Rev Bras Farma; 24(2014): 282-287

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.journals.elsevier.com/revista-brasileira-de-farmacognosia

Original article

Synthesis and evaluation of artificial antigens for astragaloside IV

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ARTICLE INFO

Article history:
Received 9 March 2014
Accepted 19 May 2014

Keywords:
Astragaloside IV
Artificial antigen
Sodium periodate method

A B S T R A C T

The objective of this study was to produce artificial antigens for astragaloside IV that could be used to prepare antibodies against astragaloside IV screened in *Radix astragali* (*Astragalus membranaceus* (Fisch) Bunge, Fabaceae) and its preparations, using an indirect ELISA. Astragaloside IV was coupled to carrier proteins, bovine serum albumin and ovalbumin using the sodium periodate method and was then evaluated using SDS-PAGE, MALDI-TOF MS and animal immunizations. The coupling ratio of astragaloside IV to bovine serum albumin ratio was determined to be thirteen, and the indirect ELISA demonstrated that three groups of mice immunized with astragaloside IV-bovine serum albumin produced anti-astragaloside IV- bovine serum albumin-specific antibody, with a minimum serum titer of 1:9600. A method for synthesizing highly immunogenic astragaloside IV artificial antigens was successfully developed thus indicating its feasibility in the establishment of a fast immunoassay for astragaloside IV content determination in *Radix astragali* and its products.

Introduction

The saponin, astragaloside IV, a 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl cycloastragenol (**1**), was extracted from *Radix astragali*, the dried root of the Chinese medicinal herb *Astragalus membranaceus* (Fisch.) Bunge, Fabaceae. Pharmacological studies indicate that astragaloside IV possesses antihypertensive, positive inotropic, anti-inflammatory, antinociceptive, hepatoprotective, neuroprotective, anti-infarction, and antiviral activities, and may be effective in treating viral myocarditis. In addition, astragaloside IV has been shown to increase T, and B lymphocyte proliferation and antibody production *in vivo*, and inhibits the production of IL-1 and TNF-alpha from peritoneal macrophages *in vitro* (Duan and Sun, 2011). Astragaloside IV is increasingly being explored as an active ingredient in therapeutics; thus, there is a need to develop an effective method for its quantitation in pharmaceutical samples. Analytical methods currently used for astragaloside IV detection, include high performance liquid chromatography (HPLC), thin-layer chromatography scanning (TLCS), and fluorescence methods (Ouyang et al., 2009). However, these

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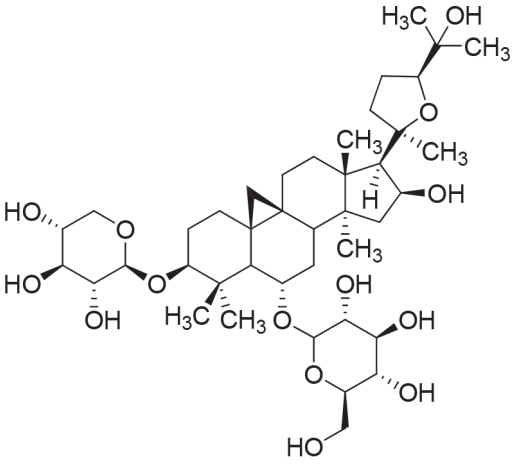
0102-695X/$ - see front matter
http://dx.doi.org/ 10.1016/j.bjp.2014.07.003

methods are complicated, mute sensitivity, require expensive instrumentation and are time-consuming. Immunoassays offer a simple, rapid, and cost-effective alternative to the traditional methods listed above, particularly when high efficiency and on-site screening tests are required. The successful preparation of artificial antigens is the first critical step in establishing any immunoassay method. Antigens (haptens) with a molecular weight (MW) less than 1000 are generally not immunogenic, and require conjugation with a carrier protein to elicit an immune response. Astragaloside IV is a relatively small molecule, with a molecular weight of 784; thus, it is not immunogenic; failing to elicit an immune response because it lacks epitopes recognized by T cells to stimulate antibody production. However, astragaloside IV, like other haptens can be chemically modified at an appropriate position to introduce a spacer with an active group at the end. The modified hapten is coupled to a macromolecular carrier to form a hapten-carrier conjugate (*e.g.* artificial antigen). The artificial antigen can make use of T cell epitopes to indirectly induce B cell proliferation and differentiation to generate specific antibodies against small molecular compounds (Jiao et al., 2004).



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In the present study, astragaloside IV was coupled to BSA to synthesize the artificial antigen, AST-BSA. The formation of AST-BSA was confirmed by SDS-PAGE and MALDI-TOF MS. An indirect ELISA was used for specificity analysis and for the determination of anti-AST-BSA specific antibody titers in serum. The results of this study laid the foundation for the preparation of monoclonal antibodies against astragaloside IV, and for the rapid detection of astragaloside IV in *Radix astragali* and its preparations using indirect ELISA.

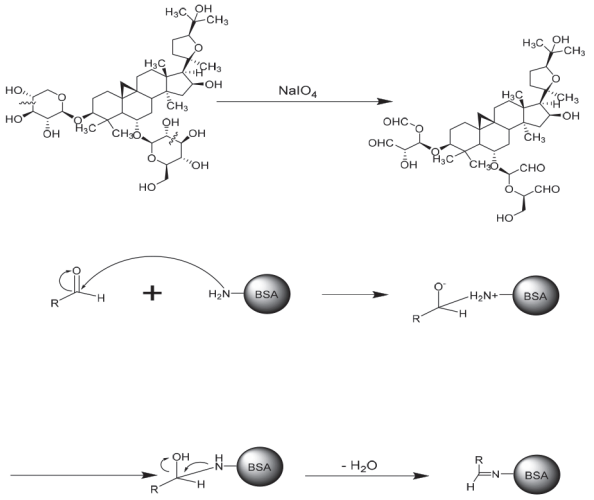
Materials and methods

Reagents and animals

Astragaloside IV standards (AST) were purchased from Chengdu Mansite Bio-Technology Co., Ltd. TMB was purchased from Solarbio Co., Ltd. Horseradish peroxidase HRP-conjugated goat anti-mouse IgG was purchased from Wuhan Boster Biological Technology Co., Ltd. Bovine serum albumin (BSA), ovalbumin (OVA), Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FICA) were purchased from Sigma-Aldrich. The animal experimentation protocols were approved by the Animal Care and Use Committee of Yangzhou University, and all experiments were conducted according to the guidelines of Yangzhou University (Institutional Ethical Committee Number SCXK (su) 2009-0002). Female Balb/C mice (6-7 weeks old) were purchased from the Laboratory Animal Service Center of the Yangzhou University (China). The mice were kept in an animal facility in controlled conditions, temperature at 22 ± 2°C and relative humidity of 50 ± 10%. Food and water were available *ad libitum*.

Preparation of AST-carrier protein conjugates

AST-BSA and AST-OVA were prepared by the sodium periodate method (Zhao et al., 2007). Briefly, 1 ml of AST in methanol (10 mg/ml) was added by dripping to 0.06 M $NaIO_4$, stirring at room temperature. After 1 h, BSA (35.7 mg BSA in 10 ml carbonate buffer, pH 9.6) was slowly added during stirring, and the reaction incubated for 6 h at room temperature. The conjugates were lyophilized following dialysis against distilled water for three days at 4°C, and stored at -20°C. AST-OVA were prepared as for AST-BSA. Scheme 1 illustrates the synthesis of the hapten derivatives for AST.



Scheme 1 – Synthesis of hapten derivatives for astragaloside IV (R).

Analysis of AST-carrier protein conjugates by polyacrylamide gel electrophoresis

Conjugates were analyzed under denaturing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), using an adaptation of the method of Joseph and David (2001). Briefly, 20 µl of conjugate (0.5 g/l) was mixed with an equal volume of 2×SDS protein loading buffer (1M Tris-HCl; pH 6.8, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 10% (v/v) glycerol, and 0.02% (v/v) bromophenol blue) and let to boil for 5 min. The denatured