ANALYSIS OF THE STRUCTURAL CHANGES CAUSED DURING THE SEPARATION OF PEGYLATED CONJUGATES BY REVERSED PHASE CRHOMATOGRAPHY

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Introduction. The *in vivo* effectiveness of protein and peptide drugs may be reduced due to poor physiological solubility at neutral pH, neutralization by host antibodies, or rapid elimination by the organism. The covalent attachment of a polymer such as polyethylene glycol (PEGylation) to a protein drug can increase its water solubility, mute a host immune response, and reduce renal elimination rates. The PEGylation product obtained depends strongly on the conditions under which the reaction takes place; depending on the protein and the attachment chemistry, a family of species characterized by a distribution in number and position of PEG groups may result (1). The separation and characterization of the isomers of this heterogeneous mixture have gained importance because only one or a few of the PEGylated species may provide optimal biological properties (2). Size Exclusion Chromatography (SEC) is the natural choice for this purpose, but it is not sufficient to resolve micro-heterogeneity, the processing time is long and the product dilution is significant. Reversed phase chromatography (RPC) is a powerful separation technique that has been used in the resolution of proteins with small differences in physical properties, including single amino acid variants. But in some situations, RPC results in low recoveries caused by denaturation in the mobile phase and on the stationary phase, and the appearance of many types of complex behaviors (3).

The objective of this project is to analyze and to understand how the process of purification by reversed phase chromatography affects the structure of the PEGylated proteins, using bovine pancreatic ribonuclease A (RNase A), a potential therapeutic (4), as a model protein.

Methodology. RNase A used in this study was purchased from (Sigma). The PEGylation reaction was performed as previously described by Kinstler and co-workers (5). The PEGylated products were analyzed by SEC and mass spectrometry. The mixture of conjugated RNase A species was separated by RPC (Akta Explorer, Amersham Biosciences) and the eluted material was collected for its analysis by circular dichroism (CD) using the CDPro software (6).

Results and discussion. The analysis by SEC and mass spectrometry shows that the predominant products of the PEGylation reaction are mono and di-PEGylated species. The separation of the PEG conjugated species and the native protein was achieved using C18 media with a gradient formed from mobile phase A 50mM $(NH_4)_2SO_4$ 0.1% TFA and mobile phase B 80% acetonitrile and 20% mobile phase A (Fig. 1). The native RNase A presented multiple peaks during RPC even when pure, wild type protein was injected. The analysis by CD shows that the mobile phase used during RPC increases the fraction of unordered structure in both the wild type and PEGylated proteins, with greater perturbations evident for the wild type protein. An increase in unordered structure is indicative of a denaturation process. The separation of the PEG-conjugates with RPC took less time and had better resolution than with SEC.

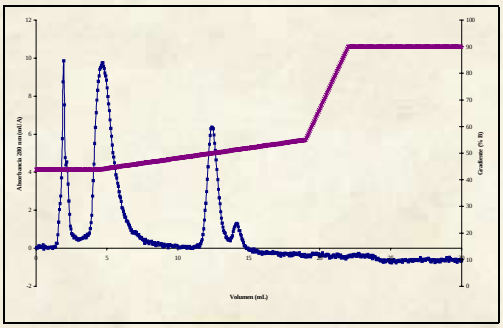

Fig. 1. Separation of the conjugated species of RNase A by RPC.

Conclusion. PEGylation confers some structural stability to the protein. However, structural changes in PEG-RNase A conjugates do occur during the process of purification by RPC. Understanding the structural changes that appear during the protein separation by RPC helps to develop purification processes with better yields.

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