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Transient conformational modification of immunoglobulin G during purification by protein A affinity chromatography

Pete Gagnon*, Rui Nian, Denise Leong, Aina Hoi

Bioprocessing Technology Institute, 20 Biopolis Way, Centros #06-01, Singapore 138668, Singapore

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ABSTRACT

Exposure of three native IgG1 monoclonal antibodies to 100 mM acetate, pH 3.5 had no significant effect on their hydrodynamic size (11.5 ± 0.5 nm), while elution from protein A with the same buffer created a conformation of 5.5 ± 1.0 nm. Formation of the reduced-size conformation was preceded by the known destabilization of the second constant domain of the heavy chain (Cγ2) by contact with protein A, then compounded by exposure to low pH, creating extended flexibility in the hinge-Cγ2 region and allowing the Fab region to fold over the Fc region. The reduced-size conformation was necessary for complete elution. It persisted unchanged for at least 7 days under elution conditions. Physiological conditions restored native size, and it was maintained on re-exposure to 100 mM acetate, pH 3.5. Protein A-mediated destabilization and subsequent restoration of native size did not create aggregates, but the reduced-size conformation was more susceptible to aggregation by secondary stress than native antibody. Protein A-mediated formation of the reduced-size conformation is probably universal during purification of human IgG1 antibodies, and may occur with other subclasses and IgG from other species, as well as Fc-fusion proteins.

1. Introduction

Potential negative consequences of exposing IgG to low pH have made protein A elution conditions an object of controversy for decades. Many investigators have expressed concern that exposure to low pH causes conformational changes leading to aggregation, and they have cited formation of turbidity upon pH neutralization of protein A elutes as proof [1–13]. Others have favored the hypothesis that IgG conformation is unaffected by such exposure [14,15], based on their observation that removal of turbidity by microfiltration had negligible effect on antibody recovery. Consistent with the latter perspective, a recent study showed that formation of turbidity was mediated through pH-dependent insolubility of chromatin-associated host cell contaminants in the eluted IgG fraction [16].

Direct characterization of domain stability with high-resolution 2-dimensional NMR has shown that the second constant domain (Cγ2) of IgG collapses entirely at pH 3.1 [17]. This makes elution conditions a legitimate concern for affinity chromatography media that are commonly eluted in the range of pH 2–3, like protein G and protein L [13], but most antibodies elute from protein A at pH 3.5 or higher, especially on current-generation recombinant protein A ligands that bind IgG exclusively at the Fc region [18]. NMR shows that domain integrity of purified IgG is virtually unperturbed at pH 3.5 [17].

These findings create an expectation that protein A affinity chromatography should be completely benign as a purification method, but the controversy surrounding elution at low pH has been screening another phenomenon that challenges that conclusion. X-ray crystallography studies have shown that the residence of protein A in its primary binding site between the Cγ2 and Cγ3 domains creates instability in the Cγ2 domain [19,20]. This suggests it must also affect IgG conformation during purification by protein A, but there is no standard for extrapolating chromatographic behavior from protein interactions constrained in a crystal lattice. The present study addresses the conformation of IgG immediately following elution from protein A and its subsequent neutralization.

2. Materials and methods

2.1. Reagents and equipment

Buffers, salts, and reagents were obtained from Sigma-Aldrich (St. Louis, MO), except allantoin, which was obtained from Merck Millipore (Darmstadt, Germany). Toyopearl AF-rProtein A-650F was obtained from Toso Bioscience (Tokyo). UNOsphere™ Q was

* Corresponding author. Tel.: +65 6407 0941; fax: +65 6478 9561.
E-mail address: pete.gagnon@bti.a-star.edu.sg (P. Gagnon).

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obtained from Bio-Rad Laboratories (Hercules, CA, USA). Capto adhere was obtained from GE Healthcare (Uppsala, Sweden). Chromatography media were packed in XK or Tricorn™ series columns (GE Healthcare). Chromatography experiments were conducted on an ÄKTA™ Explorer 100 or Avant 25 (GE Healthcare).

2.2. Experimental methods

Three prospective biosimilar IgG1 monoclonal antibodies (Herceptin™, Avastin™, and Humira™) were expressed by mammalian cell culture in Chinese hamster ovary (CHO) cells using a tricistronic vector developed by Ho et al. [21]. They were produced in 15–30 day fed-batch cultures, harvested at 30–50% cell viability.

IgG used to support most of the study was highly purified to minimize interference with analytical methods. Cell culture harvest was clarified by adding caprylic acid to cell-free culture harvest to a final concentration of 0.4%, and allantoin to a final concentration of 2%. pH was adjusted to 5.3 with 1 M acetic acid, and the mixture stirred for 2 h. UNOsphere Q pre-equilibrated with 50 mM MES, 150 mM NaCl, pH 5.3 was added at a proportion of 5% (v/v) and mixing continued for at least 4 h. Solids were removed by centrifugation and/or microfiltration. Protein A affinity chromatography media was equilibrated with 50 mM Hepes, 120 mM NaCl, pH 7.0. Sample was loaded and the column washed with 10 column volumes (CV) of equilibration buffer, then eluted with a step to 100 mM acetic acid, pH 3.5, or the same buffer with NaCl or arginine added. Aggregates, antibody fragments, DNA and residual host cell proteins were further removed by titrating the protein A eluate to pH 8.0, adding NaCl to 1 M, loading it onto Capto adhere, and eluting with a step to 50 mM MES, 0.35 M NaCl, pH 6.0. Antibodies purified by this process contained <10 ppm host cell protein (HCP), less than 1 ppm DNA, and less than 0.1% aggregates. For the remainder of the discussion, this material is referred to as *highly purified IgG*.

Experimental controls were conducted in some cases with IgG purified by protein A affinity chromatography where the cell culture harvest was clarified only by centrifugation and microfiltration.

Experimental controls were also conducted in some cases with antibody purified without exposure to protein A. In brief, harvest was clarified with the caprylate-allantoin-solid phase adsorbent system described above, then fractionated by anion exchange chromatography in void exclusion mode (VEAX) [22], in 50 mM Tris, pH 8.0. NaCl was added to the IgG-containing VEAX void fraction to a final concentration of 1.0 M then applied to Capto adhere and eluted as described above.

2.3. Analytical methods

IgG purity was documented according to the methods described fully in [16]. In brief, host cell protein (HCP) content was estimated by ELISA with a Generation III CHO HCP kit from Cygnus Technologies Inc. (Southport, NC). DNA was measured using a QX100™ Droplet Digital™ PCR System (Bio-Rad Laboratories). Aggregate content was measured by analytical size exclusion chromatography (SEC) with a G3000SWxl column (Tosoh Bioscience) on a Dionex Ultimate™ 300 HPLC system (Thermo Scientific) operated at a flow rate of 0.6 mL/min, using a buffer formulation of 50 mM MES, 20 mM EDTA, 200 mM arginine, pH 6.0. Sample injection volume was 100 μL.

Solute size distributions in free solution were characterized by dynamic light scattering (DLS) using a Zetasizer ZS (Malvern Instruments, Worcestershire, UK). The sample (200 μL) was mixed gently for 10 seconds on a vortex before being placed into a quart cuvette (ZEN2112, Malvern Instruments) using a gel loading tip to avoid bubbles. Viscosity of the carrier solution was determined using a SV-10 viscometer (A&D Company, Tokyo). The backscattered light at 173° was measured and 3 measurements were averaged. Attenuation index was maintained at a value of 7–8. Analysis of the data was performed using version 7.02 of the Dispersion Technology Software provided by the manufacturer.

Circular dichroism (CD) spectroscopy was performed with a JASCO J-810 spectropolarimeter (JASCO Corp., Tokyo). Far-UV spectra (190-260 nm) were obtained with highly purified IgG at a concentration of 0.2 mg/mL using a quartz cuvette with a path length of 0.1 cm. Near-UV spectra (250–350 nm) were obtained with highly purified IgG at a concentration of 1.0 mg/mL using a quartz cuvette with a path length of 1.0 cm. For both, 32 scans were accumulated with a scan rate of 100 nm/min and time constant of 0.125 s. All Spectra were corrected by subtracting the buffer baseline and averaged 32 times. All experiments were conducted at room temperature. Relative amounts of random coil, α-helix, and β-sheet were calculated using K2D2 software [23].

Other experimental details are described or reiterated for clarity in the following section.

3. Results and discussion

3.1. Size characterization by DLS

DLS indicated hydrodynamic size of the highly purified IgG monoclonal antibodies under physiological conditions was 11.5 ± 0.5 nm. They maintained that size when titrated to pH 3.5. When highly purified native antibodies were applied to protein A and eluted with 100 mM acetic acid, pH 3.5, hydrodynamic size of the eluted IgG was 5.5 ± 1.0 nm (Fig. 1).

Addition of NaCl increased size of the protein A-eluted IgG, as did increasing pH (Fig. 2). Exposure to physiological conditions restored native size. Restored antibody maintained native size at all tested pH values from 3.5 to 8.5, and NaCl concentrations from

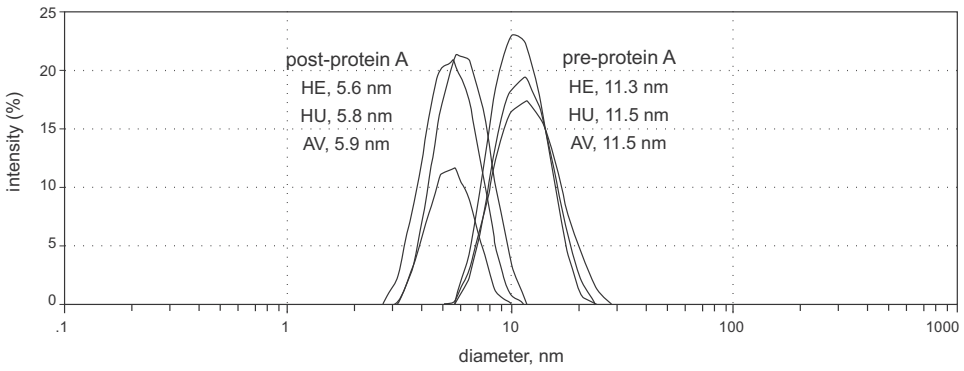

Fig. 1. DLS data showing hydrodynamic size of IgG before and after elution from protein A. Protein A-eluted samples still in 100 mM acetate, pH 3.5. HE, HU, and AV refer to biosimilar IgG1 clones for Herceptin, Humira, and Avastin.