Monitoring Protein Unfolding and Refolding

TSKgel
APPLICATION NOTE

Introduction

Proteins exist in a highly ordered, folded state. This highly ordered structure of a protein is integral to the efficacy and safety of a protein-based biotherapeutic. Protein unfolding and refolding are an indicator of protein stability. As a protein begins to unfold, the hydrodynamic radii of the protein species change.

Denaturation of proteins may range from slight and reversible conformational changes to a drastic loss of solubility, leading to irreversible aggregation. Monitoring the stability of the different protein conformations is important in protein purification. A variety of denaturants are used during different stages of purification. Denaturation of proteins through the use of concentrated guanidine hydrochloride (Gdn HCl) is one of the primary techniques in evaluating protein structure, folded vs. unfolded or partially folded structures.

A change in the conformation of a protein can be detected by the change in the intrinsic fluorescence of the tryptophan-containing proteins. Here we report the use of a 3 µm particle size, 30 nm pore size, TSKgel® UltraSW Aggregate size exclusion chromatography (SEC) column for monitoring folded (native) and unfolded (denatured) states of a monoclonal antibody using fluorescence detection (FLD).

Materials and Methods

Columns:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Instrument:	Agilent 1200 with FLD
Mobile phase:	100 mmol/L sodium phosphate/100 mmol/L sodium sulfate, pH 6.7 + NaN_3
Flow rate:	1.0 mL/min
Detection:	FLD (λ_{ex}: 280 nm, λ_{em}: 350 nm)
Temperature:	30 ºC
Injection vol.:	15 µL
Sample:	TBL mAb 01 (4 mg/mL) TBL mAb 01 is an IgG_1 based monoclonal antibody with an approximate molecular weight of 150 kDa. The number of intrinsic fluorophores is unknown as the amino acid sequence of this compound is proprietary information.

Buffer salts and reagents purchased from Sigma-Aldrich.

Results and Discussion

Figure 1 demonstrates the separation of a monoclonal antibody (mAb) under different concentrations of guanidine hydrochloride, a common additive used to analyze proteins by SEC. This additive promotes denaturation by breaking non-covalent bonds that are part of the higher order structure of proteins. Guanidine hydrochloride disrupts the protein structure to a randomly coiled (larger) structure.

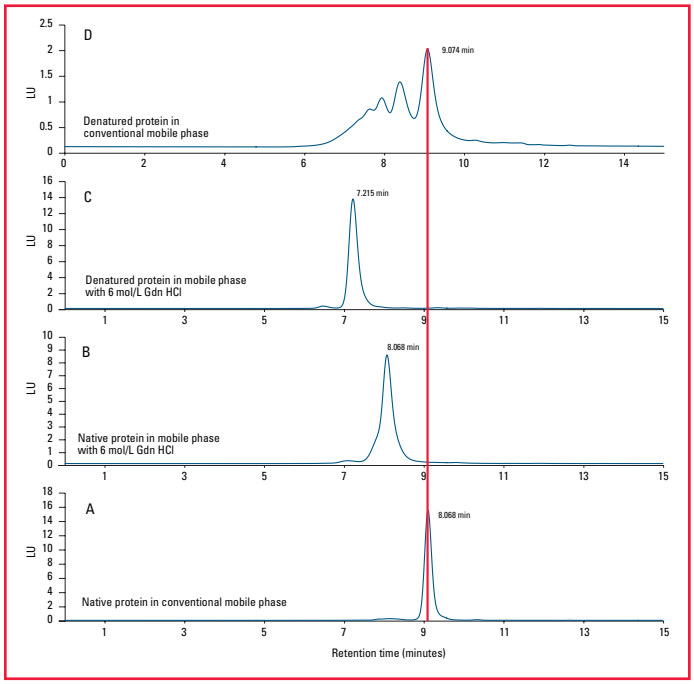
Panel A shows the separation of native monoclonal antibody TBL mAb-01 equilibrated with mobile phase that does not contain Gdn HCl. Panel B shows the separation of the same native mAb equilibrated with Gdn HCl. As seen in *Figure 1*, the peak eluted earlier with an increase in intensity.

Panels C and D represent the separation of the mAb denatured with DTT. Panel C shows further reduction in retention time when the denatured mAb is separated when equilibrated with Gdn HCl. Panel D demonstrates the separation of the denatured mAb when equilibrated with mobile phase that does not contain Gdn HCl. Since the sample volume is small compared to the column volume, the protein gets a chance to refold while traveling through the column, and as a result, the denaturant is separated in the total inclusion volume of the column.

The possible explanation why the monomer elutes earlier in panels B and C than in panels A and D is due to the partial unfolding of the mAb, resulting in a larger hydrodynamic radii. One would expect the retention time to show a more dramatic shift towards earlier elution with the use of DTT due to the full reduction of internal disulfide bonds. This would lead to an even greater increase in the hydrodynamic radii of the protein.

The separation of the fully denatured protein in the mobile phase not containing Gdn HCl (as seen in panel D) results in greater protein aggregation. The formation of aggregates during refolding of a protein, except in very dilute conditions, are known to occur and the extent of aggregation depends on the nature of the protein and other chromatographic conditions.

Figure 1. Separation of Monoclonal Antibody under Different Concentrations of Guanidine Hydrochloride



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Conclusions

The use of SEC-FLD in the evaluation of protein stability through denaturation is a simple and effective technique which allows for real-time monitoring of protein unfolding. Combined with columns having a high exclusion limit, such as the TSKgel UltraSW Aggregate, very large proteins and their aggregates can be accurately analyzed for protein stability.

The use of guanidine hydrochloride is an effective denaturant for the evaluation of protein structure, folded vs. unfolded or partially folded structures. SEC is an inert technique and doesn't shift the equilibrium between the native, denatured, and partially folded states of protein. This study demonstrates the effectiveness of the TSKgel UltraSW Aggregate, 3 µm SEC column for monitoring protein unfolding and refolding in protein stability studies.

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TOSOH BIOSCIENCE LLC
3604 Horizon Drive, Suite 100
King of Prussia, PA 19406
Tel: 800-366-4875
email: info.tbl@tosoh.com
www.tosohbioscience.com

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