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High-performance affinity monolith chromatography for chiral separation and determination of enzyme kinetic constants

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ABSTRACT

A new kind of immobilized human serum albumin (HSA) column was developed by using the sub-micron skeletal polymer monolith based on poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) [poly(GMA-EDMA)] as the support of high-performance affinity chromatography. Using the epoxide functional groups presented in GMA, the HSA immobilization procedure was performed by two different means. The affinity columns were successfully adopted for the chiral separation of D,L-amino acids (AAs). Then this method was shown to be applicable to the quantitative analysis of D-tryptophan, with a linear range between 12.0 μM and 979.0 μM, and a correlation coefficient above 0.99. Furthermore, it was used for the analysis of urine sample. This assay is demonstrated to be facile and relatively rapid. So it allows us to measure the enzyme catalytic activity in the incubation of D,L-AAs with D-AA oxidase and to study the kinetics of the enzyme reaction. It implied that the affinity monolithic columns can be a useful tool for studying DAAO enzyme reaction and investigating the potential enzyme mechanism requirement among chiral conversion.

1. Introduction

High-performance affinity chromatography (HPAC) has held an impressively strong position in the separation and analysis of chemicals. It has many advantages, such as high specificity, ease of automation, and reuse of the same ligand for multiple applications [1–4]. In this approach, an HPLC column contained an immobilized ligand capable of specifically binding the analyte or group of analytes. Up till now, the new protein- and biomolecule-based stationary phases have been widely investigated for special recognition sites and biocompatible properties [5]. Among those biomolecules, human serum albumin (HSA) has been frequently used in silica-based HPLC columns to separate various chiral analytes [3,6] and study drug–protein binding processes [7]. Furthermore, it was also proved to be effective for the separation of amino acid enantiomers (AAs) [8].

AAs are of high biological interest, both as single components and constituents of peptides and proteins. Especially, D-AAs play an important role in the regulation of many processes, such as aging, neural signaling, and hormone secretion [9]. According to pharmacokinetics studies, oxidative determination of D-AAs can be catalyzed by D-AA oxidase (DAAO) to yield hydrogen peroxide and an imino acid. The latter is further non-enzymatically hydrolyzed to an α-ketoacids and ammonium [10]. An assay was established for analysis the DAAO activity in mammalian tissues with the substrate D-tryptophan (D-Trp) analogs by a RP-HPLC system with a fluorescence detector [11,12]. In addition, chiral ligand-exchange capillary electrophoresis of D,L-AAs were studied to determine the enzyme kinetic constants in our lab [13]. However, as a valuable technology of enantiomer separation, HPAC has not been used in the application of quantitative analysis of the real D-AA samples in DAAO enzyme reaction.

Among the various supports of HPAC, polymer monoliths are of particular interest because of good biocompatibility, lack of diffusion resistance during mass transfer and excellent pH stability [14–16]. They have been employed in several previous studies to create affinity monoliths [17,18] and used in such applications as sample purification [19], chiral separation [8] and ultrafast immunoextractions [20]. However, conventional polymer monoliths made by free radical polymerization are often constructed by irregular microglobules and aggregated clusters, which easily lead to low permeability and limited surface area [21,22]. In order to solve this problem, many different types of polymerization have been developed. High internal phase emulsion (HIPE) is a promising method for the preparation of highly porous materials [23]. A novel kind of sub-micron skeletal polymer monolith was devel-

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oped in our lab based on HIPE and block copolymer chemistry [24]. These new monoliths presented good permeability, larger surface area and subsequently larger protein binding capacity [25]. Their unique properties make these supports superior as supports for HPAC.

In this work, the sub-micron skeletal polymer monoliths based on poly(glycidyl methacrylate-co-ethylene glycol dimethacrylate) [poly(GMA-EDMA)] were explored for the preparation of high-performance affinity monolithic columns containing HSA as a chiral stationary phase. Then it was successfully used for the enantioseparation of D,L-AAs. In addition, the new kind of affinity column was applied to the measurement of DAAO enzyme kinetic constants and analysis of the real D-AA samples in enzyme reaction for the first time. It also hinted that the method is potentially adaptable to the study of enzyme mechanism.

2. Experimental

2.1. Materials

Glycidyl methacrylate (GMA) and ethylene glycol dimethacrylate (EDMA) were purchased from Acros Company (New Jersey, USA). Pluronic F127 (PF127) was obtained from Chuang Qi Company (Beijing, China). Tris hydroxymethyl aminomethane (Tris) was from Fuchen Chemical Plant (Tianjin, China). DAAO (from porcine kidney), all D- and L-AA standards and D,L-Trp, D,L-phenylalanine (Phe), D,L-tyrosine (Tyr) were purchased from Sigma Chemical (St. Louis, USA). HSA was from Beijing Xin Jing Ke Biotechnology Company (Beijing, China). Potassium persulfate, tetrahydrofuran (THF), anhydrous calcium chloride, diethylamine and ethanol were from Beijing Chemical Company (Beijing, China). Other reagents were all of analytical reagent grade. Water was obtained from a triple distilled water system and solutions were filtered through a 0.45-μm membrane before use. A human urine sample was provided by a healthy volunteer. Then 20-fold volumes of MeOH was added and mixed for 2 min. These mixtures were centrifuged at 4500 × *g* for 5 min and the supernatants were collected.

2.2. Equipment

Chromatographic investigations were carried out with a Shimadzu LC-10A HPLC system (Shimadzu, Japan) consisting of a binary LC-10AT HPLC pump and a SPD-10A UV–vis detector. Data processing was performed with a HW-2000 chromatography workstation (Nanjing Qianpu Software, China).

2.3. Preparation of sub-micron skeletal polymer monoliths

In the preparation procedure, the reactor was charged with GMA 2.1 mL, EDMA 1.2 mL, and PF127 (5.5%, v/v), followed by stirring at 400 rpm. To this mixture, an aqueous solution containing potassium persulfate (the initiator, 0.2%, w/v based on H_2O) and anhydrous calcium chloride (the electrolyte, 1.0%, w/v based on H_2O) in deionized water was added dropwise. Stirring was continued for 30 min whereupon a white, milky emulsion was formed. The emulsion was purged with nitrogen for 5 min, transferred to stainless-steel column and cured at 60 °C for 36 h. After cooling to room temperature, the column was connected to the HPLC system to remove the PF127 template and the unreacted monomers by pumping deionized water (50.0 mL) and ethanol (30.0 mL) through the column.

2.4. Immobilization of HSA on sub-micron skeletal polymer monoliths

HSA was immobilized on a 5.0 cm long sub-micron skeletal monolithic column using two different immobilized means. Before immobilization, the columns were equilibrated for 10 min by washing with immobilization buffer (50 mM Tris–HCl buffer at pH 8.7 for epoxy mean and 50 mM Tris–HCl buffer at pH 7.6 for EDA mean containing no HSA). In the dynamic method, the immobilization solution was pumped through the monoliths using a syringe.

2.4.1. Epoxy means

At first, HSA was covalently immobilized on the monolith for 24 h. A 2.0 mg mL^{-1} solution of trypsin was freshly prepared in 50 mM Tris–HCl buffer (pH 8.7). After equilibrating the monoliths with this buffer for 1 h, the protein solution was pumped through the monolith at a flow rate of 0.05 mL min^{-1}. After 1–6 days, the monolith conjugated with HSA was washed with 50 mM Tris–HCl buffer including 0.5 M NaCl to eliminate nonspecific physically adsorbed protein. The residual epoxide groups were blocked by 1 mg mL^{-1} aspartic acid in 50 mM Tris–HCl buffer for 1 h. The immobilization process was performed at room temperature.

2.4.2. EDA means

A mixture of diethylamine and THF (1/1, v/v) was continuously pumped through the monoliths for 7 h at 80 °C, 0.2 mL min^{-1}. Thereafter, the column was washed routinely with THF and deionized water. A solution of 10% (v/v) glutaraldehyde in 100 mM phosphate buffer (pH 8.0) was flushed through the monolithic column for 12 h at room temperature. Then HSA was immobilized on the activated support by continuously pumping 2 mg mL^{-1} HSA in 50 mM Tris–HCl buffer (pH 7.6) containing 5 mg mL^{-1} sodium cyanoborohydride ($NaCNBH_3$) for 1–6 days at room temperature. Subsequently, the nonspecifically adsorbed protein and the residual aldehyde groups on the surface of the support were depleted by pumping with 50 mM Tris–HCl buffer (pH 7.6) for 5 h. When not in use, the affinity column should be stored in 50 mM Tris–HCl buffer (pH 7.6) containing 10 mM $CaCl_2$ and 0.02% NaN_3 at 4 °C.

2.5. Permeability properties of the affinity column

The permeability behavior of the affinity column was described by pressure drop of monolithic column at different flow rates using pure water as mobile phase. The values of the system pressure were measured at each flow rate without and with the column, and the difference between the two values was calculated as the pressure drop across the column.

3. Results and discussions

3.1. Investigation of immobilization means

For coupling of proteins onto the surfaces, epoxy and EDA means were tested for covalent binding, which eliminated any leakage of the immobilized enzyme. In certain condition, the epoxide functionalities could react directly with the amino groups of the protein molecule (epoxy means). Furthermore, epoxide group also could be modified with a diamine followed by activation using a glutaraldehyde and then protein molecule was grafted (EDA means). The procedures of HSA immobilization on the monolithic column are shown in Fig. 1.

For comparing the efficiency of two immobilization means, affinity columns were applied in chiral separation of D,L-AAs. During the pilot experiments with both immobilization means, no any significant difference have been observed either in the separation results or stability of the prepared protein columns. However, the