Analytica Chimica Acta 903 (2016) 149–155

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Sandwich enzyme-linked immunosorbent assay for naringin☆

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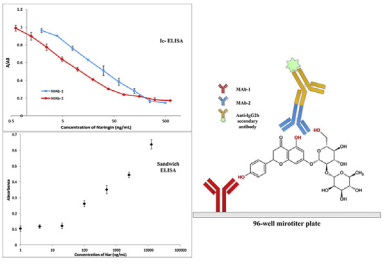
HIGHLIGHTS

- We developed a sandwich ELISA for detecting the small molecule naringin (Nar).
- The sandwich ELISA exhibited higher specificity for Nar than icELISA.
- The new method represents an improved analytical approach for Nar detection.

GRAPHICAL ABSTRACT



A true sandwich ELISA for naringin (MW:580 Da)

ARTICLE INFO

Article history:
Received 18 June 2015
Received in revised form
25 September 2015
Accepted 27 September 2015
Available online 14 November 2015

Keywords:
Naringin
Monoclonal antibody
Sandwich enzyme-linked immunosorbent assay

ABSTRACT

Among the currently used immunoassay techniques, sandwich ELISA exhibits higher specificity, lower cross-reactivity, and a wider working range compared to the corresponding competitive assays. However, it is difficult to obtain a pair of antibodies that can simultaneously bind to two epitopes of a molecule with a molecular weight of less than 1000 Da. Naringin (Nar) is a flavonoid with a molecular mass of 580 Da. The main aim of this study was to develop a sandwich ELISA for detecting Nar. Two hybridomas secreting anti-Nar monoclonal antibodies (mAbs) were produced by fusing splenocytes from a mouse immunised against Nar-bovine serum albumin (BSA) conjugated with a hypoxanthine–aminopterin–thymidine (HAT)-sensitive mouse myeloma cell line; a sandwich ELISA for detecting Nar was developed using these two well-characterised anti-Nar mAbs. The performance of the sandwich assay was further evaluated by limit of detection (LOD), limit of quantification (LOQ), recovery, and interference analyses. A dose-response curve to Nar was obtained with an LOD of 6.78 ng mL^{-1} and an LOQ of 13.47 ng mL^{-1}. The inter-assay and intra-assay coefficients of variation were 4.32% and 7.48%, respectively. The recovery rate of Nar from concentrated *Fructus aurantii* granules was 83.63%. A high correlation was obtained between HPLC and sandwich ELISA. These results demonstrate that the sandwich ELISA method has higher specificity for Nar than indirect competitive ELISA.

Abbreviations: BSA, Bovine Serum Albumin; CBS, Carbonate Buffer Solution; CR, cross-reactivity; ELISA, Enzyme-linked immunosorbent assay; GPBS, 1% gelatine in PBS; HAT, hypoxanthine–aminopterin–thymidine; HRP, Horseradish Peroxidase; icELISA, indirect competitive ELISA; Ig, immunoglobulin; LOD, Limit of Detection; LOQ, Limit of Quantification; mAb, Monoclonal Antibody; MIP, molecular imprinting polymer; Nar, naringin; OD, Optical Density; OVA, Ovalbumin; PBS, Phosphate-Buffer Saline; TMB, Tetramethyl Benzidine; TCM, Traditional Chinese Medicine.

☆ This manuscript has been thoroughly edited by a native English speaker from an editing company. An editing certificate will be provided upon request.

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http://dx.doi.org/10.1016/j.aca.2015.09.058
0003-2670/

1. Introduction

Enzyme immunoassay is one of the most popular analytical methods for the quantification and specific separation for target compounds from a complicated matrix. Among the immunoassay techniques, sandwich ELISA exhibits higher specificity, lower cross-reactivity, and a wider working range compared to the corresponding competitive assays [1,2]. Sandwich ELISA utilises two distinct antibodies that combine with two antigen-binding sites on the desired analyte. However, it is difficult to obtain a pair of antibodies that can simultaneously bind to two epitopes of a molecule with a molecular weight of less than 1000 Da. In such cases, steric hindrance may impede the antigen–antibody reaction because of the small size of the haptens [3]. A study [4] that used HVA-spacer-HIS conjugates to explore the limits of sandwich immunoassays of small molecules indicated that if the linker between the two epitopes is shorter than 5 Å (the chain length of 5 carbon atoms), sandwich formation has little chance of succeeding. Therefore, sandwich ELISA is widely used to detect macromolecular antigens such as proteins and polypeptides [2], and the indirect competitive ELISA (icELISA) method is more popular for the specific detection and quantification of naturally occurring active small molecules [5,6]. To the best of our knowledge, only two true sandwich assays for naturally occurring small molecules have been reported to date: angiotensin II (1048 Da) [7] and tacrolimus (804 Da) [1].

Naringin (Nar) is a well-known flavanone glycoside that is found in grapefruit and other common citrus fruits. Naringin has many potential health benefits [8,9]. Nar is a hapten with a molecular mass of only 580 Da. RIA [10] and icELISA for Nar have been developed; however, a non-competitive ELISA for Nar has not been reported to date.

In our previous study, we obtained two mAbs against Nar with two different isotypes. These two mAbs could simultaneously bind two epitopes of Nar. In this paper, we describe a method for the preparation of anti-Nar mAbs and the development of a sandwich ELISA for Nar. Nar is the smallest naturally occurring molecule for which a two-site immune metric assay has been developed. Our results indicate that this sandwich ELISA for Nar might have several practical applications.

2. Materials and methods

2.1. Chemicals and reagents

Fructus aurantii (*Citrus aurantium* L.) was purchased from Beijing Tong Ren Tang Medicinal Materials Co., Ltd (batch no: 20131019, 201033289, 401054259, and 201506204; Beijing, China) and Anguo Lulutong Co., Ltd (batch no: 121201J, 121201Z, and 121001J; Hebei, China). *Exocarpium citri grandis* (*Citrus grandis* L.) was purchased from Beijing Tong Ren Tang Medicinal Materials Co., Ltd (batch no: 201506291; Beijing, China). Concentrated *F. aurantii* granules were purchased from Beijing Tcmages Pharmaceutical Co., Ltd. (Beijing, China). Naringin (purity 98%) was purchased from the National Institutes for Food and Drug Control (NIFDC; Beijing, China). Horseradish peroxidase (HRP)-labelled goat anti-mouse immunoglobulin G (IgG) 2b was purchased from GE Healthcare (New Jersey, USA), and 96-well immunoplates were purchased from Corning Incorporated (New York, USA). Gelatine and 3,3′,5,5′-tetramethylbenzidine (TMB) were purchased from Sigma–Aldrich Co. Ltd. (Beijing, China). All other chemicals and reagents were of analytical grade and were obtained from Sinopharm Chemical Reagents Beijing Co., Ltd. (Beijing, China).

2.2. Instruments

Absorbance was measured on a Multiskan™ MK3 spectrophotometric microplate reader purchased from Thermo Fisher Scientific (USA). Immunoreactions were carried out in a DRP-9082 electro-heating standing-temperature cultivator from Samsung Instrument Co., Ltd. (Shanghai, China).

2.3. Buffers and solutions

Phosphate buffered saline (PBS) consisted of NaCl (137 mmol L^{-1}), $Na_2HPO_4 \cdot 12H_2O$ (10 mmol L^{-1}), KCl (2.68 mmol L^{-1}), and KH_2PO_4 (1.47 mmol L^{-1}). The washing buffer (pH 7.4) consisted of 0.05% Tween-20 in PBS (PBST). The blocking solution contained 1% gelatine in PBS. The TMB substrate solution consisted of 0.1 mol L^{-1} citrate buffer, 0.75% H_2O_2, and 2 g L^{-1} TMB; 2 mol L^{-1} H_2SO_4 was used as the stop solution.

2.4. Production and characterization of anti-Nar mAb

2.4.1. Synthesis of the antigen conjugate

Naringin-bovine serum albumin conjugate (Nar–BSA) was synthesised using a periodate oxidation procedure based on a previously reported method with some modifications [11,12]. Briefly, 50 mg Nar was dissolved in water to a final concentration of 1 mg mL^{-1}. Then, 1 mL of freshly prepared sodium periodate solution (0.1 M) was added drop wise into 50 mL of Nar solution. The mixture was stirred at room temperature for 1 h. Carbonate buffer (50 mM, pH 9.6, 2.0 mL) containing BSA (40 mg) was added to the above reaction mixture. The pH of the mixture was adjusted to 9 with 1 M Na_2CO_3 solution, and the mixture was stirred at room temperature for an additional 6 h. The reaction mixture was dialysed six times against PBS for 3 days. The Nar-ovalbumin (OVA) conjugate was synthesised in a similar manner.

2.4.2. Production of anti-Nar mAbs

Hybridomas secreting mAbs against Nar were generated as described previously [13]. The hybridomas were injected intraperitoneally into BALB/c mice to produce ascitic fluids. Individual anti-Nar mAbs were purified on a Protein G FF column (0.46 × 11 cm; GE Healthcare, USA). The pH of the ascites (50 mL) containing mAbs was adjusted to 7 with 1 M Tris solution, and the ascitic solution was loaded onto the column. The column was then washed with 20 mM phosphate buffer (pH 7.2), and the adsorbed antibody was eluted with 0.1 M glycine buffer (pH 2.7). The eluted antibody was neutralised with 1 M Tris solution, dialysed against 50 × volumes of water for five changes at 4 °C, and then lyophilised.

2.4.3. Determination of isotypes of anti-Nar mAbs

The isotype of an mAb has no relation to the specificity of the antibody, but it is closely related to the purification and application of the antibody. Determination of the mAb isotypes was carried out as described by us previously [14].

2.4.4. Sensitivity of anti-Nar mAbs using icELISA

Sensitivity of the two antibodies was examined by icELISA performed in 96-well microtiter plates coated with 100 μL of Nar-OVA conjugate (0.1 μg mL^{-1}) per well. The plates were incubated for 2 h at 37 °C. Unbound antigen was washed out of the wells three times with PBST, and the plates were stored at 37 °C with 1% gelatine in PBS (GPBS) to block non-specific binding sites. After incubation for 2 h, the plates were washed with PBST. Either 50 μL of Nar or the samples mixed with an equal volume of anti-Nar mAb (0.2 μg mL^{-1}) was added to the wells. After the mixtures were incubated for 1 h at 37 °C, the plates were washed, and 100 μL of