Preparation of Monoclonal Antibody against Ginsenoside Rf and Its Enzyme Immunoassay

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Summary

A rapid and sensitive indirect competitive enzyme immunoassay method has been developed for quantitating ginsenoside Rf (Rf) in crude total *Panax ginseng* saponins and in rat plasma using high titer mouse monoclonal antibody (mAb) raised against a conjugate of Rf and bovine serum albumin (BSA). The isotype of mAb against Rf was IgG_3 with a κ chain. The presence of Rf inhibited the binding of the mouse anti-Rf mAb to a Rf-BSA solid phase coating antigen. The working range was 0.01-10 ng/assay and detection limits were 20 pg in various ginseng extract fractions or 34 pg in rat plasma per assay. The anti-Rf mAb cross-reacted with ginsenoside Rg_2 by 57.5%, but not with other ginsenosides. However, this anti-Rf mAb did not cross-react with BSA or cellobiose, which is a carbohydrate component of Rf. Using this standard curve, we could measure the amount of Rf in ginseng total extract, ginseng total saponins, protopanaxadiol saponins, and propanaxatriol saponins. We could also measure the amount of Rf in rat plasma after the oral administration of Rf and found that Rf reached a maximum level in rat plasma after 16 h. These results indicate that the anti-Rf mAb could be useful for the quantitation of Rf in crude ginseng fractions and in body fluids.

Introduction

Ginseng, the root of *Panax ginseng* C.A. Meyer, has been used as a traditional herbal medicine for several thousands of years in the Far East. The biologically active main components of ginseng are ginsenosides, which are glycosides of dammarane[1]. About 30 ginsenosides have now been isolated and identified from *Panax ginseng*. They claim to have many medicinal functions such as anticancer, antihypertension, antidiabetic, antinociception, antistress, facilitating learning, and improving weak body

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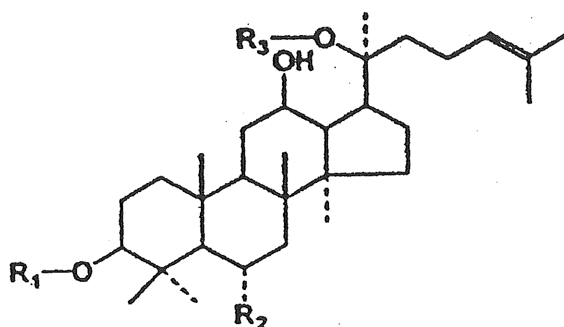
conditions as tonics[2].

Recent studies show that a certain type of individual ginsenoside more potently exerts its physiological or pharmacological activity than other ginsenosides. For example, ginsenoside Rf (Rf) exerted more inhibition of voltage-dependent Ca^{2+} channels in sensory neurons through an unidentified pertussis toxin sensitive G protein than other ginsenosides tested, such as Rb_1, Rc, Re, and Rg_1[3,4]. Ginsenoside Rg_2 and Rf also more strongly inhibited the release of catecholamine from chromaffin cells stimulated by acetylcholine than other individual ginsenosides[5].

In a previous study, we reported the development of an enzyme immunoassay (EIA) for the quantitation of Rf using a polyclonal antibody against Rf-BSA conjugates from rabbit[6]. We also showed the possibility that this polyclonal antibody could be used for the quantitation of ginsenoside Rf as well as Rg_2 as shown in Fig. 1[6]. However, we found that there were some difficulties using this polyclonal antibody for the quantitation of Rf in body fluids or in various ginseng extract fractions with other components, since this polyclonal antibody partially cross-reacted with other plasma proteins, making it difficult to precisely quantitate Rf in plasma. Thus, there are several limitations to use this polyclonal antibody against Rf. Therefore, the aim of this study was to produce a monoclonal antibody (mAb) against Rf and to develop EIA for the quantitation of Rf in various fractions of ginseng extract and in body fluids. Here, we report that we produced mAb against Rf and that this mAb was not interfered with matrices in plasma or other ginseng components.



Ginsenoside	R1	R2	R3
Rb_1	Glc-Glc	H	Glc-Glc
Rf	H	O-Glc-Glc	H
Rg_1	H	O-Glc	Glc
Rg_2	H	O-Rha-Glc	H
Rg_3	Glc-Glc	H	H
Rh_1	H	O-Glc	H
Rh_2	Glc	H	H

Fig. 1 Structures of the 5 representative ginsenosides
They differ at 3 side chains attached to the common steroid ring. Abbreviations for carbohydrates are as follows : Glc, glucopyranoside ; Ara (pyr), arabinopyranoside ; Rha, rhamnopyranoside.

MATERIALS AND METHODS

Materials All purified ginsenosides and other ginseng related compounds were obtained from the Korea Ginseng and Tobacco Research Institute. Anti-mouse IgG_1 from goat-biotinylated and Streptavidin-horseradish peroxidase conjugate were purchased from Amersham. Fetal bovine serum (FBS) and Dulbecco's modified Eagles medium (DMEM) were purchased from Gibco. Myeloma cells (SP2/O-Ag14 mouse myeloma cell line) were kindly provided by the National Veterinary Research Institute. Sprague-Dawley rats and 8-week-old Balb/c mice were purchased from Dae-Han laboratory animal center (Chungju, Korea).

Preparation of Rf-BSA Conjugate Rf was coupled to BSA by the previous procedure[6]. The molar ratio of Rf and BSA for the conjugation reaction was 60 : 1. A fresh solution of $NaBH_4$ was added dropwise and the whole mixture was stirred for 3 h. Thereafter, the pH of the reaction mixture was adjusted to pH 8.5 with 1 N NH_4OH. The reaction mixturewas dialyzed against H_2O for 8 d and lyophilized. The reaction product was dissolved in PBS (pH7.4), and insoluble material was removed by centrifugation. After dialysis, the conjugation solution was lyophilized and stored at-70℃.

Immunization and Cell Fusion Pre-immune serum was collected from each mouse before immunization. For the first injection, 1mg Rf-BSA was dissolved in 1ml saline and the solution was mixed and emulsified with 1 ml of Freund's complete adjuvant in the same ratio. On the third day after the final administration of the antigen, the mouse with the highest titer was sacrificed and its spleen was isolated. Splenocyte pellet was suspended in 5ml serum-free DMEM. After centrifuging, the cell pellet resuspended in 5ml serum-free DMEM. After myeloma cells (SP2/O-Ag14 mouse myeloma cell line) were washed, they were added to the splenocytes suspension at the ratio of 1 : 2. The fusion cell suspension was centrifuged at 800 x g for 5 min. The cell pellet was resuspended in HAT medium containing 15% of FBS and seeded into 96-well cell culture plates with the abdominal macrophages of ICR mouse in HAT medium before 1 d.

Indirect EIA for Detection of Antibody-Producing Clones The plates were screened for the production of antibodies at 2 weeks after the cell fusion. After washing with PBS 3 times, 100μl of diluted biotinylated goat anti-mouse IgG1 was added to each well and incubated for 2 h at 37℃. After washing, 100μl of diluted avidin-horse-radish peroxidase (HRP) was added to each well and incubated for 1 h at 37℃. After washing, 200μl of TMB substrate solution was added to each well. Following incubation for 30 min at room temperature, the reaction was stopped by adding 100μl of 0.5 M H_2SO_4. The activity of the enzyme bound to the solid phase was measured at 450 nm using an ELISA reader (Dynatech MR 4000). Clones showing a positive response against Rf-BSA, but showing negative response against BSA, were selected and subcloned by limiting dilution. Finally, a single clone with a positive response against Rf-BSA but with a negative response against BSA was re-tested using the same method as above.

Ascite Production Balb/c mice were injected with 0.5ml of pristane before 10 d, and single cloned hybridoma cells (1 × 107/ml) in PBS were injected into the abdominal cavities of Balb/c mice. Ascites were collected from the mice swollen just like pregnancy. Collected ascites were incubated for 1 h at 37℃ and then overnight at 4℃. After centrifugation at 6,200 x g for 10 min, the supernatant was collected and kept at the − 70℃ until use.

Isotyping Isotype determination was performed on ascites fluid. The method was based on a sandwich ELISA, which used a Mouse Monoclonal antibody Isotyping Kit.

Competitive Indirect EIA for Determination of Rf A microplate was coated with 50μl of Rf-BSA conjugate dissolved in 50mM carbonate buffer (pH 9.6) overnight at 4℃. After washing with T-PBA as above, 50μl diluted ascites in C-PBS and 50μl standard solution diluted in C-PBS were mixed in each tube, then added into each microtiter well and incubated for 2 h at 37℃. After washing as above, 100μl of diluted biotinylated goat anti-mice IgG_1 was added to each well and incubated for 2 h at 37℃. After washing as above, 100μl of diluted avidin-HRP was added to each well and incubated for 1h at 37℃. After washing, 200μl of TMB substrate solution was added to each well. Following incubation for 30min at room temperature, the reaction was stopped by adding 100μl of 0.5 M H_2SO_4. The activity of the enzyme bound to the solid phase was measured at 450 nm using an ELISA reader (Dynatech MR 4000).

Quantitation of Rf in Various Ginseng Fractions Crude ginseng total extracts, ginseng total saponins, protopanaxadiol saponins, and protopanaxatriol saponins (1mg/ml stock solution) were diluted into 10ng per 50μl C-PBS and then mixed with 50μl of diluted ascite fluid in a tube and added into the well. Other procedures were the same as the above competitive indirect EIA. HPLC for the quantitation of Rf was performed according to method of Yoon *et al.* (1998)[6].

Quantitation of Plasma Rf after Oral Administration The extraction of plasma Rf after oral administration was performed using the method of Akao *et al.* (1996) with slight modifications[7]. Rf was administered orally at a dose of 100mg/kg to rats deprived of food but given free access to water for 18 h before the experiments. We collected rat plasma (n=4) at 0.25, 0.5, 1, 2, 4, 8, 16, 24, or 48h after Rf administration. Collected plasma 100μl and 400μl of methanol were mixed for 30 s by vortexing, then centrifuged at 1,800 x g for 10 min to remove excessive plasma proteins. 200μl of the supernatant was vacuum dried and then appropriately diluted into C-PBS before use for the ELISA quantitation of Rf.

RESULTS AND DISCUSSION

Assay Optimization The number of ginsenoside Rf molecules bound to the Rf-BSA conjugate was 14[6]. Throughout several experiments, we obtained optimal results when we coated 10ng of Rf-OVA conjugate per well, prepared a 100,000-fold dilution of ascites fluid, a secondary antibody dilution of 1 : 1,500, and an avidin-HRP dilution of 1 : 2,000 for the detection of Rf by indirect EIA. In isotyping

experiments the mAb against Rf produced IgG_3 with a κ chain.

Standard Curve for Rf Following optimization of the assay procedure, standard curves for the Rf concentration in working buffer and in rat plasma were obtained, respectively (Fig. 2). When the absorbance of each standard was plotted versus its Rf concentration on a linear-log scale, a sigmoidal curve was obtained. Plotting logit (B/Bo) versus log standard Rf concentration in a working buffer and in rat plasma yielded a good linear response (r=0.996). The assay range for Rf using this EIA was 10pg -10ng per well. The detection limits found was 20pg in working buffer and 34pg in rat plasma per assay.

Assay Specificity The specificity of mAb against Rf was evaluated by cross-reactivity assays using several ginsenosides, BSA, and cellubiose. BSA is a carrier protein of Rf. Cellobiose is the carbohydrate portion of Rf. The mAb did not cross-react with either BSA or cellobiose. This mAb against Rf also did

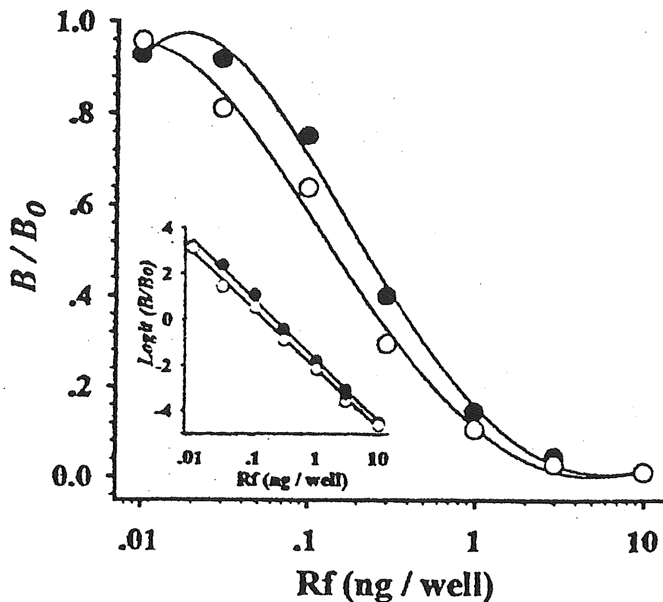


Fig. 2 EIA Standard Curve of Rf in Working Buffer (●) and in Rat Plasma (○)
B and B_0 are the percentages of binding in the presence and absence of Rf. Coating antigen concentration (200ng/ml), monoclonal antibody (1 : 100000 dilution of ascite fluid), secondary antibody (1 : 1500), and avidin-HRP (1 : 2000) were used for the detection of Rf by indirect EIA. We collected rat plasma (n = 4) at 0.25, 0.5, 1, 2, 4, 8, 16, 24, or 48 h after Rf administration. The rat plasma was mixed with methanol at a ratio of 1 : 4 by vortexing, then centrifuged at 1,800 x g for 10min to remove excessive plasma proteins. An aliquot of the supernatant was vacuum dried and then appropriately diluted into C-PBS before use. *Inset* : linearized standard curve for the EIA of Rf (r = 0.996 in both working buffer and in rat plasma). *Logit* (B/B_0) indicates *In* $[(B/B_0)/(1-B/B_0)]$.

not show any significant cross-reaction with Rb_1, Rg_1, Rg_3, Rh_1, and Rh_2. However, mAb showed a relatively high cross-reactivity with Rg_2 by 57.5%, since it has a structure similar to Rf except for having a rhamnose, instead of a second glucose, at the C-6 position on the protopanaxatriol moiety (Fig. 1) (Table 1). The accuracy of an assay method can also be assessed by spiking samples with known concentrations of samples and comparing the recovered amounts with input amounts. Thus, in the spiking experiment we obtained a mean analytical recovery of 88.3 ± 5.0% (mean ± S.E.M) for Rf with known quantities of Rf (1, 2, or 4ng/ml) (n=4).

Table 1. Cross-reactivity Studies of mAb against Rf

Compound	Cross-reaction (%)
Rf	100
Rb_1	0
Rg_1	0
Rg_2	57.5
Rg_3	0.003
Rh_1	0.002
Rh_2	0
BSA	0
Cellobiose	0

Cross-reaction ratio=(Rf concentration to induce 50% inhibition of antibody binding/sample concentration to induce 50% inhibition of antibody binding) × 100

Comparison of Rf Quantitation in Various Ginseng Extract Fractions Using EIA and HPLC, and Quantitation of Rf in Rat Plasma after Oral Administration We also performed comparison experiments for Rf quantitation using EIA and HPLC in various ginseng extract fractions such as crude ginseng total extracts, ginseng total saponins, and protopanaxadiol or protopanaxatriol saponins. We found in the assay using EIA that ginseng total extract, ginseng total saponins, protopanaxadiol saponins, and protopanaxatriol saponins contain 7.9, 14.1, 1.3, and 58μg/mg, respectively. Interestingly, in the assay using HPLC we observed that ginseng total extract and protopanaxatriol saponins appeared to contain more Rf than those of EIA. However, we could not detect Rf in crude ginseng total extract and protopanaxadiol saponins (n=4) (Table 2). Since we found in assay specificity that mAb against Rf did not cross-react with BSA, we tested whether it is possible to quantify Rf in rat plasma after the oral administration of Rf (100mg/kg). Interestingly, we found that Rf was slightly detected in plasma after 8 h oral administration, that Rf reached a maximum concentration in plasma after 16 h (17.24 ± 6.6ng/ml), and that Rf decreased with time. We could not detect Rf after 48 h (Fig. 3).

In this experiment we found that mAb against Rf has several characteristics different from the previous polyclonal antibody against Rf. First, mAb against Rf did not cross-react with BSA, which makes

Table 2. Quantitation of Rf Various Ginseng Fractions Using EIA and HPLC

Fractions	Concentration ($\mu g/mg$)	
	EIA[a)]	HPLC
GTE (4)	7.90 ± 3.0	ND
GTS (4)	14.10 ± 4.0	23.5 ± 6.0
PD (4)	1.30 ± 0.8	ND
PT (4)	57.90 ± 6.3	80.20 ± 9.7

GTE (ginseng total extract), GTS (ginseng total saponins), PD (protopanaxadiol saponins), and PT (protopanaxatriol saponins). The number of tests is shown in parenthesis and the number is mean ± S.E.M. ND: not detected. [a)]EIA has cross-reactivity of 57.5% with Rg_2, as shown in Table 1. HPLC analysis for the quantitation of Rf was performed according to the method of Yoon et al. (1998)[6)]

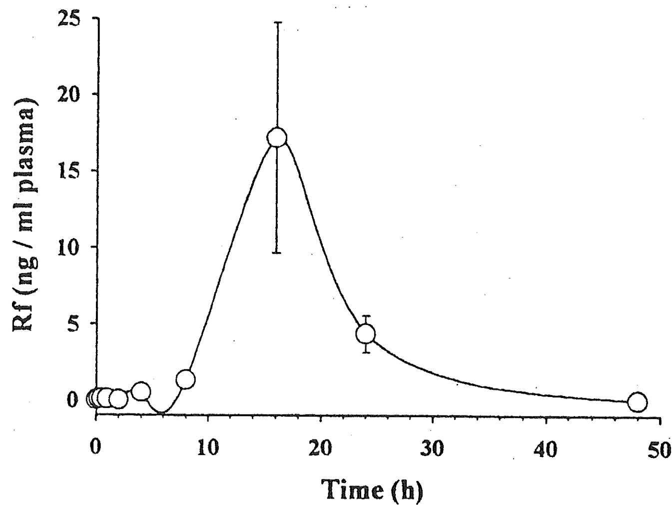


Fig. 3 Rat Plasma Rf Concentration after Oral Administration of Rf (100mg/kg) (n = 4)

possible the quantitation of Rf in body fluid (Table 1). Second, mAb was not interfered with other ginseng components, except Rg_2 in various fractions of ginseng extract. Thus, this also makes possible the quantitation of Rf in various ginseng preparations without further purification of ginseng total extracts prior to analysis, which is in contrast with the HPLC method that requires an additional purification step before Rf detection (Table 2). We also observed that the amount of Rf was increased with fractionation steps from ginseng total extract to protopanaxatriol saponin fractions in EIA assay. In an assay using HPLC, we observed that ginseng total extract and protopanaxatriol saponins contained

more Rf than those of EIA. This may be due to the difference in resolution of these two assays. Moreover, we also observed that a trace amount of Rf could be detected in protopanaxadiol fraction in EIA but not with HPLC (Table 2), although Rf belongs to protopanaxatriol saponins. We could not explain this result currently, but could not exclude the possibility of contamination of a trace amount Rf or Rg_2 that was not detected with HPLC during fractionation. Third, mAb was more selective for Rf than Rg_2, since mAb showed about 50% less cross-reaction with Rg_2 comparing to the previous polyclonal antibody [6].

Interestingly, we found in the Rf absorption experiment that Rf could be first detected after 8 h following oral administration, and that Rf reached a peak at 16 h. But we could not detect Rf after 48 h. These results suggest that the absorption of Rf is slow and that the amount of Rf absorbed is quite a low (Fig. 3). In a previous report, compound K, which is a metabolite of ginsenoside Rb_1, was detected in less than 1 h after oral administration in an experiment using EIA [7]. On the other hand, in experiments of Rg_1 using the TLC analysis method, Rg_1 was detected at 15min, reached a peak at 30min, and was hardly detected after 6 h [8]. Thus, these results show that the absorption rate and amount of individual ginsenosides may be different from each other.

In summary, we produced a mAb against Rf and were able to use this mAb for the quantitation of Rf together with Rg_2 in biological fluid as well as in various ginseng extract fractions and commercial ginseng products.

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