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Short Communication

Antibody purification using affinity chromatography: A case study with a monoclonal antibody to ractopamine

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ABSTRACT

The application of antibodies to small molecules in the field of bioanalytics requires antibodies with stable biological activity and high purity; thus, there is a growing interest in developing rapid, inexpensive and effective procedures to obtain such antibodies. In this work, a ractopamine (RAC) derivative, *N*-4-aminobutyl ractopamine (ABR), was synthesized for preparing new specific affinity chromatography to purify a murine monoclonal antibody (mAb) against RAC from ascites. The performance of the new specific chromatography was compared with four other purification methods in terms of recovery, purity and biological activity of mAb. These four purification methods were prepared by using specific ligands (RAC and RAC-ovalbumin) and commercial ligands (protein G and protein A), respectively. The results showed that the highest recovery (88.1%) was achieved using the new chromatography; in comparison, the recoveries from the other methods were all below 70%. The purity of the mAbs from the new chromatography was 88.3%, while, the highest purity of 97.6% was from protein G chromatography and the lowest purity of 84.7% was from protein A chromatography. The biological activity of the purified mAb from all of the chromatography methods was comparable in enzyme-linked immunosorbent immunoassay (ELISA).

1. Introduction

Mammalian ascites represents a remarkable and economical source of monoclonal antibodies (mAbs) against small molecules that are widely used for diagnostic and therapeutic applications. Monoclonal antibodies are routinely used as coatings or labeling reagents for the detection of target analytes in a variety of immunoassays and as specific ligands for immunoaffinity columns (IAC) [1–5]. Obviously, using Abs from ascites influences the reproducibility and stability of immunoassays and bioconjugation; thus, antibody purification is becoming critical to guarantee its reliable application. Non-chromatographic and chromatographic methods are currently the two main categories of techniques used to purify mAbs [6]. The non-chromatographic methods normally include Ab after precipitation with inorganic salts or alcohol, such as ammonium sulfate and ethanol [7–9] or aqueous two-phase extraction (ATP) and high-performance tangential flow filtration [6,10,11]. There are many examples of chromatographic methods for Ab purification, such as protein A or protein G chromatography [12], ion-exchange chromatography [13], hydrophobic interaction chromatography [14], hydroxyapatite chromatography [15], and membrane chromatography [16]. In research and in industrial practice, protein A and G resins are the most commonly used ligands in chromatographic methods [17]. However, these ligands are species and isotype dependent, and thus, adsorptions sometimes exhibit very low capacity. Moreover, proteins A and G cannot tolerate harsh conditions for cleaning procedures because of their low chemical and proteolytic stability. Recently, certain new ligands have been provided to compensate for the above limitations of protein A and protein G. For example, mannan-binding lectin (MBL) is suitable for IgM [18], while protein L and protein P can bind κ light chains of antibodies and are commercially available [19]. However, these ligands are expensive, and antibodies purified by these materials inevitably contain some non-specific antibodies.

Recently, specific affinity chromatography methods based on the bound interaction between antigen (or hapten) and antibody have become good alternatives for antibody purification, in

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particular for antibodies to small molecules. The specific ligands employed in specific affinity chromatography are generally organic molecules that are commercially available or easily synthesized. More importantly, the organic ligands provide high specific affinity and sufficient robustness and diversity compared with their biological counterparts, such as protein A and protein G.

In this study, we used mAbs toward ractopamine (RAC) as the model and a new RAC derivative as the specific ligand to verify the advantage of specific affinity chromatography. The purity, recovery and biological activity of this system were compared with other purification methods.

2. Materials and methods

2.1. Materials and equipments

RAC and *N*-(4-bromobutyl) phthalimide were obtained from Sigma Chemical Co. (St. Louis, Mo., USA). CNBr-activated Sepharose 4B, HiTrap protein G Sepharose 4 Fast Flow, and HiTrap Mabselect SuRe was purchased from GE Healthcare (Uppsala, Sweden). A Bradford Protein Assay Kit was obtained from Beyotime Institute of Biotechnology (Haimen, China). Pre-coated silica gel 60 GF254 glass sheets for thin layer chromatography (TLC) were purchased from Qingdao Haiyang (Qingdao, China). The antigen RAC-OVA was prepared in our laboratory.

A Multiscan MK3 reader was obtained from Thermo Inc. (Shanghai, China). A UPLC system coupled to a Micromass Quattro Premier XE triple quadrupole mass spectrometer was obtained from Waters (Manchester, UK). ^{1}H NMR spectra were recorded with a Bruker DPX300 spectrometer and an internal reference: TMS (δ 0.000 ppm for ^{1}H). A mini-PROTEAN II apparatus was purchased from Bio-Rad laboratories, Inc. (Hercules, CA, USA).

2.2. Synthesis of the N-4-aminobutyl derivative of RAC

RAC (Fig. 1A) was derivatized to *N*-4-aminobutyl ractopamine (ABR) prior to conjugation with the CNBr-activated Sepharose 4B. The chemical reaction process (Fig. 1A) was as follows: a mixture of *N*-(4-bromobutyl) phthalimide (0.296 mmol), triethylamine (0.888 mmol), and RAC (0.296 mmol) in DMF (5 mL) was stirred at 110 °C for 14 h and cooled to 20 °C, and the organic solvent was evaporated by rotary evaporation. The residue was partitioned between ethyl acetate (15 mL) and water (15 mL); the organic fraction was washed with water (2 × 7.5 mL) and brine (7.5 mL) and then dried; and the organic solvent was evaporated by rotary evaporation. The residue was purified by TLC and eluted with methanol-dichloromethane (v/v, 3:50) to give RAC phthalimide (110 mg, 10%) as a clear oil. The clear oil (0.0199 mmol) and hydrazine hydrate (0.0996 mmol) in 2 mL of dry ethanol were stirred at RT for 24 h. Filtration followed by evaporation of the filtrate gave the ABR (11.9 mg) as an oil in a quantitative yield. The purity and confirmation of ABR were checked by TLC (methanol–dichloromethane, v/v, 3:50), ^{1}H NMR and mass spectrometry [20]. ^{1}H NMR (300 MHz, MeOD) δ: 8.16 (dd, 1H), 7.59 (dd, 1H), 7.02–6.95 (m, 3H), 6.69–6.54 (m, 3H), 4.37 (dt, 1H), 2.80–2.71 (m, 3H), 2.48–2.31 (m, 6H), 1.70 (dd, 2H), 1.52 (dd, 2H), 1.19 (s, 2H), 1.01–0.80 (m, 3H). HRMS (+EI) calcd. $C_{22}H_{32}N_2O_3$ (M^+) for 373.30, found 373.3 (Fig. 1B).

2.3. Preparation of immunoaffinity adsorbents

For this purpose, 750 mg of CNBr-activated Sepharose 4B (1 g of powder results in approximately 3.5 mL of gel) was mixed to form a gel in 5 mL of HCl (1 mM) and poured into a sintered-glass funnel (40 μm). The gel was washed with 10 mL of HCl (1 mM) and then with 10 mL of conjugating solution (100 mM sodium bicarbonate buffer containing 0.5 M NaCl, pH 8.3). After this procedure, the gel was divided into three equal aliquots and mixed with 10 mM of RAC, ABR or 4 mg of RAC-OVA ligand in 2 mL of conjugating solution, respectively. After 2 h incubation at RT, the remaining reactive groups were blocked by incubating the gel in 2 mL of 1 M ethanolamine (pH 9.0) for 2 h. The coupled gel was washed three times with 0.1 M sodium acetate (containing 0.5 M NaCl, pH 4.0) and 0.1 M Tris–HCl (containing 0.5 M NaCl, pH 8.0).

2.4. Preparation of immunoaffinity column chromatography

For this purpose, 1 mL of adsorbent was washed by 5 mL of 0.1 M sodium acetate (containing 0.5 M NaCl, pH 4.0) and then 5 mL of reaction buffer (0.1 M Tris–HCl containing 0.5 M NaCl, pH 8.0). After washing, the absorbent was packed into a glass column (10 mm × 0.8 mm i.d.) and equilibrated with 2–3 column volumes of the reaction buffer. The prepared column was stored in ethanol/water (v/v, 20:80) until use. Blank packing was also prepared according to the immobilization procedure in Section 2.3, except for the ligand-coupling step.

After a fivefold dilution of ascites with 10 mM PBS (containing 150 mM NaCl, pH 7.4), the ascites was filtered with a 0.45 μm filter. Then, 2 mL of the treated fluid was applied to the prepared immunoaffinity columns at a flow rate of 1 mL/min. The unbound proteins in the columns were washed out with 0.1 M sodium acetate (containing 0.5 M NaCl, pH 4.0) and 0.1 M Tris–HCl (containing 0.5 M NaCl, pH 8.0). Next, the antibodies were eluted with 0.1 M glycine–HCl (pH 2.7) in all chromatography columns [21]. One milliliter fractions of the eluent solution were collected in tubes and detected by UV-Vis Spectrometry at 280 nm. After elution, the column was regenerated by subsequently washing with 10 mL of binding buffer, 10 mL of water, and 5 mL of 20% ethanol solution.

2.5. Determination of purity, recovery, and activity of purified mAb

Recovery is calculated by Eq. (1). Protein concentrations were determined by the Bradford assay with BSA as a standard [22].

$$\text{Recovery (in\%)} = \frac{\text{Yield of purified protein}}{\text{Yield of ascites}} \quad (1)$$

where yield is the amount of mAb (mg) per mL ascites or purified mAb per mL of ascites.

The purity of the bound fractions from the affinity columns was checked by SDS-PAGE, performed under reducing conditions on a 1 mm separation gel. Approximately 7 μg of total protein was analyzed by performing electrophoretic runs on the Mini-PROTEAN II apparatus (595 nm). Protein bands were detected using the Brilliant Blue Coomassie R-250 staining method, and the purity was determined using the Scion Image software (Scion Corporation, USA). The sum of the density of the two bands (50 and 25 kDa) in one lane divided by the density of all of the bands in the lane gave the purity of the mAb (in %).

The activity of the mAb was determined using indirect ELISA. For the detailed procedure and the data analyses, refer to our previous report [23].

3. Results and discussion

3.1. Preparation of the RAC derivative and affinity columns

The agarose beads of the CNBr-activated Sepharose 4B are made of monodisperse hydrophilic material and bear free cyano groups on their surface that are responsible for reacting with amino groups of the target ligand. A new derivative of RAC with an amine group,