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A monoclonal antibody-based enzyme-linked immunosorbent assay for the determination of chlorogenic acid in honeysuckle

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ABSTRACT

The edible flower buds of honeysuckle are traditionally used as herbal medicine or food additives in China. Chlorogenic acid (CGA) serves as the quality control marker of honeysuckle for its high content and antipyretic property. In this paper, a specific monoclonal antibody (mAb2E2) against CGA was produced. After optimization, mAb2E2-based indirect competitive enzyme-linked immunosorbent assay (icELISA) was developed. The concentrations of CGA producing 50% inhibition and the calibration range of the icELISA were 0.39 and 0.10–1.51 ng/mL, respectively. The icELISA had cross-reactivity with 3,5-Dicaffeoylquinic acid (17.53%), and the cross-reactivity levels with other analogs were all below 5%. The average recoveries obtained by standard CGA addition to honeysuckle samples were from 88.4 to 104.8%. The icELISA was applied to CGA detection in different honeysuckle samples and the results were confirmed by high-performance liquid chromatography analysis. The correlation coefficient between the two assays was 0.97. The proposed icELISA provides a feasible analytical method for highly sensitive and specific, simple, fast, and high-throughput determination of CGA in honeysuckle samples.

1. Introduction

Recently, scientists have developed an interest in dietary polyphenols for their health benefit of preventing chronic degenerative diseases [1–3]. Chlorogenic acid (CGA), a natural phenolic acid, is famous for its potent antioxidative and free radical-scavenging activities [4,5]. CGA exhibits protective effect against chronic diseases such as cardiovascular disease, diabetes and cancer [6–8]. Many other health benefits such as antifungal, antiviral, neuroprotective and lung protective effects have been reported [9–12]. Moreover, the food industry is very interested in CGA serving as a new antioxidant among natural products to protect processed foodstuffs from rancidity and off flavors [13]. CGA is receiving considerable attention for its wide distribution, biological effects, low toxicity and side effects and highly potential commercial value.

CGA is widespread in plant materials, including fruits, grains and vegetables. The flower buds of several *Lonicera* species, also named honeysuckle, are known for their high CGA content in China. As an edible and medicinal cognate, the edible flower buds of honeysuckle have traditionally been used as a health food or food additive in the folk diet, such as in teas, beverages, wine, and acidophilus milk [14]. In recent years, different kinds of CGA-rich honeysuckle teas have become popular in China. Honeysuckle is one of the major ingredients and plays a significant role in the flavor, taste and hot alexipharmic efficacy of tea. However, the quality of honeysuckle varies with species, cultivation region, harvest time and processing technology. CGA has been officially used as the quality control marker for its high content and antipyretic property. Thus, a reliable and accurate method is required to determine the CGA content of honeysuckle samples for the purpose of quality control.

Several analytical methods, including chemiluminescence [15], high-performance liquid chromatography (HPLC) [16], liquid chromatography coupled with mass spectrometry (LC–MS) [17], proton nuclear magnetic resonance spectroscopy (NMR) [18], near-infrared spectroscopy (NIR) [19], capillary electrophoresis (CE) [20], electroanalytical methods [21], and biosensors, have been reported

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for the detection of CGA [22]. Methods based on instrumental analysis such as HPLC, LC–MS, NMR, and NIR are time-consuming and require expensive and specialized equipment, while the biosensors are low-throughput. As for immunosorbent assays, Jin Xue [23] had prepared a polyclonal antibody (pAb) against CGA and developed an indirect competitive enzyme-linked immunosorbent assay (icELISA) with the linear range of 15.6–250 ng/mL. However, pAb, obtained from the serum of immunized mice, is a complex mixture. It usually has more cross-reactions with analogs and lower sensitivity compared to monoclonal antibodies (mAb) [24]. icELISA is a useful analysis method for small molecular compounds that is characterized as simple, sensitive, rapid and high throughput [25]. Thus, the icELISA method based on mAb may supply an alternative analytical method for CGA detection, and there were no published reports about anti-CGA mAb and icELISA for the analysis of CGA.

In this study, the complete antigen was synthesized by coupling the CGA with carrier proteins via the active ester method. A specific anti-CGA mAb was produced and characterized. An icELISA method was used for the determination of CGA content in honeysuckle samples, and the results were confirmed by HPLC.

2. Materials and methods

2.1. Reagents

CGA and its analogs were obtained from the National Institutes for Food and Drug Control (Beijing, China).Reagents that were used for antigen synthesis and antibody production were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Antigen synthesis

The complete antigen was synthesized following the active ester method [26]. Briefly, CGA (6.4 mg), *N*-hydroxysuccinimide (NHS, 2.5 mg) and dicyclohexylcarbodiimide (DCC, 4.5 mg) were dissolved in 1 mL, 100 μL and 100 μL of absolute *N,N*-dimethylformamide (DMF), respectively. NHS was added to the CGA solution slowly under constant stirring and DCC was subsequently added 30 min later. Two hours later, the action solution was stored at 4 °C for 20 h and then centrifuged. The BSA/OVA protein solutions (20.0 mg) were dissolved in 2.0 mL of PBS buffer (0.01 M phosphate buffer composed of 0.15 M NaCl, pH 7.5). The resulting mixture was added to the protein solutions dropwise while stirring. The coupling reaction was stored at 4 °C overnight. After dialysis with 0.01 M PBS for 72 h, CGA-BSA and CGA-OVA were stored at −40 °C for further use.

2.3. Immunization, mAb production and characterization

Female Bal b/c mice (6–7 weeks old) that were used for immunization and ascites production were obtained from the Laboratory Animal Center of the Institute of Genetics (Beijing, China), and all procedures were in compliance with the guidelines of Animal Care Committee of China Agricultural University. After acclimation for 2 weeks, the mice were immunized with CGA-BSA. For the first injection, the CGA-BSA (1 mg/mL, dissolved in PBS) was emulsified with equal amounts of Freund's complete adjuvant. A CGA-BSA solution that was emulsified with incomplete Freund adjuvant was used for the next four injections at two-week intervals. Four days after the fifth injection, blood samples of each mouse were collected. After centrifugation, the serum was obtained and its specificity was detected by icELISA as described below. The positive mice were boost immunized with 0.1 mg/mL CGA-BSA without adjuvant four days before fusion. SP2/0 (ATCC, Manassas, VA, USA) was fused with positive splenocytes by the polyethylene glycol method. After fusion, the hybridomas were fed with HAT medium in a CO_2 incubator (Thermo, Franklin, MA, USA) at 37 °C. Ten days later, positive hybridomas were cloned by limiting dilution, and then the positive clones were tested by icELISA and expanded for ascites production. Ammonium sulfate precipitation was used to purify the antibody. After dialysis against H_2O for 48 h, the antibody was lyophilized and its characteristics including titer, dissociation constant (K_d), and isotype were determined.

2.4. Indirect competitive ELISA (icELISA) procedure

The buffers and solutions including carbonate buffer, PBS, washing buffer, sample dilution buffer, substrate solution, and a stop solution used here were the same as we previously reported [27]. All plates were washed four times with washing buffer by the same method, and all reactions were incubated in a constant-temperature incubator (ZXDR-2800, Shanghai, China) at 37 °C. First, 100 μL of CGA-OVA (1.0 μg/mL, dissolved in carbonate buffer) was added to a microplate (Corning, NY, USA) and incubated for 3 h. After being washed, CGA standard (50 μL, 0.02–4.0 ng/mL) in the sample dilution buffer and 50 μL of mAbs were added to each well in the proper order. The plate was incubated for 0.5 h and then washed. One hundred microliters of goat anti-mouse IgG-HRP conjugate solution (0.1 μg/mL, dissolved in sample dilution buffer) was added to each well and then incubated for 0.5 h. Substrate solution (100 μL) was added to each well for color development after the plate was washed. After a few minutes, the stop solution (50 μL) was added to terminate the reaction, and the optical density values were measured at 492 nm with the Multiskan FC microplate reader (Thermo, Franklin, MA, USA).

2.5. Assay precision and variation

To determine the precision and variation of this icELISA method, the measuring range (0.02–4 ng/mL) of CGA was determined. For intra-assay precision (well to well), the range was measured three times in a day, while for inter-assay precision (plate to plate and day to day), the range was measured for three consecutive days.

2.6. Assay specificity

A cross-reactivity (CR) experiment was used to evaluate the specificity of the mAb. Namely, IC_{50} values with CGA and its structural analogs as the inhibitors were detected by the developed icELISA described above and then calculated as follows: CR (%) = (IC_{50} of CGA)/IC_{50} of CGA analogs) × 100%

2.7. Sample preparation and extraction

The honeysuckle samples were collected from eight provinces of China, and then dried at 60 °C in an oven before being ground to a fine powder. Honeysuckle was officially listed in the China Pharmacopoeia [28] and the extracted method was well defined. Each honeysuckle sample was powdered and accurately weighted (0.1 g). Ten milliliters of 50% methanol (v/v) was added to each sample and weighted before extraction using an ultrasonic bath. After extraction for 40 min, the extract was weighted again and 50% methanol was added for the weight lost. The supernatant was splited into two equal parts after centrifugation. One part of resultant residue was detected by icELISA after dilution with the sample dilution buffer, and the other one was used for HPLC analysis after being filtered through a 0.22 μm Millipore membrane. The dilution ratios were 1:200 000 and 1:5 (dilution with 50% methanol) for icELISA and HPLC, respectively.