Determination and Isolation of Ginsenosides from Araliaceous Plants by Using Eastern Blotting Fingerprinting

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Abstract

In the eastern blotting method, a new fashion to separate the ginsenoside molecule into two functional parts using a simple and well-known chemical reaction was developed. In principle, the sugar moieties were oxidized by NaIO₄ to form dialdehydes, which reacted with amino groups of the protein and covalently bound to the adsorbent PVDF membrane. The MAb bound to the aglycon part of the ginsenoside molecule for immunostaining. Double staining of eastern blotting for ginsenosides using anti-ginsenoside -Rb₁ and –Rg₁ MAbs promoted complete identification of ginsenosides in Panax species. As an application, we analyzed several Araliaceous plants by eastern blotting and enzyme-linked immunosorbent assay (ELISA) using anti-ginsenoside Rb₁, MAb leading to the investigation of ginsenoside Rb₁, from Kalopanax pictus and Acanthopanax koreanum. On the other hand, by immunoassay-guided fractionation and chromatography separation on the methanol extract of American ginseng, two new minor ginsenosides were isolated. As another application, identification of two known ginsenosides was achieved from the P. japonicas extract using eastern blotting and immunoaffinity column combined with anti-ginsenoside Rb₁, MAb.

Introduction

Ginseng saponins, commonly called ginsenosides, are the principal components in Panax species and have been occurred in several other Araliaceous plants. According to different aglycones, ginsenosides can be classified into three types: the 20(S)-protopanaxadiol type such as ginsenosides Rb₁, Rc, Rb₂, and Rd, the 20(S)-protopanaxatriol type such as ginsenosides Rg₁ and Re, and the oleanolic acid type including ginsenoside Ro and polyacetylene Ginsenoside Ro, respectively [1]. These compounds have been widely investigated for their effects on disturbances of the central nervous system, hypothermia and tumor metastasis, and for their antioxidant, antiinfective, antiaging and radioprotective activities [2]. Especially, ginsenoside Rb₁, one of major ginsenosides, exhibited remarkable effects on the central nervous system [2,3] and drug-induced memory impairment [4]. In addition, the regulation of ChAT, NfI, and trkA mRNA expression by ginsenoside Rb₁ in the rat brain was observed [3]. Therefore, ginsenosides including ginsenoside Rb₁ have played as important resources in the development of new drugs [5].

For qualitative and quantitative analyses of ginsenosides, thin layer chromatography (TLC) [6], high performance liquid chromatography (HPLC) [7,8], and liquid chromatography-mass spectrometry (LC-MS) [9] have been routinely used. Recently, an enzyme-linked immunosorbent assay (ELISA) system has been opened for natural product analysis as the most promising methodology. We have developed the preparation of monoclonal antibodies (MAbs) against ginsenosides Rb₁, [10], -Rg₁, [11], and -Re [12]. Furthermore, we set up the ELISA using individual MAbs and applied for the quantitative analysis of ginsenosides [13,14]. On the other hand, regarding immunostaining we succeeded to immunostain the steroidal alkaloid glycosides using anti-solamargine MAb [15] in the first stage, then established the immunostaining methods for ginsenosides-Rb₁ and -Rg₁ [13]. According to development of new staining method for glycosides, we named this methodology as eastern blotting for immunostaining of glycyrrhizin [16].

Eastern blotting fingerprint for ginsenosides

The ginsenoside Rb₁, Rc, Rd, Rg₁ mixture was applied to TLC plates and developed with n-BuOH-EtOAc-H₂O (15:1:4, v/v/v). One TLC plate developed was sprayed and stained with H₂SO₄. Another TLC plate developed was blotted on the PVDF membrane by heating at around 120°C for short period, the PVDF membrane was treated with NaIO₄ solution to release aldehyde groups in sugar moieties, then reacted with proteins such as bovine serum albumin (BSA) resulting in ginsenoside-BSA conjugates which can fix on the PVDF membrane. On the other hand, an aglycone and a part of sugar moiety as an antigen can be stained by MAb like western blotting. Therefore, it became evident that ginsenosides having small cross-reactivities for anti-ginsenosides Rb₁ and anti-ginsenoside Rg₁, MAbs could be stained in the case of eastern blotting for ginsenosides, suggesting that the specific reactivity of the sugar moiety in the ginsenoside molecule against MAb might be modified by NaIO₄ treatment of the ginsenoside on the membrane, resulting that other ginsenosides having protopanaxatriol as an aglycone such as ginsenosides Re, Rf and Rg₁ can be detectable by eastern blotting using anti-ginsenoside Rg₁, MAb although 3.3% of cross-reactivity for ginsenoside Re [13] (Figure 1). Likewise, ginsenosides possessing protopanaxadiol as an aglycone like ginsenoside Rc, Rd and Rb₁ can be stained using anti-ginsenoside Rb₁, MAb [17] (Figure 2). This finding is important for the surveys of saponins having two types of aglycone like ginsenosides.

Analysis of ginsenoside by eastern blotting fingerprinting

Determination of ginsenoside Rb₁ from Acanthopanax koreanum Nakai: Acanthopanax koreanum (Araliaceae), which is a perennials shrub and distributed in Northeast Asia, has been used as a tonic and for treatment of rheumatism, allergies, hepatitis, and diabetes [18,19].

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Bioactive constituents of *A. koreanum* have been reported including several lignans, diterpenes [20,21], and lupane-type triterpene glycosides, which are considered as major constituents [22,23]. Since we have established the combination of ELISA and eastern blotting methods using anti-ginsenoside Rb1 MAb for the identification of ginsenoside Rb1 in *Panax* species and traditional Chinese medicines [14] as a high sensitive and rapid method, the finding and determination of ginsenoside Rb1 in *A. koreanum* will be reviewed in this section.

Figure 3 showed the double eastern blotting staining (A) and the H2SO4 staining (B) profiles of ginsenoside standards (right) and crude extracts of *A. koreanum* (left) using anti-ginsenoside Rb1 and anti-ginsenoside Rg1 MABs. Although H2SO4 staining detected clearly all standard ginsenosides without color differences, the TLC profile of *A. koreanum* crude extract revealed complicated fingerprinting patterns indicating that ginsenosides are ambiguously determined. From double eastern blotting we confirmed that no ginsenoside having protopanaxatriol as an aglycone was contained in the *A. koreanum* crude extract because no purple spot appeared. On the other hand, blue spots were detected meaning that protopanaxadiol type ginsenosides are contained in *A. koreanum*. It is clear that Rf value on TLC reflects the sugar number in general. From these evidences it is easily suggested that *A. koreanum* contains small amount of ginsenoside Rb1, and a more polar ginsenoside which cannot be longer analyzed due to its trace amount [24].

We analyzed *A. koreanum* leaves crude extract by competitive ELISA using anti-ginsenoside Rb1 MAB in order to confirm the existence and concentration of ginsenoside Rb1 resulting in 0.000016% dry wt. of ginsenoside Rb1. The roots and stems were also analyzed separately by the same manner finding concentrations of 0.000039% and 0.000014% dry wt., respectively. The concentrations of ginsenoside Rb1 in the sample is extremely low, therefore, it has been suggested that chromatographic purification and analyses of ginsenoside Rb1 have been unaffordable to date. To our knowledge, this is the first evidence of ginsenoside Rb1 in *Acanthopanax* species [24]. The results further support potential and promising application of MAB such as eastern blotting and ELISA for surveying ginsenoside sources.

**Determination of ginsenosides from *Panax japonicus***: *P. japonicus*, which is widely distributed in Japan and China, is morphologically different from the other *Panax* species. Regarding its constituents, Yahara et al. reported that no ginsenoside Rb1 was found and isolated several dammarane-type saponins structurally related to ginsenoside like chikusetsusaponin I–VI and oleanane-type saponins named as chikusetsusaponins as major components [25]. In addition, Morita et al. examined the varieties of *P. japonicus* by chemical analysis of saponins [26]. From these results, the concentration of ginsenoside Rb1 might be at trace levels. However, higher concentration compared with previous reports was determined by ELISA [13], although relatively half the concentration of ginsenoside Rb1 was detected by HPLC analysis compared with ELISA. In order to confirm these differences, immunooaffinity column chromatography was employed for immunooaffinity concentration of ginsenoside Rb1. The crude root extract of *P. japonicus* was subjected to the immunoaffinity column and first washed with the washing solvent (PBS; fraction 1) and then with elution solvent (ACOH buffer and 20% MeOH; fraction 2) [14].

Figure 4 shows the H2SO4 staining (A) and the eastern blotting (B) profiles of the two fractions separated by the immunooaffinity
compared with ginsenoside Rb1, as indicated by their panaxadiol. Moreover, this compound might have the same sugar components compared to the unknown ginsenoside has protopanaxadiol as an aglycone and three (Figure 5).

Isolation of ginsenosides by using eastern blotting fingerprinting

Isolation of ginsenoside Rb1 from Kalopanax pictus Nakai

A number of Araliacea species, including Panax species, have been used as tonics in Asian folk medicine. On the basis of phytochemical study, Araliaceous plants have been documented to contain similar constituents such as ginsenoside, depending on their chemotaxonomical classification. Various analytical methods have been used to analyze ginsenosides, and of them, ELISA appears to be the most promising methodology, considerably. This section reviewed the distribution of ginsenosides in Araliaceous species and isolation of ginsenoside Rb1 from the bark of Kalopanax pictus using ELISA and eastern blotting monitoring. Since the bark of K. pictus (Figure 6 line 13) indicated a positive band, the crude extract was analyzed by competitive ELISA using anti-ginsenoside Rb1 MAb, resulting in 0.0009% dry wt. of ginsenoside Rb1. The crude extract was subjected to repeated silica gel column chromatography guided with eastern blotting using anti-ginsenoside Rb1 MAb to afford compound 1. Compound 1 was consistent with that of ginsenoside Rb1 by eastern blotting, and the physical and spectroscopic data (1H- and 13C-NMR) of compound 1 resembled to those of authentic ginsenoside Rb1. Further analyses of the stem bark and leaves by competitive ELISA revealed that a higher concentration of ginsenoside Rb1 was occurred in the leaves (0.0037% dr. wt.) than in the bark (0.0009% dr. wt.) [27]. To our knowledge, this is the first isolation of ginsenoside Rb1 from K. pictus although the isolation of various oleanane saponins was reported [28,29]. These findings suggested that K. pictus might be a new resource of ginsenoside Rb1, and moreover the immunoaffinity column conjugated with anti-ginsenoside Rb1 MAB [13] is readily applicable for purification of ginsenoside Rb1 in the final stage of the separation process.

Isolation of new ginsenosides from Panax quinquefolium: American ginseng (Panax quinquefolium L.), which is mainly cultivated in the USA, Canada and China, has been widely used as a tonic and functional foods in various forms such as decoction, powder, tea, capsule, etc. like Asian ginseng (P. ginseng, CA. Meyer). These conventional ginseng products have been reported to have a wide array of pharmacological and physiological actions including antiaging, antidiabetic, anticarcinogenic, analgesic, antiptic, antioxidant, and antifatigue, respectively [30-33]. The dammarane-type saponins (ginsenosides) are the major active constituents in American ginseng and with the development of modern chromatography, there are more than 30 ginsenosides such as ginsenosides Rb1, Rb2, Rc, Rd, Re and reported [34,35]. However, in comparison with a number of researches on Asian ginseng, the study on American ginseng and its constituents is much less extensive.

Figure 7 showed the double eastern blotting staining (A) and H2SO4 staining (B) profiles of crude extracts of Panax species including American ginseng (line 5) using anti-ginsenoside Rb1 and anti-ginsenoside Rg1 MABs. In general, since H2SO4 staining detected all compounds, the TLC profile stained by this reagent showed complicated fingerprinting patterns so that ginsenosides are
ambiguously indicated. On the contrary, eastern blotting obviously and selectively revealed positive spots regarding ginsenosides. The crude extracts of American ginseng clearly indicated ginsenosides Rg1, Re, Rd, Rc and Rb1, as major ginsenosides. Besides, there are minor spots with the similar color indicating lower Rf value than ginsenoside Rg1 and ginsenoside Rb1 (Figure 7-A line 5) in respect to ginsenoside Rb1, (blue) or ginsenoside Rg1, (purple), suggesting more polar ginsenosides. Accordingly, two minor dammarane-type saponins, namely quinquenosides Ja (1) and Jb (2), were isolated from the American ginseng extract for the first time by immunoassay-guided fractionation and chromatography separation (Figure 8). Their structures were elucidated as 6-O-[α-L-rhamnopyranosyl(1→2)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl(1→4)-β-D-glucopyranosyl]-3β,6α,12β,20β-tetrahydroxydammar-24-ene (1) and 3-O-[β-D-glucopyranosyl(1→2)-β-D-glucopyranosyl]-20-O-[α-L-arabinofuranosyl(1→6)-β-D-glucopyranosyl](1→6)-β-D-glucopyranosyl]-3β,12α,20β-trihydroxydammar-24-ene (2) on the basis of chemical and spectroscopic methods [36]. The results further supported potential and promising application of MAb such as eastern blotting for surveying new ginsenoside sources.

Conclusions

It is well known that ginsenosides possesses wide pharmacological activities, and one of them, ginsenoside Rg1, is now an anti-cancer drug in China suggesting that the other ginsenosides may have the possibility of drug development. However, since such ginsenosides can be supplied from ginsengs resulting in high cost performance of ginsenosides, a new supplement system of ginsenosides is needed. From these aims in this review the eastern blotting using anti-ginsenoside Rb1 MAb was applied to analyze the distribution of ginsenosides in Araliaceous plants, like A. koreanum and K. pictus. From A. koreanum we determined ginsenoside Rb1 by ELISA and separated ginsenosides having the same aglycone with ginsenoside Rb1, and finally determined two ginsenosides. This biotechnological system has a great possibility to widely apply for quick separation of ginsenosides. In fact we succeeded to separate all solasodine glycosides in Solanum khasianum fruits by once immunoaffinity column conjugated with anti-solamargine MAb [37]. The results further used the immunoaffinity column conjugated with anti-ginsenoside Rb1 and separated ginsenosides having the same aglycone with ginsenoside Rb1, and finally determined two ginsenosides. This biotechnological system has a great possibility to widely apply for quick separation of ginsenosides. In fact we succeeded to separate all solasodine glycosides in Solanum khasianum fruits by once immunoaffinity column conjugated with anti-solamargine MAb [37]. The results further
Reference supported potential and promising application of MAb such as eastern blotting for surveying pharmacologically active resources.

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