Determination of Ginsenoside Rf and Rg_2 from *Panax ginseng* Using Enzyme Immunoassay

So-Rah YOON,[a] Jin-Ju NAH,[a] Si-Kwan KIM,[b] Seok-Chang KIM,[b] Ki-Yeul NAM,[b] Da-Woon JUNG,[a] and Seung-Yeol NAH*,[a]

Department of Physiology, College of Veterinary Medicine Chonnam National University,[a] Kwangju Korea 500–757 and Korea Ginseng and Tobacco Research Institute,[b] Tae-Jon Korea.
Received February 16, 1998; accepted April 9, 1998

We have developed an enzyme immunoassay (EIA) to quantify trace amounts of ginsenoside Rf (Rf), one of the glycosides of protopanaxatriol from *Panax ginseng*. A carrier protein of bovine serum albumin (BSA) was coupled to the carbohydrate component of Rf using the periodate oxidation method. Antibodies were raised in rabbits using Rf–BSA conjugate as the immunogen and competitive indirect EIA was used for the determination of Rf. The working range was 0.01—10 ng per assay. The anti-Rf antiserum cross-reacted with ginsenoside Rg_2 (105%), which is also a component of *Panax ginseng* and has a very similar chemical structure to Rf. These results suggest that the anti-Rf antiserum could also be used for the quantitation of ginsenoside Rg_2 as well as ginsenoside Rf. In a comparison of EIA and HPLC the linear regression equation and correlation coefficient for the two methods were y(EIA) = 1.31x (HPLC) − 11.48 and 0.98, respectively.

Key words ginsenoside Rf; hapten; periodate oxidation; immunogenic conjugates; cross-reaction; enzyme immunoassay

Recent studies show that ginseng saponins or ginsenosides are the main biologically active components of *Panax ginseng*.[1] About thirty ginsenosides have now been isolated and identified from *Panax ginseng*.[2] Although many investigators have used total ginseng saponins in many previous studies, these individual ginsenosides are also known to have their own specific biological activity that other ginsenosides do not have. For example, ginsenoside Rf (Rf) exerts more inhibition of voltage-dependent Ca^{2+} channels in sensory neurons than other ginsenosides tested such as Rb_1, Rc, Re, and Rg_1.[3] Among various ginsenosides ginsenoside Rf and Rg_2 also strongly inhibit the release of catecholamine from chromaffin cells stimulated by acetylcholine.[4] However, until now there was no method of measuring trace amounts of ginsenoside Rf or Rg_2 from *Panax ginseng* using enzyme immunoassay (EIA) (Fig. 1). Therefore, the aim of this study was to develop a method for the quantification of ginsenoside Rf using EIA. We found that anti-Rf antiserum could also be used for the quantitation of ginsenoside Rg_2 as well as ginsenoside Rf.

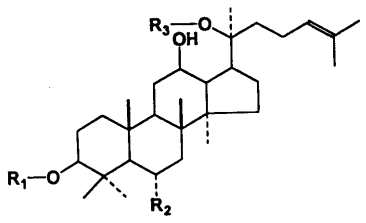
Experimental

Materials All purified ginsenosides were obtained from the Korea Ginseng and Tobacco Research Institute. Bovine serum albumin (BSA), ovalbumin (OVA), complete and incomplete forms of Freund's adjuvant, tetramethylbenzidine (TMB) substrate solution, and casein were purchased from Sigma. Anti-rabbit IgG-peroxidase from sheep was obtained from Boehringer Mannheim. Microtiter plates (flat-bottomed) were purchased from Nunc. All other agents were obtained from Sigma.

Preparation of Rf–BSA Conjugate Rf was coupled to BSA by the procedure used for colchicoside with a slight modification.[5] Briefly, to a solution of Rf (200 mg, 0.25 mmol) in 80% ethanol (9.375 ml), $NaIO_4$ (428 mg, 2 mmol) dissolved with 5 ml H_2O was added dropwise over 40 min under stirring at room temperature. Subsequently, three drops of 50% aqueous ethylene glycol were added to decompose excess $NaIO_4$. After 30 min of further stirring, the precipitate was removed by centrifugation. The supernatant was added dropwise to a solution of BSA (280 mg, 4.2 μmol) in H_2O (30 ml) under stirring at room temperature and the pH was kept at pH 9.0—9.5 by adding 5% aqueous K_2CO_3 during a further 90 min period. The molar ratio of Rf and BSA for the conjugation reaction was 60 : 1. A fresh solution of $NaBH_4$ (147.25 mg, 3.9 mmol) in H_2O (15 ml) was added dropwise and the whole mixture was stirred for 3 h. Then, 1 N HCOOH (pH 6.5) was added to decompose excess $NaBH_4$ followed by one hour of stirring. Thereafter, the pH of the reaction mixture was adjusted to pH 8.5 with 1 N NH_4OH. The reaction mixture was dialyzed against H_2O for 8 d and lyophilized. The reaction product was dissolved in phosphate buffered saline (PBS) (pH 7.4) and insoluble material was removed by centrifugation. The soluble fraction was again dialyzed against H_2O for 4 d. After dialysis, the conjugation solution was lyophilized and stored at −70 °C.

Determination of the Number of Rf Molecules Coupled to One BSA Molecule in the Rf–BSA Conjugate The number of Rf molecules linked to one BSA molecule was determined according to the method of Kanaoka *et al.* using trinitrobenzenesulfonic acid (TNBS).[6] The number of Rf molecules bound to the Rf–BSA conjugate was fourteen.

Preparation of Rf–OVA Conjugate To a solution of Rf (100 mg, 0.125 mmol) in 80% ethanol (9.375 ml), a solution of $NaIO_4$ (374 mg, 7.75 mmol) in H_2O (5.625 ml) was added dropwise over 45 min under



Ginsenoside	R_1	R_2	R_3
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 Ginsenosides

They differ at three side-chains attached to the common ring. Abbreviations for carbohydrates are as follows: Glc, glucopyranose; Rha, rhamnopyranoside.

* To whom correspondence should be addressed.

stirring at room temperature. Subsequently, three drops of 50% aqueous ethylene glycol were added after 30 min of further stirring and the precipitate was removed by centrifugation. The supernatant was added dropwise to a solution of OVA (56.2 mg, 1.25 μmol) in H_2O (15 ml) under stirring at room temperature and the pH was kept at 9.0—9.5 by adding 50% aqueous K_2CO_3. The molar ratio of Rf and OVA for the conjugation reaction was 100 : 1. After 110 min of further stirring, a fresh solution of $NaBH_4$ (147 mg, 3.9 mmol) in H_2O (15 ml) was added dropwise and the mixture was stirred for a further 3 h. 1 N HCOOH (pH 6.5) was added to decompose excess $NaBH_4$ followed by 1 h of stirring. Thereafter, the pH of the reaction mixture was adjusted to pH 8.5 with 1 N NH_4OH and then dialyzed against H_2O for 8 d, lyophilized, and then used as a coating antigen.

Immunization and Antiserum Production Five rabbits were used for immunization with Rf–BSA conjugate. Preimmune serum was collected from each rabbit before immunization. For the first injection 1 mg Rf–BSA conjugate was dissolved in 1 ml saline and the solution was mixed and emulsified with Freund's complete adjuvant in the same ratio. This emulsion was injected subcutaneously into a rabbit at multiple sites on its back. The same dose of immunogen mixed in the same ratio with Freund's incomplete adjuvant was used as a booster once every 2 weeks for two months and given subcutaneously at multiple sites on the back. Then, half of the initial dose of immunogen was used every two weeks for an additional two months and given subcutaneously and intramuscularly at multiple sites on the back and legs. The rabbit was bled one week after the last booster injection of half the initial dose. The serum was separated and stored at −70 °C until required.

Measurement of Antisera Titer by Indirect EIA A microplate was coated with 50 μl Rf–OVA conjugate dissolved in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C. After washing 3 times with PBS (pH 7.4), the plate was post-coated with 200 μl 0.1% casein in PBS (C-PBS) for 2 h at 37 °C. Then the plate was washed 3 times with PBS. The antiserum serially diluted 2-fold with C-PBS was added to each well of the microtiter plate and incubated for 2 h at 37 °C. After washing 3 times with PBS, 50 μl of diluted peroxidase-labeled anti-rabbit IgG was added each well and incubated for 2 h at 37 °C. After washing 4 times with PBS, 200 μl TMB peroxidase substrate solution was added to each well. Following incubation for 30 min at room temperature, the reaction was stopped by adding 100 μl 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).

Competitive Indirect EIA for the Determination of Rf A microplate was coated with 50 μl Rf–OVA conjugate dissolved in 50 mM carbonate buffer (pH 9.6) overnight at 4 °C. After washing 3 times with PBS (pH 7.4), the plate was post-coated with 200 μl C-PBS for 2 h at 37 °C and then the plate was washed 3 times with PBS. Following this 50 μl diluted antiserum in C-PBS and 50 μl standard solution diluted in C-PBS were added to each well of the microtiter plate and incubated for 2 h at 37 °C. After washing 3 times with PBS, 100 μl diluted peroxidase-labeled anti-rabbit IgG was added to each well and incubated for 2 h at 37 °C. After washing 4 times with PBS, 200 μl TMB peroxidase substrate solution was added to each well. Following incubation for 30 min at room temperature, the reaction was stopped by adding 100 μl 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. The concentration of Rf used was in the range 10 pg—100 ng/50 μl per well in methanol. The final concentration of methanol was less than 5%.

HPLC Conditions A Waters 515 pump was used with a Waters™ 486 UV spectrophotometric detector for peak detection (203 nm) coupled to a Waters 746 data module integrator (Attn: 256, PT: 166). A octadecyl silica (ODS) column (YMC-pack, 4.6 × 250 mm, i.d.) was used, and the mobile phase was MeOH–CH_3CN–H_2O (6 : 1 : 3) at a flow rate of 0.3 ml/min. The retention time of Rf was 26 min. Each sample (15, 50, 100, 150, 200, 250 μg/ml) of Rf was assayed three times by HPLC. The same samples diluted 1000—10000 fold were used for EIA.

Results and Discussion

Preparation of the Rf–BSA Conjugate In preparing the aldehyde forms at two vicinal hydroxyl groups of glucose(s) at the C-6 position of ginsenoside Rf, the addition of sodium periodate might produce at least three different types of cleavages from the carbohydrate moiety of ginsenoside Rf. The first type of reaction product could be a cleavage of the first glucose at the C-6 position to give the dialdehyde forms. The second type of reaction product could be a cleavage of the second glucose at the C-6 position. The third type of reaction product could be the cleavage of both glucoses at the C-6 position, although the possibility that the second glucose at the C-6 position could be hydrolyzed during the reaction cannot be excluded. Then, the addition of these three different types of reaction product of Rf to BSA solution might produce at least two different types of Rf–BSA conjugate if the second glucose moiety at the C-6 position was not hydrolyzed. BSA could be conjugated to the dialdehyde form of the first glucose moiety at the C-6 position to produce the monoglycoside–BSA conjugate attached to a second glucose, since the second glucose at the C-6 position is not hydrolyzed. BSA could also be conjugated to the second glucose moiety at the C-6 position to produce a diglycoside–BSA conjugate. However, if the second glucose moiety at the C-6 position is hydrolyzed during the reaction, there will be only one form of monoglycoside–BSA conjugate of ginsenoside Rf. Subsequently, the addition of sodium borohydrate to these Rf–BSA conjugates would be resulted in more stable forms of the conjugates and the uncoupled free Rfs would be removed by dialysis following the final reaction.

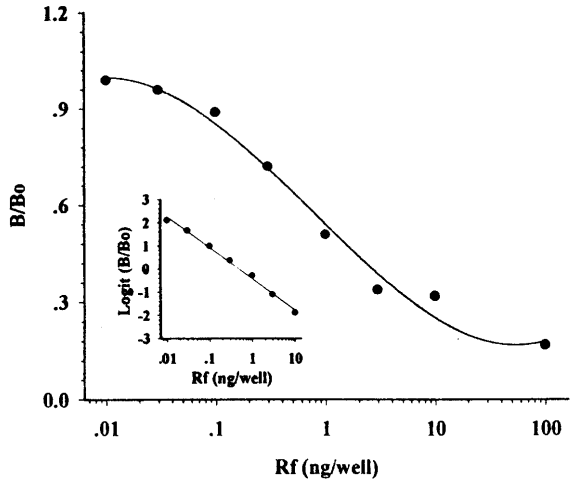


Fig. 2. EIA Standard Curve of Rf

B and *Bo* are the percentage of binding in the presence and absence of Rf, respectively. The concentration of coating antigen was 10 ng/50 μl/well. The dilution of anti-Rf antiserum was 1 : 80000. The dilution of peroxidase-labeled anti-rabbit IgG was 1 : 1000. Inset: Linearized standard curve for the EIA of Rf ($r = 0.99$). Logit (B/Bo) indicates $\ln[(B/Bo)/(1 - B/Bo)]$.

Assay Sensitivity Optimal assay conditions depend on the quantity of coating antigen bound to the surface of the microtiter wells and also on the antibodies dilution. In several experiments we obtained optimal results when we coated 10 ng OVA–Rf conjugate per well, prepared an 80000-fold dilution of the first antibody against Rf and a second antibody dilution of 1 : 1000 for the detection of Rf by indirect EIA. Following optimization of the assay procedure, a standard curve for the Rf concentration was obtained. When the absorbance of each standard was plotted *versus* its Rf concentration on a linear-log scale, a sigmoidal curve was obtained (Fig. 2). Plotting logit (B/Bo) *versus* log standard Rf concentration yielded a

Table 1. Cross-Reactivity Studies of Anti-Rf Antibodies

Compound	Cross-reaction (%)
Rf	100
Rb_1	0.01
Rh_1	0.02
Rh_2	0.02
Rg_1	0.01
Rg_2	105
Rg_3	0.02
Cholesterol	<0.01

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

linear response ($r=0.99$) (Fig. 2, inset). The assay range for Rf using this EIA was 10 pg—10 ng.

Assay Specificity The antibody specificity of anti-Rf antiserum was evaluated by cross-reactivity assays using several ginsenosides structurally related to Rf. Interestingly, the antiserum cross-reacted with Rg_2 (105%), which has the same structure as Rf except for having a rhamnose instead of a second glucose at the C-6 position on the protopanaxatriol moiety. These results show that anti-Rf antiserum can react with Rf as well as Rg_2 and can also be used for the simultaneous quantitation of ginsenoside Rf as well as Rg_2. However, the anti-Rf antiserum did not cross-react to any significant extent with Rb_1 (0.01%), Rh_1 (0.02%), Rh_2 (0.02%), Rg_1 (0.01%), Rg_3 (0.02%), and cholesterol (0.01%) (Table 1). Thus, anti-Rf antiserum showed a generally low cross-reactivity with structurally related ginsenosides except for Rg_2 compared with previous reports.[6,7] This difference might be due to the position of the carbohydrate(s) in each ginsenoside. For example, anti-Rb_1 antiserum, which was raised against Rb_1 with BSA conjugated at the C-26 position of ginsenoside Rb_1, showed some cross-reactivity with Rb_2, Rc, and Rd. In fact, these three ginsenosides share common carbohydrates with Rb_1 at the C-3 position and only differ at the C-20 position (Fig. 1). Similarly, anti-Rg_1 antiserum, which was raised against Rg_1 with BSA also conjugated at the C-26 position of ginsenoside Rg_1, showed low cross-reactivity with Rc, Rd, Re, and Rf. Ginsenoside Rg_1 has one glucose at the C-6 and C-20 positions. These four ginsenosides also share common carbohydrate component(s) with ginsenoside Rg_1. Thus, ginsenoside Rc has a glucose at the first sugar of the C-20 position. Ginsenoside Rd also has a glucose at the C-20 position. Ginsenoside Re has a glucose at first sugar of the C-6 position and has a glucose at the C-20 position. Ginsenoside Rf also has a glucose at the second sugar of the C-6 position. On the other hand, anti-Rf antiserum showed very low cross-reactivity with ginsenosides that have carbohydrate component(s) at the C-3 or C-20 positions, since ginsenoside Rf has no carbohydrates except at the C-6 position (Fig. 1).

In a previous study a method for detecting ginsenoside Rb_1 or Rg_1 using EIA or RIA was also developed and reported.[6,7] In those studies BSA as a carrier protein was introduced at the C-26 position of the unsaturated side-chain in ginsenoside Rb_1 or Rg_1, since ginsenoside Rb_1 or Rg_1 has another carbohydrate component at the C-20 position besides the carbohydrate component at the C-3 or C-6 position, respectively. The procedure for coupling ginsenoside Rb_1 or Rg_1 to BSA required several steps and analysis. In present study we demonstrated that the periodate oxidation method could be used to couple Rf to carrier protein to produce antibodies against Rf, which does not have any carbohydrate at the C-20 position as mentioned above. In addition, this procedure did not require many steps to couple Rfs to carrier proteins such as BSA and OVA and the isolation of Rf–BSA conjugates from free Rf could be achieved by dialysis alone. This procedure has also been used to couple BSA to glycosides such as digoxin and cholchicoside.[5,8]

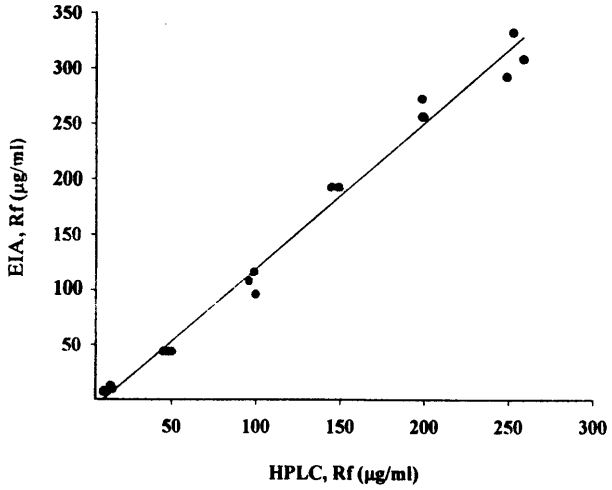


Fig. 3. Correlation between the Values of Rf Obtained by EIA and HPLC

We have also developed a competitive and indirect EIA method for the determination of ginsenoside Rf, one of the trace components of ginseng, which exhibits a number of biological activities. The EIA involved competitive inhibition by Rf of the binding of the anti-Rf polyclonal antibody to an OVA-Rf solid phase coating antigen on a microtiter plate. The binding of the polyclonal antibody to the well was monitored using a peroxidase-labeled anti-rabbit IgG and TMB substrate solution.

The accuracy of this EIA for quantification of Rf using anti-Rf antiserum was evaluated by comparing the results obtained by EIA and by HPLC. Figure 3 shows that there was a good correlation between the values determined by the two methods. The linear regression equation and correlation coefficient for the two methods were $y(\text{EIA}) = 1.31x(\text{HPLC}) - 11.48$ and 0.98, respectively. Using a standard curve we could extend the linear range from 10 pg to 10ng Rf. Therefore, we could measure ginsenoside Rf over the range 10 pg—10 ng. This immunoassay exhibited little cross-reactivity with Rb_1, Rh_1, Rh_2, Rg_1, Rg_3, and cholesterol but interestingly, this immunoassay showed high cross-reactivity with Rg_2 (Table 1). In fact, ginsenoside Rf and Rg_2 are closely related each other as far as their chemical structure is concerned. Moreover, the biological activity of these two ginsenosides is also similar.[4] We also tested the cross-reactivity with steroid hormones such as estrogen and hydrocortisone, since the back-bone structure of these steroid hormones is similar to that of Rf. However, we were unable to detect any

cross-reactivity with these hormones (data not shown). In summary, this sensitive and specific EIA is useful for the quantitation of very low amounts of Rf as well as Rg_2 from *Panax ginseng*.

Acknowledgements This work was supported in part by the Korea Ginseng and Tobacco Research Institute. We thank Dr. Kyung-Keun Kim, Dept. of Pharmacology Chonnam National Medical School for valuable help with the antibody preparation.

References

1) Kaku T., Miyata T., Uruno T., Sako I., Kinoshita A., *Arzneim-Forsch./Drug Res.*, **25**, 539—547 (1975).
2) Nah S. Y., *Korean J. Ginseng Sci.*, **21**, 1—12 (1997).
3) Nah S. Y., Park H. J., McCleskey E. W., *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 8739—8743 (1995).
4) Tachikawa E., Kudo K., Kashimoto T., Takahashi E., *J. Pharmacol. Exp. Ther.*, **273**, 629—636 (1995).
5) Poulev A., Deus-Newmann B., Bombardelli E., Zenk M. H., *Planta Med.*, **60**, 77—83 (1994).
6) Kanaoka M., Kato H., Shimada F., Yano S., *Chem. Pharm. Bull.*, **40**, 314—317 (1992).
7) Sankawa U., Sung C. K, Han B. H., Akiyama T., Kawashima K., *Chem. Pharm. Bull.*, **30**, 1907—1910 (1982).
8) Butler V. P., Chen J. P., *Proc. Natl. Acad. Sci. U.S.A.*, **57**, 71—77 (1967).