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Demonstrating β-glucan Clearance in CHO- and Yeast-Produced Monoclonal Antibodies during Downstream Purification Processes

Fengqiang Wang[1]*, Hong Li[2], Zhi Chen[1], John P Welsh[2], Douglas Richardson[3], Jennifer M Pollard[2], Daisy Richardson[1] and Mohammed Shameem[1]

[1]*Sterile Product and Analytical Development, Merck Research Laboratories, Kenilworth, NJ, USA*
[2]*Process Development and Engineering, Merck Research Laboratories, Kenilworth, NJ, USA*
[3]*Bioprocess Technology and Expression, Merck Research Laboratories, Kenilworth, NJ, USA*

Abstract

Biologics production using yeast or CHO with Yeastolate as cell culture additives often introduces β-glucan, which could potentially pose immunogenicity risk, if not adequately removed. Although a previous study has shown the effective clearance of Yeastolate-derived β-glucan by Protein A chromatography, the clearance pattern of yeast cell derived β-glucan remains unknown. In this study, we characterized the β-glucan clearance patterns during downstream processing of three monoclonal antibody (mAb) products, one mAb fragment from *Pichia pastoris* (mAb A) and two full mAbs from CHO expression system (mAb B and mAb C), by Glucatell assay. We demonstrated effective β-glucan clearance in both small (100 L) and large scale (5000 L) batches of mAb A as well as in one batch of mAb B. Protein A purification step removed an average of 97.74% (1.7 $\log_{10}$ reduction) of β-glucan detected in the two batches of mAb A microfiltration permeates (MFP) and 99.99% (3.9 $\log_{10}$ reduction) of β-glucan detected in mAb B clarified culture fluid harvest (HCCF). Residual β-glucans post Protein A purification in the two batches of mAb A were further removed by the two polishing chromatography steps (94.76% reduction on average). Residual β-glucan measured in the mAb A and mAb B drug substance ranges from 7.8 to 19 pg/mg, which is unlikely to alter physiological concentrations significantly in healthy adults when administered with typical intravenous doses. However, in mAb C, after almost complete removal (99.99%) by Protein A purification step, β-glucan level increased more than 20 fold in Viral Filtration (VF) product sample, indicating that it can be introduced from materials used in downstream process, such as cellulose-based filters and membranes. Our study results suggest that although β-glucan can be cleared by Protein A and other chromatographic steps such as AEX, monitoring β-glucan clearance during downstream process development remains very important to identify and avoid potential contaminations to the drug substance.

Keywords: β-glucan; Yeastolate; Chromatography; Process clearance; Biotherapeutics; Monoclonal antibody

Introduction

Monoclonal antibodies (mAbs) account for a significant portion of biotherapeutics because of their target specificity and their ability to engage a wide variety of targets [1,2]. mAbs are commonly produced by genetically-engineered Chinese Hamster Ovary (CHO) cells, with other host cells of non-human origin, such as *E. coli* and yeast, also being used [3-5]. During mAbs production, the cell culture media and subsequent feed media play important roles in maintaining cell growth and promoting protein production [6]. Traditionally, cell culture media are supplemented with animal serum to promote cell growth; however, safety concerns associated with the use of serum in biotherapeutics production have triggered the use of serum-free or animal origin-free media [7]. Although chemically-defined media has gained popularity, plant- or yeast-derived hydrolysates are still commonly used in cell culture media due to their vital roles in promoting cell growth [8-10]. However, the use of yeast-derived hydrolysates or yeastolate in cell culture media as well as the use of yeast cell for mAbs production has sparked potential safety concerns due to the introduction of β-glucan into the production process.

Beta-glucans are polysaccharides of D-glucose monomers linked by β-glycosidic bonds, with the most common forms being β-1, 4 glucans (also known as cellulose) and β-1, 3 glucans. Beta-1, 3 glucans often have side-chains of D-glucose attached at the (1, 4) or (1, 6) position, known as β-1, 3/1, 4 glucans or β-1, 3/1, 6 glucans. Beta-1, 3/1, 4 glucans are usually water soluble fiber commonly found in oats (*Avena sativa*), which are often used as dietary fiber supplements [11-13]. The most active forms of β-glucans are β-1, 3/1, 6 glucans, which are known as "biological response modifiers" because of their ability to activate the immune system [14-19]. These β-glucans are commonly found in yeast cell wall and yeast extract, and are considered process-related impurities with potential immunogenicity risk in biotherapeutics. Although previous studies have shown that β-glucans introduced in raw materials can be easily cleared by Protein A chromatography [20], potential contamination of β-glucans during downstream purification processes has also been observed, with sources of contamination including raw materials used in chromatography mobile phases, formulation buffers, and cellulose-based filters [21]. Therefore, it is important to characterize the process clearance of β-glucans during downstream process development to ensure product safety according to the Q6B guidelines from the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH).

CHO-produced monoclonal antibody purification often follows

***Corresponding author:** Fengqiang Wang, Associate Principal Scientist, Sterile Product and Analytical Development, Merck Research Laboratories, Kenilworth, NJ, Mailstop K15-4-E214, 2000 Galloping Hill Rd., Kenilworth, NJ 07033, USA, Tel: 908-740-5106; Fax: 908-740-4366; E-mail: fengqiang.wang@merck.com

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similar downstream steps such as harvest by centrifugation and filtration followed by capture using Protein A chromatography, low pH for viral inactivation, polishing steps by AEX or CEX chromatography, Viral Filtration (VF), and Ultrafiltration/Diafiltration (UFDF) [22-24]. For biotherapeutics produced from *Pichia pastoris* or *E. coli*, both viral inactivation and viral filtration steps are not needed since these hosts are not suitable for amplification of viruses that are infectious for humans. To characterize the β-glucan clearance patterns during downstream processing, process-intermediates retained from a monoclonal antibody fragment (mAb A) produced from *Pichia pastoris* and two full monoclonal antibodies (mAb B and mAb C) produced from CHO expression system were analyzed by Glucatell assay. The production of both mAb B and mAb C uses cell culture feed media containing Yeastolate, which has been considered as a source of β-glucan [20]. The downstream process steps used for purifying mAb A, mAb B, and mAb C are illustrated in Figure 1. Since β-glucans normally do not bind to Protein A resin, it is expected that β-glucans released into cell culture from the use of yeast cell or yeast extract can be removed during Protein A chromatography step. However, the risk of introducing additional β-glucans from raw materials or cellulose-based filters used in downstream process steps persists [21]. Therefore, in this study, we analyzed the β-glucan levels in all process-intermediates retained from downstream processing of both yeast-produced mAb A and CHO-produced mAb B and mAb C.

Materials and Methods

Materials

Microfiltration Permeate (MFP), Quenched ProA Pool (QPAP), Mix-Mode Chromatography product (MMC), AEX product (AEX), and UF retentate (UF) were collected from mAb A downstream processing at Fujifilm Diosynth Biotechnologies; Clarified Culture Fluid Harvest (HCCF), Neutralized Viral Inactivated Product (NVIP), AEX product (AEX), Viral Filtration product (VF), and UFDF product retains were collected from mAb B and mAb C downstream purification steps at Merck & Co. Inc. For mAb A, process-intermediates from both a small scale (100 L) and a large scale (5000 L) production were retained. These samples were used for residual β-glucan testing by Glucatell assay. The downstream processes used for mAb A, B, and C purification and the steps where retains were collected are shown in Figure 1. All samples were shipped frozen from Kenilworth, NJ to Associates of Cape Cod in East Falmouth, MA and stored at -70°C until analysis. The concentrations of process-intermediates described above are listed in Tables 1 and 2.

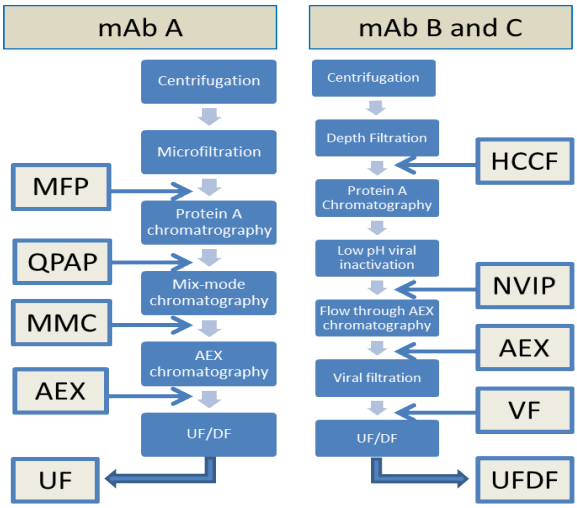

Figure 1: Schematic illustration of the downstream purification processes and the process-intermediates collected for mAb A, B, and C. Left: downstream purification process and process-intermediates collected for mAb A; Right: downstream purification process and process-intermediates collected for mAb B and mAb C.

Beta-glucans analysis

The Glucatell-kinetic chromogenic method was used to determine (1, 3)-β-D-glucan concentration in three mAbs' process intermediates and their corresponding buffer matrices. The test was performed at Associates of Cape Cod Inc (East Falmouth, MA) using the Glucatell kit following similar procedure as previously reported [20,21]. Briefly, five β-glucan calibration standards in a series of 2-fold dilution (from 50 pg/mL to 3.125 pg/mL) were added to a 96-well plate in duplicate. Limulus Amebocyte Lysate (LAL) Reagent Water (LRW) was used as negative control, while samples were diluted with LRW in serial dilutions ranging from 1:2 to 1:2,000,000 and tested in duplicate to find minimum, non-interfering dilutions. A parallel series of sample dilutions described above were spiked with 12.5 pg/mL β-glucan standard as positive controls. The plate was placed in the plate reader preheated to 37°C (±1°C), shaken for 10 seconds and the absorbance's at 405 nm were measured every 15 seconds for 1 h. The chromogenic onset time (seconds) was then plotted against standard concentration to draw a linear curve. For system suitability, the recovery % of the positive control must be > 50% and < 200%; the relative differences of the duplicated standards must be < 25%; and the correlation coefficient (R) of the linear curve must be ≥ 0.980); the negative control values must be lower than the values observed in the lowest standard concentration (3.125 pg/mL).

Results

Beta-glucan clearance patterns in mAb A

A monoclonal antibody fragment (mAb A) was produced from a glyco-engineered yeast cell line, *Pichia pastoris*. The downstream purification steps of mAb A are illustrated in Figure 1. Beta-glucan concentrations in process-intermediates collected from one small scale (100 L, Lot 1) and one large scale production batch (5000 L, Lot 2) were tested by the Glucatell assay. Testing results were expressed as both pg/ml in the sample solution and pg/mg relative concentration to the protein content in the testing samples. MFP samples from both small scale and large scale production have relatively high amount of β-glucans, with 8,847 pg/mL detected in the small scale MFP sample and 15,181 pg/ml detected in the large scale MFP sample (Table 1). The relative concentrations of β-glucans in these two samples are at 11,580 and 14,188 pg/mg of protein content, respectively, which have a relative difference of 20.2% (Table 1). After ProA purification, β-glucans were removed to lower than 1 ng/mg (1 ppm) in both cases, with 1.9 $\log_{10}$ reduction observed in Lot 1 and 1.5 $\log_{10}$ reduction observed in Lot 2 (Table 1). The scaling up of production may have contributed to the slightly increased amount (18.4%) of β-glucan in MFP and relatively lower reduction factor by Protein A chromatography in Lot 2. Additionally, the polishing chromatography steps further removed β-glucans by a total of 0.8 and 1.3 $\log_{10}$ in Lot 1 and Lot 2, respectively, as calculated by the pg/mg concentration (Table 1). The final β-glucan concentrations in mAb A Drug Substance (DS) were 10 pg/mg (Lot 1) and 19 pg/mg (Lot 2), respectively, which are not considered

Samples	Protein (g/L)		β-glucan (pg/mL)		β-glucan (pg/mg)		Log_{10} reduction	
	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2	Lot 1	Lot 2
MFP	0.76	1.07	8,847	15,181	11,580	14,188	-	-
QPAP	8.30	10.98	1,335	4,878	161	444	1.9	1.5
MMC	5.36	7.78	330	172	62	22	0.4	1.3
AEX	4.84	7.72	128	167	26	22	0.4	0.0
UF	85.1	81.8	856	1,588	10	19	0.4	0.1

Table 1: β-glucan concentrations measured in *P. pastoris* produced mAb A process-intermediates and the log_{10} reduction from each purification step.

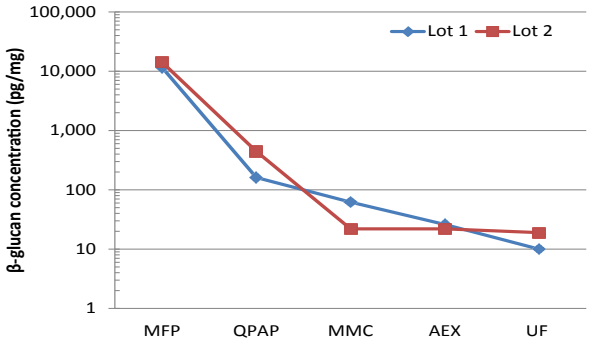

Figure 2: β-glucan clearance patterns during mAb A purification process. The majority of β-glucans found in the MF permeate of mAb A was removed by ProA affinity purification and mix-mode chromatography purification steps.

Samples	Protein (g/L)		β-glucan (pg/mL)		β-glucan (pg/mg)		Log_{10} reduction	
	mAb B	mAb C	mAb B	mAb C	mAb B	mAb C	mAb B	mAb C
HCCF	2.3	2.7	73,928	152,059	32,143	56,318	-	-
NVIP	8.9	17.5	38.2	79.6	4.3	4.6	3.9	4.1
AEX	8.2	8.9	70.7	44.6	8.6	5.0	- 0.3	- 0.0
VF	7.8	5.4	80.7	626	10.3	116.0	- 0.1	- 1.4
UFDF	66.4	50.2	517	3,974	7.8	79.2	0.1	0.2

Table 2: β-glucan concentrations measured in CHO-produced mAb B and mAb C process-intermediates and the log_{10} reduction from each purification step.

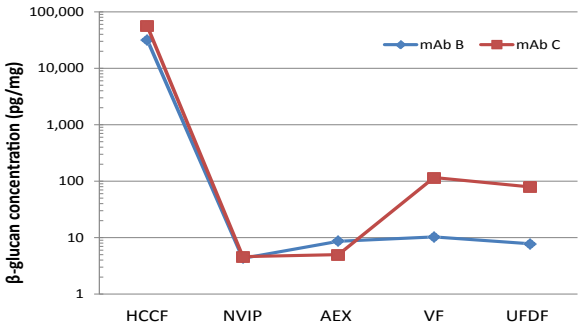

Figure 3: β-glucan clearance patterns during mAb B and mAb C purification process. The majority of β-glucans found in the HCCF of mAb B and mAb C was removed by ProA purification, however, further introduction of β-glucans in mAb C VF sample was observed.

significantly different given the accuracy (50%-200%) of the Glucatell method. The pattern of β-glucan clearance in mAb A downstream process is also shown in Figure 2.

Beta-glucan clearance patterns in mAb B

Monoclonal antibody B was produced in genetically engineered CHO cells. Similar to the downstream purification processes of mAb A, mAb B was first harvested and centrifuged to remove cell debris, filtered and then captured on Protein A chromatography, followed by low pH viral inactivation, AEX chromatography, viral filtration and UFDF steps (Figure 1). Process-intermediates testing results show that β-glucans observed in the HCCF sample of mAb B (32143 pg/mg), were almost completely removed by Protein A chromatography step, with only 4.3 pg/mg of β-glucan detected in the NVIP sample (3.9 log_{10} reduction, Table 2). Beta-glucan concentrations measured in AEX, VF, and UFDF process-intermediates (Figure 1) range from 7.8 pg/mg to 10.3 pg/mg, which are approximately twice of that in NVIP, but within the method variation of the Glucatell assay. It appears that Protein A and viral inactivation steps of mAb B purification almost completely removed β-glucans detected in HCCF, with no further removal of residual β-glucans observed during flow through AEX, viral filtration, and UFDF steps. The pattern of β-glucan process clearance during mAb B downstream process is shown in Figure 3.

Beta-glucan clearance patterns in mAb C

Monoclonal antibody C was also produced in CHO cells and purified following a similar downstream process to mAb B (Figure 1). Similarly, β-glucans detected in the HCCF sample of mAb C were almost completely removed by Protein A chromatography, with a 4.1 log_{10} reduction of β-glucans observed in the NVIP sample as compared to the HCCF sample (Table 2). However, β-glucan levels increased more than 22 times from 5 pg/mg to 116 pg/mg after viral filtration step (Table 2). A previous study by Gefroh, E. and colleagues has shown that β-glucans can be introduced into the purification process through raw materials containing β-glucan or leachables from filters made from

Samples	Test 1 (pg/mg)	Test 2 (pg/mg)	Mean value (pg/mg)	Relative difference (%)
VF	116	98.5	107.3	16.3
UFDF	79.2	70.7	75.0	11.3

Table 3: Repeated β-glucan testing results for VF and UFDF process-intermediates from mAb C.

cellulose [21]. The observed increase of β-glucan concentrations after viral filtration in mAb C suggests a contamination or leak of β-glucans from the viral filtration filter used. To rule out the possibility of testing error, VF and UFDF samples from mAb C were re-tested. Less than a 20% difference in amount of β-glucans was found between the results from the two tests (Table 3). The β-glucan concentrations remained at high levels after UFDF, which confirms the lack of β-glucan removal capability at the UFDF step (Figure 3). Since low levels of beta-glucans have also been found in raw materials such as sucrose and cellulose filters used [21], the Millipore V pro virus filter used for mAb C viral filtration could be the potential source of contamination, pending confirmation from future studies.

Discussions and Conclusions

This study reports the β-glucan testing results in process-intermediates retained from both yeast-produced mAb A and CHO-produced mAb B and mAb C where yeast hydrolysate was used as cell culture additive. Protein A chromatography step removed the majority of β-glucans from all three monoclonal antibody products. However, the extent of β-glucans removal by Protein A chromatography step differs significantly between *P. p*astoris-produced product (mAb A) and CHO-produced products (mAb B and mAb C). Both our results (Table 2) and data presented from a previous study [20] show close to 4 $\log_{10}$ reduction of β-glucans by Protein A chromatography step in monoclonal antibody products produced in engineered CHO cells, where Yeastolate or Yeast Hydrolysate has been used as culture media additive. In contrast, for mAb A, Protein A purification step only reduced the amount of β-glucans by ~ 2 $\log_{10}$ (Table 1). Significant amount of β-glucans remained after Protein A purification in mAb A, which was further cleared by polishing chromatography steps. Although, the exact cause of β-glucan clearance difference observed above remains unclear, both the differences in host cells and downstream processing may have contributed to this difference. First of all, the structure and linkage differences between β-glucans leaked from yeast cell wall and those in Yeastolate may contribute to the observed β-glucan clearance differences by Protein A purification. A previous report shows that the proteins with internal repeats (PIR proteins) are covalently linked to the *β* 1,3-glucan of the yeast cell wall via alkali-sensitive ester linkage [25]. Other yeast cell wall constituents such as chitin, manno proteins also play important roles in cell wall integrity and have interactions with β-glucans [26]. Therefore, the interaction of β-glucans with other cell wall components may help them non-specifically bind to the Protein A resin and thus only free β-glucans were removed. Secondly, the downstream processing of mAb B and C includes a viral inactivation step after Protein A elution, which is not part of mAb A purification process. It is unclear but also unlikely the low pH viral inactivation step removes β-glucan. However, as indicated in the β-glucan clearance pattern of mAb A, β-glucans not removed by Protein A purification can be further removed through polishing chromatography steps. Residual β-glucan levels in the UF/DF samples of mAb A and mAb B were both at very low levels after downstream purification processes, ranging from 7.8 pg/mg to 19 pg/mg.

As previously reported [20,27], the average serum level of β-glucan in healthy adults was found to be 17 pg/mL ± 34 pg/mL. Given the average circulating blood volume of 77 mL/Kg in healthy adults, a dose of up to 207 mg/Kg (69 mg/Kg ± 138 mg/Kg) drug substance containing 19 pg/mg residual β-glucan can be injected into healthy adults to have β-glucans remained at physiological levels. Therefore, the downstream process removal of β-glucans in mAb A and mAb B is sufficient to assure the residual β-glucan levels will not affect product safety. Due to the effective removal of β-glucans by Protein A chromatography, risks related to β-glucan contamination in cell culture raw materials are not a significant concern. However, contaminants introduced during the polishing chromatography steps or leached from the filtration steps are of more concern since later steps have limited capability in β-glucan removal. The sources for these β-glucan contaminants can in general be divided into two categories: β-glucan contaminants introduced through chemicals used in the chromatography and formulation buffers, and β-glucan contaminants introduced directly into the production solution through interactions with product-contacting filters that leach β-glucan [20,21]. Additional assessment of the above mentioned β-glucan sources and hence their control will have to be performed unless the downstream processes show robust clearance of β-glucan by Protein A chromatography and no further contaminations are introduced in downstream purification steps.

Both mAb B and mAb C downstream processing follow a similar platform approach as shown in Figure 1. However, the filter used in viral filtration step of mAb C is different from what is used for mAb B. Planova 20 N filter was used for mAb B viral filtration, while Millipore VPro was used at mAb C viral filtration step. The potential introduction of β-glucan by Millipore VPro filter is currently under investigation. Additional testing on raw materials may be necessary if the filter investigation results show no leachable β-glucans.

In summary, β-glucan testing results in the process-intermediates of three mAb products provide insights on its clearance patterns during downstream processing. This study also indicates the importance of β-glucan testing and control during the process characterization of biotherapeutics.

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