Characterization Studies of PEGylated Lysozyme using TSKgel HPLC Columns

TSKgel
APPLICATION NOTE

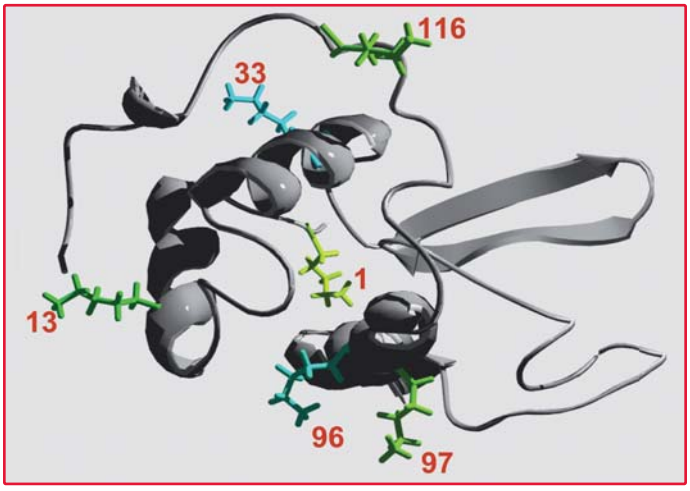
Jessica Christel, Werner Conze, Achim Sprauer, Volker Noedinger and Egbert Mueller, Tosoh Bioscience GmbH

Introduction

Chemical modification of therapeutic proteins in order to enhance their biological activity is of increasing interest. One of the most frequently used protein modification methods is the covalent attachment of poly (ethylene glycol), which is referred to as PEGylation. This polymeric modification changes the biochemical and physicochemical properties of the protein, which can result in several important benefits, among them more effective target delivery, slower in vivo clearance, and reduced toxicity and immunogenicity of therapeutic proteins. After PEGylation reaction the mixture has to be purified in order to remove non-reacted protein and undesired reaction products. Liquid chromatography is the most common purification method of PEGylation reaction products. Since the extent of PEGylation influences masking and shielding effects of the covalently linked PEG molecule, there is increasing demand for chromatographic methods to separate the modified isoforms from the native protein. This application note describes the use of size exclusion and ion exchange chromatography for the characterization of PEGylated lysozyme.

Lysozyme is a well known standard protein and is often used to determine the dynamic binding capacity of Ion Exchange Chromatography (IEC) resins; therefore we decided to use PEG-lysozyme as a model protein in our study. PEGylated lysozyme was synthesized from methoxy PEG aldehyde (with a MW of 5kDa, 10kDa and 30kDa) and chicken egg white lysozyme in phosphate buffer in the presence of sodium cyanoborohydride ($NaCNBH_3$) as a reducing agent. The PEGylation reaction takes place between the aldehyde group of methoxy PEG aldehyde and the free amino acid group (NH_2-group) of lysine residues within the lysozyme molecule (*see Figure 1*).

Figure 1. Lysozyme has six lysine residues as possible PEGylation reaction sites



The product mixture was analyzed with a TSKgel G3000SW_{XL} size exclusion column, SDS-PAGE (not shown), a TSKgel SP-5PW strong cation exchange column, a TSKgel SP-NPR strong cation exchange column and subsequent MALDI-TOF MS analysis (not shown).

Materials and Methods

PEGylation of egg white lysozyme:
5, 10, 30kDa methoxy PEG aldehyde; 100mmol/L phosphate buffer (Na_2HPO_4, NaH_2PO_4) pH 6.0;
PEGylation by reductive alkylation; 20mmol/L $NaCNBH_3$ to reduce a Schiff base; 100mmol/L HCl to stop PEGylation reaction

Size Exclusion:
Column: TSKgel G3000SW_{XL}, 5µm, 7.8mm ID x 30cm
Mobile phase: 0.1mol/L phosphate buffer, 0.1mol/L Na_2SO_4, pH 6.7
Flow rate: 1.0mL/min
Detection: UV@280nm
Injection vol.: 20µL

Ion Exchange (low pressure):
Column: TSKgel SP-5PW, 20µm, 6.6mm ID x 22cm
Buffer A: 25mmol/L phosphate buffer, 0.1mol/L Na_2SO_4, pH 6.0
Buffer B: A + 0.5mol/L NaCl
Flow rate: 0.85mL/min
Detection: UV@280nm
Injection vol.: 100µL

Ion Exchange:
Column: TSKgel SP-NPR, 2.5µm, 4.6mm ID x 3.5cm
Buffer A: 25mmol/L phosphate buffer, 0.1mol/L Na_2SO_4, pH 6.0
Buffer B: A + 0.5mol/L NaCl
Flow rate: 1.0mL/min
Detection: UV@280nm
Injection vol.: 5µL

Results

PEGylation of lysozyme
Figure 2 shows a typical chromatogram of a reaction mixture of PEGylated lysozyme separated on a TSKgel SP-5PW column. PEG chain lengths of 5kDa and 30kDa are shown. The profiles indicate a similar reaction characteristic. Non-reacted lysozyme remained in the reaction mixture; mono-PEGylated lysozyme as well as poly-PEGylated lysozyme was formed during the reaction.

Size exclusion chromatography was performed as shown in *Figure 3*. The retention volumes of PEGylated lysozymes were used to assign the peaks in *Figure 3* based on a standard calibration curve.

Figure 2. Separation of PEGylated lysozymes on an analytical TSKgel SP-5PW column: mPEG5-aldehydes (A) and mPEG30-aldehydes (B). Peaks were identified by MALDI-TOF analysis. Identical sizes were numbered consecutively.

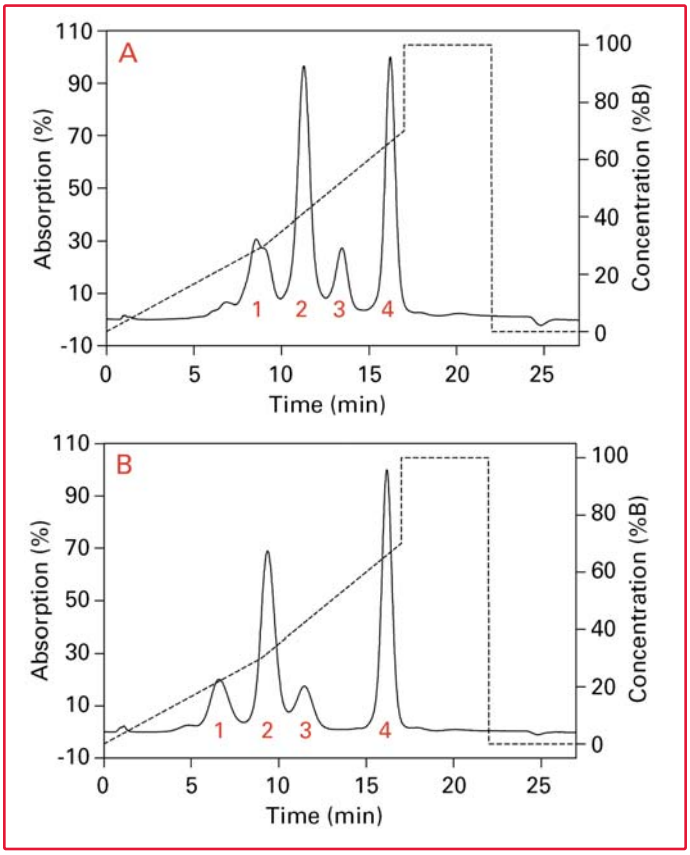
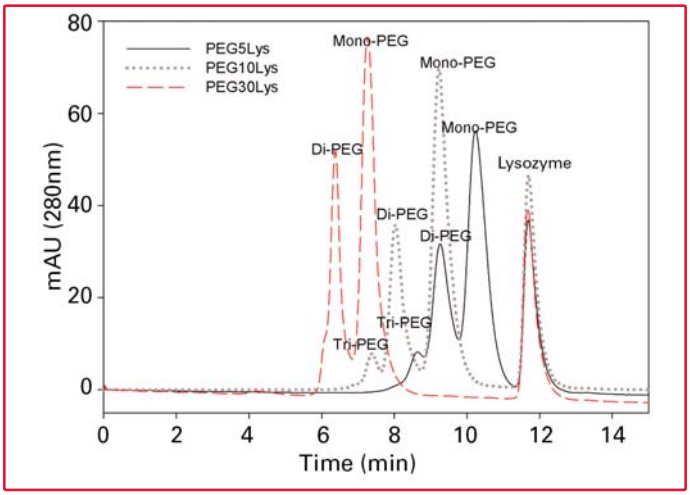


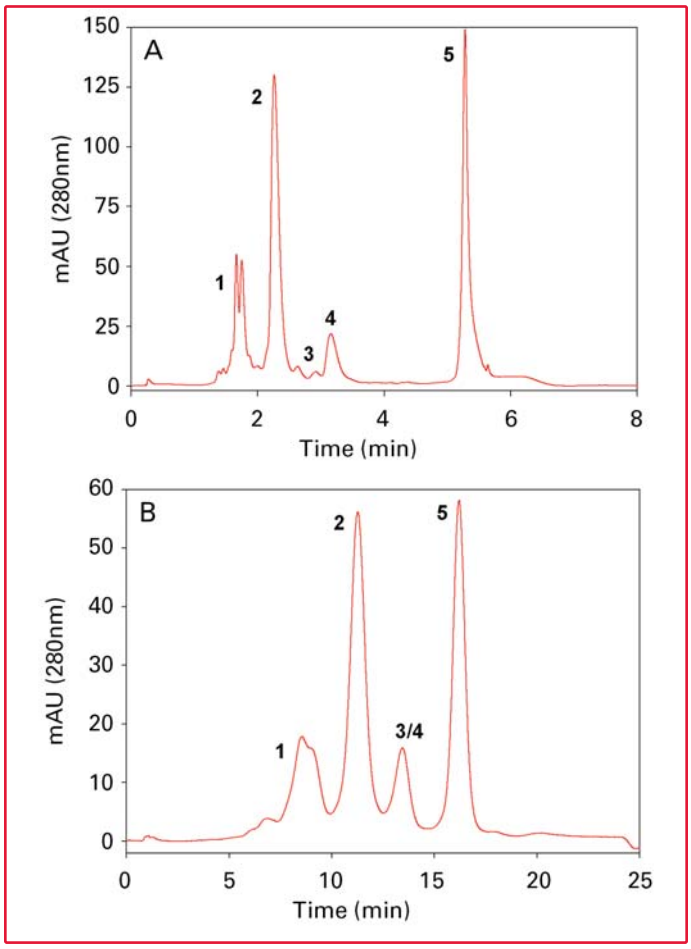
Figure 3. SEC analysis of reaction mixtures performed with a TSKgel G3000SW$_{XL}$ column. Lysozyme and PEGylated lysozyme derivates are shown.



Selectivity

As expected, particle size greatly influenced resolution, while differences in particle and ligand chemistry contributed to selectivity differences. The non-porous particle resin of the prepacked TSKgel SP-NPR column in particular showed very high resolution; with a number of mono-PEGylated isoforms resolved while two isoforms were visible for di-PEGylated lysozyme. TSKgel SP-5PW (20µm) is a polishing resin with a particle size that is almost ten times larger than the TSKgel SP-NPR matrix and also a much higher binding capacity as the TSKgel SP-5PW particles are fully porous. As shown in *Figure 4*, although the resolution on the TSKgel SP-5PW column is lower, two mono-PEGylated isoforms remained visible.

Figure 4. Resolution dependency on particle size shown with 5kDa PEGylated lysozyme reaction mixture. (A) TSKgel SP-NPR, (B) TSKgel SP-5PW; (1) poly-PEG5Lys, (2) 1-mono-PEG5Lys, (3) 2-mono-PEG5Lys, (4) 3-mono-PEG5Lys and (5) lysozyme



Discussion

Lysozyme, as the model protein, was PEGylated to examine the behavior of PEGylated proteins in cation exchange chromatography. A random PEGylation of lysozyme using methoxy PEG aldehyde of sizes 5kDa, 10kDa and 30kDa was performed.

As a result of PEGylation, we observed a large increase in the size of lysozyme by size exclusion chromatography. The SEC elution position of lysozyme modified with a 30kDa PEG was equivalent to that of a 450kDa globular protein. There was a linear correlation between the theoretical MW of PEGylated protein and the MW calculated from SEC. This result illustrates the strong effect that PEG has on the hydrodynamic radius of the resulting PEGylated protein.

Selectivity comparison
Cation exchange chromatography was capable of resolving the PEGylated isomers which are products of the random PEGylation. Best resolution was obtained with a non-porous TSKgel SP-NPR column. This is due to the faster mass transfer kinetics for large molecules on small, non-porous particles. Despite lower resolution, a porous resin with larger particle size for the first chromatographic step was useful because of higher capacity and better pressure-flow characteristics.

Conclusion

The selectivity of various cation exchange resins were evaluated with random PEGylated lysozyme (chicken egg white). It is shown that the selectivity for PEG-modified proteins depends on particle size of the resin. All PEGylated lysozyme species could be resolved on a TSKgel SP-NPR column with a particle size of 2.5µm and on a TSKgel SP-5PW column packed with 20µm particles.

Acknowledgement

We thank our colleagues from Institute of Bioprocess Engineering, Friedrich-Alexander University Erlangen-Nuernberg, for carrying out the MALDI-TOF analysis. This work was supported by the Federal Ministry of Education and Research (BMBF), Germany.

Reference

A. Moosmann et al.; J. Chromatogr. A (2010) 1217 (2): 209-215

TOSOH

TOSOH BIOSCIENCE

TOSOH Bioscience LLC
3604 Horizon Drive, Suite 100
King of Prussia, PA 19406
Orders & Service: (800) 366-4875
Fax: (610) 272-3028
www.separations.us.tosohbioscience.com
email: info.tbl@tosoh.com