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Purification of human IgG by negative chromatography on ω-aminohexyl-agarose

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ABSTRACT

The ω-aminohexyl diamine immobilized as ligand on CNBr- and bisoxirane-activated agarose gel was evaluated for the purification of human immunoglobulin G (IgG) from serum and plasma by negative affinity chromatography. The effects of matrix activation, buffer system, and feedstream on recovery and purity of IgG were studied. A one-step purification process using Hepes buffer at pH 6.8 allowed a similar recovery (69–76%) of the loaded IgG in the nonretained fractions for both matrices, but the purity was higher for epoxy-activated gel (electrophoretically homogeneous protein with a 6.5-fold purification). The IgG and human serum albumin (HSA) adsorption equilibrium studies showed that the adsorption isotherms of IgG and HSA obeyed the Langmuir–Freundlich and Langmuir models, respectively. The binding capacity of HSA was high (210.4 mg mL^{-1} of gel) and a positive cooperativity was observed for IgG binding. These results indicate that immobilizing ω-aminohexyl using bisoxirane as coupling agent is a useful strategy for rapid purification of IgG from human serum and plasma.

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1. Introduction

Polyamines are polycations that are able to interact with proteins, nucleic acids, and phospholipids [1–5]. Due to their structure, polyamines interact with negatively charged molecules by electrostatic binding. Despite their polycationic nature, polyamines also have the potential for hydrophobic interactions, depending on the length of alkyl chain. Numerous studies have indicated that polyamines are selective and efficient immobilized ligands for purification of proteins with affinity for amino groups [1].

Different polyamine-grafted chromatographic matrices have been used as affinity adsorbents for purification of many proteins. Like pseudobiospecific ligands, polyamines are low cost and have high stability, capacity, simplicity, and selectivity [1]. They can be easily immobilized after derivatization of different matrices with various activating agents such as cyanogen bromide (CNBr), epichrorohydrin, bisoxiranes, or divinylsulfones.

Diamines such as 1,5-diaminopentane (aminopentyl) and 1,6-diaminohexane (aminohexyl) have been immobilized as ligands on CNBr- or divinylsulfonyl-activated agarose for purification of amine oxidases [6,7]. As lactoferrin (a protein with a high isoelectric point) binds to diamines by affinity interactions, it has been purified using aminohexyl immobilized on divinylsulfone-activated agarose [8]. Plasminogen binds to lysine-Sepharose and can be purified in one step using an α-carboxy-aminoalkyl gel [9]. More complex polyamines such as spermidine, spermine, and TREN (Tris(2-aminoethyl)amine)-grafted chromatographic matrices have been used for the purification of spermine synthase, ryanodine receptor, and human immunoglobulin G (IgG), respectively [10–12].

TREN, a polyamine with four amino groups (four nitrogen atoms, three of which are primary in nature and the fourth one is tertiary), adsorbed human serum proteins (probably by electrostatic interactions) and human IgG could be purified by negative chromatography. In the negative chromatographic mode, the impurities or contaminants are adsorbed by the adsorbent and the product is collected in the flowthrough and washing fractions [12–14].

Although immobilized polyamines have been used successfully for purification of different proteins, the effect of amino residues content, the nature of the amino group (primary, secondary, or tertiary amino group), and the length of the alkyl chain in protein purification are not yet known.

Thus, the aim of this work was to study whether a simpler polyamine such as an aliphatic amine would be better for use in human IgG purification than the more complex polyamines (such as TREN) used in the reported studies. This work evaluated the feasibility of using the ligand aminohexyl (1,6-diaminohexane) immobilized on agarose gel for the purification of IgG from human serum or plasma solutions by negative chromatography. The effects of the spacer arm and operating conditions (type of feed, pH, buffer system, concentration of IgG in feed solutions) on the capacity and selectivity of the system were also investigated. The breakthrough curve and dynamic capacity of serum proteins were determined to obtain data useful for the development of large-scale

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processes for purification of human IgG in the negative chromatography mode. IgG and the HSA binding were also studied and the adsorption isotherms were analyzed with the Langmuir and Langmuir–Freundlich models. Parameters pertinent to the adsorption processes such as the apparent dissociation constant (K_d), the maximum binding capacity (Q_m), and the cooperativity were analyzed and discussed.

2. Experimental

2.1. Materials

The ω-aminohexyl immobilized on CNBr and on epoxy-activated agarose (cross-linked 4% beaded), referred to in this work as ω-aminohexyl-agarose and ω-aminohexyl-bisoxirane-agarose, respectively, were purchased from Sigma (USA).

For SDS-PAGE analysis, acrylamide, bis-acrylamide, sodium dodecyl sulfate (SDS), and dithiotrietol were purchased from Bio-Rad (USA). High molecular mass markers for SDS-PAGE (myosine, 212 kDa; α2-macroglobulin, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa; glutamic dehydrogenase, 53 kDa) were purchased from GE Healthcare (USA). Coomassie Brilliant Blue and Morpholinoethane sulfonic acid (Mes) were purchased from Merck (Germany). Morpholinopropane sulfonic acid (Mops), Hydroxyethylpiperazine ethanesulfonic acid (Hepes), crystalline bovine serum albumin (BSA), and prepurified human serum albumin (HSA, 99% purity) were provided by Sigma (USA). Prepurified human immunoglobulin G (with 98.3% IgG according to nephelometric analysis of IgG, IgM, IgA, albumin (HSA), and transferrin (Trf) 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. Human serum and plasma samples

Blood samples from a healthy donor were collected in Vacuette® serum tubes or in Vacuette® plasma tubes (Greiner BioOne, Austria). The serum tubes were kept at room temperature for about 2 h in order to allow agglutination to obtain serum. The samples collected in serum and plasma tubes were centrifuged at 4 °C for 5 min at 3000 rpm 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 or plasma samples with the proper adsorption buffer.

2.3. Chromatographic experiments

All chromatographic experiments were carried out using an automated chromatography system (ÄKTA Prime Plus, GE Healthcare, USA) at 25 °C at a flow rate of 0.5 mL min^{-1} (linear flow rate of 38.2 cm h^{-1}). For studies concerning the influence of the buffer on human serum and human plasma protein adsorption, the following loading buffers at 25 mmol L^{-1} covering a pH range from 6.5 to 8.2 within their respective buffering ranges were used: Mops, Mes, and Hepes.

The ω-aminohexyl-agarose and ω-aminohexyl-bisoxirane-agarose gels were 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 3.0 mL. The column was equilibrated with equilibration buffer (25 mmol L^{-1} Mops, Mes, or Hepes buffer). The human serum or plasma was diluted 20 times with the equilibration buffer and loaded into the column. After protein injection, the column was washed with equilibration buffer until protein was no longer detected in the column out-stream by absorption at 280 nm. Elution was performed with the loading buffer containing 1.0 mol L^{-1} NaCl.

During the wash and elution steps, absorbance at 280 nm was monitored and fractions of 1.0 and 2.0 mL, respectively, were collected. The Bradford method [15], 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 mmol L^{-1} NaOH, followed by water, and finally by the loading buffer to restore it to its initial conditions (regeneration) for carrying out the next experiment.

2.4. Adsorption breakthrough curves

These experiments were carried out at 25 °C with an automated chromatography system (ÄKTA Prime Plus, GE Healthcare, USA). After equilibration of ω-aminohexyl-bisoxirane-agarose with the loading buffer (25 mmol L^{-1} Hepes buffer at pH 6.8), 106.0 mL of human serum diluted 20 times in Hepes buffer at pH 6.8 (359.58 mg of total serum protein) 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 mmol L^{-1} Hepes buffer at pH 6.8, containing 1.0 mol L^{-1} 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 mmol L^{-1} NaOH, followed by water and the loading buffer.

Protein concentrations in the retained and nonretained fractions were determined by the Bradford method [15] and nephelometric analysis and analyzed by SDS-PAGE under nonreducing conditions. The ratio of the outlet total protein concentration (C) to that in the feedstream (C_0) was plotted in a breakthrough curve as a function of the volume of protein solution throughput.

2.5. Protein adsorption studies

Experiments to determine the adsorption isotherms of HSA and human IgG on ω-aminohexyl-bisoxirane-agarose at 25 °C were carried out (in duplicates) in Eppendorf tubes (as stirred tanks) with 0.05 mL of gel. The gel had been previously equilibrated with degassed 25 mmol L^{-1} Hepes buffer at pH 6.8 and the aliquots of 1.0 mL HSA or IgG solutions were added to the tubes. The initial protein concentrations of HSA and IgG were in the range of 0.5–50.0 mg mL^{-1} and 0.5–36.0 mg mL^{-1}, respectively. The tubes were agitated for 3 h to allow equilibrium to be established. After this, the protein equilibrium concentration in the liquid phase (C) was quantified. The concentration of protein was measured based on absorbance at 280 nm (UV–vis spectrophotometer, Beckman DU 650, USA). The difference in unbound protein concentrations between the experiments did not exceed on average 2.0% and 0.5% for IgG and HSA, respectively. The mass of protein adsorbed per volume of gel (mg mL^{-1}), Q, was calculated as the difference between the amount of protein added and that remaining in the liquid phase after equilibrium divided by the volume of the adsorbent. Plotting Q as a function of C yielded the equilibrium isotherm. The Langmuir (Eq. (1)) and Langmuir–Freundlich (Eq. (2)) isotherm models [16,17] were used to fit the data:

$$Q = \frac{Q_m C}{K_d + C} \quad (1)$$

$$Q = \frac{Q_m C^n}{K_{dLF} + C^n} \quad (2)$$

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