Purification and Characterization of lysozyme modified with Poly Ethylene Glycol (PEG)

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Introduction

PEGylation is a strategy that has been used to improve the biochemical properties of proteins and their physical and thermal stabilities. In this study, hen egg white lysozyme (HEWL) was PEGylated using N-terminal specific mPEG propionaldehyde (PEG aldehyde, 20KDa) in presence of cyanoborohydrate. The PEGylation reactions were carried out at 1:7 molar ratio of lysozyme to PEG at 4°C for 16 hours. A method to purify mono PEGylated lysozyme was developed using Agilent 1260 Infinity BioLC and Agilent ZORBAX Semi-preparative 300 SB-C18, 9.4 x 250mm, 5 µ column using fraction collection. The homogeneity of purified PEG lysozyme was determined using Agilent 2100 Bioanalyzer using high sensitivity Protein 250 Kit and Size Exclusion Chromatography (SEC) on Bio SEC-3, 300Å, 7.8 x 300 mm, 3 µm column. The biological activity of lysozyme and its conjugate were determined by turbidometric method using *Micrococcus lysodecticus* cells as substrate. The results of our analysis indicated that lysozyme was mono PEGylated in a site specific manner. However, there was a significant decrease in the biological activity of PEG lysozyme compared to that of unconjugated lysozyme; it is more than offset by other advantages of PEGylation such as increased half-life, decreased immunogenicity and decreased antibody binding, which we hope will improve the efficacy of intravenously administered therapeutic protein for the treatment of systemic infections.

Experimental

Lysozyme PEGylation

A 10mg/ml solution of lysozyme was prepared in 50mM Sodium Phosphate buffer, pH 7.0 and stoichiometric amounts of lysozyme: mPEG-propionaldehyde was added at 1:7 (w/w) ratios. The mixtures were incubated at 4°C for overnight containing 20mM sodium cyanoborohydrate.

Purification of PEG Lysozyme

Agilent 1260 Bio-inert Infinity Quaternary LC System and Agilent ZORBAX Semi-preparative 300 SB-C18, 9.4 x 250mm packed with 5µm particles were used. Parameters used were Mobile phase A (Water + 0.1 % TFA), Mobile phase B (Acetonitrile + 0.09 % TFA); Flow rate, 3mL/min; injection volume, 50µL; Gradient (0 to 100% B in 12 minutes); Data acquisition,214 and 280nm; sample thermostat 5°C, column oven 50°C; Fraction Collection: Peak detector mode (Threshold only & 50 mAU),Fraction trigger mode (Peak-based with a maximum peak duration of 2 minutes)

Software: Agilent Chemstation B.04.02 (or higher)

Experimental

Agilent 2100 Bioanalyzer

Protein analysis was done on the Agilent 2100 Bioanalyzer with the Agilent Protein 250 kit. Protein loading and on-chip sample analysis were performed as described in the Protein 230 Kit Guide. The Agilent 2100 Expert software was used for run control and data analysis.

SEC-HPLC

Agilent 1260 Bio-inert Infinity Quaternary LC System and Agilent Bio SEC-3, 300Å, 7.8 x 300 mm packed with 3 µm particles was used. Parameters used were; Mobile phase A (150mM Sodium phosphate(pH=7.0) containing 150mM Sodium chloride); TCC Temperature,30°C; Injection volume ,5µL(Lysozyme) ,20µL (PEGylated Lysozyme); flow rate,0.8ml/min ; UV detection,220 and 280nm) and run time of 20 minutes.

LC MS conditions

The intact and PEGylated lysozyme and the PEG reagent were analyzed using Agilent 1290 infinity HPLC-coupled to Agilent 6530 Accurate-Mass Q-TOF LC/MS. Refer Poster 731 (HPLC 2013) for more details.

Enzyme assay: Lytic activity with *M. lysodeikticus*

The enzymatic activity of lysozyme and its conjugate was determined by measuring turbidity changes in *M. lysodeikticus* bacterial cell suspensions (0.5 mg/ml) in 50mM of pH 7.0 phosphate buffer using Agilent Cary 60 spectrophotometer. Absorbance of the suspension was measured at 450nm, and a decrease in absorbance of 0.001 was defined as 1 unit of lysozyme activity.

Results and Discussion

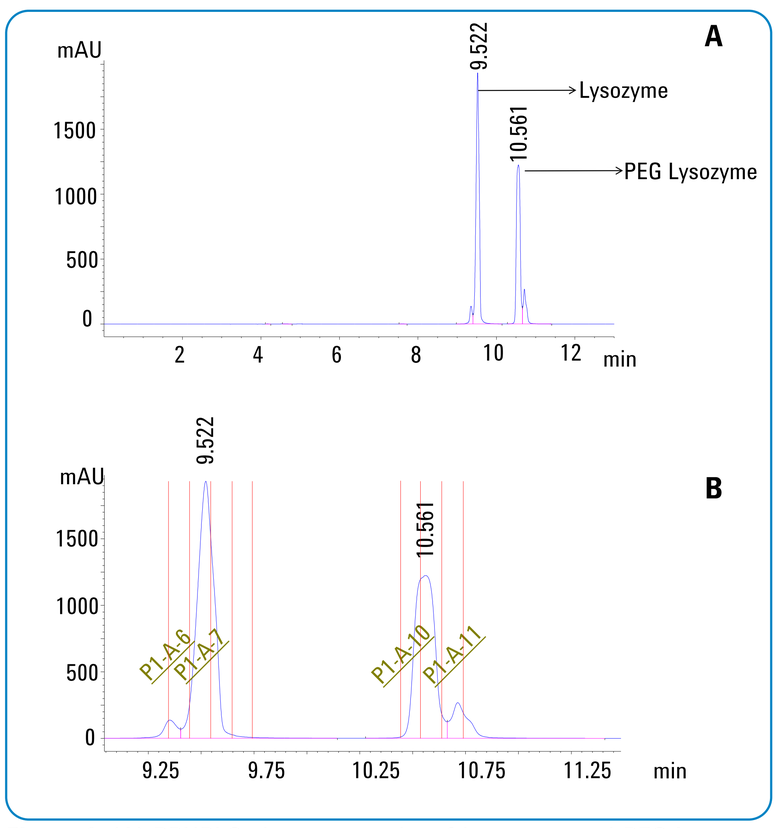

Figure 1: (A) RPHPLC chromatography of Lysozyme and PEG-lysozyme reaction mixture on ZORBAX Semi-preparative 300 SB-C18, 9.4 x 250mm, 5µm column. (B) Peak based Fraction collection.

On a semi preparative column lysozyme eluted at 9.5 min RT and PEGylated lysozyme eluted at 10.5 min RT Figure 5 shows lysozyme and modified lysozyme collected in A7 and A10, A11 wells of the fraction collection plate respectively.

Results and Discussion

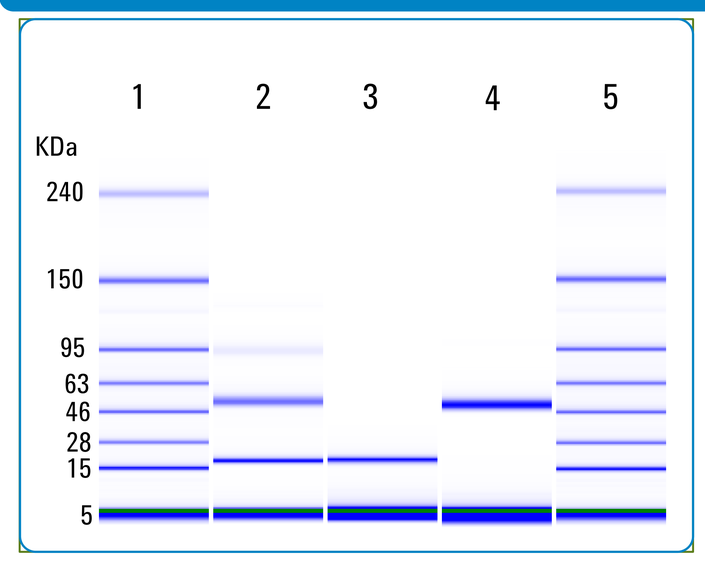

Figure 2: Lane 1-Molecular weight ladder, Lane 2- Lysozyme-PEG reaction mixture, Lane 3- Lysozyme Peak (Semi-prep RP HPLC), Lane 4- PEG Lysozyme Peak (Semi-prep RP HPLC).

As demonstrated in Figure 2 the Bioanalyzer results clearly distinguished between the series of PEGylation species, which was not achieved with SDS-PAGE (data not shown). This data clearly show the advantages of using the Bioanalyzer over SDS-PAGE.

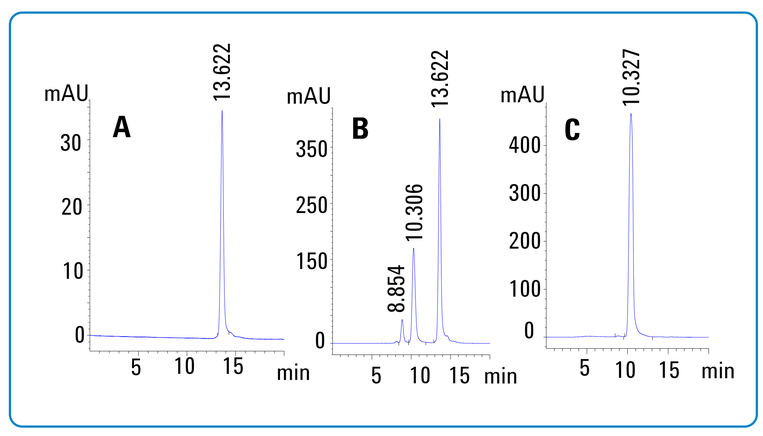

Figure 3: (A) Native Lysozyme (B) Reaction mixture and (C) Purified PEG lysozyme

SEC profiles of lysozyme PEGylation and purification (Figure 3) indicates that the purified PEG lysozyme was homogenous.

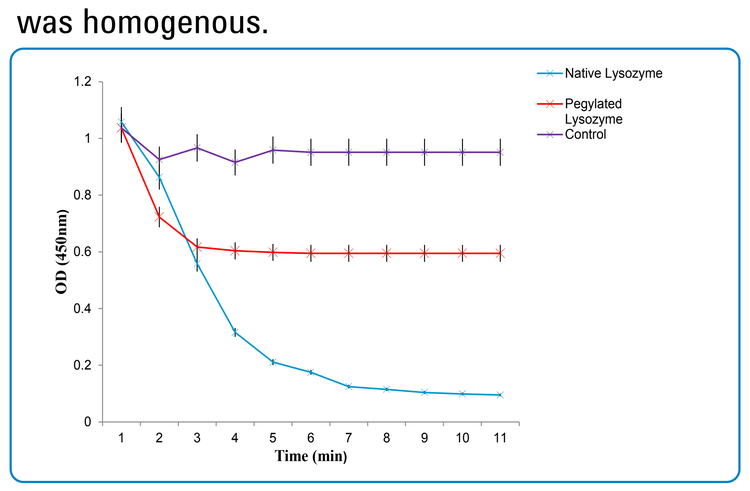

Figure 4: Killing activity of lysozyme and PEGylated lysozyme toward substrate M. lysodeikticus

PEG lysozyme retained 50% activities as compared to unmodified lysozyme (Figure 4). Although the *M. lysodecticus* activity of PEG-lysozyme is reduced compared to unconjugated lysozyme, it is more than offset by other advantages of PEGylation such as the enhancements to serum protein concentrations, increased serum protein half-life, and decreased antibody binding, which we hope will improve the efficacy of intravenously administered therapeutic protein for the treatment of systemic infections.

Conclusions

- PEG CHO was used to conjugate lysozyme in a site specific manner.
- A protocol for the purification of PEG lysozyme was developed using semi prep RP HPLC method.
- Purified PEG lysozyme was found to be homogenous as determined by Bio A, SEC and LC MS.
- Activity assay on *M.lysodecticus* showed that purified PEG lysozyme retained 50% acivity as compared to native lysozyme