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Visual detection of saikosaponins by on-membrane immunoassay and estimation of traditional Chinese medicines containing Bupleuri radix

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Abstract

The purpose of this study was to describe the simple, rapid, and environmental-cost effective determination method for saikosaponins in complicated samples like Bupleuri radix and traditional Chinese medicines (TCM). Saikosaponin standards, extracts of Bupleuri radix and TCM, were applied to a polyethersulphone (PES) membrane and developed by acetonitrile–water (1:4, by volume). Saikosaponin a (SSa), SSc, and SSd were visually detected by an immunostaining method (called Eastern blotting technique) using a monoclonal antibody (MAb) against SSa. At least 62.5 ng of SSa, SSc, and SSd were clearly detectable individually. These coloring spot areas of saikosaponins on PES membrane were calculated by using the NIH Imaging software and three saikosaponins can be analyzed quantitatively between 62.5 ng and 1.0 μg. Saikosaponins in Bupleuri radix and TCM were determined and these results of SSa and total saikosaponin concentrations were in good agreement with those from the ELISA analysis.
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Keywords: Saikosaponins; Bupleuri radix; Traditional Chinese medicines; On-membrane immunoassay; NIH Imaging analysis

Bupleuri radix (*Bupleurum* spp. root) is one of the most important crude drugs used in many TCM, prescribed with other crude drugs for many diseases. It is believed that a part of its pharmaceutical properties is due to saikosaponins belonged to triterpenoid saponin (Fig. 1). A major saponin, saikosaponin a (SSa), has anti-cancer [1], anti-inflammatory [2], corticosterone secreting [3], and plasma-cholesterol decreasing activities [4]. SSb_2 performs phagocytosis-increasing activity on macrophages [5]. SSd shows liver protective effect [6], anti-inflammatory [7], immunoregulatory [8], virus inactivating [9], and anti-cancer activities [10]. Furthermore, in a prescription of TCM (Sho-saiko-to) containing Bupleuri radix, SSa, SSb_2, SSc, and SSd showed anti-tumor effects [1].

Recently, we prepared a specific MAb against SSa [11] and a MAb showing wide cross-reactivity to saikosaponins [12], and the developments of enzyme-linked immunosorbent assay (ELISA) system for measurement of SSa and total saikosaponins. However, it is difficult to gain each content of SSa, SSb_2, SSc, and SSd by these competitive ELISA methods. In pharmacological investigation of TCM, it is very important to grasp each bio-active compound content in each crude drug prescribed individually, because individual saikosaponins have significant effects as indicated above.

In addition we have investigated the immunostaining of naturally occurring bioactive glucosides in our ongoing studies of preparation of MAbs and their applications. We have developed improved Western blotting techniques with new immunostaining methodology for the identification of glucoalkaloids [13], ginsenoside-Rb1, -Rc, and -Rd [14–16], and Eastern blotting technique of glycyrrhizin [17] which make it possible to visualize small molecule compounds on a polyvinylidene difluoride (PVDF)

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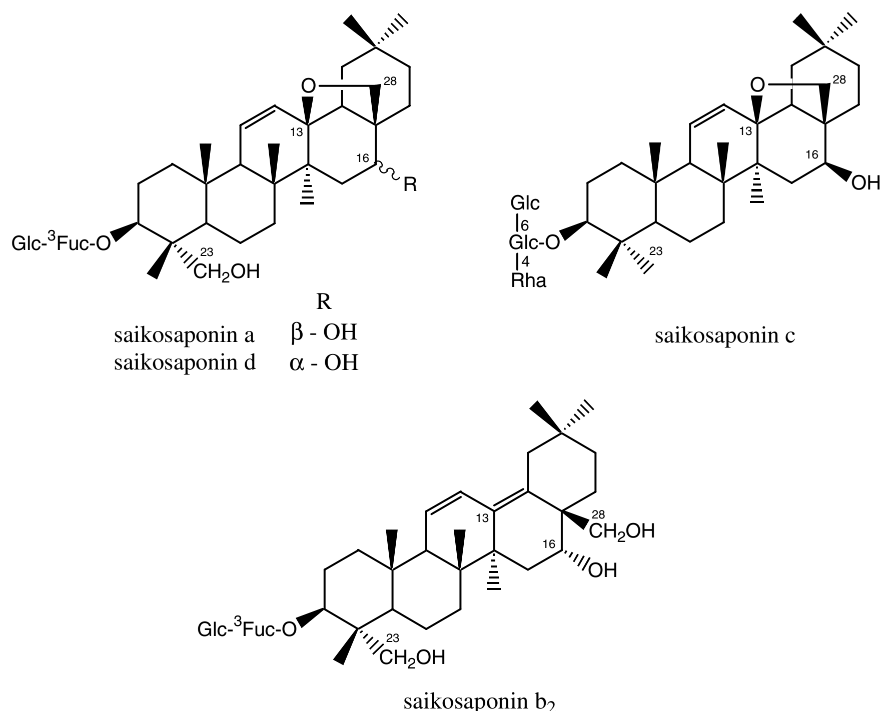

Fig. 1. Chemical structures of saikosaponins.

membrane. In this method, we blotted glucosides from a thin-layer chromatography (TLC) plate developed by solvent system to a PVDF membrane and separated the glucoside molecule into two functional parts, the epitope part and the conjugation function to membrane by sugar part. The sugar parts in glucosides were oxidatively cleavaged to give aldehyde groups which were conjugated with carrier protein to fix on a PVDF membrane. However, since the transfer efficiency was difficult to control, this method could not be applied for the quantitative immunoassay.

In this article, we describe a new approach, visual detection of SSa, SSc, and SSd by the newly developed Eastern blotting technique using a single MAb, anti-SSa MAb and apply to the quantitative immunoassay arranged by NIH Imaging software for the estimation of TCM containing Bupleuri radix.

Materials and methods

Chemicals and immunochemicals. Saikosaponin a, b_2, c, and d were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was provided by Pierce (Rockford, IL, USA). Peroxidase-labeled anti-mouse IgG was obtained from ICN Biomedicals, Inc. (Aurora, OH, USA). Mustang™ E positive-charged polyethersulphone (PES) membrane was purchased from Pall Corporation (East Hills, NY, USA). DAB Peroxidase Substrate Kit was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). All other chemicals were standard commercial products of analytical grade.

Plant materials. The roots of *Bupleurum* spp. were purchased from Nakai Koshindo Co., Ltd. (Kobe, Japan). The commercial TCM manufactured by Tsumura Co. (Tokyo, Japan) and Teikoku Seiyaku Co., Ltd. (Kagawa, Japan) were kindly provided by Mr. T. Somehara, Saga Medical School.

Sample preparation. Dried powders of various Bupleuri radix (20 mg) and TCM (50 mg) were extracted five times with 0.5 ml methanol with sonication and filtered using a Cosmonice filter W (0.45 μm filter unit, Nacalai Tesque, Kyoto, Japan). The combined extracts were diluted with methanol for ELISA and NIH Imaging analyses.

MAbs against SSa and saikosaponins and their developments of ELISA system. Immunizations, hybridizations, and purifications of MAbs against SSa (1G6) and saikosaponins (3G10), and their developments of ELISA system for determination of SSa and total saikosaponins have been established and described previously [11,12].

Visual detection of SSa, SSc, and SSd by on-membrane immunoassay using an Eastern blotting technique. Eastern blotting was performed as reported previously [13] except for separation by TLC plate and transfer to a PVDF membrane as follows. Saikosaponins and the extracts of Bupleuri radix and TCM were applied onto a PES membrane. After drying, this membrane was hung in the tank and immersed and developed by acetonitrile–water (1:4, by volume). The developed PES membrane was dried and dipped into water containing $NaIO_4$ (10 mg ml^{-1}) for 1 h. After washing with water, 50 mM carbonate buffer solution (pH 9.6) containing BSA (1%) was added and was stirred at room temperature for 3 h. After washing PES membrane with phosphate-buffered solution (PBS), the membrane was treated with PBS containing 5% skimmed milk (S-PBS) for 2 h to reduce nonspecific adsorption. The PES membrane was immersed in anti-SSa MAb (hybridoma supernatant was used) and stirred at room temperature for 2 h. After washing the PES membrane twice with PBS containing 0.05% Tween 20 (T-PBS) and water, a 1:1000 dilution of peroxidase-labeled goat anti-mouse IgG in PBS containing 0.02% gelatin was added, and the mixture was stirred at room temperature for 1 h. Then the PES membrane was washed twice with T-PBS and water and then exposed in Tris–HCl buffer (pH 7.5) containing 1 mg ml^{-1} 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.02% H_2O_2 for 30 min at room temperature. The reaction was stopped by washing with water, and the immunostained PES membrane was allowed to dry.

Image analysis system and image acquisition. A graphic analysis system, which consisted of a personal computer (Macintosh Power Book G4, Apple Computer Inc., Irvine, CA, USA), a public domain program NIH Imaging 1.62 (developed at the US National Institutes of Health and

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