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Modeling on-column reduction of trisulfide bonds in monoclonal antibodies during protein A chromatography

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ABSTRACT

Trisulfides can be a common post-translational modification in many recombinant monoclonal antibodies. These are a source of product heterogeneity that add to the complexity of product characterization and hence, need to be reduced for consistent product quality. Trisulfide bonds can be converted to the regular disulfide bonds by incorporating a novel cysteine wash step during Protein A affinity chromatography. An empirical model is developed for this on-column reduction reaction to compare the reaction rates as a function of typical operating parameters such as temperature, cysteine concentration, reaction time and starting level of trisulfides. The model presented here is anticipated to assist in the development of optimal wash conditions for the Protein A step to effectively reduce trisulfides to desired levels.

1. Introduction

Recombinant monoclonal antibodies (mAbs) have established themselves as the most dominant and successful class of biotherapeutics due to their high specificity and long half-life [1–3]. mAbs are large proteins (150 kDa) that are relatively homogeneous in structure that being comprised of two copies of light and heavy chain, each with multiple intra and inter-chain disulfide bonds [4]. The number of interchain disulfide bonds is variable based on the class of immunoglobulin (IgG) and has been extensively characterized in the literature [5,6]. In addition, the susceptibility of these disulfide bonds to various modifications such as thioether crosslinking, disulfide bond scrambling, glutathionylation, oxidation and cysteinylation lead to further product heterogeneity within an IgG subclass [7–10]. These product-related variants need to be controlled during an antibody manufacturing process to ensure consistent product quality over time.

In the recent years, another disulfide related post-translational modification, the formation of trisulfide bonds, has been reported in the literature for mAbs. Trisulfides are a variant of disulfides that contain an extra sulfur atom, creating an S-S-S bridge between two cysteine residues [11]. Initially, trisulfide modifications were reported for a handful of proteins such as *E. coli*-derived recombinant growth hormone [12], recombinant truncated interleukin 6 [13], superoxide dismutase [14] and a few others. Interestingly, trisulfide modification has been increasingly mentioned by several companies for all subclasses of antibodies independent of their glycosylation state [15,16]. Gu and researchers [15] performed extensive characterization of this variant using LC–MS based peptide mapping analysis of non-reduced protein samples and showed that trisulfides are present only in the heavy-light and heavy–heavy inter-chain linkages that connect the four subunits, and not in any of the intra-chain disulfides. Their work also showed that the presence of high levels of trisulfides had no observable effect on the function or stability of the antibody.

Nevertheless, controlling trisulfide levels during an antibody manufacturing process is very important as this modification can be a significant source of product heterogeneity. Gu et al. [15] have shown that trisulfide levels can vary significantly (<1% to 39%) based on variations in cell culture conditions. Kshirsagar et al. [17] have studied the impact of cell culture conditions on trisulfide formation and shown that cysteine concentration in the feed medium can have a direct correlation to the trisulfide level in the product. Following up on this thought, Biogen has pioneered the use of a reducing agent (namely cysteine) during the wash step of Protein A chromatography to consistently reduce trisulfide levels to below detection limits [18]. Protein A affinity chromatography currently forms the framework for all antibody platform purification processes [19,20] and incorporating this reduction reaction during the capture step provides a manufacturing friendly way of

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eliminating the trisulfide/disulfide product heterogeneity. This is particularly important for demonstration of comparability during process scale-up or transfer to multiple manufacturing sites. Currently, all the possible causes for trisulfide level variation often seen in the upstream process are not fully understood, hence having a cysteine wash in the downstream process provides an easy lever to reduce this variability.

In this manuscript, we have further investigated this trisulfide reduction during the Protein A wash step to develop generic conditions for our antibody purification platform. A systematic study was carried out to evaluate the effect of operating parameters such as reaction time, cysteine concentration and operating temperature on the rate of trisulfide reduction. Several antibodies were used in this study to determine the general applicability of this approach across a variety of antibodies. Furthermore, a simple empirical model was built correlating the final trisulfide level to the various operating parameters. Kinetic models have been reported in the literature for on-column protein aggregation during Protein A chromatography and was shown to be useful for comparing conditions that minimize aggregation [21]. Recently, Mazzer et al. have tried to model IgG4 aggregation post Protein A chromatography by using an exponential decay function to capture the decrease of monomer concentration over time [22]. In this paper, the decrease of trisulfide concentration over time is modeled using a second-order rate equation to determine rate constants at a particular cysteine concentration. This is then combined with an empirical functional relationship between rate constant and cysteine concentration. The model developed here can be an extremely useful powerful tool for process development and help in quick selection of optimal conditions for the on-column trisulfide reduction reaction.

2. Materials and methods

2.1. Materials

Monoclonal antibodies (mAbs) A, B, C, D, E and F were used as model proteins for this study. They are all recombinant humanized monoclonal IgG_1 antibodies produced in Chinese hamster ovary (CHO) cells at Biogen. Harvested cell culture fluid (HCCF) was used as the load material for all Protein A runs.

MabSelect SuRe LX Protein A resin was obtained from GE Healthcare (Uppsala, Sweden) and packed in 6.6 mm diameter Omnifit glass columns obtained from Diba Industries (Danbury, CT, USA).

All buffer chemical components used were purchased from Sigma (St. Louis, MO, USA) and J.T. baker (Phillipsburg, NJ, USA) unless stated otherwise.

2.2. Equipment

An ÄKTA Explorer 100 purification station from GE Healthcare was used for all chromatographic experiments. This system had an in-built C–900 pH/conductivity meter, UV-900 detector with a 2 mm flow cell and P-950 pump system.

A Waters e2695 HPLC system was used for analytical Protein G assay for titer measurement in HCCF. A Solo UV Spectrophometer (C-technologies, NJ, USA) was used to measure protein concentration in Protein A purified samples.

2.3. Experimental methods

2.3.1. Protein A experiments

Protein A chromatography was carried out using a 0.66 cm D × 20 cm L column that was sequentially perfused with Equilibration buffer (5CV), HCCF, EQ-Wash (3CV) with Equilibration buffer, Wash I buffer (5CV), Wash II buffer (3CV) and Elution buffer (5CV).

Table 1
Operating parameters for the Protein A wash I step.

mAb	Process temperature (°C)	Column Volume	Static hold	Total Contact Time (min)
A	20	5	None	30
B	20	5	None	30
C	20	5	None	60
D	20	5	None	30
E	20	5	None	30
F	5	5	1 h	90

Following elution, the column was cleaned with 0.1N NaOH and stored in the storage buffer. Wash I buffer contained cysteine for the reduction and its concentration was varied as described in the Results section. The flowrate of the Wash I step was also varied to achieve the different reaction times. All other operating parameters were kept constant for an even comparison. Column loading was kept constant at 55 g/L. The Protein A elution pool was collected, adjusted to a neutral pH and assayed for tri-sulfide content.

2.3.2. Analysis

Analytical protein G assay was used to quantify the antibody concentrations in HCCF using 30 mm L × 2.1 mm (i.d.) Poros G 20 μm column (Life Technologies, MA). Standard and test samples (containing 50–100 ug IgG) were injected onto the column that was pre-equilibrated with the buffer containing 25 mM sodium phosphate and 200 mM NaCl, pH 6.5. IgG in the samples that had bound to the column was eluted at pH 2.0 containing 150 mM NaCl.

Antibody concentrations for purified samples were determined based on UV absorbance at 280 nm with a Solo VPE UV Spectrophometer (C-technologies, NJ, USA).

Trisulfide levels in the samples were measured by using a non-reduced tryptic and endoprotease Lys-C peptide mapping assay, as described by Aono et al. [18].

3. Results and discussion

As mentioned during Introduction, Aono et al. [18] were the first to report the use of a cysteine wash during Protein A chromatography to reduce trisulfide levels in an antibody process. Their studies reported cysteine concentrations of 1–3 mM to be optimal for the reduction reaction. Increasing cysteine concentration to >10 mM caused significant loss of intact mAb (as measured by a non-reduced, denaturing electrophoretic assay) suggesting disulfide bond reduction and breakage in addition to the trisulfide to disulfide conversion. On the other hand, cysteine concentrations lower than 0.5 mM were shown to be insufficient in promoting trisulfide reduction. The authors also showed that adding such a low concentration cysteine did not adversely affect the ability of the wash to reduce HCPs. In addition, a 1 h total contact time was employed in this study using 5 column volumes of the wash buffer at a residence time of 12 min. Based on these results, 1.3 mM cysteine was chosen for the Biogen platform as it was found to reduce trisulfide levels to below detection limit for most antibodies. However, the total wash contact time used was highly variable between antibodies and was somewhat arbitrarily selected for each antibody process. Table 1 lists the relevant operating parameters used during the wash phase of the Protein A step for several of our mAb processes. As seen in Table 1, while the wash volume and cysteine concentration was consistent between the processes, the total contact time varied within a very wide range of 30–90 min. It was also surmised that mAb F needed a static hold during the wash step to achieve desired conversion. No fundamental understanding existed about the root cause for such a wide range in contact time as well as the necessity of the static hold. In a typical mAb manufacturing process, the Protein A capture step is usually performed over