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Short communication

Towards the identification of alkaline phosphatase binding ligands in Li-Dan-Hua-Shi pills: A Box-Behnken design optimized affinity selection approach tandem with UHPLC-Q-TOF/MS analysis

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ABSTRACT

Alkaline phosphatase conjugated magnetic microspheres were synthesized via amide reaction, and employed as an effective adsorbent in affinity selection of binding ligands followed by UHPLC-Q-TOF/MS analysis. The analytical validity of the developed approach was evaluated under optimized conditions and the following figures of merit were obtained: linearity, 0.01–0.5 g L^{-1} with good determination coefficients (R^2 = 0.9992); limits of detection (LODs), 0.003 g L^{-1}; and limits of quantitation (LOQ), 0.01 g L^{-1}. The precision (RSD%) of the proposed affinity selection approach was studied based on intra-day (0.8%) and inter-day (1.3%) precisions. Finally, the adsorbent was successfully applied to identification of binding ligands in Li-Dan-Hua-Shi pills and good recoveries were obtained in the range from 96.9 to 99.4% (RSDs 1.6–3.0%).

1. Introduction

Alkaline phosphatase (AP) is a hydrolase enzyme responsible for removing phosphate groups from many types of molecules, including nucleotides, proteins, and adenosine triphosphate (ATP) [1]. Accumulated evidence revealed that alkaline phosphatase plays a direct role in organ damage [2,3]. Therefore, inhibitors of alkaline phosphatase recently emerged as drug for treating organ damage. All mammalian alkaline phosphatase isoenzymes are inhibited by homoarginine. Other known examples of alkaline phosphatase inhibitors are levamisole and (−)-*p*-bromotetramisole. Several inhibitors were found to be more potent than levamisole [4]. Some of them were patented.

As a Chinese patent drug, Li-Dan-Hua-Shi pills (LDHSP) have the function of purging liver and gallbladder, clearing heat and freeing strangury. The recipe of LDHSP contains multiple herbs, including *Lysimachia christinae*, *Artemisia capillaries, Polygonum cuspidatum*, *Citrus medica* and so on [5]. Animal experiments showed that the preparation has obvious inhibitory effect on albumen induced toe swelling model and xylene induced acute ear inflammatory response [6]. Moreover, the preparation has a strong antibacterial activity in vitro [6]. However, all of previous studies on the preparation were not highly correlated with its indication, i.e. liver and gallbladder diseases. Herein, we speculated that the preparation may protect the liver and gallbladder by acting on alkaline phosphatase.

Though alkaline phosphatase had been successful immobilized on fibrin scaffold [7], chitosan nanoparticles [8] and naked magnetic nanoparticles [9], the application of AP conjugated magnetic microspheres (MMPs) to ligand fishing has not been reported yet. In the present work, AP conjugated MMPs was synthesized by amide linkage on the surface of carboxyl-terminated MMPs. The as-prepared adsorbent was successfully applied for affinity selection of AP binding ligands in medicinal plant samples.

2. Experimental

2.1. Chemicals and materials

Alkaline phosphatase (from bovine intestinal mucosa, EC Number:3.1.3.1), N-hydroxysuccinimide (NHS), 2-(N-morpholino)ethanesulfonic acid (MES) and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide(EDC) were purchased from Sigma Co. Li-Dan-Hua-Shi pills were provided by Jiangsu Province Hospital of traditional Chinese medicine. Bradford kit and alkaline phosphatase testing kit were obtained from Beyotime Co. Monodispersed carboxyl functionalized MMPs

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were provided by Tianjin BaseLine ChromTech Research Centre. HPLC-grade acetonitrile was obtained from Merck. Caftaric acid was purchased from Shanghai Rongbo Biotechnologies Co. Gardenoside, caffeic acid, paeoniflorin, vitexin, naringenine and pinoresinol diglucoside were purchased from Sichuan Weikeqi Biological Technology Co. Azelaic acid, sodium orthavanadium, theophylline and forsythiaside were obtained from Shanghai Yuanye Bio-Technology Co. *p*-Methoxybenzaldehyde was provided by Shanghai Aladdin Biochemical Technology Co. 96-well microtiter plates were purchased from Corning Inc. All solutions and dilutions were prepared with ultrapure water from a milli-Q water purification system.

2.2. Apparatus

X-ray diffractometer (XPert PRO), transmission electron microscopy (FEI Tecnai G2 F20 S-TWIN), vibration sample magnetometer (PPMS-9), fourier transform infrared spectroscopy (Bruker Optics), ZetaMaster3000 zeta potential laser particle size analyzer (Marven), Agilent 1100 LC-UV system (Agilent), Shimadzu UHPLC system (Shimadzu), Q-TOF 5600-plus mass spectrometer(AB sciex), PB-10 Sartorious pH meter (Sartorious), Synergy 2 multimode reader (Biotek).

2.3. Preparation of AP conjugated magnetic microspheres

AP was conjugated to MMPs by amide linkage according to our approach described before [10]. Box-Behnken designs for optimization of AP immobilization are shown in Table S1.

2.4. Morphology characterization

The crystalline structure of MMPs was identified using powder X-ray diffractometer. The morphology of AP conjugated MMPs was characterized by using transmission electron microscopy (TEM). Size distributions were monitored by using zeta potential laser particle size Analyzer. Fourier transform infrared spectroscopy (FTIR) was acquired on a Bruker infrared spectrometer. Magnetization curve was measured in a vibration sample magnetometer.

2.5. Optimization conditions for affinity selection of AP binding ligands

One well-recognized AP inhibitor, i.e. caffeic acid (CA), was employed to optimize the experimental conditions. At first, different wash times (1–4 times) were attempted for the washing procedure. Second, methanol–water (10, 30, 50, 70 and 90%, v/v) and different ratios of acetonitrile-water (10, 30, 50, 70 and 90%, v/v) were investigated for the elution step. Third, incubation temperature (25, 30, 37, 40 and 45 °C) and time (5, 15, 30, 45 and 60 min) were varied to study the effect of pH and ion strength on the extraction efficiency. Fourth, a gradient pH (7.0, 7.5, 8.0, 8.5 and 9.2) and a gradient concentration (50, 100, 200, 350 and 500 mM) of Tris-HCl buffers were also interrogated.

The reaction mixtures of the model component (20 μL, 0.1 mg mL^{-1}) and AP conjugated MMPs (20 μL) were prepared in Tris-HCl buffer to reach a final volume of 200 μL, and incubated for appropriate time at proper temperature. The supernatant was stored, and the AP conjugated MMPs were washed multiple times with 200 μL of the buffer and subsequently incubated in 200 μL denature solvent for 10 min to dislodge specific bound compounds. The dissociated ligands were collected and sent to UHPLC-Q-TOF/MS for analysis.

2.6. Validation of the established approach

The chromatographic separation was performed on a reversed-phase XBridge-C_{18} column (250 mm × 4.6 mm, 5 μm, Waters) with the column temperature set at 30 °C. The mobile phase consisted of water containing 0.1% (V:V) formic acid (A) and acetonitrile (B). A gradient program was used according to the following profile: 0 min, 5% B; 45 min, 100% B. The flow rate was 1 mL min^{-1} and the injection volume was 10 μL. The UV spectra were recorded from 190 to 400 nm while the chromatogram was acquired at 280 nm.

Selectivity was assessed by comparing the chromatograms of mixture sample spiked with four standards, dissociated binders by using the AP conjugated MMPs based affinity selection approach and dissociated binders by using blank MMPs based affinity selection approach. The affinity selection procedure was carried out as described in the former section.

The calibration curves were constructed by plotting the peak area of CA to its gradient concentrations (0.01–0.5 g L^{-1}) on the basis of a linear regression model. The accuracy and precision of the approach were evaluated by repeated analyses of QC samples (0.125 g L^{-1} for CA) on three consecutive days. Matrix effects were investigated on crude extract of LDHSP, by calculating the ratio of the peak area of CA in the presence of crude extract of LDHSP to the peak area in absence of crude extract of LDHSP at three different QC concentrations. The reusability of AP conjugated MMPs was evaluated by conducting the solid-phase extraction after incubating with CA at the same conditions for ten consecutive times. "denature solvent" (30% methanol–water) was used to unbound the CA. The MMPs were reused after rinsed with phosphate buffer solution thoroughly. The fourth eluates of the ten consecutive cycles were collected and analyzed using HPLC-DAD.

2.7. Application to Li-Dan-Hua-Shi pills

1 g of LDHSP was ultrasonicated in 50 mL of methanol for an hour. Second, the supernatant was then filtered. Third, the filtrate was concentrated and dried under vacuum and the residue was reconstituted in 2 mL Milli-Q water for the next experiment. The affinity selection procedure was performed according to the approach described above in Section 2.5.

The chromatographic separation of UHPLC was performed on a Syncronis C_{18} column (100 mm × 2.1 mm, 1.7 μm, Thermo) with the column temperature set at 35 °C. The mobile phase consisted of water containing 0.1% (V:V) formic acid (A) and acetonitrile (B). A gradient program was used according to the following profile: 0–2 min, 5% B; 8 min, 30%B; 13 min, 95% B; 15 min, 98% B, 17 min, 5%B. The flow rate was 0.3 mL min^{-1} and the injection volume was 5 μL.

The acquisition parameters for quadrupole-time-of-flight mass spectrometry were as below: collision energy, −35 eV; ion spray voltage, −4.5 kV; nebulizer gas (gas 1), 55 psi; declustering potential, −60 V; heater gas (gas 2), 55 psi; turbo spray temperature, 550 °C; and curtain gas, 35 psi. The Q-TOF/MS scan range was set as m/z 100–1500.

2.8. AP inhibitory assay

AP inhibitory activity was measured according to the approach described in the Ref [11]. Sodium orthavanadium was used as the positive control.

2.9. Compound binding studies

The following compounds were subjected to the binding studies to AP: aftaric acid, gardenoside, caffeic acid, paeoniflorin, *p*-methoxybenzaldehyde, azelaic acid, vitexin and naringenine.