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Investigation of Ginsenoside Rb1 from *Acanthopanax koreanum* by Eastern Blotting and ELISA Analyses

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Abstract

Ginsenosides such as ginsenoside Rb_1 are the principal components in *Panax* spp. and have been found in some other Araliaceae plants. In our survey of ginsenoside from Araliaceous species based on eastern blotting and ELISA methods using anti-ginsenoside Rb_1 monoclonal antibody, ginsenoside Rb_1, one of the principal ginseng saponins, was investigated with concentrations of 0.000016, 0.000014, and 0.000039% dry weight in the leaves, stem, and roots of the well-known medicinal plant *Acanthopanax koreanum* respectively. The obtained results further support potential and promising application of MAb including eastern blotting and ELISA for surveying ginsenoside sources including ginsenoside Rb_1.

Keywords: *Acanthopanax koreanum*; Araliaceae; Ginsenoside; Ginsenoside Rb_1; Monoclonal antibody; Eastern blotting

Introduction

Acanthopanax koreanum which is a shrub belonging to the Araliaceae family and is distributed in Northeast Asia, has been used as a tonic and to treat rheumatism, allergies, hepatitis, and diabetes [1,2]. Phytochemical profile of *A. koreanum* have been documented with several lignans and diterpenes [3,4] along with, especially, various lupane-type triterpene saponins, which are considered as major constituents [5,6].

In the case of qualitative and/or quantitative analytical approaches of ginsenosides, thin layer chromatography (TLC) [7], high performance liquid chromatography (HPLC) [8,9], and liquid chromatography-mass spectrometry (LC-MS) [10] are used frequently. Recently, it becomes evident that an enzyme-linked immunosorbent assay (ELISA) using monoclonal antibody (MAb) against small molecular has been developed for natural products as a highly sensitive, specific, and simple methodology. In this regard, we have succeed in the preparation of MAb against ginsenoside Rb_1 (G-Rb_1) [11]. ginsenoside Rg_1 [12], and G-Re [13], and established a new immunostaining method, eastern blotting for G-Rb_1 and -Rg_1 [14]. Additionally, in our previous study, the combination of ELISA and eastern blotting methods using anti-G-Rb_1 MAb was applied for the identification of G-Rb_1 in *Panax* species and traditional Chinese medicines containing lower concentrations of G-Rb_1 [15]. This paper herein deals with the finding and determination of G-Rb_1 in *A. koreanum* of Araliaceae family by using ELISA and eastern blotting monitoring.

Materials and Methods

Materials

Polyethersulfone (PES) membranes (Immobilon-N) and glass microfiber filter sheets were from Millipore Corporation (Bedford, MA, USA), and bovine (BSA) and human (HSA) serum albumins were from Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was from Organon Teknika Cappel Products (West Chester, PA, USA). Standard G-Rb_1, -Rc, -Rd, -Re and -Rg_1 were from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All chemicals and solvents used were of analytical grade and water was filtered by a Millipore ultrapure water system (Milli-Q Direct, Merk Millipore, Germany).

Sample preparation

A. koreanum samples were colleted by Dr. Susumu Isoda from Herbal Garden in Faculty of Pharmaceutical Science, Showa University, and authenticated by one of the authors (YS). Dried samples of the plant (roots, stems, and leaves) (50 mg individually) were powdered then extracted with methanol (5 mL) under sonication five times. They were then filtered and the combined extract was diluted with 20% methanol for ELISA and eastern blotting.

Eastern blotting and double staining

After adaptation with procedures from Tanaka et al. [15], *A. koreanum* extracts were loaded onto two TLC plates and developed with *n*-BuOH–EtOAc–H_2O (15:1:4). Of which, one developed TLC plate was dried and stained with H_2SO_4 and another plate was dried followed by spraying with a blotting solution mixture of isopropanol-methanol-H_2O (1:4:16, v/v/v). Next, the treated TLC plate was placed on a stainless steel plate then covered with a PES membrane sheet. After covering with a glass microfiber filter sheet, it was pressed evenly for 70 s with a 120°C hot. The PES membrane was separated from the plate and dried then dipped in water containing $NaIO_4$ (10 mg/mL) while stirring at room temperature for 1 h. After washing with water, 50 mM carbonate buffer solution containing BSA was added followed by stirring for 5 h. The PES membrane was then washed twice with PBS containing 0.05% Tween-20 (TPBS) for 5 min then washed with water. It was then immersed in anti-G-Rb_1 MAb and shaken at 4°C overnight. In the next day, the membrane was washed twice with TPBS-water and 1000 times diluted of peroxidase-labeled goat anti-mouse IgG in PBS containing 0.2% gelatin (GPBS) was then added followed by stirring at room temperature for 1 h. The obtained membrane was then rinsed twice with TPBS and water, and treated with 1 mg/mL 4-chloro-1-naphthol-0.03% H_2O_2 in PBS solution freshly prepared before use for

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10 min at room temperature.

For the staining by anti-G-Rg_1 MAb, the blotted PES membrane was treated in the same procedure as anti-G-Rb_1 MAb except that it was exposed to 2 mg/10 mL 3-amino-9-ethylcarbazole-0.03% H_2O_2 in acetate buffer (0.05 M, pH 5.0) containing 0.5 mL of *N,N*-dimethylformamide.

Competitive ELISA

G-Rb_1 concentrations in *A. koreanum* were analyzed by ELISA [14]. G-Rb_1-HSA (100 µL of 1 µg/mL) was adsorbed onto the wells of a 96-well immunoplate and treated with 300 µL PBS containing 5% skimmed milk (S-PBS) for 1 h to reduce non-specific adsorption. Different concentrations of G-Rb_1 (50 µL) or samples diluted in 20% MeOH were incubated with 50 µL G-Rb_1 MAb solution for 1 h. The sample plates were washed three times with T-PBS and incubated with 100 µL of a 1000-fold dilution of peroxidase-labeled goat anti-mouse IgG for 1 h. Next, the plates were washed again plates with T-PBS and then 100 µL substrate solution (100 mM citrate buffer, pH 4.0, containing 0.003% H_2O_2 and 0.3 mg/mL of 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Wako Pure Chemical, Osaka, Japan) was added to each well followed by incubation for 15 min. The absorbance was recorded on micro-plate reader (Immuno Mini NJ-2300, Nalge Nunc, Roskilde, Denmark) at 405 nm. All reactions were carried out at 37°C.

Results and Discussion

While PES membranes treated with $NaIO_4$ solution and reactive BSA protein enhanced the fixation of ginsenoside-BSA conjugation, small cross-reactivities against anti-G-Rb_1 MAb like G-Rc (0.024%) and G-Rd (0.02%) were also observed [11]. It is noticeable that only G-Rb_1, G-Rc and G-Rd having protopanaxadiol as an aglycone could be stained and this became evident that the specific reactivity of the sugar part in the ginsenoside molecule against MAb might be modified by $NaIO_4$ treatment of the ginseng saponin on the membrane. This phenomenon is important for the surveys of saponins having the same aglycone like ginsenosides. Furthermore, we used two MAbs, anti-G-Rb_1 MAb and anti-G-Rg_1 MAb for eastern blotting as previously reported [16]. In this case, two types of ginsenoside having protopanaxadiol and protopanaxatriol as an aglycone can be stained separately. Purple color and blue color indicate ginsenosides having protopanaxatriol and protopanaxadiol as aglycone, respectively as indicated in previous paper [16].

As shown in Figure 1, although H_2SO_4 staining detected clearly all standard ginsenosides without changing color, the TLC profile of *A. koreanum* crude extract revealed complicated spot patterns indicating that ginsenosides are ambiguously determined. However, the evidence of double eastern blotting unambiguously indicated that no ginsenoside having protopanaxatriol as an aglycone was detected in the *A. koreanum* crude extract because of no purple spot appeared. On the other hand, blue spots were observed meaning that protopanaxadiol type ginsenosides are occured in *A. koreanum*. In addition, it is clear that R_f value on TLC reflects the sugar number in general. Taken together, based on these evidences, it is suggested that *A. koreanum* contains small amount of G-Rb_1 and a more polar ginsenoside which cannot be longer analyzed due to its trace amount (Figure 1).

Next, we analyzed *A. koreanum* leaves crude extract by competitive ELISA using anti-G-Rb_1 MAb (Figure 2) in order to confirm the existence and concentration of G-Rb_1 resulting in 0.000016% dry wt. of G-Rb_1. The roots and stems were also analyzed separately by the same

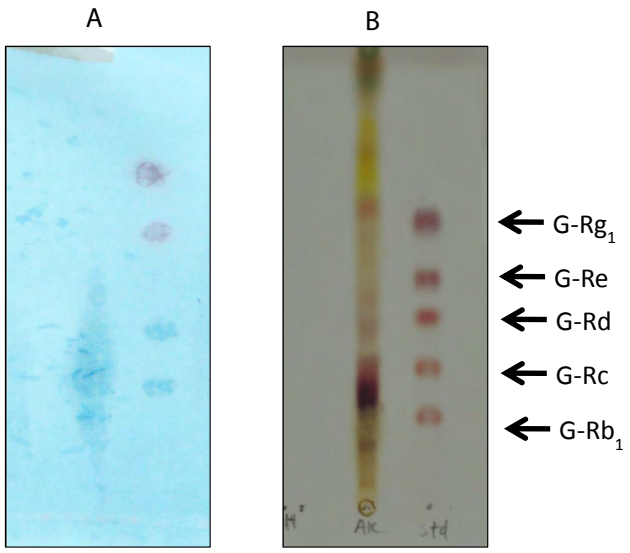

Figure 1: (A) Eastern blotting profile for the standard ginsenosides and MeOH extract of *A. koreanum* (leaf) (B) TLC plate treated with 10% H_2SO_4.

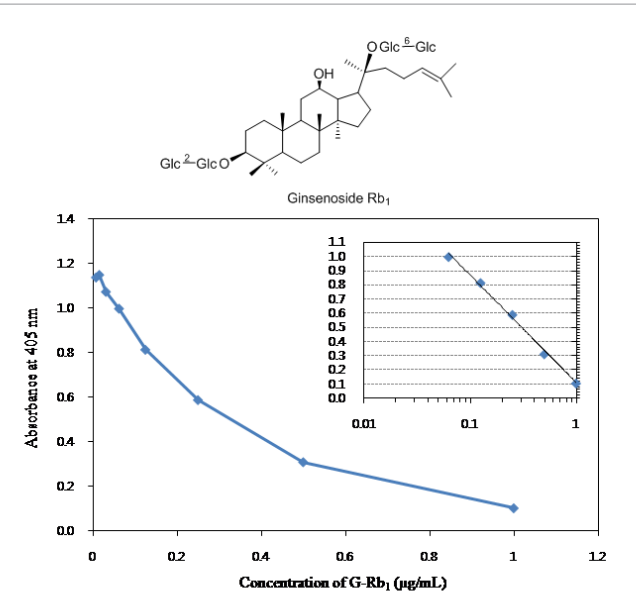

Figure 2: Calibration curve for G-Rb_1 various concentrations of G-Rb_1 were incubated with MAb in wells pre-coated with G-Rb_1 HSA (1 µg/mL). After washing with PBS containing 0.05% of Tween 20, the wells were incubated again with peroxidase-labeled anti-mouse IgG. Absorbance was measured at 405 nm.

manner finding concentrations of 0.000039% and 0.000014% dry wt., respectively. In the view point of naturally occurring compounds, the concentrations of G-Rb_1 in *A. koreanum* is extremely low, therefore, it has become evident that chromatographic purification and analyses of G-Rb_1 have been unaffordable to date. To the best of our knowledge, occurrence of G-Rb_1 and dammarane-type saponins in the *Acanthopanax* genus had not been reported previously.

In conclusion, it is found that *A. koreanum* might be a new resource of G-Rb_1 and more analytical studies are suggested to accumulate G-Rb_1 from the medicinal materials. This study further support potential and

promising application of MAb such as eastern blotting and ELISA for surveying ginsenoside sources.

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