

# The Radio-Immunoassay Method for Ginsenoside Rg<sub>1</sub> of Korean Ginseng

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In order to develop the radio-immunoassay procedure for the ginsenoside Rg<sub>1</sub>, we prepared the Rg<sub>1</sub>-BSA conjugate and Rg<sub>1</sub>-tyramine conjugate by condensing the Rg<sub>1</sub>-azide, which was prepared by a series of six step chemical modification of the Rg<sub>1</sub>-side chain, with bovine serum albumin(BSA) or with tyramine. Rabbits were immunized by repeated injection of Rg<sub>1</sub>-BSA conjugate with Freund's Complete Adjuvant for 5 month long to obtain very potent anti-Rg<sub>1</sub> serum. The radio-labelled haptene was prepared by direct radio-iodination (125<sub>I</sub>) of Rg<sub>1</sub>-tyramine according to the chloramine-T method. The radio-immunoassay procedure was successfully furnished by using DCC method (dextran coated charcoal) and the anti-body titer of the anti-serum was found as being 1600~3200 by using 15000cpm tracer per test. Calibration test using non-labelled Rg<sub>1</sub> showed linear competitive binding response in the (8-300) × 34pg. range of non-labelled Rg<sub>1</sub>. The cross reaction test using 19 ginsenoside analogues enabled us a full structure-activity analysis on the antigen-antibody reaction that the anti-body in the serum would recognize the full structure of ginsenoside Rg<sub>1</sub> except the side chain moiety.

The ginsenoside Rg<sub>1</sub> and its related glycosides in Korean ginseng have been known as the bio-active substances by the chemical<sup>1,2)</sup> and biological<sup>3,4)</sup> studies. Now the recent trends of the so called ginseng sciences are developing into a new phase of metabolic studies<sup>5)</sup> and clinical studies<sup>6)</sup> on the components. This newly provoked problems require a very sensitive, specific, and simple assay procedure for the ginsenosides in the presence of the biological fluids or in the presence of the other plant

components. Although several assay procedures for the ginsenosides such as colorimetric method<sup>7)</sup> or GLC<sup>8)</sup> or HPLC<sup>9)</sup> -methods have already been published, they are less feasible to apply to the metabolic and clinical studies due to their poor sensitivity and specificity. RIA-method would suffice those requirements, however, presently available methods are not directly applicable to the ginsenosides.

Recently we developed a RIA method for the ginsenoside Rg<sub>1</sub> which will be applicable to

any other ginsenosides and to any tetracyclic triterpenoids and their glycosides having unsaturated bond in their side chain. Followings are a list of organic substances on which RIA methods have already been described.

#### **RIA in Practice:**

1. Hormones; peptides, steroids, thyroids c-AMP, prostaglandins
2. Plasma proteins; LDL, HDL, fetoprotein
3. Bioamines; catecholamine serotonin
4. Vitamins; A, B<sub>6</sub>, B<sub>12</sub>, folate
5. Antibiotics; penicilins, CM, tetracyclines
6. Drugs; barbiturate, sulfonamides, aspirin, chlorpromazin
7. Toxins; carcinogens, insecticides
8. Normal cell components, sugar, cholesterol
9. Alkaloids; morphine, strychnine, reserpine, colchicine, nicotine, LSD
10. Cardiac glycosides; digitoxin, digoxin
11. Plant H. 3-IAA, gibberellic acid.

This list covers almost every organic substances of biological interest ranging from macromolecular substance to low molecular substances. The macromolecules such as peptide hormones and plasma proteins are highly immunogenic by itself and they usually contain tyrosine residues on which the radio-iodination can be easily accomplished. The low molecular substances such as steroid hormones and various plant constituents are not immunogenic by itself, but they turn to highly immunogenic substances when they are bound to high molecular protein as bovine serum albumin by covalent bonding.

#### **Our Studies Comprises Following Four Categories:**

1. The synthesis of Rg<sub>1</sub>-antigen by condensing the Rg<sub>1</sub>-molecule to the amino group of BSA. In this synthesis some spacing group is

necessary between Rg<sub>1</sub> and the BSA-moiety. The optimum size of the spacing group is told as being six methylene units.

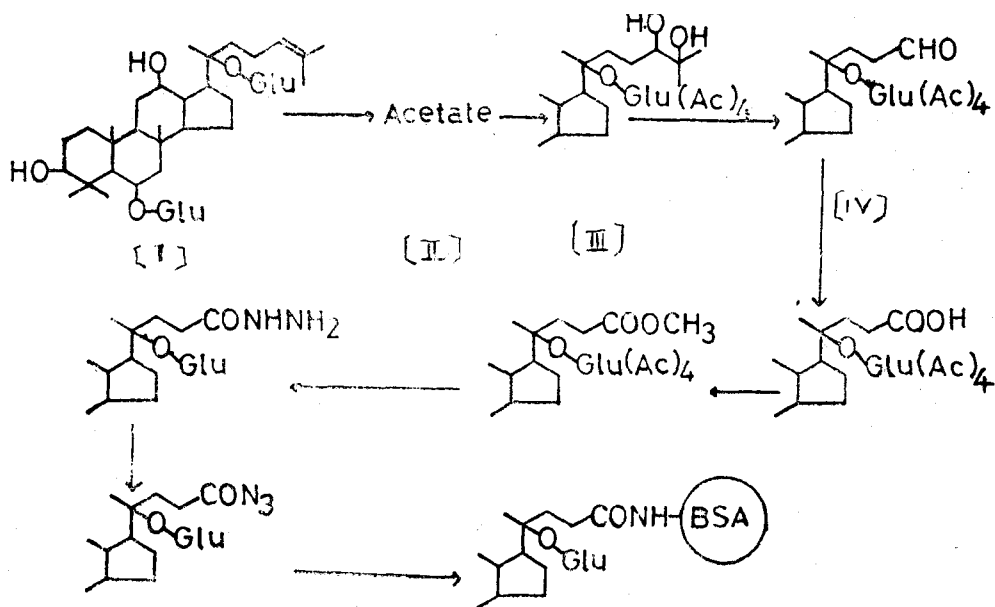
2. The synthesis of the radio-iodinated tracer, that is, the synthesis of Rg<sub>1</sub>-tyramine and then labelling by the radio-iodination.

3. The immunization of rabbits with the Rg<sub>1</sub>-antigen and the production of anti-serum.

4. The establishment of the detailed RIA-procedure and the examination of the specificity and the sensitivity of the RIA-methods.

The ginsenoside Rg<sub>1</sub> was employed as the starting material in this study. It would be able to bind this molecule to BSA by employing either the side chain or sugar moieties as the anchoring site. Choosing the anchoring site is very important to increase the size of the region of structure specific recognition in the antigen-antibody reaction. The structure specific distinction of the ginsenosides is located largely on the structure of polycyclic skeleton and of sugar moiety rather than on the structure of the side chain. If we choose the sugar moiety as the anchoring site, the greater part of the polycyclic skeleton will not be recognized by the anti-body, since six methylene unit close to the binding site will act as the spacing group. Based on this consideration we choose the side chain of the ginsenoside Rg<sub>1</sub> as the anchoring site in the binding with BSA.

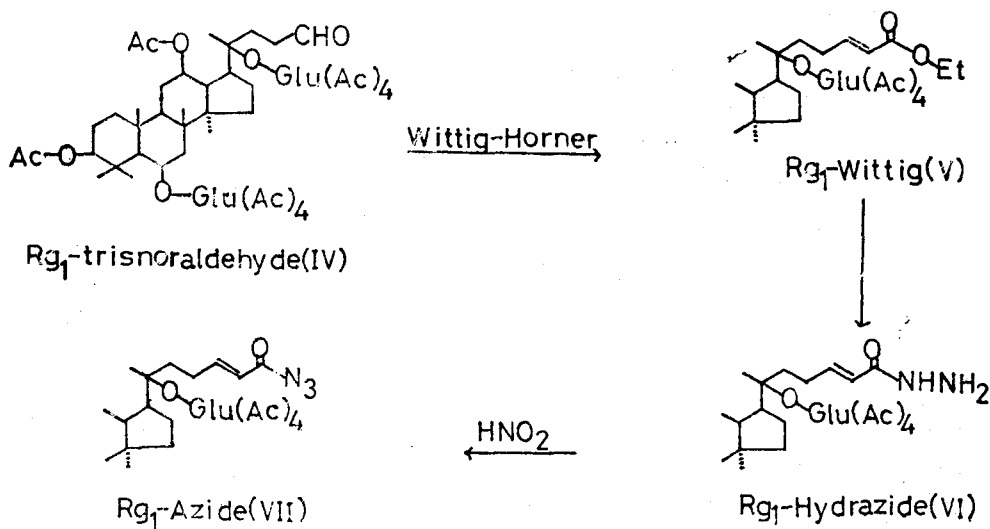
This scheme shows our preliminary approach to conjugate the Rg<sub>1</sub> to BSA. In the organic chemical sense, it is the easiest way to derive a carboxylic acid from unsaturated hydrocarbon chain and then to bind this carboxylic acid to protein by mixed anhydride method or by the aid of dicyclohexyl carbodiimide. However, in case of glycosides as the Rg<sub>1</sub> those methods are not applicable, since the glycoside will self polymerize by this process rather than conjugate to BSA due to their polyvalent hydroxyl group.



Scheme I. Synthetic approach-I

At present, we can not find any example in which the side chain of the saponin has been employed as the ligand in the binding to the protein. In our experiment, the side chain of  $Rg_1$  was modified through this process to carboxylic acid and then to reactive intermediate  $Rg_1$ -azide. Azido-compound of carboxylic acid has already been known to react selectively with amino group to form acid amide in the presence of hydroxyl group. Through this

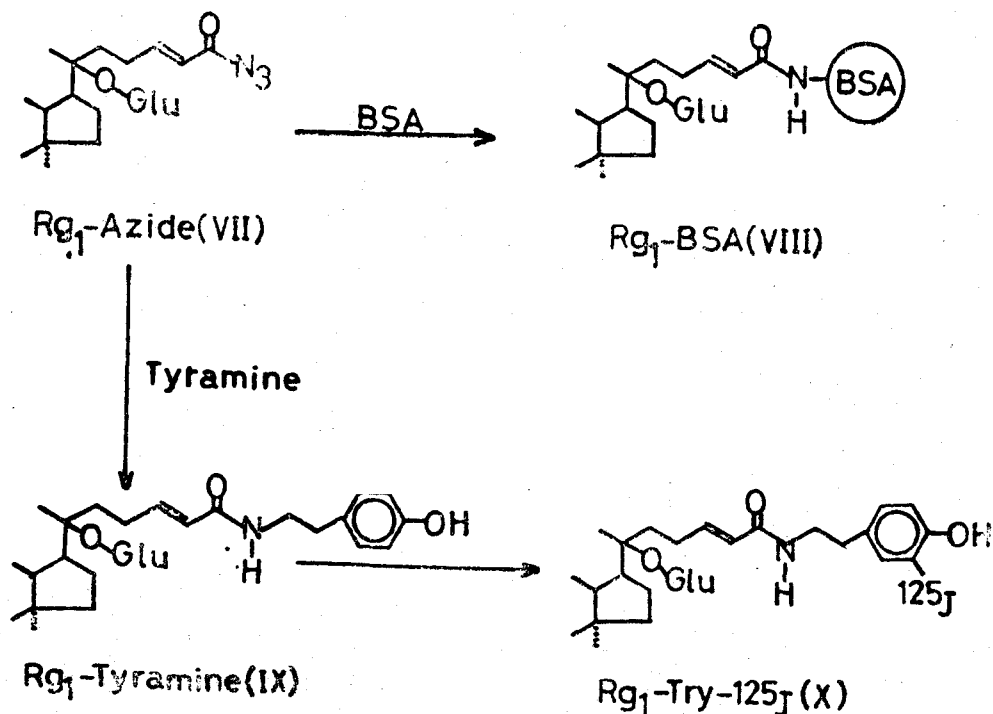
experiment we could see that this approach was very successful in the binding of  $Rg_1$  to BSA. However, when we count back six methylene unit from this carboxylic acid group, two asymmetric carbons near the branching point form polycyclic skeleton will be included in the six methylene unit which will not be recognized by antibody. Then finally this will result in the reduction of the specificity of the RIA.



Scheme II. Preparation of  $Rg_1$ -azide

Starting from the previously mentioned  $Rg_1$ -trisoraldehyde, the side chain could be easily elongated by Wittig-Horner synthesis.<sup>10)</sup> The resulting product was subjected to hydrazinolysis and then converted to  $Rg_1$ -azide. Every reaction intermediates except hydrazide and

azide was pure isolated and identified by spectrometry. One half of the reaction mixture containing azide was condensed with BSA to produce  $Rg_1$ -BSA conjugate and the other one half was condensed with tyramine to produce  $Rg_1$ -tyramine.

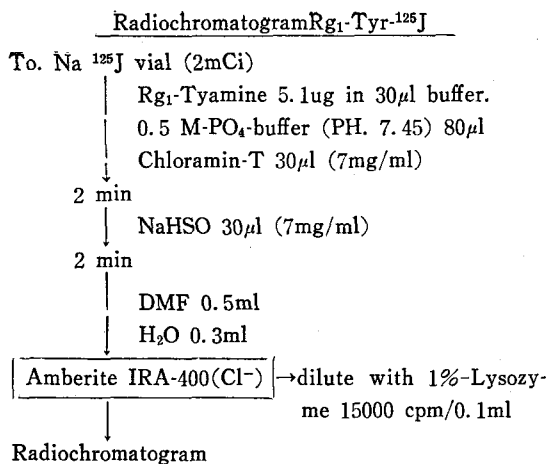


**Scheme III.** Synthesis of  $Rg_1$ -immunogen and  $Rg_1$ -tracer ( $^{125}J$ )

$Rg_1$ -BSA conjugate was dialyzed sufficiently against phosphate buffer to remove unreacted  $Rg_1$ -components and then lyophilized. In order to ensure the binding of  $Rg_1$  to BSA, the  $Rg_1$  content in the conjugate was assayed, as being 3.4mol/mol conjugate by the analysis of sugar content<sup>11)</sup> and 4.6mol by the decrease in protein content.<sup>12)</sup>

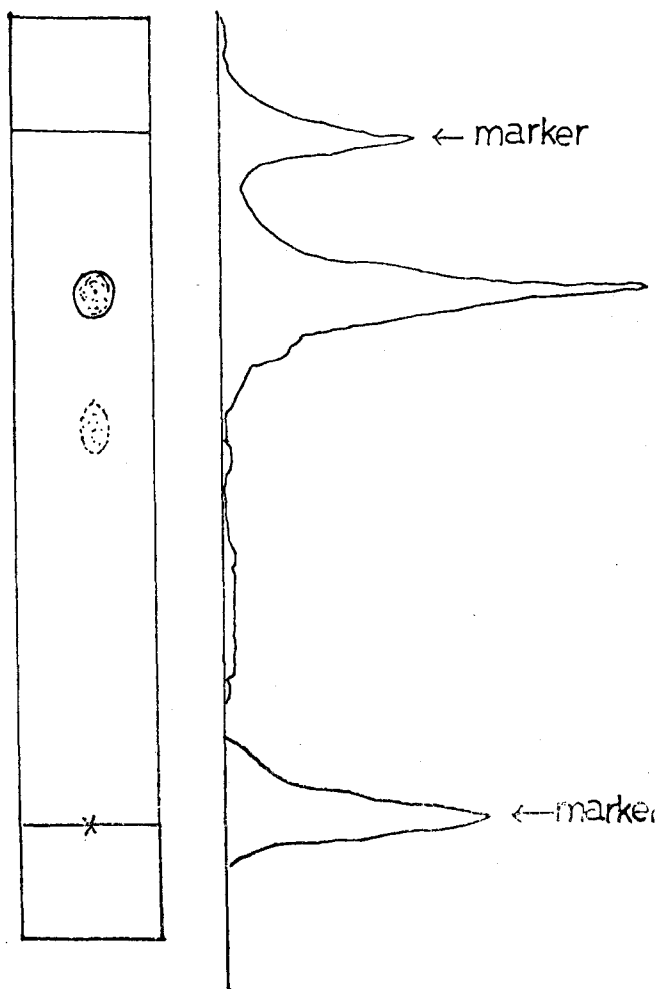
$Rg_1$ -BSA conjugate which was synthesized by the latter process was used as antigen. Rabbits were immunized by the injection of  $Rg_1$ -BSA conjugate 1mg/week for 5 month long with Freund's Complete Adjuvant<sup>13)</sup> and obtained the antiserum by usual way.

The reaction mixture of  $Rg_1$ -Azide with tyramine showed six Pauli<sup>14)</sup> reaction positive spots on TLC and one of them showed also Lieberman-Buchard reaction.<sup>15)</sup> The  $Rg_1$ -tyramine was isolated from the reaction mixture by silicagel column chromatography and it was identified by its NMR spectrum. The NMR spectrum of the  $Rg_1$ -tyramine showed characteristic six angular methyl signals due to the  $Rg_1$ -sapogenin moiety and aromatic protons at 6.64ppm and 6.96ppm and two methylene signals at 2.74 and 3.36ppm due to tyramine moiety.



This scheme shows the experimental details of radio-iodination of R<sub>G1</sub>-tyramine. 5μg R<sub>G1</sub>-tyr. was treated with 2mCi <sup>125</sup>J by chloramine-T method.<sup>16)</sup> In order to remove unreacted iodine, the reaction mixture was treated seven times with the chloride form of Amberite IRA-400. To check the radiopurity of the product, the reaction mixture was chromatographed over pre-coated silica-gel plate, and then the radioactivity distribution was checked by Radiochromatogram scanner.

**Scheme IV.**



**Fig. 1. Radiochromatogram**

As shown in this Fig, the final product seems to be very pure in its radio-chromatogram. In order to minimize the decomposition of the tracer by radiolysis, the final product was

immediately diluted so as to contain 15,000cpm /0.1 ml solution by the addition of 0.1%-human serum albumin.

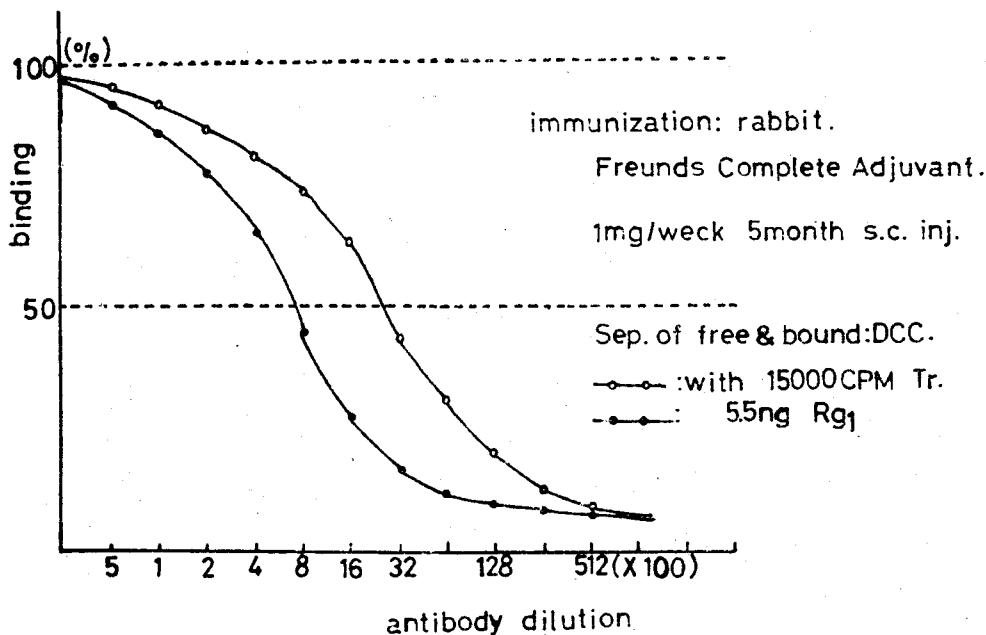


Fig. 2. Antibody titer

An appropriately diluted antibody is mixed with 15,000cpm tracer, buffer, and then incubated. The separation of bound form from the free form of tracer was performed by dextran coated charcoal method.<sup>17)</sup> Free form is selectively adsorbed in the charcoal. Centrifugation, aspiration and  $\gamma$ -counting are all necessary work. In order to determine the antibody titer of the antiserum, two-fold dilution of anti-serum was repeated. Using those two-fold diluted anti-serum, the binding ratio of the tracer was analyzed by the just mentioned process. Anti-body dilution index for 50% antibody binding of the given amount of the tracer is referred as the anti-body titer. Upper curve shows the determination of antibody titer and the lower curve shows the competitive binding of non-labelled Rg<sub>1</sub> between the labelled tracer. In one experiment the antibody titer was

shown as being 3200. This lower curve was obtained by the addition of 5.5 nano-gram of nonlabelled Rg<sub>1</sub>. This curve shows clearly a very sensitive competition by non-labelled Rg<sub>1</sub>. From this curve we could see that our study was very successful.

Fig. 3 shows the calibration curve and the cross reaction test by other ginsenoside analogus. Using 800-fold or 1,600-fold diluted anti-serum and 15,000cpm tracer per tube, the effect of nonlabelled Rg<sub>1</sub> on the antibody binding ratio of tracer was checked. As shown in the Fig. 3, the antibody binding ratio of the tracer was linearly decreased in proportion to the added non-labelled Rg<sub>1</sub> in a range of 270pg-10,000pg. The Fig. 3 shows also the results of cross-reaction test using the structurally closely related ginsenoside analogues. As shown in the Fig. 3, all the other ginsenosides do not compete

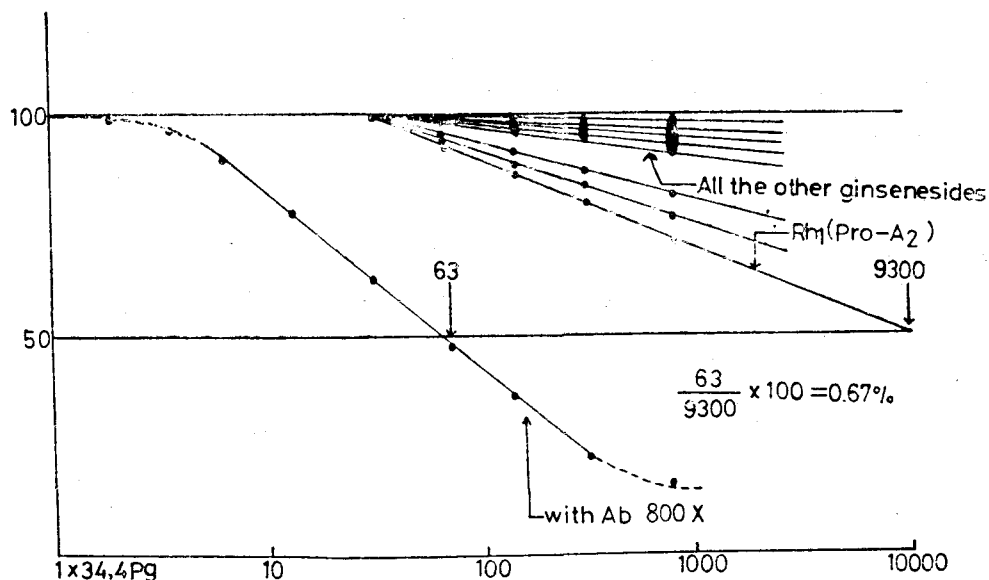
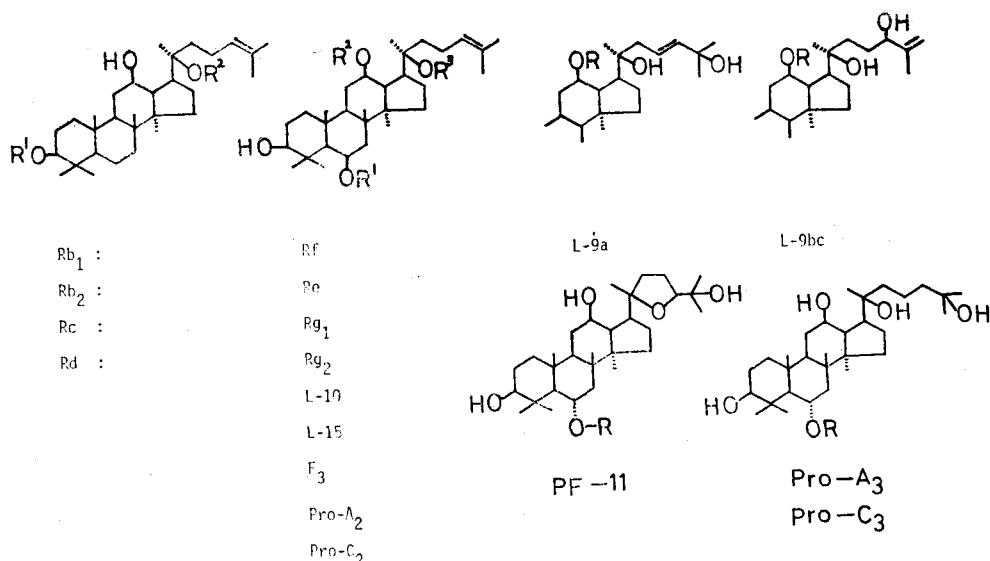


Fig. 3. Calibration & Cross reaction test



Scheme V. Ginsenoside used in cross reaction test

with tracer even at very high concentration.

The above shown are the structures of ginsenoside used in the cross reaction tests. We employed 18 kinds of the ginsenosides which differ each other in their aglycone structure and their glycosyl moiety. Summarizing the above results, we can clearly say that the antibody elicited by our synthetic antigen

recognize the full structure of  $Rg_1$  except side chain.

The highly structure specific recognition of  $Rg_1$ -antiserum is strongly contrasted to the results of digoxin antiserum<sup>17)</sup> which was reported to recognize the ring-C, D and side chain of the digoxin structure. Present  $Rg_1$ -antiserum seems to have sufficient specificity in

antigen recognition and sufficiently higher antibody titer to be useful in the the metabolic studies and in the quality control of the ginseng preparations.

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