



Use of 3 μm Analytical Size Exclusion Chromatography (SEC) Column for Monitoring Protein Unfolding and Refolding in Stability Studies of Proteins and Monoclonal Antibodies

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Introduction

- Denaturation of proteins may range from slight and reversible conformational changes to a drastic loss of solubility, leading to irreversible aggregation. Monitoring stability of the different protein conformations is important in protein purification.
- A variety of denaturants are used during different stages of purification.
- The use of additives in size exclusion chromatography (SEC) for protein analysis are considered in cases where protein solubility is limited, where non-specific interaction is suspected between the sample and column matrix, and where the addition of another buffer or water-soluble organic solvent is not effective.
- Common additives used to analyze proteins by SEC include guanidine hydrochloride, sodium dodecyl sulfate (SDS), and urea.
- These additives promote denaturation by breaking non-covalent bonds that are part of the higher order structure of proteins. Different additives work with different mechanisms of action.
- Guanidine hydrochloride and urea disrupt the protein structure to a randomly coiled (larger) structure.
- 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.



Introduction: Protein Folding and Unfolding

- Protein unfolding and refolding are indicators of protein stability.
- 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.
- Solvent-induced unfolding may result in conformational changes, even if the protein is not completely denatured.
- As a protein begins to unfold, the hydrodynamic radii of the protein species change.
- Different proteins are susceptible to solvent-induced unfolding to a different extent.
- It is possible to identify a change in the conformation of a protein due to solvent-induced unfolding by the associated volume changes that occur in an SEC analysis. Conformational changes can be detected by the change in the intrinsic fluorescence of the tryptophan-containing proteins.
- Size exclusion chromatography is useful for buffer exchange, aggregate removal and to perform a folding reaction.
- As expected, it is easier to monitor the volume changes associated with the unfolding of a protein with a larger molecular exclusion limit column, provided the conformation of the unfolded state is stable under the chromatographic conditions of the analysis.



Introduction: Protein Folding and Unfolding

- Diafiltration and dialysis using ultra-filtration membranes have been used to reduce high denaturant concentrations for refolding of proteins.
- An alternative methodology of refolding is the use of gel filtration chromatography (GFC).
- One of the major causes of low refolding yields is aggregate formation due to too high of protein concentrations during the refolding process.
- GFC restricts the available pore volume for various protein forms in the gel matrix, thus, facilitating the separation of correctly folded and aggregated species¹.
- Intrinsic tryptophan fluorescence of the proteins and antibodies are generally used for detection using a fluorescence detector.
- A TSKgel[®] G3000SW SEC column has been used for the precise measurement of the volume change associated with solvent-induced protein denaturation and for monitoring the folded and unfolded state of the proteins².
- Here we report the use of a 3 μm particle size, 30 nm pore size, TSKgel UltraSW Aggregate SEC column for monitoring folded (native) and unfolded (denatured) states of 3 different proteins using fluorescence detection (FLD).

¹M. Li et al. / Protein Expression and Purification 33 (2004) 1–10

²Roche RS, Biochemistry 1984, 23(8),1888-94

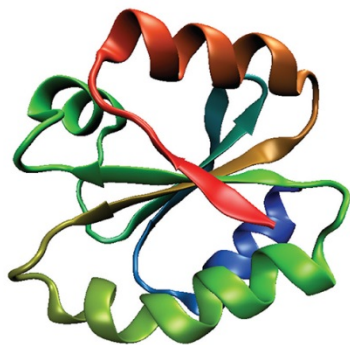


Materials and Methods

- Column: TSKgel UltraSW Aggregate, 3 μm , 7.8 mm ID \times 30 cm
- Instrument: Agilent 1200 with FLD
- Mobile phase: 100 mmol/L sodium phosphate/100 mmol/L sodium sulfate, pH 6.7 + NaN_3 (unless otherwise noted)
- Flow rate: 1 mL/min
- Detection: FLD (λ_{ex} : 280 nm, λ_{em} : 350 nm)
- Temperature: 30 $^{\circ}\text{C}$
- Injection vol.: 15 μL
- Samples: thioredoxin (*E. coli*, 1 mg/mL)
IgM (human, 1 mg/mL)
TBL mAb 01 (4 mg/mL)

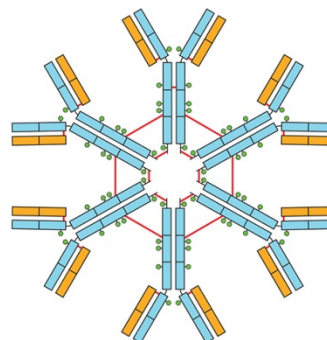
All samples, buffer salts, and reagents purchased from Sigma-Aldrich.

Figure 1: Proteins Used in this Study



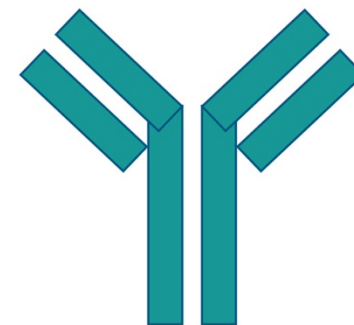
Thioredoxin

- 12 kDa protein found in most organisms
- typically facilitates protein reduction through cysteine thiol-disulfide exchange
- the *E. coli* variant contains two tryptophan residues - Trp 28, Trp 31



Immunoglobulin M (IgM)

- 970 kDa antibody
- typically composed of 5 immunoglobins covalently bonded by disulfide bonds
- each heavy-light chain fragment of IgM contains 14 tryptophan residues



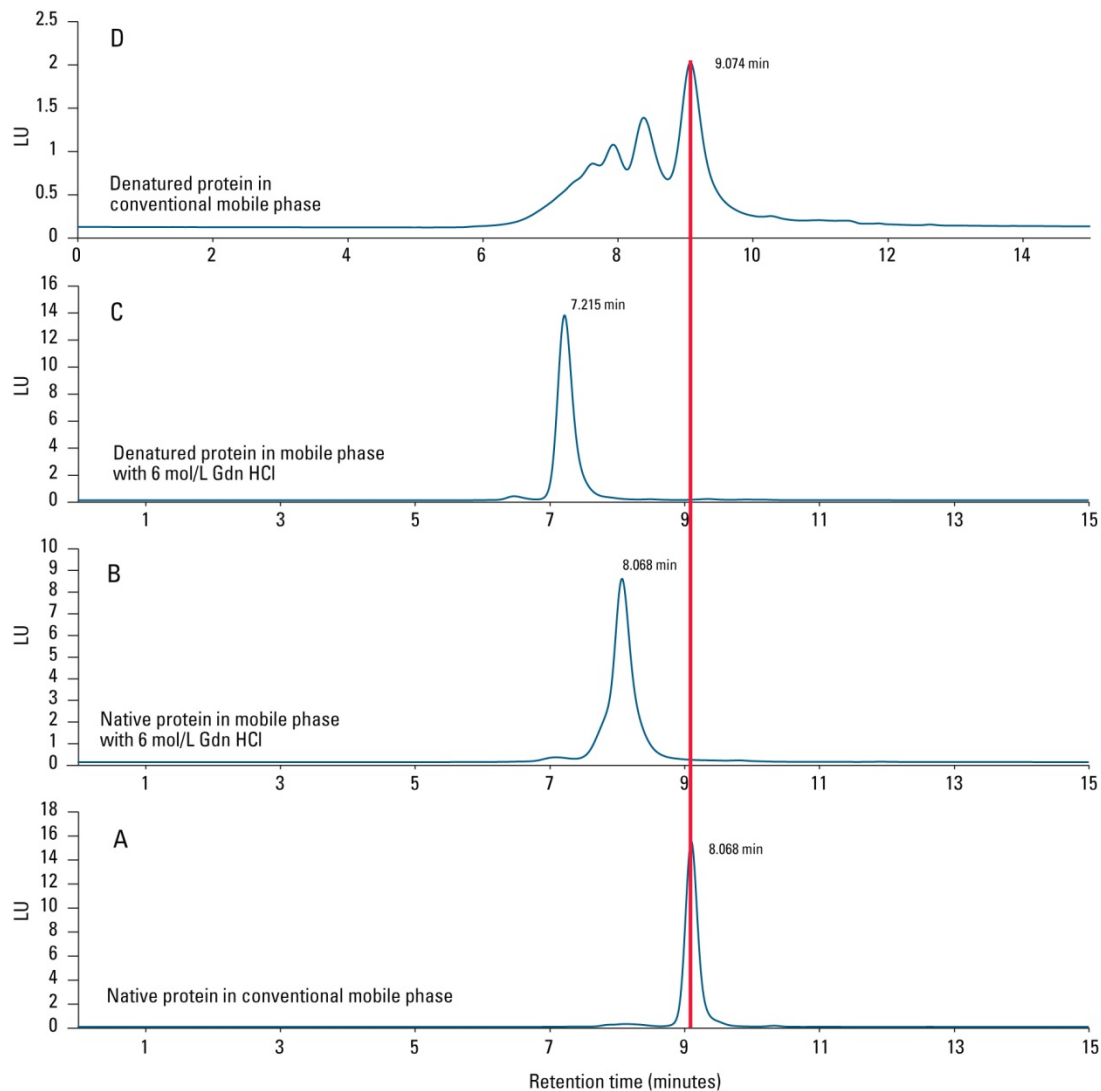
IgG

- TBL mAb 01 is an IgG₁ based monoclonal antibody
- MW: 150 kDa (approx.)
- # of intrinsic fluorophores – unknown as the amino acid sequence of this compound is proprietary information

The effect of denaturants, such as guanidine hydrochloride, is different depending on the types and nature of the protein as well as the availability of water surrounding the protein structure. We have used the above 3 different proteins to determine if a 3 um SEC column can be used to monitor changes in retention volume.



Figure 2: Separation of Monoclonal Antibody under Different Concentrations of Guanidine Hydrochloride





Separation of Monoclonal Antibody under Different Concentrations of Guanidine Hydrochloride: Conclusions

- Panel A shows the separation of native monoclonal antibody mAb-01 through the column equilibrated with mobile phase that does not contain Gdn HCl.
- Panel B shows the same native mAb separated through the column equilibrated with Gdn HCl. The peak eluted earlier with an increase in intensity.
- Panel C shows further reduction in retention time when the denatured mAb is separated through the column equilibrated with Gdn HCl.
- Panel D shows the separation of denatured protein through the column 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 the denaturant is separated in the total inclusion volume of the column.
- Retention time of the monomer in panel A and panel D are the same.
- Panel D shows the presence of a number of aggregate species which were not seen in panels B and C.
- The possible explanation why the monomer in panels B and C elutes earlier 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.



Separation of Monoclonal Antibody under Different Concentrations of Guanidine Hydrochloride: Conclusions

- 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.
- The effects of additives such as guanidine hydrochloride on the refolding behavior of a specific protein is dependent on the subtle changes in the structure of water around the protein.
- Pressure was closely monitored due to the increased viscosity of the 6 mol/L Gdn HCl-enriched mobile phase, but was found to only contribute approximately 0.3 MPa to the conventional mobile phase operating conditions.
- The following figures show the separation of thioredoxin and IgM under the same conditions as the mAb analysis using this TSKgel UltraSW Aggregate, 3 μ m SEC column.



Figure 3: Separation of Thioredoxin under Different Concentrations of Guanidine Hydrochloride

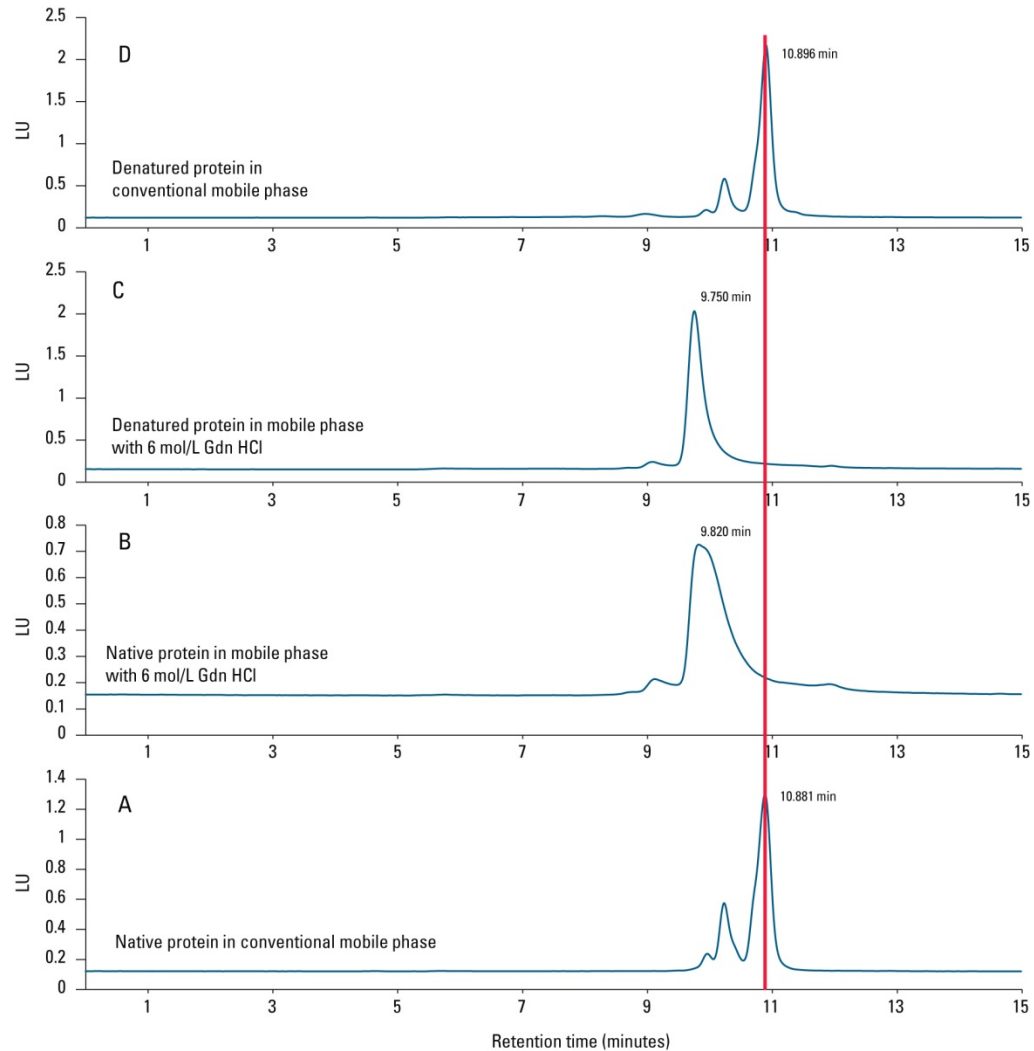




Figure 4: Separation of IgM under Different Concentrations of Guanidine Hydrochloride

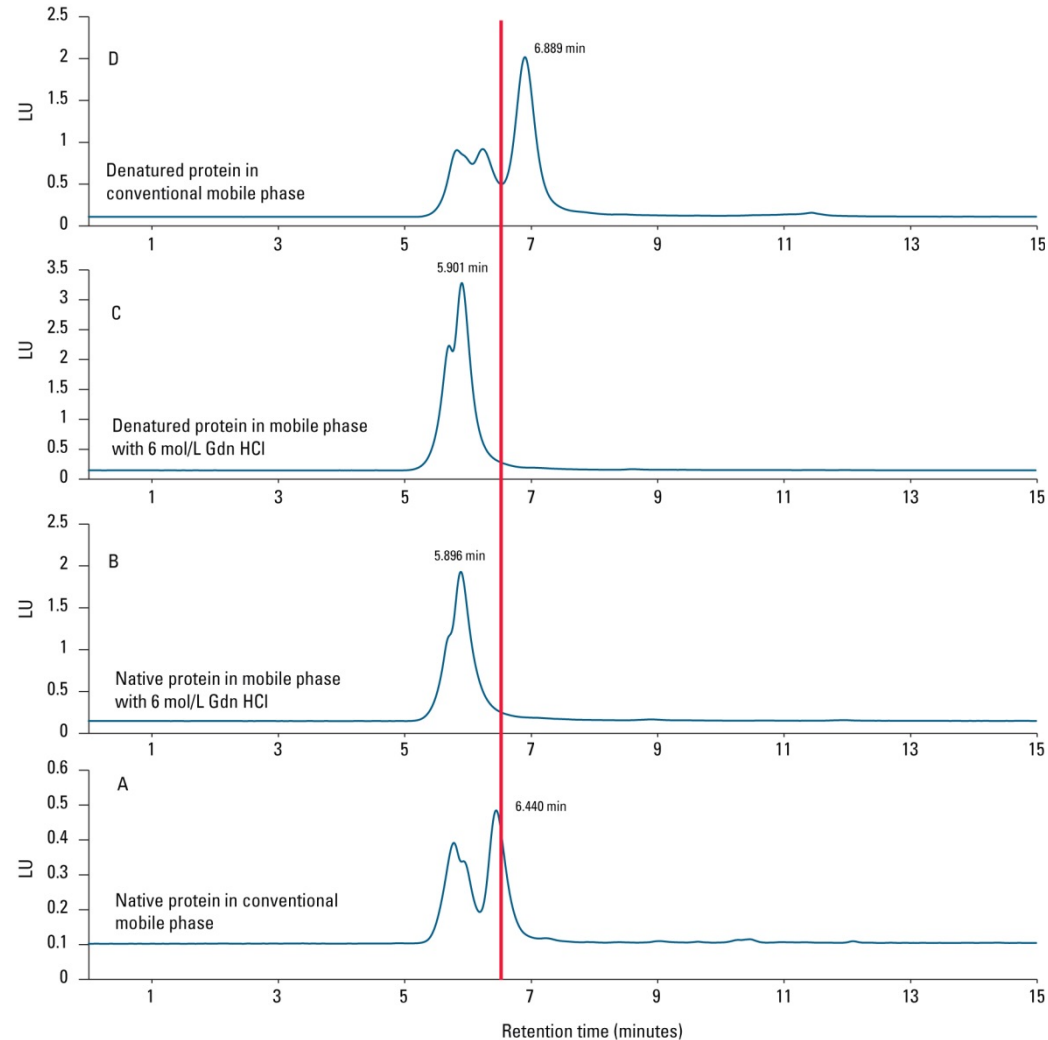
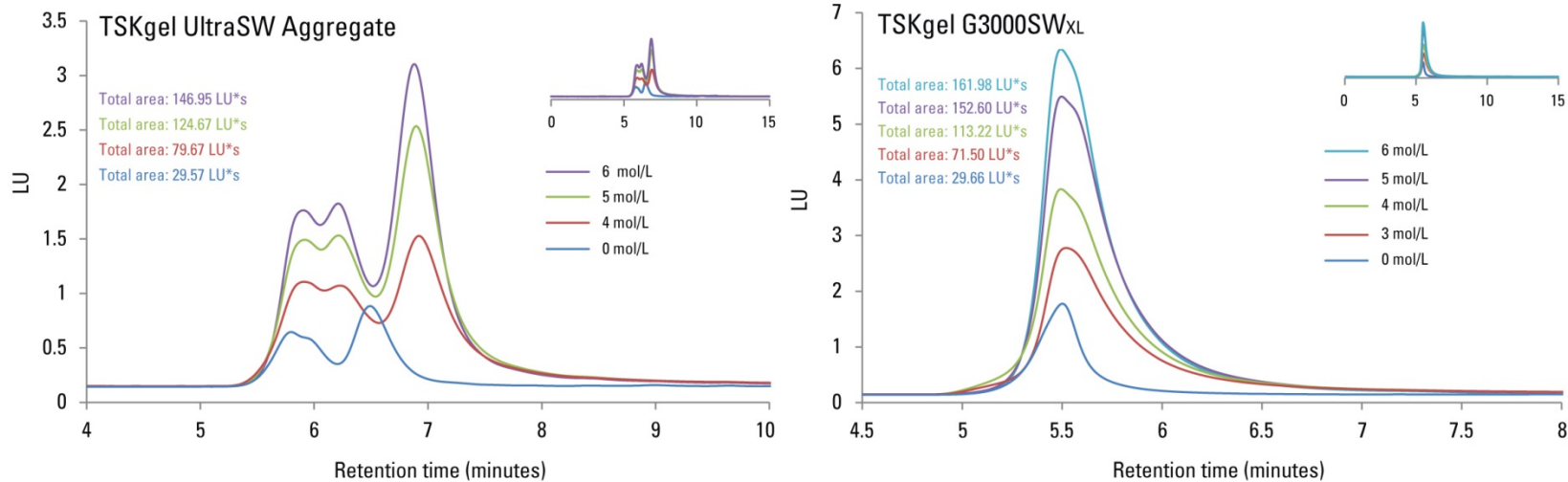


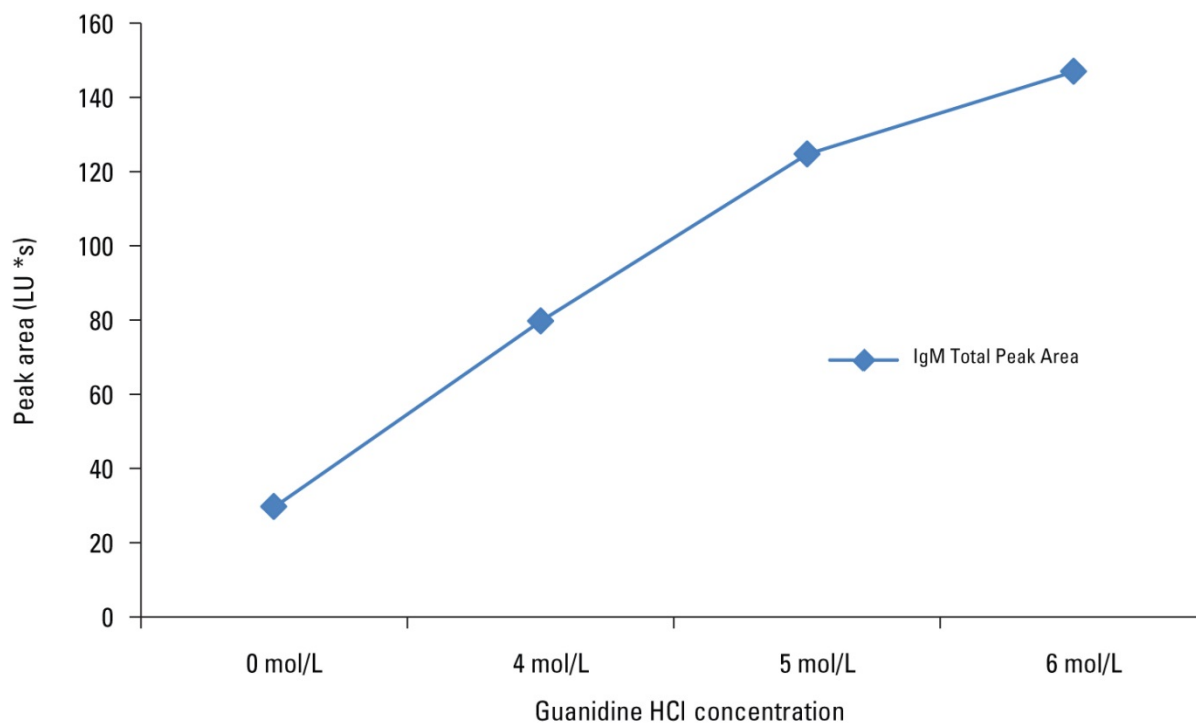
Figure 5: Separation of Denatured IgM Variants



- 970 kDa native IgM (blue) was similarly evaluated against denatured IgM (4-6 mol/L Gdn HCl) using normal mobile phase without guanidine hydrochloride.
- A significant increase in peak areas, as well as increased fluorescent intensity of the heterogenic species, is observed with increasing Gdn HCl concentration.
- Similar analysis performed under identical chromatographic conditions using the TSKgel G3000SWXL column did not yield the separation of the heterogenic species which could be seen with the TSKgel UltraSW Aggregate column.
- The TSKgel G3000SWXL column has an exclusion limit of 500 kDa whereas; the TSKgel UltraSW Aggregate column has an exclusion limit of 2,500 kDa.
- The higher exclusion limit of the TSKgel UltraSW Aggregate column separated the native IgM and its aggregate variants.



Figure 6: Denaturation of IgM using Gdn HCl



The IgM peak area is increased linearly up to 6 mol/L Gdn HCl as a function of increased exposure of the tryptophan residues.



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, but the unfolding kinetics of various proteins can differ significantly.
- 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.