Introduction

Ginseng saponins, commonly called ginsenosides, are the principal components in Panax species and have been occurred in several other Araliaceae plants. According to different aglycones, ginsenosides can be classified into three types: the 20 (S)-protopanaxadiol type such as ginsenosides Rb1, Rc, Rd, and Rf, the 20 (S)-protopanaxatriol type such as ginsenosides Rg and Re, and the oleanolic acid type including ginsenoside Ro and polyacetyleneginsenoside 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, antiadiposity, 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, NGF 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, -Rg1, and -Re [10]. 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 -Rg [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, Rg 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-ginsenosides Rg 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 Rg, can be detectable by eastern blotting using anti-ginsenoside Rg 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 Rb, can be stained using anti-ginsenoside Rb 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 perennal shrub and distributed in Northeast Asia, has been used as a tonic and for treatment of rheumatism, allergies, hepatitis, and diabetes [18,19].

Keywords: Araliaceae; Acanthopanax koreanum; Kalopanax pictus; Panax quinquefolium; Ginsenoside; Ginsenoside Rb1; Monoclonal antibody; Eastern blotting
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 of Araliaceae family by using ELISA and eastern blotting monitoring will be reviewed in this section.

Figure 3 showed the double eastern blotting staining (A) and the H$_2$SO$_4$ staining (B) profiles of ginsenoside standards (right) and crude extracts of A. koreanum (left) using anti-ginsenoside Rb1, and anti-ginsenoside Rg1 MABs. Although H$_2$SO$_4$ 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 japonicas: P. japonicas, 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, immunoaffinity column chromatography was employed for immunoaffinity 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 H$_2$SO$_4$ staining (A) and the eastern blotting (B) profiles of the two fractions separated by the immunoaffinity
compared with ginsenoside Rb1, as indicated by their panaxadiol. Moreover, this compound might have the same sugar components compared to the unknown ginsenoside having protopanaxadiol as an aglycone and three (Figure 5).

Evidence revealed that an unknown compound contained in fraction 2 resembled molecular structure and a similar cross-reaction with TLC as indicated in (Figure 4). The unknown compound is proposed to resemble ginsenoside Rb1 and likely a ginseng saponin having an aglycone of G-Rb1, and with the development of modern chromatography, there are more than 30 ginsenosides such as ginsenosides Rb1, Rb2, Rg1, Rc, Rd, and Re reported [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, Rg1, Rc, Rd, and Re reported [34,35]. However, in comparison with a number of researches reported [34,35], it is much less extensive.

Isolation of new ginsenosides from Panax quenquefolium: American ginseng (Panax quenquefolium 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 (Panax ginseng, CA. Meyer). These conventional ginseng products have been reported to have a wide array of pharmacological and physiological actions including antiaging, antidiabetic, anticancerogenic, analgiesic, antipyretic, antistress, 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, Rg1, Rc, Rd, and Re 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.

Isolation of ginsenosides by using eastern blotting fingerprinting

Isolation of ginsenoside Rb1 from Kalopanax pictus Nakai: A number of Araliaceae 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% dry wt.) than in the bark (0.0009% dry 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.

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Figure 5. Identified compound 1 and 2 by using immunoaffinity column and eastern blotting and their structures together with ginsenosides Rb1, Rc and Rd in Panax japonicas.

Figure 4: Purification and determination of ginsenosides of P. japonicas by immunoaffinity column and eastern blotting.

Figure 5. Identified compound 1 and 2 by using immunoaffinity column and eastern blotting and their structures together with ginsenosides Rb1, Rc and Rd in Panax japonicas.

Column. Fraction 1 eluted with the washing solvent showed two spots in the eastern blotting profile. A clear unknown spot appeared in fraction 2 eluted with the elution solvent. However, ginsenoside Rb1 was not detected by eastern blotting although it was determined by TLC as indicated in (Figure 4). The unknown compound is proposed to resemble molecular structure and a similar cross-reaction with ginsenoside Rb1, and likely a ginseng saponin having an aglycone of protopanaxadiol. Moreover, this compound might have the same sugar fragments in the molecule, but possess an additional sugar moiety compared with ginsenoside Rb1, as indicated by its Rf values. These evidences revealed that an unknown compound contained in fraction 2 is chikusetsusaponin VI comparing with the authentic sample directly (Figure 5).

The eastern blotting fingerprinting of fraction 1 indicated that an unknown ginsenoside has protopanaxadiol as an aglycone and three sugar components compared to the Rf value of ginsenoside Rc. Therefore, we determined that the unknown compound contained in fraction 1 is chikusetsusaponin III compared with the authentic sample (Figure 5). From this investigation we confirmed that the saponins having protopanaxadiol as an aglycone can be separated by the immunoaffinity column conjugated with anti-ginsenoside Rb1 MAb. Moreover, we concluded that the combination of immunoaffinity concentration and ELISA is suitable for samples containing lower concentrations of ginsenoside Rb1 since the immunoaffinity column can concentrate the ginsenoside Rb1 concentration [14].
ambiguously indicated. On the contrary, eastern blotting obviously and selectively revealed positive spots regarding ginsenosides. The crude extracts of American ginseng clearly indicated ginsenosides Rg, Re, Rg, Rc, and Rb, as major ginsenosides. Besides, there have minor spots with the similar color indicating lower Rf value than ginsenoside Rg and ginsenoside Rb, (Figure 7A-line 5) in respect to ginsenoside Rb, (blue) or ginsenoside Rg, (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 immunoadsorbent-guided fractionation and chromatography separation (Figure 8). Their structures were elucidated as 6-O-[α-L-rhamnopyranosyl(1→4)-β-D-glucopyranosyl]-20-O-[β-D-glucopyranosyl(1→4)-β-D-glucopyranosyl]-3β,6a,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,12β,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 Rg, 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 Rb and/or anti-ginsenoside Rg MAb was applied to analyze the distribution of ginsenosides in Araliaceous plants, like A. koreanum and K. pictus. From A. koreanum we determined ginsenoside Rb, by ELISA and eastern blotting using anti-ginsenoside Rb, MAb. P. quinquefolium were tested, and the structure elucidations of two new ginsenosides from P. quinquefolium were succeeded. The combination of eastern blotting and immunoaffinity column conjugated with anti-ginsenoside Rb, MAb makes it evident to separate ginsenosides having small cross-reactivity with anti-ginsenoside Rb, MAb. In the case of P. japonicus we used the immunoaffinity column conjugated with anti-ginsenoside Rb, and separated ginsenosides having the same aglycone with ginsenoside Rb, 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


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References


