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Separation of PEGylated from unmodified ribonuclease A using sepharose media

Mayra Cisneros-Ruiz [a], Karla Mayolo-Deloisa [a], Todd M. Przybycien [b,c], Marco Rito-Palomares [a,*]

[a] Departamento de Biotecnología e Ingeniería de Alimentos, Centro de Biotecnología, Tecnológico de Monterrey, ITESM, Ave. Eugenio Garza Sada 2501-Sur, Monterrey, NL 64849, Mexico
[b] Department of Chemical Engineering, Carnegie Mellon University, Pittsburgh, PA 15213, USA
[c] Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA 15213, USA

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ABSTRACT

PEGylation, used to mitigate some of the 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. This study presents the use of a mildly amphiphilic support, Tris-capped CH Sepharose 4B as an alternative for separating PEGylated proteins from their unmodified counterparts. The effects of parameters such as pH, salt type and salt concentration upon the chromatographic behavior of native, mono-PEGylated and di-PEGylated ribonuclease A on this media were characterized. The separation of the native protein from the PEGylated species was achieved using a gradient elution between a high ionic strength mobile phase (3 M ammonium sulfate in 25 mM potassium phosphate, pH 7.0 or 2 M potassium phosphate, pH 7.0) and a low ionic strength phase (25 mM potassium phosphate, pH 7.0). The pH of the mobile phases as well as the addition of PEG_{600} (as a potential mobile phase modifier) to the low ionic strength phase had no significant influence on chromatographic behavior of the species. This media provides a simple and practical chromatographic method for the separation of unmodified proteins from their corresponding PEG conjugates.

1. Introduction

The covalent attachment of polyethylene glycol (PEG) molecules to pharmaceutical proteins – a reaction process known as "PEGylation" – can mitigate factors that adversely affect therapeutic effectiveness, including susceptibility to enzymatic degradation, short circulation time, low solubility and immunogenicity [1,2]. However, this can present a challenging separation problem due to the fact that PEGylation reactions often result in a population of conjugate species in addition to the residual unmodified protein. The resulting conjugate species can differ in terms of the number of attached PEG chains and their locations and can differ in biological activity [3]. Chromatographic operations are often used to resolve PEGylation reaction mixtures, with size-based size exclusion and charge-based ion exchange modes most frequently used [4]; comparatively little work has been done to explore other modes such as the hydrophobic interaction mode [5,6].

Hydrophobic interaction chromatography (HIC) is intriguing with respect to the separation of PEGylated protein reaction mixtures as PEG itself exhibits lower critical solution temperature behavior: it can adopt collapsed configurations at higher temperatures and/or salt concentrations that are comparatively more hydrophobic than the relatively extended configurations that occur at lower temperatures and/or salt concentrations [7]. Very little work has been done on exploiting hydrophobic interactions for the separation of PEGylated proteins [4]. HIC consists of injecting a protein sample in a column packed with a hydrophobic media under conditions of high salt concentration to drive hydrophobic interactions between the proteins and the media, analogous to a salting-out process. Bound proteins are eluted by lowering the salt concentration. In the case of a strongly bound protein, it can be eluted by the use of a chaotropic agent or an organic modifier [8]. The most common hydrophobic ligands used in HIC are linear chain alkanes or simple aromatic groups. However, these hydrophobic ligands may promote strong hydrophobic interactions that sometimes result in irreversible adsorption of the proteins or denaturation during adsorption and subsequent elution with harsh mobile phases. Ligands with milder hydrophobic characters can be attractive as they can provide moderate binding strength and bound species can be eluted by simply decreasing the salt concentration of the eluent [9].

In this work, we were able to completely separate PEGylated ribonuclease A (RNase A) species from unmodified RNase A using a Tris-capped CH Sepharose 4B media with salt gradient elution. RNase A was chosen as a model protein as it is well-characterized, has potential therapeutic application as an aspermatogenic and

* Corresponding author. Tel.: +52 81 8328 4132; fax: +52 81 8358 1400.
E-mail address: mrito@itesm.mx (M. Rito-Palomares).

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antitumor agent and studies have shown an improvement of the therapeutic effect when RNase A is conjugated with PEG [10]. RNase A was PEGylated with an aldehyde-activated PEG of 20 kDa nominal molecular weight. The behavior of unmodified RNase A and mono- and di-PEGylated species previously separated using size exclusion chromatography (SEC) was characterized on the proposed Sepharose-HIC separation system as were protein PEGylation reaction mixtures. pH, salt type and salt concentration were the parameters selected to define conditions under which the separation of the unmodified protein from the PEGylated species can be achieved. Free PEG with a nominal molecular weight of 600 Da was also examined as a potential mild mobile phase modifier.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (cat. no. R5000, Lot 093K0765) was purchased from Sigma–Aldrich (St. Louis, MO). Methoxy-poly(ethylene glycol)-butyraldehyde with a nominal molecular weight of 20 kDa came from Nektar Therapeutics (Huntsville, AB). Sodium cyanoborohydride was purchased from ICN Biomedicals Inc. (Costa Mesa, CA). Activated CH sepharose 4B (cat. no. 17-0490-01 Lot 307571) and the chromatography column, a Tricorn 5/100, came from Amersham Biosciences (now GE Healthcare, Uppsala, Sweden). Conjugation and purification buffers were prepared from sodium phosphate monobasic and dibasic salts (Fisher Scientific, Pittsburgh, PA), and potassium chloride (Sigma–Aldrich Company, St. Louis, MO). All HPLC grade reagents were obtained from Fisher Scientific. Other salts and solvents used in this research were of reagent grade. All reagents were used as received.

2.2. Preparation of PEGylated protein

PEGylated RNase A was prepared according to the procedure of Daly et al. [11]. Briefly, a solution of RNase A (2.0 mL) at 3.0 mg/mL in a pH 5.1, 100 mM sodium phosphate buffer with 20 mM sodium cyanoborohydride was added to a vial containing 30 mg of the nominal 20 kDa methoxy poly(ethylene glycol) butyraldehyde. The reaction mixture was stirred rapidly for 17–19 h at 4 °C. Experiments were conducted both with the final reaction mixture and with species purified using size exclusion chromatography (SEC).

2.3. Analysis and separation of PEGylated protein mixture by size exclusion chromatography (SEC) and mass spectrometry

The reaction mixture (2.0 mL) was analyzed via size exclusion chromatography with an Amersham Pharmacia Akta Explorer system (now GE Healthcare, Uppsala, Sweden) using a Sephacryl S-300 column (1.6 cm inner diameter, 60 cm length, Amersham Pharmacia, Uppsala, Sweden) with an isocratic mobile phase of 10 mM sodium phosphate buffer, pH 7.2, containing 150 mM potassium chloride at a flow rate of 0.5 mL/min. The column was pre-equilibrated with one-half column volume of distilled water and two column volumes of mobile phase. Fractions having an absorbance at 280 nm were collected and concentrated in an Amicon (Beverly, MA) stirred cell fitted with a YM 10 membrane (molecular weight cutoff 10 kDa) and then diafiltered with 5 volumes of 25 mM sodium phosphate buffer, pH 7.2.

Each SEC peak was analyzed by mass spectrometry (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry, MALDI-TOF/MS). Prior to analysis, the samples were desalted using centrifugal microconcentrators (Microcon YM-3, Millipore). MALDI-TOF/MS was performed with a PerSeptive Voyager STR mass spectrometer fitted with a standard 337 nm nitrogen laser. The spectra were recorded with the analyzer in the positive-ion linear mode of detection. The ion accelerating potential was 25 kV. A saturated solution of α-3,5-dimethoxy-4-hydroxycinnamic acid (Fluka, Buchs, Switzerland) in 50% acetonitrile was used as a matrix solution. The sample crystals were prepared by mixing 1.0 μL of sample with 1.0 μL of matrix. The product peaks were identified as diPEGylated, monoPEGylated and unmodified RNase A species.

2.4. Preparation of chromatography media

Sepharose was used as the base matrix for the construction of a mild amphiphilic support. Activated CH Sepharose 4B (Amersham Biosciences, Uppsala, Sweden, Lot: 307571) was modified with a capping reaction using tris(hydroxyethyl)aminomethane (Tris) as shown in Fig. 1. The matrix was prepared according to supplier instructions (Amersham Biosciences). About 1.3 g of activated Sepharose were washed with about 250 mL of 1 mM HCl and the washed medium was transferred to 0.1 M Tris–HCl buffer, pH 8. After 1 h, the modified media was washed with three cycles of alternating pH (0.1 M acetate buffer, pH 4.0 containing 0.5 M NaCl followed by 0.1 M Tris–HCl buffer, pH 8 containing 0.5 M NaCl). Based on the activated media product literature, 15 μmol/mL media is an upper bound on the Tris capping group density.

2.5. Separation of PEGylated species from unmodified RNase A

Chromatographic experiments were carried out with the Akta Explorer 100 integrated chromatography system operating at room temperature. Protein samples collected from SEC experiments or from the final PEGylation reaction mixture were applied to a Tricorn 5/100 column (Amersham Biosciences, 4.6 mm × 150 mm) packed with about 3.5 mL of the modified Sepharose media. The column had about 65 theoretical plates, as determined by elution peak width analysis of injections of 1% (v/v) acetone pulses at 25 °C. Gradient elutions were carried out for protein separations at a flow rate of 1 mL/min with a solvent A (3 M ammonium sulfate in 25 mM potassium phosphate pH 7.0, or 2 M potassium phosphate, pH 7.0 or 3 M ammonium sulfate in 25 mM acetate buffer, pH 5.0) and a solvent B (25 mM potassium phosphate, pH 7.0 or 25 mM acetate buffer, pH 5). The following gradient profile was selected: 0% B for 6.5 mL, 0–100% B over 29.5 mL, and 100% B for 15 mL. Peak elution was monitored via the UV absorption at 280 nm. 100 μL of sample was injected at room temperature. The concentrations were 3, 1.5 and 0.97 mg/mL for unmodified, monoPEGylated and diPEGylated RNase A, respectively. The flow rate was 1 mL/min.

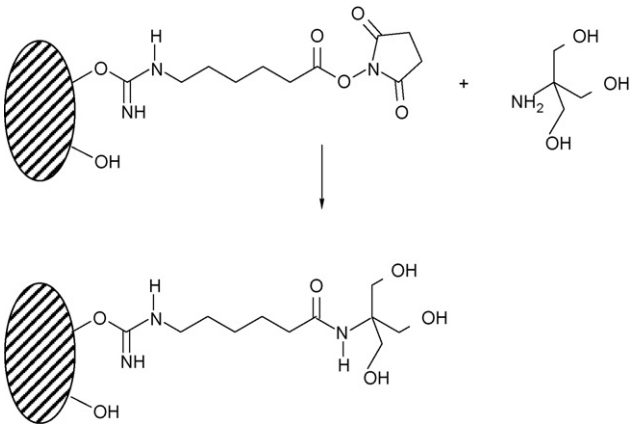

Fig. 1. Reaction scheme for the modification of activated CH Sepharose 4B by capping with tris(hydroxyethyl)aminomethane.