC MAb [11]. The cross-reactivities of the anti-GC MAb against glycyrrhetic acid-3-*O*-glucuronide and glycyrrhetic acid were 0.585 % and 1.865 %, respectively. The other related compounds (deoxycholic acid, ursolic acid, and oleanolic acid) were all less than 0.005 %. Moreover, we established competitive ELISA and Eastern blotting method using anti-GC MAb [11,49].

The immunoaffinity column against GC was prepared by coupling the purified 60 mg of the anti-GC MAb to 25 ml of an Affi-Gel Hz gel [11]. To eliminate GC from licorice extract, 12 mg of licorice crude extract (GC content: 1275.0 μg) in loading buffer (5 % MeOH) was applied on the anti-GC MAb immunoaffinity column, and then the loading buffer was continuously circulated through the column to enhance the binding efficiency. After overnight circulation at 4 °C, the unbound fraction was separated. The column was washed with washing buffer (5 % MeOH) and then eluted with elution buffer (20 mM phosphate buffer containing 30 % MeOH). After separation, each fraction was deionized and the solvent was lyophilized. Figure 7 showed the recovery ratio of GC checked by ELISA. In the unbound fraction, 3.50 μg of GC (0.27% of the applied GC) was detected. On the other hand, 1269.26 μg of GC (99.55% of the applied GC) was obtained in the bound fraction. These data indicate that the anti-GC column could eliminate 99.55 % of the loading GC. Thus, we named this unbound fraction “GC-knockout (GC-KO) extract” [50].

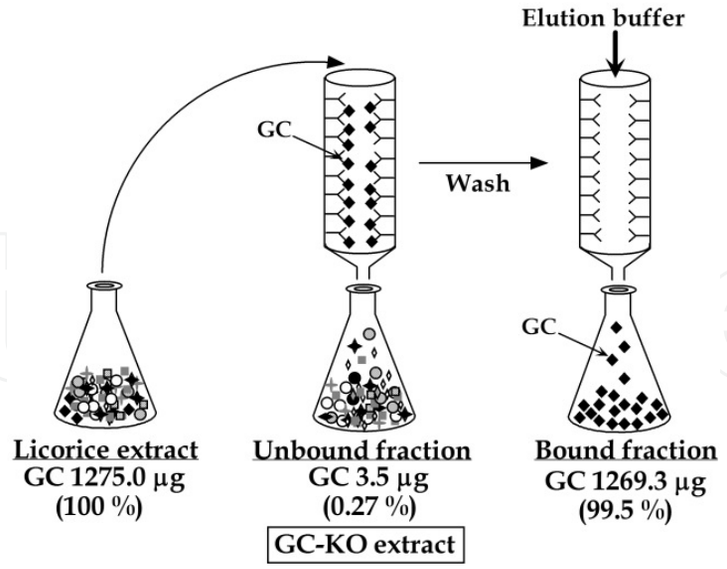

Figure 7. Preparation of GC-KO extract from licorice extract by anti-GC immunoaffinity column.

To further characterize GC-KO extract, the TLC analysis and Eastern blotting were performed [50]. As shown in Figure 8A, several spots including GC were detected in licorice extract (Lane B). However, the spot of GC was completely disappeared in GC-KO extract, although all other spots were clearly detected (lane C). Eastern blotting by anti-GC MAb indicated that GC was detected in licorice extract (Figure 8B, lane B), but the spot of GC was disappeared in GC-KO extract (Figure 8B, lane C). Therefore, these data suggest that GC was specifically eliminated from licorice extract by anti-GC MAb immunoaffinity column.

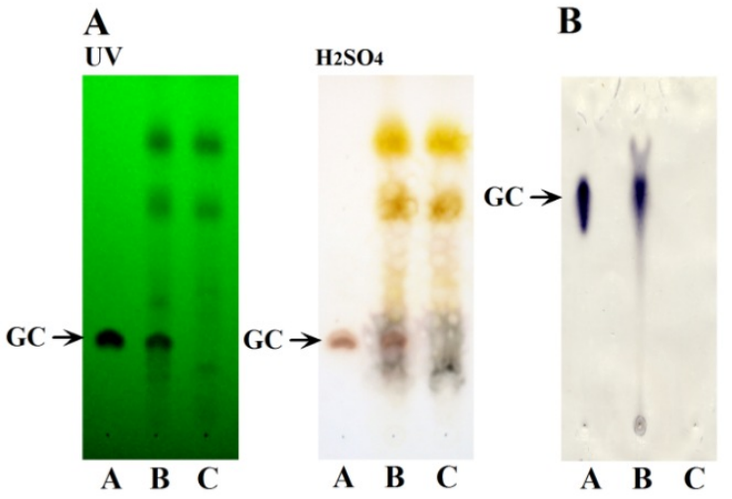

Figure 8. TLC profiles (A) and Eastern blotting by anti-GC MAb (B). Lane A, B, and C indicate GC, licorice extract, and GC-KO extract, respectively.

3.2. *in vitro* Assay by GC-KO extract prepared by anti-GC immunoaffinity column

Nitric oxide (NO), synthesized by NO synthase (NOS) from L-arginine, is an important regulatory/modulatory mediator for several physiological processes [51]. However, during inflammatory process, a large amount of NO is produced by inducible NOS (iNOS) stimulated by bacterial lipopolysaccharide (LPS) and inflammatory cytokines participates in the pathogenesis of inflammatory diseases [52]. Overproduced NO synthesized by iNOS triggers a variety of inflammatory diseases including sepsis, psoriasis, arthritis, multiple sclerosis, and systemic lupus *erythematosus* [53]. Therefore, inhibiting NO production by blocking iNOS expression may be useful strategy to treat a variety of inflammatory diseases.

In LPS-treated mouse RAW264 macrophages, licorice extract inhibited NO production and iNOS expression. At 100 μg/mL of licorice extract, iNOS protein and mRNA were completely suppressed [50].

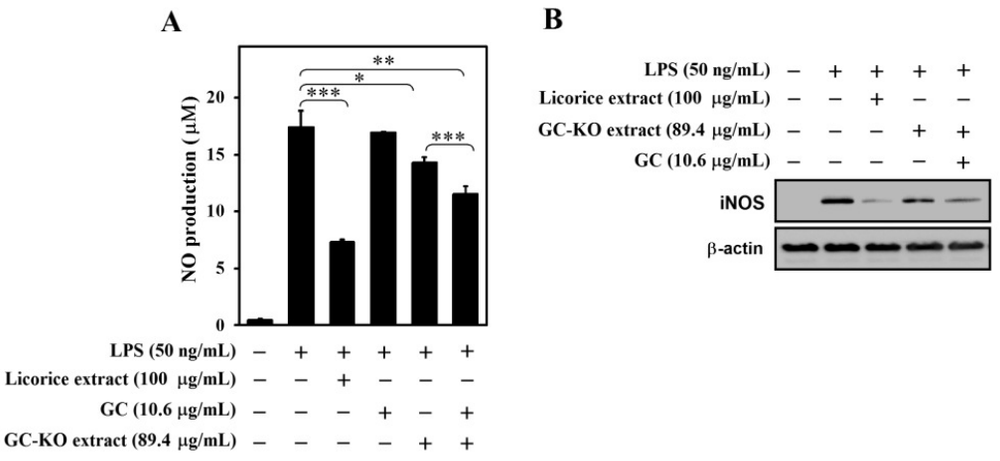

Figure 9. Effect of GC-KO extract and the combination of GC-KO extract and GC on NO production (A) and iNOS protein expression (B) in LPS-treated RAW264 cells.Each bar indicates the mean S.D. of four individual experiments. **P*<0.05, ***P*<0.01, ****P*<0.001 indicate significant differences from the LPS alone

We next examined the inhibitory effect of GC alone, GC-KO extract and the combined treatment with GC and GC-KO extract on NO production [50]. Since 100 μg of licorice extract contains 10.6 ±0.618 μg of GC,the cells were pre-treated with licorice extract (100 μg/ml), GC-KO extract (89.4 μg/ml), or the combination of GC-KO extract (89.4 μg/ml) and GC (10.6 μg/ml). Figure9A indicated that the treatment of licorice extract led to a marked suppression of NO production as compared to LPS treatment [inhibition ratio (IR) 57.7%]. Interestingly, the inhibitory effect of GC-KO extract was lower (IR 17.8%) compared with licorice extract although GC alone could not block NO production as indicated above. On the other hand, the combined treatment with GC-KO extract and GC significantly improved the inhibitory ability (IR 33.5%). To determine whether the combinational effect of GC-KO extract and GC was related to iNOS expression, we performed Western blotting. As shown in Figure 9B, the treatment of GC-KO extract diminished the inhibitory ability of LE on iNOS expression, and addition of GC to GC-KO extract could improve it. These data suggest that GC alone cannot

suppress iNOS expression, but combinational inhibition of iNOS expression may occur when GC coexists with the other constituents contained in licorice extract. The *in vitro* and in *vivo* analysis by using KO extract prepared by immunoaffinity column is a useful approach for determination of potential function of natural compound on *in vitro* and in *vivo* assays.

4. Conclusion

In this chapter, we introduce the unique strategy of one-step purification of target compounds from crude extract by anti-natural compound specific MAb-conjugated immunoaffinity column. The immunoaffinity column conjugated with anti-G-Rb_1 MAb could purify the G-Rb_1 from *P. ginseng* extract, and the washing fraction contained all compounds expect only G-Rb_1, which was named G-Rb_1-KO extract. By the use of the cross-reactivity of MAb, the anti-G-Rb_1 immunoaffinity column can identify new unknown compounds related to target compound of MAb and determine their structures. Furthermore, our data suggest that the combination of the immunoaffinity column and ELISA by using MAb provided a reliable and high sensitivity analysis for target compound in various TCMs and crude extract. We also demonstrated the in vitro assay by using GC-KO extract prepared by anti-GC immunoaffinity column from licorice extract. The KO extract may be able to support the pharmacological investigation for finding out a really active compound in a TCM and crude drug.

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