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Preparation and characterization of chitosan microparticles for immunoaffinity extraction and determination of enrofloxacin

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ABSTRACT

The use of chitosan microparticles as chromatographic support has received much attention. In this study, the effects of process parameters, namely, chitosan molecular weight, chitosan concentration, molar ratio of amino group to aldehyde group, volume ratio of water to oil phase and stirring speed on the size and size distribution of chitosan microparticles and their application for immunoaffinity extraction were extensively investigated. Size distribution analysis indicated that the average diameter of the microparticles was 124 μm with Span value of 1.1. The obtained microparticles exhibited low non-specific adsorption and kept stable in the pH range 4.0–10.0. Immunoaffinity chromatography (IAC) column was prepared by coupling antibody against enrofloxacin (ENR) with chitosan microparticles. Further characterization indicated that the binding capacity of the column was 4392 ng ENR/mL gel and the variation of ENR extraction efficiency among columns was less than 5.2%. When challenged with ENR-fortified bovine milk samples, recoveries of ENR by immunoaffinity extraction were found to be in the range of 85.9% to 101.9%, demonstrated the feasibility of the prepared IAC columns for sample clean-up in ENR residue determination.

1. Introduction

Immunoaffinity chromatography (IAC) takes the advantage of the specific and reversible interaction between antibody and antigen, and provides a selective method to isolate and concentrate target analytes from complex sample matrices and can also be used as an alternative means of purifying chemical residues [1,2]. The application of IAC for sample pretreatment for residue analysis of mycotoxin, veterinary drugs, pesticides, phycotoxins, process and environmental contaminants and vitamins, has been reported [3–7]. The most commonly used support for immunoaffinity chromatography separation was agarose. However, agarose is rather expensive; moreover, the highly toxic cyanogen bromide (CNBr) is usually used in its activation and regeneration. Therefore, it would be worth to find a new and cheap matrix or support, which can be activated and regenerated easily.

Chitosan is a natural product derived from deacetylation of chitin which is known to be the second most abundant polysaccharide in nature [8]. For the presence of free amino and hydroxyl groups on its polysaccharide chain, which provides active reaction sites for coupling of various ligands, chitosan has been considered as one of the potential chromatographic support, and it is, therefore, interesting not only as an abundant resource but also as a novel type of functional materials [9–11]. Compared with agarose, the mostly usually used support for chromatography separation, chitosan is rather cheap and presents the similar chemical properties to agarose, such as biodegradability, low toxicity, and good biocompatibility [8,12]. Moreover, with respect to potential microbial contamination, chitosan also exhibits excellent antimicrobial activity towards most of the microorganisms [13]. For these reasons, the development and application of chitosan based chromatography adsorbents is recently gaining much interest [14].

Low mechanical property of chitosan polymer imposes restriction on its further development [15]. Cross-linking and microparticle reinforcement are the two possible methods to improve the mechanical properties of chitosan. To date, there are quite a few articles related to the preparation of chitosan microparticles by various crosslinking methods and evaluation its application as a type of chromatography matrix support [16–19]. The major drawbacks associated with chitosan microparticles prepared in these articles is that the size and size distribution is non-uniform and this would result poor chromatographic separation. Therefore, the work aimed to: (1) optimize cross-linking parameters for chitosan microparticles preparation; (2) develop a

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novel immunoaffinity chromatography adsorbents by coupling the obtained chitosan microparticles with polyclonal antibody (pAb) against enrofloxacin (ENR), which is a synthetic fluoroquinolone and has been strictly regulated in stock farming due to its adverse effect on human health; (3) characterize the prepared IAC columns and evaluate their potential application to ENR detection in real samples.

2. Materials and methods

2.1. Chemicals and materials

Three different molecular weight chitosan (MW = 5×10^4, 1×10^5 and 6×10^5 Da) with deacetylation degree from 85% to 95% were supplied by Golden-Shell Biochemical Co., Ltd. (Yuhuan, China). Enrofloxacin, *N*-hydroxysuccinimide (NHS), 1-ethyl-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), *N,N*-Dimethylformamide (DMF), triethylamine, bovine serum albumin (BSA), complete Freund's adjuvant (CFA) and incomplete Freund's adjuvant (IFA) were purchased from Sigma-Aldrich (St. Louis, USA). High-performance liquid chromatography (HPLC)-grade methanol was purchased from Fisher Scientific, Inc. (Pittsburgh, USA). HRP-conjugated goat anti-rabbit IgG was obtained from Shanghai Shangon Co., Ltd (Shanghai, China). All other chemicals were of analytical grade and supplied by Sinopharm Chemical Reagent Co., Ltd (Shanghai, China).

2.2. Preparation of chitosan microparticles

A total of 0.2 g chitosan was dissolved in 10 mL of acetic acid (2%, v/v) solution. The resulted chitosan solution was emulsified by mechanical stirring for 3 h in 100 mL of paraffin oil containing 2% span-80. Then, 3 mL glutaraldehyde saturated toluene (GST) was slowly dropped into the mixture to crosslink chitosan droplets at a stirring speed of 500 rpm for 3 h, with the pH being maintained between 9 and 10 throughout. The crosslinked chitosan was filtered and washed thoroughly with petroleum ether and ethyl alcohol. Followed by washed with distilled water, 5 mol/L $NaBH_4$ solution was added to quench the aldehyde groups and hydrogenate C=N bonds. The obtained chitosan microparticles were stored in 20% ethyl alcohol at 4 °C.

To achieve suitable size and narrow size distribution of chitosan microparticles, the preparation parameters including chitosan molecular weight (50, 100 and 600 kD), chitosan concentration (1%, 2%, 3% and 4%), the molar ratio between amino groups and aldehyde groups (4:1, 2:1, 1:1 and 1:2), volume ratio of water to oil phase (1: 5, 1: 8, 1: 10 and 1: 12) and stirring speed (200, 300, 400 and 500 rpm) were examined in terms of particle size and size distribution.

The shape and surface morphology of the microparticles was visualized using a JSM-7001F scanning electron microscope (JEOL, Japan). The particle size and size distribution was measured using a Bettersizer-2000 laser particle size analyzer (Dandong, China). The particle size distribution was referred to as the Span value and was calculated as follows [20,21]:

$$Span = \frac{D_{90} - D_{10}}{D_{50}},$$

where D_{90}, D_{50} and D_{10} are the volume size diameters at 90%, 50% and 10% cumulative volumes, respectively. The smaller span value indicates the narrower size distribution.

2.3. Non-specific protein adsorption of chitosan microparticles

BSA was chosen as representative protein to test the non-specific protein adsorption of the prepared chitosan microparticles according to the previously described method [22]. Chitosan microparticles (10 mg) were suspended in 1 mL of 1 mg/mL BSA solution dissolved in 0.01 mol/L PBS buffer (pH 7.4) and kept at 25 °C for 24 h to achieve adsorption equilibrium. After centrifugation at 3000 rpm for 5 min, BSA content in supernatant was determined by Bradford method to calculate the non-specific protein adsorption. The amount of BSA absorbed was calculated by $q = (C_0 - C)/W$, where C_0 (mg/mL) is initial BSA concentration; C (mg/mL) is BSA concentrations after adsorption; W (mg) stands for the weight of the particles.

2.4. pH stability of chitosan microparticles

The pH stability of chitosan microparticles was evaluated in the following solutions: 50 mmol/L HAc-NaAc (pH 4.0), 50 mmol/L phosphate buffer saline (pH 6.0), 50 mmol/L Tris-HCl (pH 8.0), and 50 mmol/L $NaHCO_3$-Na_2CO_3 (pH 10.0). Crosslinked chitosan microparticles were suspended in these buffers and stored at 4 °C for 30 h, respectively. Afterwards, particle structure was observed by scanning electron microscopy.

2.5. Generation and purification of polyclonal antibody

The polyclonal antibody was generated as previously described [23]. Briefly, two male New Zealand white rabbits (about 2 kg each) were used to generate antisera. For each rabbit, 1 mg of ENR-BSA conjugate was dissolved in 1 mL of 0.9% NaCl solution and emulsified with CFA (1:1, v/v).The emulsion was injected intradermally at multiple sites on the back of the rabbit. For booster immuniations, immunogen was dissolved in 0.9% NaCl solution and emulsified with IFA (1:1, v/v). The booster immunizations were given every three weeks for a total of 4 booster immunizations. A week after the final booster injection, blood was drawn from ear vein and the serum was isolated by centrifugation. The anti-ENR polyclonal antibody was purified from antiserum using a caprylic acid and ammonium sulfate precipitation method.

2.6. Preparation of immunoaffinity column

The immunoaffinity column was prepared by coupling the purified pAb with chitosan microparticles according to the following procedure [16]. In brief, about 0.4 g wet chitosan microparticles suspended in 5 mL of coupling buffer (0.1 mol/L $NaHCO_3$ containing 0.5 mol/L NaCl, pH 8.3) was mixed with 2 mL of 0.15 mg/mL purified pAb solution followed by gentle agitation for 3 h at room temperature. Then epichlorohydrin was added to the above solution to the final concentration of 20% and mechanically stirred for another 5 h at 37 °C. The resultant immunosorbent was washed thoroughly with distilled water and transferred to a column (10 mm × 65 mm) and stored in PBS containing 0.02% NaN_3 at 4 °C.

2.7. ELISA procedure

For enzyme linked immunosorbent assay (ELISA), polystyrene microtiter plate was coated with coating antigen (ENR-OVA) in 0.05 M carbonate buffer (pH 9.6) by overnight incubation at 4 °C. Unoccupied sites were blocked with 0.2% gelatin/PBS solution for 1 h at 37 °C. After the washing procedure, the plate received 50 μL/well of enrofloxacin followed by 50 μL/well of pAb and incubated at 37 °C for 1 h. After the washing procedure, 100 μL of peroxidase-conjugated goat anti-rabbit IgG was added to each well and the plate was then incubated for another 1 h at 37 °C. The color development was initiated by adding 50 mL of the substrate (TMB/H_2O_2 in acetate buffer pH 5.5) for each well and incubated for