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Adsorption of human serum proteins onto TREN-agarose: Purification of human IgG by negative chromatography

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ABSTRACT

Tris(2-aminoethyl)amine (TREN) – a chelating agent used in IMAC – immobilized onto agarose gel was evaluated for the purification of IgG from human serum by negative chromatography. A one-step purification process allowed the recovery of 73.3% of the loaded IgG in the nonretained fractions with purity of 90–95% (based on total protein concentration and nephelometric analysis of albumin, transferrin, and immunoglobulins A, G, and M). The binding capacity was relatively high (66.63 mg of human serum protein/mL). These results suggest that this negative chromatography is a potential technique for purification of IgG from human serum.

1. Introduction

Human immunoglobulin G (IgG), the major class of serum glycoproteins, constitutes an important therapeutic protein for a number of malignancies [1] and is also required for immunodiagnostic and immunochromatographic (downstream-processing) purposes. Therefore, there is a high demand for high purity IgG, free from other serum proteins. Processes for the purification of human IgG typically involve precipitation with ethanol followed by chromatographic techniques (such as ion exchange chromatography, gel permeation chromatography, or affinity chromatography) [2,3].

The proteins A, G, and L are the most frequently biological specific ligands used in affinity adsorption for IgG. Due to their high affinity for the Fc antibody domain (proteins A and G) and the variable domain of the human kappa chain (protein L), these ligands can be employed in a one-step adsorption process for IgG purification or extracorporeal removal of autoimmune IgG from biological fluids [4–9]. However, protein A, G, and L adsorbents are expensive and the desorption of IgG involves drastic and denaturing elution conditions and cannot withstand the harsh conditions of cleaning procedures. In addition, these proteins are susceptible to degradation and leakage after some purification cycles [1,10]. These drawbacks have stimulated the development of more stable and less expensive adsorbents in recent years.

Pseudospecific affinity ligands such as nonbiological molecules (metal chelates, thiophilic, and dye ligands) and biological molecules (like the amino acid histidine) have been studied for human IgG purification by many research groups [11–16]. Generally, the pseudobiospecific ligands are low cost and have high stability, capacity, simplicity, and selectivity [17]. Among these ligands, the immobilized amino acid histidine is an interesting alternative for human IgG purification [18,19]. As an example, histidine grafted aminohexyl-Sepharose 4B was used as a negative affinity adsorbent for purification of IgG from human plasma diluted 20 times in Mops buffer at pH 7.2 [20]. The negative chromatographic mode (as cited by many authors [21–25]) aims at allowing the product to pass through the column, retaining only the contaminants or impurities [26]. The adsorption of serum proteins in histidine grafted aminohexyl-Sepharose seems to be due to the electrostatic interaction of albumin and other proteins of human serum by the free remaining cationic α-NH_3^+ of histidine. The unprotonated imidazole group of histidine is also involved in the retention of serum protein around neutral pH [20].

The nonbiological ligand TREN (Tris(2-aminoethyl)amine) is a quadridentate chelating ligand used in IMAC with four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary. TREN chelated with copper and nickel ions has been employed in protein purification [16,27–29]. Due to its high amine residue content, TREN (without chelated metal ion) can serve as

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an anion exchanger. At a pH lower than 10.0, TREN is positively charged (according to the manufacturer, Sigma–Aldrich) and can adsorb negatively charged molecules, so, this ligand could be an excellent candidate for the purification of IgG from serum proteins. However, in the literature there is no study describing TREN as a ligand for IgG purification.

Therefore, the purpose of this study is to evaluate the feasibility of using the ligand TREN immobilized onto agarose through an ether linkage (epichlorohydrin-activated gel) for the purification of IgG from human serum by negative chromatography. The main advantages of this unconventional proposed process is the use of nonbiological ligand that costs less and is more stable than ligands traditionally used in affinity chromatographies. Experimental studies were conducted aiming to find the least favorable conditions – pH and buffer system (phosphate, Tris–HCl, Bis–Tris, Mes, and Mops) – for human IgG adsorption. The breakthrough curve and dynamic capacity of serum proteins were determined since they are the basis for process design, scale-up, and optimization of large-scale negative chromatographic mode separation processes.

2. Experimental

2.1. Materials

The TREN-agarose gel (cross-linked 4% beaded agarose, activated with epichlorohydrin), 3-(N-morpholino)propanesulfonic acid (Mops), bis(2-hydroxyethyl)amino–tris(hydroxymethyl) methane (Bis–Tris), crystalline bovine serum albumin (BSA), and human serum albumin (HSA, 98–99% purity) were obtained from Sigma (USA). The electrophoresis calibration kit for molecular mass determination (myosine, 210 kDa; α_2-macroglobulin, 170 kDa; β-galactosidase, 110 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) was provided by GE Healthcare (USA). Tris(hydroxymethyl amino methane) and 2-(N-morpholino)ethanesulfonic acid (Mes) were purchased from Merck (Germany). Prepurified human immunoglobulin G (containing 98.3% IgG according to the nephelometric analysis of IgG, IgM, IgA, albumin (HSA), and transferrin done in our laboratory) was provided by Aventis Behring (Germany). The nephelometric reagents were purchased from Beckman Coulter (USA). The water used in all experiments was ultrapure water obtained using a Milli-Q System (Millipore, USA). All other chemicals were of analytical reagent grade.

2.2. Preparation of human serum

Blood samples from a healthy donor were collected without anticoagulant. These samples were centrifuged at 4 °C for 5 min at 1000 × *g* and the supernatant was used without further treatment. To ensure that proper ionic strength and pH were maintained for optimal binding, it was necessary to dilute serum samples with the proper adsorption buffer.

2.3. Analysis of serum proteins

Total protein concentration was determined by the Bradford method [30] using BSA as reference protein. The concentrations of IgG, IgA, IgM, HSA, and transferrin in the fractions collected in the chromatographic experiments were determined nephelometrically using an Array Protein System (Beckman Coulter, USA), in accordance with the method provided by the manufacturer.

2.4. Sodium dodecyl sulfate polyacrylamide gel elecrophoresis (SDS-PAGE) and isoelectrofocusing (IEF)

The chromatographic fractions were analyzed by SDS-PAGE (7.5% polyacrylamide gels) under nonreducing conditions using a Mini-Protean III System (Bio-Rad, USA) in accordance with Laemmli [31] and stained with silver nitrate in accordance with Morrissey [32]. The PhastSystem (Pharmacia, Sweden) and pH 3–9 gradient gels (GE Healthcare, USA) were used for IEF and stained with silver nitrate in accordance with the method provided by the manufacturer.

2.5. Chromatographic experiments

All chromatographic procedures were carried out with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA) at 25 °C at a flow rate of 0.5 mL min^{-1}. For studies concerning the influence of the buffer on human serum protein adsorption, the following loading buffers at 25 mM covering a pH range from 5.5 to 9.0 within their respective buffering ranges were used: Mops, Mes, Tris–HCl, Bis–Tris–HCl, and sodium phosphate.

The TREN-agarose gel was suspended in the loading buffer described above, degassed, and packed into columns (10.0 cm × 1.0 cm I.D., GE Healthcare, USA) to give bed volumes of approximately 1.0 or 3.0 mL.

Human serum (0.1 or 0.3 mL) and its solutions diluted 5, 10, or 20 times with an appropriate loading buffer were injected into the column, which had been previously equilibrated with loading buffer. For the experiments with prepurified human IgG, protein samples containing 2.0 mg of IgG diluted in 2.0 mL of equilibration buffer was loaded into the column (bed volume of 3.0 mL). For both experiments, after protein injection, the column was washed with loading buffer until the absorbance values at 280 nm of eluate were close to 0. Elution was performed with the loading buffer containing 0.4 M NaCl.

During the wash steps and elution, absorbance at 280 nm was monitored and fractions of 1.0 and 2.0 mL, respectively, were collected. The Bradford method [30], nephelometric analysis, SDS-PAGE, and IEF were used to analyze the proteins in nonretained and retained fractions. After each experiment, the column was washed with 50 mM NaOH, followed by water, and finally by the loading buffer to restore it to its initial conditions (regeneration) for carrying out the next experiment.

2.6. Adsorption breakthrough curves

These experiments were carried out at 25 °C with an automated chromatography system (Econo Liquid Chromatography System, Bio-Rad, USA). After equilibration of TREN-agarose with the loading buffer (25 mM Mes buffer, pH 6.5), human serum diluted 20 times with the loading buffer was pumped through the column at a flow rate of 0.5 mL min^{-1} (residence time, t_R of 36 s, calculated by dividing the bed interstitial volume by the flow rate). The column outlet absorbance at 280 nm was continuously monitored. The loading of the protein solution was stopped when absorbance at 280 nm at the column outlet became constant after an initial increase. The unabsorbed protein was washed out of the column with loading buffer. The adsorbed proteins were eluted with a 25 mM Mes buffer, pH 6.5, containing 0.4 M NaCl. The effluents were monitored as described previously (measurement of absorbance at 280 nm). After elution had been completed, the column was regenerated by sequentially washing with 50 mM NaOH, followed by water and the loading buffer.