SEPARATION OF PEGYLATED RNase A PROTEINS FROM UNMODIFIED PROTEIN USING A MILD HYDROPHOBIC INTERACTION SUPPORT

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Introduction. PEGylation, used to mitigate some problems that affect the effectiveness of therapeutic proteins, often results in a heterogeneous population of conjugated species and unmodified protein that presents a protein separations challenge. Hydrophobic interaction chromatography (HIC) is a purification technique used to separate proteins on the basis of their surface hydrophobicity. However, very little work has been done on utilizing this technique for the separation of PEGylated proteins (1). The combination of aqueous two-phase extraction (ATPE) and HIC procedures has been used to develop new mild hydrophobic ligands less denaturing to proteins than those utilized in traditional HIC. ATPE is a technique extensively described by Albertsson (2) that exploits mild hydrophobic interactions. The systems are composed of either two polymers or a polymer and a salt. This study presents the use of a mild hydrophobic support (polyethylene glycol MW 5 kDa immobilized on sepharose) as an alternative to separate PEGylated proteins from their unmodified species. This media provides a simple and practical chromatographic method for the separation of unmodified proteins from their PEG conjugates.

Metodology. RNase A used in this study was purchased from (Sigma). The PEGylation reaction was performed as previously described by Kinstler and co-workers (3). The PEGylated products were analyzed and separated by SEC and mass spectrometry. PEG (Nektar Therapeutics, mPEG-NH2) immobilized on sepharose (activated CH sepharose 4B, Amersham Biosciences, Uppsala, Sweden) was used as a mild hydrophobic support. The mPEG-NH2 was coupled to the matrix according to supplier instructions. Chromatographic experiments were carried out with an Akta Explorer 100 integrated chromatography system (Amersham Pharmacia Biotech, Piscataway, NJ) at room temperature. Protein samples collected from SEC experiments were applied to a Tricorn 5/100 column (Amersham Biosciences, 4.6 x 150 mm) packed with Sepharose-mPEG-NH2 media.

Results and discussion. In order to study the retention behavior of the proteins using the sepharose-PEG support, each protein was applied to the column with an initial concentration of 2 M ammonium sulfate (Figure 1). The PEGylated proteins are strongly retained on the support under these conditions and they can not be eluted until a very low salt concentration is achieved (100% B). However, the unmodified protein elutes as a wide peak at a volume at ~10 mL before the gradient is initiated. These results show that the PEGylated protein interacts with the PEG on the support strongly whereas the unmodified protein has only weak interactions with the media. It is notable the difference in absorbance between the mobile phases used, reason why the baseline at the end of the gradient has higher absorbance that at the beginning.

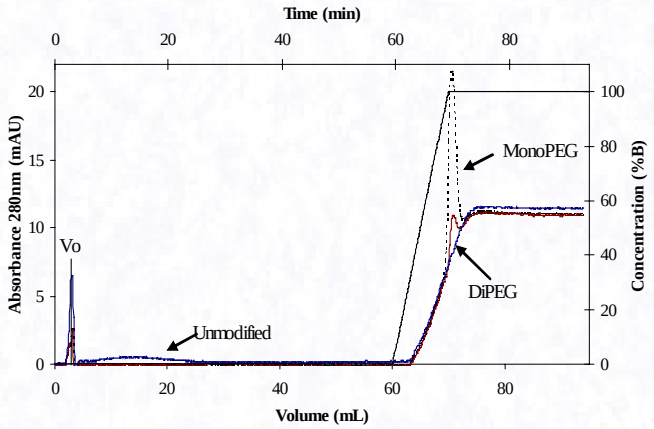

Fig 1 Retention behavior of RNase A, MonoPEG and DiPEG-RNase A on the PEG HIC media. *Mobile phase A, 2 M ammonium sulfate 25 mM potassium phosphate, pH 7.0; mobile phase B: water. The PEGylated proteins were separated by SEC and exchanged into a 25 mM potassium phosphate buffer, pH 7.0; 100 µL of sample were injected at room temperature. The flow rate was 1 mL/min.*

Conclusions. It was demonstrated that the separation system developed here can exploit the approach of combining ATPE and HIC to separate unmodified proteins from their PEGylated conjugates. Selectivity was found between the unmodified protein and its PEGylated species using a gradient elution.

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