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A Simple Mehtod for Conjugation Between Ginsenoside Rf and Bovine Serum Albumin

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Abstract : For the first time we have developed a simple method for conjugation between ginsenoside Rf (Rf) and bovine serum albumin (BSA) or ovalbumin (OVA) by a periodate oxidation method. The Rf-BSA conjugate is confirmed by ultraviolate (UV) scanning, thin layer chromatography (TLC), and SDS-gel electrophoresis. In UV scanning Rf showed three small peaks approximately at 230, 265 and 280 nm. BSA showed a peak at 280 nm. The Rf-BSA conjugate showed right shifted three peaks that BSA alone did not show. In TLC analysis, the Rf-BSA conjugate did not show mobility in silica gel but showed a slight stream of trace. In SDS-gel electrophoresis, Rf-BSA conjugate show a slight less mobility than BSA alone. Rf-OVA conjugate also showed similar patterns with Rf-BSA conjugate. These results demonstrated that periodate oxidation method could be used to produce a stable Rf-BSA or Rf-OVA conjugate and also could be applied for other ginsenoside(s).

Key words : Ginsenoside Rf, BSA, Periodate oxidation, Rf-BSA conjugate

Introduction

Recent investigations demonstrated that ginseng saponins or ginsenosides are the main biologically active ingredients of *Panax ginseng*. About thirty ginsenosides have now been isolated.[1] The physiological or pharmacological role of their individual ginsenosides has become identified at the cellular level. For example, ginsenoside 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.[2] Ginsenoside Rf is more potent in attenuating capacitance increase than other ginsenosides in rat chromaffin cells[3] and also relieves formalin-induced pains in mice.[4] Ginsenoside Rg_2 and Rf strongly inhibit the release of catecholamines from chromaffin cells stimulated by acetylcholine.[5] These results showed the possibility that ginsenoside Rf and other individual ginsenoside could be a novel ligand having various physiological or pharmacological functions in biological system.

However, it is not yet confirmed whether individual ginsenosides having cellular effects act on the cell membrane or penetrate into cells to exert for their biological effects. One way identifying action site(s) of ginsenosides is to couple ginsenoside to other large molecular weight proteins such as BSA and is to test ginsenoside-BSA conjugates for their cellular action, since BSA could not penetrate cell membrane.

On the other hand, the molecular weight of individual ginsenosides is usually in the range of 600 to 1100. Its relatively small molecular weight is not enough to elicit antigenicity when they are administered into experimental animal for antibody production against ginsenoside(s). Therefore, they need to be conjugated with other high molecular weight carrier protein(s).

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The goal of this study was to prepare the conjugate of ginsenoside Rf and BSA for further study the action of ginsenoside Rf and antibody production for ginsenoside Rf.

Materials and Methods

1. Materials

The purified ginsenoside Rf was obtained from Korea Ginseng and Tobacco Research Institute. Bovine serum albumin and ovalbumin were purchased from Sigma. TLC plate were purchased from Merck. All other agents used for conjugation were obtained Sigma.

2. Methods

Preparation of Rf-BSA conjugate: Rf was coupled to BSA by the procedure used for colchicoside with a slight modification.[6] 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 stir- ring 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 m*l*) 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, 3.9 mmol) in H_2O (15 m*l*) 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 days and lyophilized. The reaction product was dissolved in PBS (pH 7.4) and insoluble material was removed by centrifugation. The soluble fraction was again lyophilized against H_2O for 4 days. After dialysis, the conjugation solution was lyophilized and stored at −70℃. Rf-OVA also was prepared with same method used for Rf-BSA.

UV spectral analysis: the optimal density of Rf, BSA, and Rf-BSA (0.5 mg/m*l*) was measured from 200 to 400 nm by using Perkin-Elmer Lamda 18 spectrophotometer (Germany)

Thin layer chromatography : the developing solvent (chloroform : methanol : H_2O=65 : 33 : 10) was mixed well and then waited until this mixture is separated into two layers. Only lower layer was used as the developing solvent. Free Rf, BSA, Rf-BSA conjugate, or Rf+BSA mixture (10 mg/m*l*) was spotted by 3 μ*l* several times on silica precoated plate (Merck, Inc) and put on developing chamber for 3~4 hr followed dried at oven. Visualization was carried out by spraying the plate with 10% H_2SO_4 or 0.5% ninhydrin solution and heating.

SDS-gel electrophoresis: 10% SDS-polyacrylamide gel was used for electrophoresis. BSA (2 μg/lane) and Rf-BSA (10 μg/lane) were used and were stained with coomassie blue.

Results and Discussion

Fig. 1 shows the chemical structure of Rf and a possible procedure and reactions for conjugation between Rf and BSA. The addition of $NaIO_4$ oxidized the carbohydrate components of Rf at C-6 position into aldehyde form. The mixing of Rf having aldehyde forms with BSA induced a spontaneous reaction to form aldimines which are un-

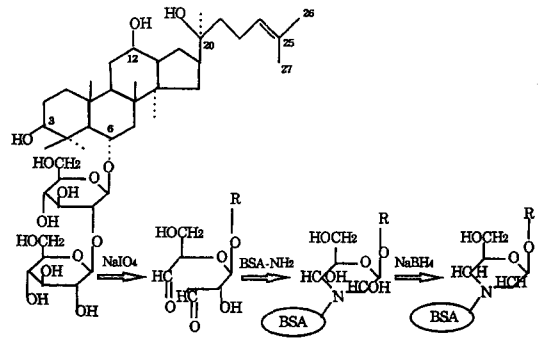

Fig. 1. The proposed scheme for conjugation of ginsenoside Rf to carrier protein (BSA) by periodate oxidation method.

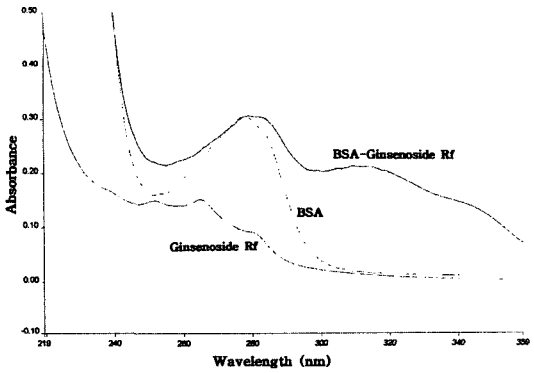

Fig. 2. Ultraviolet (UV) spectra of Rf, BSA, and Rf-BSA. The concentration of Rf, BSA, and Rf-BSA is 0.5 mg/ml, respectively. The range of UV scanning is from 200 to 400 nm. Rf-BSA showed newly formed peaks at about 280, 315, and 345 nm.

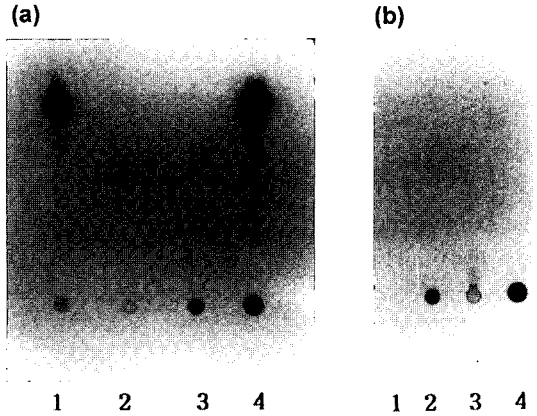

Fig. 3. Thin layer chromatography (TLC) of Rf, BSA, Rf-BSA, and Rf+BSA. A. Rf (lane 1), BSA (lane 2), Rf-BSA (lane 3), and Rf+BSA (lane 4) was visualized using sulfuric acid following development. B. Rf (lane 1), BSA (lane 2), Rf-BSA (lane 3), and Rf+BSA (lane 4) was visualized using ninhydrin following development.

stable due to hydroxyl group but the addition of reducing agent such as $NaBH_4$ produced more stable covalent form of conjugate (Fig. 1).

As shown in Fig. 2, to know that Rf-BSA conjugation is formed following periodate oxidation using $NaIO_4$, we scanned free Rf, BSA, and Rf-BSA, respectively. The concentration of three materials was 0.5 mg/ml. Interestingly, free Rf showed a small three peaks at near 230, 265, and 280 nm. BSA also showed a typical protein peak at 280 nm. In Rf-BSA conjugate, there is a slight right shift of usual protein peak and a large absorbance that was not shown in BSA between 290 nm and 350 nm with two small additional peaks. These results demonstrated that Rf molecules conjugate with BSA, since there are new peaks that was not shown in BSA alone. On the other hand, it looks that three small peaks of free Rf transferred into BSA forming new three peaks of Rf-BSA that originate from free Rf.

As additional experiments to prove Rf-BSA conjugation, we did apply free Rf, BSA, Rf-BSA conjugate, and Rf+BSA mixture onto silica gel plate. As shown in Fig. 3A, after visualization with H_2SO_4 we could compare the migration pattern of four samples. Free Rf migrated as an usual way with developing solvent (Fig 3A, lane 1). However, BSA alone did not migrate as free Rf did but there is a little trace of color with H_2SO_4 treatment (Fig 3A, lane 2). Rf-BSA conjugate also did not migrate with developing solvent but there is a typical color of ginsenoside induced by H_2SO_4 treatment on the spotted place without any trace of free Rf migration, proving that Rf is tightly conjugated into BSA and free Rf was removed during dialysis. There is no free Rf just inserted into BSA molecules (Fig 3A, lane 3). In contrast, simple Rf+BSA mixture showed two colored spots. One is a migrated free Rf and the other one is probably free Rf just inserted into BSA molecules by mixing that was not migrated (Fig. 3, lane 4).

After visualization with ninhydrin, we also could compare the four samples on the migration and color. Free Rf did not react with ninhydrin because it is not protein (Fig. 3B, lane 1). Therefore, there was no color after ninhydrin treatment. But BSA and Rf+BSA mixture showed a typical protein color (Fig. 3B, lanes 2 and 4). Interestingly Rf-BSA conjugate also showed a color but the color was much faint than that of BSA alone (Fig. 3B, lane 3). These results suggest that Rfs conjugated into BSA interfere the reaction of ninhydrin and BSA.

If Rfs were conjugated with BSA, the mobility of Rf-BSA on the gel will be less than BSA, since the conjugation of Rf with BSA will increase the molecular weight. For this experiment we performed SDS-gel electrophoresis. As shown in Fig. 4, the mobility of Rf-BSA was a slightly less than BSA alone. Interestingly, the staining by coomassie blue was not clear as much as BSA alone, probably suggesting that Rfs conjugated into lysine residue interfere the action of the dye. Again this result suggests that Rfs were conjugated into BSA.

In previous study a method for Rb_1- or Rg_1-BSA conjugation was reported.[7,8] In these studies BSA was introduced at the C-26 position of the unsaturated side chain in Rb_1 or Rg_1. The procedure for coupling Rb_1 or Rg_1 to BSA required several steps and analysis. In this study we confirmed that the periodate oxidation method could be used to couple Rf to carrier protein. In addition, this procedure did not require many steps to couple Rfs to carrier proteins such as BSA or 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.[6]

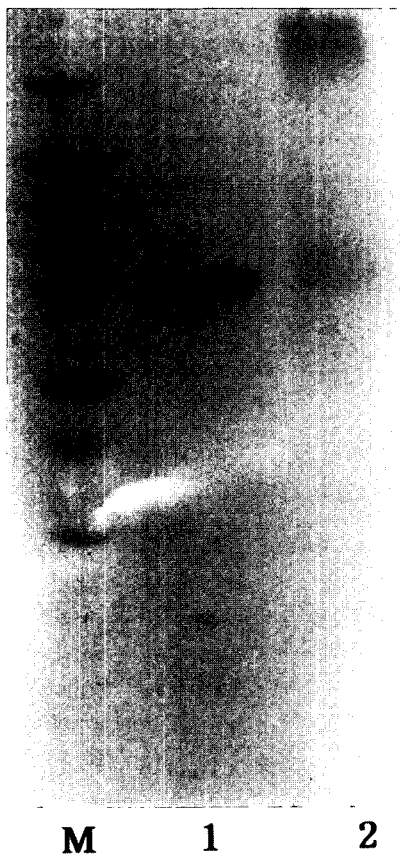

Fig. 4. SDS-gel electrophoresis of BSA and Rf-BSA. M (molecular marker), lane 1 (BSA), and lane 2 (Rf-BSA). There is a slightly less migration of Rf-BSA conjugate than that of BSA alone.

On the other hand, the addition of sodium periodate open two hydroxyl groups of glucose(s) at the C-6 position of Rf to produce aldehyde forms. Thus, this agent produce probably at least three different types of cleavages from the carbohydrate moiety of Rf. The first type product could be a cleavage of the first glucose at the C-6 position to give dialdehyde forms. The second type could be a cleavage of the second glucose at the C-6 position. The third type product could be the cleavage of both glucose 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. 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. Then, BSA could be conjugated to the dialdehyde form of the first glucose moiety at the C-6 position to produce 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 on form of monoglycoside-BSA conjugate of Rf.

In conclusion, we have demonstrated that periodate oxidation method could be used to produce a stable Rf-BSA or Rf-OVA conjugate. This method also could be applied for other ginsenosides having carbohydrate(s) at only C-3, C-6, or C-20 position.

Acknowledgments

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