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Differential binding of heavy chain variable domain 3 antigen binding fragments to protein a chromatography resins

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ABSTRACT

This work examines the binding of 15 different V_H3 IgGs and their corresponding $F(ab')_2$ fragments to two different protein A chromatography resins: MabSelect®, which utilizes a recombinant protein A ligand, and MabSelect SuRe® (SuRe), which utilizes a tetrameric Z domain ligand. The results show that V_H3 $F(ab')_2$ fragments can exhibit a variety of binding behaviours for the two resins. Contrary to previously published data, a subset of these molecules show strong interaction with the Z domain of SuRe®. Furthermore, the results show that sequence variability of residue 57 in the V_H3 heavy chain CDR2 domain correlates with binding behaviour on MabSelect® and SuRe®. Site-directed mutagenesis of this residue confers gain or loss of V_H3 $F(ab')_2$ binding to these resins in 3 mAbs, demonstrating that it plays a key role in both recombinant protein A and Z domain interaction. A fourth mAb with a longer CDR2 loop was not affected by mutation of residue 57, indicating that CDR2 domain length may alter the binding interface and lead to the involvement of other residues in protein A binding.

1. Introduction

Protein A is a 42 kDa cell wall-anchored pathogenicity factor of the bacterium *Staphylococcal aureus*. This factor has evolved to bind two distinct sites on human immunoglobulins with high affinity and specificity in order to evade or disrupt the host immune system [1–3]. One site is located at the C_H2/C_H3 interface of the Fc domain of human IgG1, 2, 4 and a subset of IgG3s [4–7]. A second protein A binding site is located in the heavy chain (HC) variable domain of the V_H3 class of IgG, IgM, IgE and IgA molecules [8–10]. The V_H3 Fab HC binding site has been localized to conserved framework regions 1 and 3 (FR1 and FR3) and complementary determining region 2 (CDR2) [8,9,11]. Nearly half of all human V_H genes belong to the V_H3 subfamily [12,13].

The interaction between protein A and the immunoglobulin Fc and V_H3 Fab domains has been examined extensively by both X-ray crystallography and NMR [5–7,10,14–16]. The N-terminal portion of protein A consists of a tandem repeat of five highly homologous domains of approximately 58 amino acids each [16,17]. Each individual domain, labelled from the N-terminus as E, D, A, B and C, consists of 3 alpha helices arranged in a compact helical bundle [16]. The Fc and Fab V_H3 binding sites on protein A are structurally distinct, with residues from helices 1 and 2 interacting with IgG Fc via predominantly hydrophobic interactions, while the opposite face of helix 2 as well as residues from helix 3 interacting with V_H3 Fab domains electrostatically through specific side-chains as well as and hydrogen bonding [5,14]. The C-terminal end of protein A consists of the XM domain, which facilitates binding to the bacterial cell wall.

Due to the high affinity interaction of protein A and the IgG Fc domain, protein A chromatography is the most commonly used capture step in the manufacture of IgG monoclonal antibodies (mAbs) and Fc fusion protein therapeutics [18,19]. Binding occurs at neutral pH, thus mAb and Fc fusion proteins can be directly captured on protein A from harvested cell culture fluid. Additionally, the strength of the interaction between protein A and the Fc domain allows for the application of relatively stringent washes, such as high concentrations of various salts, detergents, and even chaotropes such as urea to remove unwanted contaminants including host cell proteins bound to the product [20–23]. Elution of the product is achieved at acidic pH, typically at a pH between 3.0 and 4.0, depending on the product's amino acid sequence and process requirements. Many companies have developed highly platformed protein A chromatography operations, which can robustly deliver >98% purity in one step [18,19].

Protein A resins for preparative chromatography and manufacturing have evolved significantly over the last 15 years. Second generation chromatography resins, such as MabSelect®, use a

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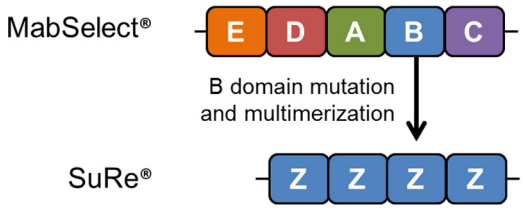

Fig. 1. Schematic of MabSelect® and SuRe® ligands.

recombinant protein A ligand with the XM domain removed, as it does not contribute to IgG binding. Other protein A analogues have been developed, such as the Z domain, a version of the B domain with two amino acid substitutions, N28A and G29A (Fig. 1) [24]. These substitutions confer some resistance to hydroxylamine cleavage, allowing for sodium hydroxide regeneration and sanitization of the resin at industrial scale [24]. Additionally, the G29A mutation has been shown to disrupt interaction with the V_H3 Fab binding site while leaving Fc binding intact [25,26]. This analogue has been used in MabSelect SuRe® (SuRe®) resin, which consists of a tetramer of Z domains that have additional arginine substitutions to further improve alkaline stability [27]. Previous studies have indicated that V_H3 Fab binds weakly or not at all to SuRe® resin, and thus allows for more moderate low pH elution conditions to be used [28].

In this paper, we compare the interaction of intact mAb and F(ab')$_2$ fragments of 15 V_H3 IgG molecules with MabSelect® and SuRe® resin. The data show that V_H3 Fabs exhibit a range of binding behaviour on these resins. In contrast with previously published data [28], some V_H3 Fabs are capable of strong binding to both resins. Others interact with MabSelect® but not SuRe®, while the remaining V_H3 Fabs do not bind either resin. We have identified a single amino acid of the V_H3 HC CDR2 domain, residue 57, which plays a key role in both recombinant protein A and Z domain binding. Site-directed mutagenesis of this residue significantly alters binding characteristics to both MabSelect® and SuRe® in 3 out of 4 V_H3 Fab fragments tested. These data demonstrate that not all V_H3 Fabs exhibit the same binding behaviour MabSelect® and SuRe® resin and that residue 57 plays a key role in determining binding behaviour to both recombinant protein A and a Z domain analogue for many V_H3 Fabs. Understanding V_H3 interactions can facilitate improvements in protein A chromatography for large scale manufacturing for V_H3-containing mAb and Fab-based therapeutics.

2. Materials and methods

2.1. Antibody and F(ab')$_2$ stocks

CHO-produced monoclonal antibodies were purified over protein A followed by 1–2 polishing chromatography steps using standard procedures. Antibody stocks were formulated at 70–120 mg/ml in10 mM sodium acetate, 9% sucrose pH 5.0.

Single point mutants in the V_H3 HC of four monoclonal antibodies were generated by PCR mutagenesis and subcloned into expression vectors using a QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA). Forward primers were as follows: mAb 2 HC T57E (ACTGGTGGTGCCACAAGCTATGCAGAC), mAb 3 HC T57E (ACTTGATGGTGGGACAACACAGTACACT), mAb 13 HC E57T (GATCGAAGTAATGAATACTATGCAGAC), and mAb 14 HC E57T (GATGGAAGTGATGAATACTATGCAGAC). Constructs were transfected into CHO cells using standard electroporation procedures. Transfected cells were subjected to selection to generate stable cell pools, which were used to perform bioreactor productions. Harvest and two column purification was performed using standard procedures. Mutations were confirmed at the construct level by DNA sequencing and the protein level by reduced tryptic peptide mapping. Mutant mAb stocks were formulated by dialysis into 10 mM sodium acetate, 9% sucrose pH 5.0.

2.2. Pepsin digestion to generate F(ab')$_2$

Antibody stocks were diluted to 2 g/L in 20 mM sodium acetate, pH 3.9 and titrated to pH 4.0 ± 0.1 with HCl if needed. Pepsin at 1 mg/ml (Worthington Biochemical, Lakewood, NJ) was added to the diluted antibody solution at a 1:500 pepsin:antibody mass ratio. Reactions were incubated in the dark at 37 °C for 24 h, after which the pepsin was inactivated by titrating the samples to pH 7.5–8.0 with 2 M Tris-acetate pH 8.0, and then stored at 2–8 °C. During the development of the pepsin reaction conditions, intact mass measurements were performed to confirm that the desired F(ab')2 fragment was generated. F(ab')$_2$ samples prepared for the current work were analyzed by analytical size exclusion chromatography (SEC) to confirm > 95% digestion of intact mAb into a 99–100 kDa F(ab')$_2$ fragment and smaller Fc fragments.

2.3. Protein A chromatography

MabSelect® and MabSelect SuRe® (SuRe) resins (GE Healthcare, Piscataway, NJ) were packed to a 5 cm bed height in 1.15 cm ID Vantage columns (Millipore, Billirica, MA). Runs were conducted on an Akta Explorer 100 (GE Healthcare) at 72 cm/h with UV monitoring at 280 and 300 nm. The flowrate was chosen to match the 4 min residence time of a 25 cm full height column run at 360 cm/h. Columns were equilibrated with 25 mM Tris, 100 mM NaCl, pH 7.4 (EQ), loaded with intact mAb or F(ab')$_2$ diluted to 2 g/L in EQ buffer, and washed with four column volumes (CV) of EQ. Elution was performed with a 20 CV pH gradient of 50 mM sodium acetate, pH 5.0 to 50 mM acetic acid, pH 3.0. Fractions were collected across the entire run. Columns were cleaned with three CV 0.1 M phosphoric acid, 1 CV EQ, and 3 CV of either 6 M Urea (MabSelect®) or 0.3 M NaOH (SuRe®), followed by storage in 50 mM sodium citrate, 2% benzyl alcohol, pH 5.0. Protein-containing fractions were analyzed by SEC to determine where the F(ab')$_2$ and Fc fragments exit the column. Columns were loaded to 2 g/L resin, to provide a tight window of elution for determining elution peak pH, and in some cases 20 g/L resin, to gauge binding capacity. The AKTA pH at the elution peak maximum was normalized to an offline pH measurement using the following: (Offline pH of pH 3.0 buffer—AKTA reading of pH 3.0 buffer) + AKTA pH at elution peak maximum.

2.4. Analytics

2.4.1. Size Exclusion Chromatography (SEC)

SEC was performed on a Waters 2695 HPLC system equipped with either a 2475 dual wavelength or 2996 PDA detector (Waters, Milford, MA), using a TSKgel G3000SW 7.8 x 300 mm, 5 μm, 300 Å column (Tosoh Biosciences, King of Prussia, PA). Runs were performed over 35 min with a mobile phase of 0.25 M NaCl, 0.1 M NaH_2PO_4, pH 6.8 and UV detection at 280 nm. Column and autosampler temperature were ambient and 4 °C, respectively. Data were analyzed using Waters Empower 2 software.

2.4.2. Reduced peptide map with mass detection

V_H3 HC mutants were confirmed by reduced peptide map analysis. Samples were denatured in a guanidine buffer, reduced with dithiothreitol (DTT) and alkylated with sodium iodoacetate. The samples were buffer exchanged into 50 mM Tris, pH 7.9 and digested with sequencing grade trypsin (Roche, Basel, Switzerland) (1:10 w/w) for 30 min at 37 °C. The peptides were separated on a Zorbax 2.1 x 150 mm, C18 RR HD, 1.8 μm column (Agilent Technologies, Santa Clara, CA) using a 0.1% TFA/Water and 0.1%