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Investigation of the interaction mechanism of the recombinant human antibody MDJ8 and its fragments with chromatographic apatite phases

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ABSTRACT

The chromatographic behaviour of a recombinant human antibody (IgG_1-subtype, κ-light chain, MW: 149.5 kD, p*I*: 9.3) was investigated as a function of the buffer pH and buffer type (HEPES, phosphate, borate) on fluoroapatite and hydroxyapatite stationary phases. HEPES buffer was used at pH 7.0, phosphate buffer at pH 8.2 and borate buffer between pH 8.5 and 11. Elution was by a double gradient method of first a salt gradient from 0 to 1 M NaCl in the corresponding buffer, followed by a step gradient to 0.4 M sodium phosphate. Regardless of the pH and buffer type, the antibody eluted in the NaCl gradient; capacity factors decreased with increasing pH. At pH 11 the antibody eluted in the flow-through. Retention was thus dominated by electrostatic interaction throughout the investigated pH-range. Investigation of antibody fragments obtained by papain digestion (fc- and fab-fragments) and deglycosylated fc-fragments showed that the sugar structures had no influence on the chromatographic behaviour. Instead the chromatographic behaviour was dominated by that of the fab-fragment. ζ-Potential measurements verified that the apatite surface bore a negative surface charge in the investigated pH range, while the antibody net surface charge switched from positive to negative as the pH increased. The corresponding isoionic point was a function of both the buffer concentration and the buffer species. However, above a pH of 8.3 the ζ-potential of the antibody generally was negative. Simulations of the molecular electrostatic potential of the antibody and the two fragments revealed the presence of a positively charged patch within the fab-fragment, which only disappeared above a pH of 10. Most likely this patch was responsible for the observed behaviour.

1. Introduction

Antibodies currently are among the most valuable and promising products of modern biotechnology [1,2]. In this context, apatite (AP) is increasingly discussed as interesting chromatographic material for specific antibody (fragment) isolation [2,3]. Ceramic apatite stationary phases for chromatography are currently available as hydroxyapatite (sum formula: $Ca_{10}(PO_4)_6(OH)_2$) and more recently also as fluoroapatite (sum formula: $Ca_{10}(PO_4)_6(F)_2$). Fluoroapatite (FAP) is prepared from hydroxyapatite (HAP) via exchange of some of the hydroxy groups by fluoride ions. Whether this exchange has consequences for the retentive properties of the apatite material remains at present unclear and requires further studies of the particular role of the OH^-- and the F^--sites in the chromatographic interaction. However, FAP has the advantage of a better stability towards acidic pH, as this material may be used down to a pH of 5.0, while HAP should not be exposed at any length to a pH below 6.5 [4].

With regard to protein binding, the AP-lattice is described to support two major adsorption mechanisms [5–10]. One contribution to retention is phosphoryl cation exchange or P-site interaction and involves the negatively charged phosphate groups on the AP-surface. Since most antibodies bear a positive net-charge at neutral pH, their interaction with AP materials is typically assumed to be dominated by electrostatic P-site interaction and their elution is often possible in a gradient of increasing NaCl-concentration [11]. However, data in the pertinent literature show that even in such cases the retention is not explained by a simple cation exchange mechanism, e.g. [3,12]. The second type of interaction mechanism supported by AP is C-site interaction with the calcium moieties. The physico-chemical basis for this type of interaction is less clear. However, it is well documented in the literature that certain biomacromolecules bearing a negative net-charge are retained by AP in spite of the net negative surface charge of the material. Such molecules typically cannot be eluted by a simple salt gradient but require increasing amounts of agents such as phosphate and fluoride, i.e. compounds that can be assumed to interact strongly with the calcium-sites in the apatite [13]. It has been proposed that 'C-site interaction' may in the case of proteins be mediated by a chelating effect of neighbouring carboxylate groups or by metal

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ion affinity interaction, e.g. between histidine side chains in the protein and the calcium site. However, experimental evidence presented in [14] shows that histidine–calcium interaction is not a major factor in the retention. The hypothesis of a chelating effect is, on the other hand, supported by the fact that EGTA (ethylene glycol bis(aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid), a calcium-chelating agent, can serve as displacer of C-site interacting proteins in hydroxyapatite displacement chromatography [15] and by the fact that the elution of C-site interacting proteins is possible by IDA (iminodiacetic acid). The contribution of H-bridges to the retention is another possibility discussed in the literature, as is the possibility of anion exchange with positively charged calcium groups [5,8–10,13]. However, to date little experimental evidence has been presented to either support or exclude these possibilities.

Most proteins carry a pattern of positively and negatively charged groups on their surface. Retention on apatite phases can thus be expected to be of a mixed mode type, where the contribution of the various possible interactions can be fine-tuned via the composition of the mobile phase in a manner not possible in single interaction mode chromatography. The complexity of the interaction constitutes a distinct advantage of AP chromatography, as it allows the development of very specific isolation procedures. However, this complexity also renders process development difficult. A more systematic process development would benefit from a better understanding of the underlying interaction mechanisms. While some work has been done in the past on the selective desorption of acidic and basic proteins by increasing ionic strength and specifically C-site interacting agents [6,8–13,16,17], comparatively little has been done, in regard to understanding the attraction of proteins to the apatite surface on a molecular basis. In the past few years, the calculation of the electrostatic interactions using protein continuum electrostatics improved significantly providing a new tool for the interpretation of adsorption/desorption-processes in chromatographic systems. Surprisingly, the effect of buffer ions (charge shielding) is typically not considered in these calculations, instead the proteins are placed in water for such molecular dynamic simulations [18–20]. However, ions bound to the protein surface will influence the initial adsorption, since they change the net charge as well as the charge pattern.

In this contribution, the interaction between a recombinant human antibody and HAP- as well as FAP-surfaces was studied at an extended pH range around and above the isoelectric point of the antibody using different buffering agents. Since the amino acid sequence of the antibody was known, molecular modelling of the structure became possible. By conducting *in silico* calculations of the molecular electrostatic potential (MEP) of the antibody, additionally information in regard to the charge distribution at the protein surface was obtained and correlated to the observed chromatographic behaviour. As far as we know this is the first time such an extensive study of the molecular basis for protein retention on apatite materials has been performed.

2. Materials and methods

2.1. Materials

Chemicals were from Acros Organics (Geel, Belgium), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), or Sigma–Aldrich (Seelze, Germany) and used as obtained. Milli-Q water was used to prepare all aqueous solutions. Buffers were filtered with a nylon membrane filter (0.2 μm pore size, Nalgene, Rochester, NY, USA) and degassed prior to use. The ceramic hydroxyapatite (CHT type I, 20 μm particle size, 600–800 Å pore size), the ceramic fluoroapatite (CFT type I, 40 μm particle size, 600–800 Å pore size) and the Unosphere S (80 μm particle size, 1000 Å pore size) stationary phases were from BioRad Laboratories (Hercules, CA, USA). Empty Tricorn high performance columns (55 mm × 5 mm) used for packing of CHT, CFT, and Unosphere S were from GE Healthcare (Uppsala, Sweden).

2.2. Antibody production

The recombinant human antibody MDJ8 (h-IgG$_1$-subtype, κ-light chain) was produced by a Chinese hamster ovary (CHO) cell line containing the appropriate expression cassettes of the full-length immunoglobulin [21]. The stable cell line was kindly provided by Prof. F. Wurm (Laboratory of Cellular Biotechnology, Swiss Federal Institute of Technology, Lausanne, Switzerland). Antibody production took place in 100 mL as well as 1 L spinner flasks as suspension culture (inoculation cell density: 2×10^5 cells/mL). The culture medium was the Pro-CHO 5 medium from Cambrex (Vervier, Belgium) supplemented with L-glutamine (final concentration 4 mM) and penicillin / streptomycin (100 U/mL and 0.1 mg/mL, respectively) (both PAA Laboratories, Linz, Austria). Cell densities were determined in a Vi-Cell XR (Beckman Coulter, Miami, FL, USA). Cell viability was assessed via Trypan blue stain exclusion assay. Spinners were harvested usually after 9–11 days, when the cell viability had declined to approximately 30% (final viable cell density: $2.18 \pm 0.2 \times 10^6$ cells/mL (100 mL spinner), $0.69 \pm 0.2 \times 10^6$ cells/mL (1 L spinner)). Cell suspensions were centrifuged for 20 min at 4200 × *g* (4 °C) (Heraeus Multifuge 3 S-R, Kendro, Osterode, Germany) to remove the cells. If necessary, cell free supernatants were stored at −20 °C until use.

2.3. Preparation of antibody fragments

For preparation of the antibody fragments via papain digestion, the antibody was isolated from the supernatant by Protein A affinity chromatography as described previously [22] (column: HiTrap rProtein A FF (GE Healthcare), loading buffer: 20 mM phosphate buffer, pH 7.0, containing 150 mM sodium chloride, eluent: 100 mM glycine–HCl, pH 3.0, flow rate: 1 mL/min, collected fractions were immediately neutralised to pH 7). Afterwards a buffer exchange of the pooled antibody fraction was conducted with a Sephadex G-25 fine column (XK 26/10, GE Healthcare) to a 20 mM Na-phosphate buffer pH 7, containing 150 mM NaCl. To reach a final antibody concentration of >1 mg/mL, the sample was concentrated by centrifugal ultrafiltration (Vivaspin 10,000 MWCO PES, Vivascience AG, Hannover, Germany). For preparation of the fab-/fc-fragments, the concentrated antibody sample was digested at 86:1 (w/w) ratio with papain (from *papaya latex*, Sigma–Aldrich) for 2 h at 37 °C according to ref. [23]. Afterwards the digest was desalted using a Sephadex G-25 fine column. Fab- and fc-fragments were separated by Protein A affinity chromatography as described above. Protein A has previously been used to separate fc- and fab-fragments, e.g. by Guerrier et al. [12]. The collected fragments (fab-fragment in the flow through and fc-fragment in the glycine step) were again desalted and concentrated to a final concentration of 0.75 and 0.52 mg/mL, respectively.

2.4. Preparation of the deglycosylated fc-fragment

The fc-fragment was deglycosylated by digestion in 20 mM ammonium bicarbonate buffer (Sigma) with 2 units of peptide *N*-glycosidase F enzyme (PNGase F, Sigma) per μg substrate for 19 h at 37 °C.

2.5. Sample preparation for chromatography

Prior to AP chromatography, the thawed antibody-containing cell culture supernatant was passed through a Minisart single use filter unit (cellulose acetate, 0.2 μm pore size, Sartorius, Göttingen,