UNLOCKING THE BLACK BOX

BSA MONOMER CONTENT AS A CRITICAL PRODUCT ATTRIBUTE

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INTRODUCTION

Bovine serum albumin (BSA) is a widely used reagent in the life science industry due to its array of functionalities and relatively low cost. BSA is most used to block nonspecific interactions with solid surfaces in diagnostic assays, as a nutrient transporter in cell culture applications, and as a high purity protein standard. Minor differences in BSA lots, grades, and manufacturers are not captured in the product specifications or certificates of analysis. These often-overlooked minor variances in fatty acid profile, endotoxin contamination, purity, protein monomeric abundance, etc. can dramatically affect BSA's performance in an application.

THIS WHITE PAPER WILL HIGHLIGHT MONOMER CONTENT AS A CRITICAL BSA ATTRIBUTE THAT SHOULD BE UNDERSTOOD AND CONSIDERED FOR MANY BSA APPLICATIONS. FURTHERMORE, THE IMPACT OF THE MANUFACTURING PROCESS ON BSA MONOMER CONTENT WILL BE EXPLAINED AND HOW DIFFERENT GRADES AND MANUFACTURERS OF BSA HAVE VARYING DEGREES OF MONOMER CONTENT.

BSA is a 66.4 kDa protein formed from a single 538 amino acid chain (Peters 1996). BSA exists in its monomer form in the circulatory system (Andersson 1966), binding and transporting biologicals components such as fatty acids, steroids, metals, etc. (Fasano, Curry et al. 2005). Within the life science industry preservation of the biological functionality of BSA is critical, particularly when BSA is used in binding studies or as a nutrient transporter in cell culture applications. The native functionality of BSA is largely preserved through purification but can be hindered as BSA molecules oligomerize. Two BSA molecules bound to each other is termed a dimer; however, trimers, quatramers, and larger polymers can also form. Polymer formation results in a heterogeneous product where differences in BSA's quaternary structure can affect final product functionality. Specifically, formation of BSA oligomers can create steric hinderance to binding sites (Nakano, Shimamori et al. 1982), change the hydrophobicity of the solvation layer (Atmeh 2007), lead to formation of soluble aggregates (Lin, Meyer et al. 2000) and affect attributes like molecular weight, which is crucial when BSA is used as a molecular weight standard. Furthermore, preserving BSA monomer content can be critical in cell culture applications where binding and transport of molecules and interaction with cell surface receptors could be affected if the BSA is not in its native monomeric state.

The process of oligomerization is complex but driven by formation and shuffling of disulfide bonds between cysteine residues (Rombouts, Lagrain et al. 2015). BSA contains one free cystine, Cys34, that is believed to participate in most oligomerization events. Cys34 is located in a structural domain containing fatty acid and metal binding sites, as well as a domain that interacts with transmembrane proteins such as the neonatal Fc receptor (FcRn) (Sand, Bern et al. 2014), which regulates albumin transport. Nakano et al. demonstrated the significance of maintaining the domain structure and inherent flexibility of BSA. It was observed that preserving BSA in a high monomeric state facilitates access to all the binding sites, whereas oligomerization leads to a loss in binding capacity due to allosteric and steric effects (Nakano, Shimamori et al. 1982). These results demonstrate that changes to binding capacity and binding site accessibility can clearly influence the kinetics and transport characteristics of BSA that are critical attributes of BSA for life science applications.

While high monomer content is beneficial for applications requiring BSA in its native state, there are situations where some oligomer content improves functionality. The presence of oligomers can be beneficial in applications where BSA is used to passivate solid surfaces, such as in diagnostics. Park et al. hypothesized that oligomeric BSA could form denser more irreversible coatings on hydrophilic surfaces such as silicon dioxide, and using surface plasmon resonance demonstrated a 10-fold improvement in blocking efficiency against FBS biofouling compared to monomeric BSA (Park, Jackman et al. 2018). The authors then performed western blots on nitrocellulose and showed BSA oligomers were more effective at reducing non-specific band signal when compared to blocking with BSA monomers.

BSA monomer content has been shown it can influence how a BSA product performs. For some applications high monomer content is beneficial, while for others some oligomer content is preferred. Thus, when using BSA in any application it is important to understand if monomer content is a critical product variable. This white paper will describe in detail a HPLC method for quantifying monomer and oligomer content of BSA. This method will then be used to analyze how monomer content varies between grades and manufacturers of BSA and how those variations are likely caused by differences in manufacturing processes.

MATERIALS & METHODS

MATERIALS - Standard Grade (Fraction V, pH 7.0) and Reagent Grade (Fatty Acid-Free) analogous grades of BSA were obtained from various manufacturers. Certified ACS grades of sodium sulfate anhydrous, sodium phosphate monobasic anhydrous, and sodium phosphate dibasic anhydrous were purchased from Fisher Scientific (Fair Lane, NJ), and dissolved in reverse osmosis water.

METHODS - A robust HPLC method to analyze the monomeric content of BSA was developed using size exclusion chromatography. A Thermo Fisher Scientific BioBasic™ SEC 300 Size Exclusion Chromatography HPLC Column (Part Number: 73505-307846) with corresponding guard column (Part Number: 73505-037821) was used for chromatographic separation. The BioBasic™ column is 300mm long and packed with 5µm silica beads having a 300Å pore size. The 300mm long Phenomenex Yarra™ SEC-3000 column (Part Number: 00H-4513-K0) packed with 3µm silica beads having a 290Å pore size gave analogous results.

Lyophilized BSA was prepared for HPLC by dissolving samples 1% (w/w) in the mobile phase, a 0.1M sodium sulfate solution buffered at pH 7.0 with 0.1M sodium phosphate buffer. To eliminate potential storage effects the Proliant BSA products were analyzed immediately following production. The dissolved BSA samples were filtered through 0.45 µm PVDF syringe filters prior to a 10uL injection onto the column. Mobile phase was passed over the column at a rate of 0.5 mL/min for 32 minutes, yielding integrable oligomer, dimer, and monomer peaks at approximately 16.1, 17.2, and 18.7 minutes, respectively. A summary of the HPLC method can be found in Table 1. Example HPLC chromatograms with oligomer, dimer, and monomer peaks labeled are shown in Figure 1.

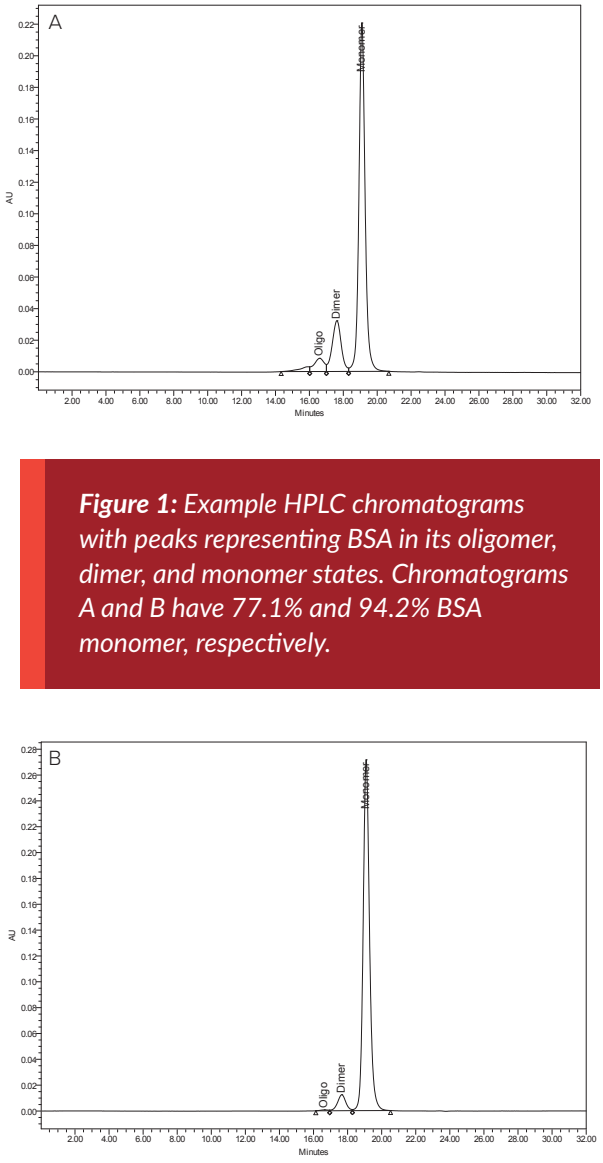

***Figure 1:** Example HPLC chromatograms with peaks representing BSA in its oligomer, dimer, and monomer states. Chromatograms A and B have 77.1% and 94.2% BSA monomer, respectively.*

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Table 1: *Overview of the HPLC method for the determination of BSA monomeric content.*

HPLC Monomer Quantification Method Parameters	
Column	Thermo BioBasic™ SEC 300 Column (300A 5µm 300 x 7.8mm) with guard column
Temperature	Ambient (or 25°C)
Mobile Phase	0.1M sodium sulfate with 0.1M sodium phosphate buffer pH 7.0
Sample Preparation	1% (w/w) protein in mobile phase
Injection volume	10.0 µL
Flow rate	0.5 mL/min
Run time	32 min
Detection	280nm

RESULTS & DISCUSSION

The industry-wide variability in BSA offerings through manufacturers presents challenges when identifying critical product specifications for various applications. The two primary grades of BSA are a "Standard" or "Diagnostic" Grade versus a "Reagent" or "Fatty Acid-Free" BSA. The primary difference between these BSA grades is the presence of residual endogenous fatty acids as well as residual fatty acids used in processing to aide in BSA purification. BSA is a primary transporter of endogenous fatty acids in the body. It has also been shown that BSA stability can be increased through binding fatty acids, which is a characteristic typically exploited to aide in the purification of BSA.

Lyophilized Standard Grade BSA products have a monomer content ranging from 70-82%, whereas lyophilized Reagent Grade BSA products typically yield 2-7% lower, dependent on manufacturer (Tables 2 and 3). The presence of fatty acids in Standard Grade will contribute to the stability of BSA, including throughout the lyophilization and storage process. However, it is also important to see that within a grade there can be large differences in monomer content. It is important to know if monomer content is a critical BSA attribute for an application and the sensitivity of the application to changing monomer content.

Proliant's Precision Grade, Ultra High Monomer Standard 30% Solution and Ultra High Monomer Reagent 30% Solution products have significantly higher monomer content than comparable products of the same grade. The higher monomer content in the liquid products demonstrate the effects lyophilization can have on monomer content. These products could function well in applications where native structures are important such as cell culture or vaccine applications or in aggregate sensitive applications. Whereas testing products such as Proliant's Reagent or Standard Grades with typical monomer contents in the mid-70% may be better for blocking applications.

Table 2: *Standard Grade – Fraction V BSA pH 7.0*

Manufacturer	Monomer%	Standard Deviation	Lots Tested
Proliant Ultra High Monomer Standard Grade 30% Solution	98.0%	0.3%	6
Proliant Standard Grade	82.3%	1.4%	6
Manufacturer A	77.7%	2.3%	4
Manufacturer B	77.2%	4.8%	6
Manufacturer C	70.4%	0.1%	2

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Table 3: *Reagent Grade – Fatty Acid-Free BSA*

Manufacturer	Monomer%	Standard Deviation	Lots Tested
Proliant Ultra High Monomer Precision Grade 30% Solution	92.3%	1.4%	6
Proliant Precision Grade	91.2%	1.0%	16
Proliant Reagent Grade	77.5%	2.0%	6
Manufacturer A	75.4%	2.8%	3
Manufacturer B	73.8%	2.5%	3
Manufacturer C	69.6%	0.1%	2

The variation in monomer content of the products can be traced back to processing differences while the BSA is in solution and during lyophilization. The freeze-drying process can significantly increase the occurrence of oligomerization depending on the lyophilization temperature. Products dried at reduced temperatures have a higher monomer content than products dried at elevated temperatures. Proliant's high monomer Precision Grade powder is subject to gentle lyophilization temperatures compared to the Standard and Reagent Grade powders. Products such as Proliant's Ultra High Monomer solutions have never been lyophilized and maintain the highest monomer content. While lyophilization temperature is the primary driver of oligomerization for the Proliant powder products, it is worth noting other processing conditions can cause oligomerization while the BSA is in solution such as organic solvents, pH extremes, high protein concentrations, and heat (Peters 1996). Stabilizing molecules may be used to minimize the formation of oligomers during processing, but the alteration of the BSA or the incomplete removal of these chemicals can affect the ultimate performance of the BSA.

CONCLUSION

Bovine serum albumin is an important component in numerous applications such as cell culture, vaccine production, in-vitro diagnostics, surface passivation, and as a protein standard. Differences in BSA grades, manufacturers, and even lots are often overlooked, and can cause performance issues dependent on the application. One of those unnoticed differences may be the percentage of BSA monomer in a final product as it varies between manufacturers and grades. The monomer content of BSA has been shown to be affected by the presence of stabilizing molecules, such as fatty acids, and processing conditions with lyophilization temperature being the primary driver of oligomerization. For protein standards, cell culture, vaccine production, and binding and transport applications it may be best to maintain the BSA in a natural monomeric state by choosing a high monomer BSA, such as Proliant's Precision Grade or Ultra High Monomer Solution products. Whereas for diagnostic applications and surface passivation a BSA with some oligomer content, such as Proliant's Standard Grade and Reagent Grade products, may be required and perform better than BSA in its natural state. Having the ability to analyze and determine monomer content adds another piece of information in determining which BSA product might best fit a certain application or in helping to troubleshoot inconsistent results.

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