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Development of an enzyme-linked immunosorbent assay based on anti-puerarin monoclonal antibody and its applications

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ABSTRACT

An enzyme-linked immunosorbent assay (ELISA) was developed, and its application in immunoaffinity column chromatography was studied using a monoclonal antibody (MAb) against puerarin. Splenocytes isolated from a female BALB/c mouse immunised with a puerarin–bovine serum albumin (BSA) conjugate were fused with SP2/0 myeloma cells. The hybridoma cell line secreting MAb against puerarin (AA9) was acquired by screening and limiting dilution. The antibody generated was highly specific for puerarin with <0.01% cross-reactivity with over 50 structurally related chemicals, except for baicalein (51.8%). Using AA9, we developed an immunoassay for puerarin with a linear detection range of 10 ng/ml to 1 μg/ml. This assay system was further validated using intra- and inter-assays and recovery experiments. In addition, puerarin levels in both formulated Chinese medicines and biological samples were determined with high sensitivity and efficiency. Finally, we developed and validated protocols for knocking puerarin out of its parent medicine completely. In conclusion, we successfully developed a reliable ELISA and an immunoaffinity column for puerarin detection and knockout, which are useful tools for exploring the role of puerarin in formulated Chinese medicines.

1. Introduction

Radix puerariae is the dried root of *Pueraria lobata* (Willd.) Ohwi, which is a semi-woody, perennial, and leguminous vine native to Southeast Asia. This root has been used for more than 2000 years as herbal medicine for the treatment of fevers, acute dysentery, diarrhea, diabetes, cardiovascular and cerebrovascular diseases [1]. To explore the underlying mechanism of its biological action, studies of its traditional applications, phytochemistry, pharmacological activities, toxicology, quality control, and potential interactions with conventional drugs have been reported [2]. There are over seventy phytochemicals that have been identified in radix puerariae, of which puerarin (PU), which is an isoflavonoid, is believed to be the major constituent responsible for the pharmacological effects of this herbal medicine. Indeed, data from animal and in vitro studies suggest that PU has beneficial effects for the Parkinson's disease (PD) [3], diabetes [4], angina pectoris [5,6], alcohol-caused liver injury [7], colon cancer [8], breast cancer [9], hepatic fibrosis [10], lead-induced hepatotoxicity and hyperlipidaemia [11], and ischemic stroke [12,13]. For PU-containing medicine, the PU injection has significant effects on angina pectoris due to coronary heart disease and it has been found to be safe with no obvious side effects [14]. In addition, this medicine effectively improves the cardiac arrhythmia and prognosis of chronic heart failure patients [15] and enhances the susceptibility of leukaemia cells to cytotoxic drugs from resistance to response [16].

Because PU has such important effects, it is vital to measure the concentration of PU from various preparations. Therefore, a rapid and sensitive method for monitoring the PU concentration in Chinese medicines or Chinese herbal compounds and pharmacological research is needed. The purification and knockout of PU from medicine is also an important issue. Commercial isolation of PU typically includes several isolation steps, such as crystallisation, column chromatography, and liquid partitioning. However, these methods are far from satisfactory for analytical purposes such as high sensitivity, reproducibility, large amounts of extraction solvents that are required, and the time-consuming nature of the methods. Therefore, it is imperative to establish a desirable approach.

Various approaches for the separation or quantification of PU in radix puerariae have been reported, such as high-performance

Abbreviations: PU, puerarin; PLL, polylysine; BSA, bovine serum albumin; MAb, monoclonal antibody; EXT, extract; KO, knockout; HPLC, high performance liquid chromatography; TLC, thin layer chromatography; BBS, blocking buffer.

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liquid chromatography (HPLC) with ultraviolet detection [14], HPLC or ultra-performance liquid chromatography (UPLC) with electrochemical detection (ECD) or diode array detection (DAD) combined with mass spectrometry (MS) [15–17], thin layer chromatography (TLC), and ultraviolet(UV) visible spectrophotometry [18,19]. To establish the fingerprints of radix puerariae, HPLC-diode array detection-flow injection-chemiluminescence coupled with HPLC-electrospray ionisation-MS methods have also been reported to separate and identify PU and other chemicals [20,21]. These methods suffer from various limitations, such as a high cost, component degradation, lengthy time, low recovery rates, or complicated pretreatment, especially for in vivo metabolism research. Therefore, it is necessary to establish a new and simple method for PU analysis.

An immunoassay with a polyclonal antibody for the determination of PU has been recently established [22,23]. However, an immunoassay with monoclonal antibodies (MAbs) for PU determination does not currently exist. In addition, we developed a hybridoma to produce the anti-PU MAb(AA9), which can then be produced in relatively large quantities as needed, without the need to raise new antibody from animals. Therefore, in the current study, we generated a hybridoma to produce the MAb against PU and developed an ELISA to measure PU in various samples and an immunoaffinity column to knock PU out from its original medicinal plants.

2. Experimental

2.1. Materials and methods

PU was purchased from the National Institute for Food and Drug Control (NIFDC, China, purity of 98%). The PU injection was purchased from Xiehe Pharmaceutical Co., Ltd. (Beijing, China), and various Chinese medicines were obtained from Beijing Tong Ren Tang Group Co., Ltd. (Beijing, China), Sanjiu Medical & Pharmaceutical Co., Ltd. (Beijing, China), Cachet Pharmaceutical Co. Ltd. (Beijing, China), AnguoshiTongli Herbal Medicine Co., Ltd. (Anguo, China), and Guangzhou Pharmaceutical Holdings Ltd. (Guangzhou, China). Sodium periodate was obtained from Sinopharm Chemical Reagents (Beijing, China). Bovine serum albumin (BSA), polylysine (PLL) and Freund's complete and incomplete reagents were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). All of the other chemicals and reagents were of analytical grade and purchased from Sinopharm. The CNBr-activated sepharose 4B and protein G FF columns were obtained from GE Healthcare Co. (USA).

2.2. Synthesis of PU–BSA conjugate

The conjugates were synthesized using a periodate oxidation procedure according to a previously reported protocol with some modifications [24,25]. Briefly, PU was dissolved in methanol at 10 mg/ml. Then, 1 ml of a freshly prepared sodium periodate solution (0.1 M) was added dropwise into 1 ml of the PU solution. The mixture was stirred at 25 °C for 1 h followed by the addition of 1 ml of glycol. After stirring for 12 h, 8 mg of BSA was added, and the final pH was adjusted to 9.0 using a 0.05 M carbonate buffer (pH 9.6). After 6 h, the mixture was dialysed six times against phosphate-buffered saline (PBS). The dialysate of the PU–BSA conjugate was stored at 4 °C for detection and immunisation. The PU–PLL conjugate was synthesized using the same method as the PU–BSA conjugate synthesis method described above.

2.3. Determination of the PU–BSA conjugate by TLC and UV

The PU–BSA conjugates were determined using TLC and UV, as previously reported [18,19]. For the TLC analysis, 5 μl of PU, BSA, or PU–BSA was loaded on the start line of the chromatography plate and then expanded using a 15:6:1 solvent mixture of chloroform, methanol and water followed by detection with a sulphuric acid–ethanol solution. With the UV approach, the characteristic spectra of PU, BSA, PU–BSA, PLL, and PU–PLL were simultaneously analysed. Conjugation of the hapten to BSA or PLL was also confirmed by comparison of their spectra.

2.4. Animal treatment

Female BALB/C mice (6 weeks old) were purchased from Vital River Laboratories (Beijing, China). The mice were fed a standard rodent diet (Keaoxieli Animal Feed Co. Ltd., Beijing, China) *ad libitum* and housed in an environmentally controlled (23 ± 2 °C; 12 h light/dark cycle) animal facility. All experimental protocols were approved by the Committee on Ethics of Animal Experiments at Beijing University of Chinese Medicine, China.

2.5. Immunisation

The immunisations were performed at 2 week intervals. The mice were intraperitoneally (i.p.) injected with an initiation shot of 50 μg of the PU–BSA conjugate in PBS emulsified with an equal volume of Freund's complete adjuvant in the initial immunisation. The second and third immunisations, which contained 50 μg of the PU–BSA conjugate in Freund's incomplete adjuvant, were injected subcutaneously at 2 and 4 weeks after the initial injection. The fourth immunisation involved injection with a solution of PU–BSA(100 μg) in PBS without adjuvant. In one day, mice blood was obtained from the tail vein, and sera were tested for their titer by indirect ELISAs using PU–PLL as a solid-phase antigen.

2.6. Cell fusion and preparation of anti-PU MAb

Three days after the final immunisation, splenocytes were isolated and fused with a hypoxanthine–aminopterin–thymidine (HAT)-sensitive mouse myeloma cell line, SP2/0 (Sciencell Research Laboratory; Carlsbad, CA, USA), according to the polyethylene glycol (PEG) method [26,27]. Briefly, 1 ml of PEG was added dropwise to the cell pellet after centrifugation of the blended splenocytes and myeloma cells (at a ratio of 5:1) and incubated for 1 min at 37 °C. Then, the HAT medium (Sigma-Aldrich) was added. The hybridoma was transferred to 96-well plates for cell culture. The cells producing MAb reactive to PU as identified by an indirect ELISA were cloned by the limiting dilution method [28,29]. The established hybridoma was cultured in HT medium.

2.7. Purification of the MAb

Ascites obtained from the abdominal cavity irritated with an established hybridoma of BALB/c mice was purified using a protein G FF column (GE Healthcare) [30]. After adjusting the solution to a pH of 7 with a 1 M Tris solution (pH 9), the ascites containing IgG were filtered through a 0.22 μm membrane and loaded on top of the column. After binding, the column was washed with PBS (pH 7.2), and the bound IgG was eluted with a 0.1 M glycine buffer (pH 2.7). The eluted fraction was neutralised with a 1 M Tris solution (pH 9) and dialysed against water (50× volume) for five cycles at 4 °C followed by lyophilisation.

2.8. Indirect ELISA using PU–PLL

The reactivity of MAb to PU–PLL was determined by an indirect ELISA. PU–PLL (1 μg/ml, 100 μl/well) dissolved in 50 mM carbonate buffer (pH 9.6) was added to the wells of a 96-well Maxisorp immunoplate and incubated for 1 h. The plate was washed three times with washing buffer (PBS containing 0.05% Tween 20, PBST)