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Hydrophobic interaction chromatography for purification of *monoPEGylated* RNase A☆

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ABSTRACT

The chromatographic methods used for the purification of *PEGylated* proteins are mainly Size Exclusion (SEC) and Ion Exchange Chromatography (IEX). Although the *PEGylation* affects the protein hydrophobicity, Hydrophobic Interaction Chromatography (HIC) has not been extensively applied for the separation of these proteins. Purification of *monoPEGylated* Ribonuclease A (RNase A) using HIC is studied in this work. The products of the *PEGylation* reaction of RNase A with 20 kDa methoxy-poly(ethylene glycol) were separated using three resins with different degrees of hydrophobicity: Butyl, Octyl and Phenyl sepharose. The effects of resin type, concentration and salt type (ammonium sulphate or sodium chloride), and gradient length on the separation performance were evaluated. Yield and purity were calculated using the plate model. Under all conditions assayed the native protein was completely separated from *PEGylated* species. The best conditions for the purification of *monoPEGylated* RNase A were: Butyl sepharose, 1 M ammonium sulphate and 35 column volumes (CVs); this resulted in a yield as high as 85% with a purity of 97%. The purity of *monoPEGylated* RNase A is comparable to that obtained when the separation is performed using SEC, but the yield increases from 65% with SEC to ~85% with HIC. This process represents a viable alternative for the separation of *PEGylated* proteins.

1. Introduction

PEG–protein conjugates, or *PEGylated* proteins, are an important class of modern therapeutic drugs. However, *PEGylated* proteins must be characterized and purified before use in order to meet the stringent regulatory requirements that demand proven clinical efficacy and safety [1]. The process of *PEGylation* involves formation of a stable covalent bond between activated PEG (polyethylene-glycol) polymers and the polypeptide drug of interest [2]. *PEGylation* changes the physical and chemical properties of the biomedical molecule, such as its conformation, electrostatic binding, and hydrophobicity; resulting in an improvement in the pharmacokinetic behavior of the drug. In general, *PEGylation* improves drug solubility and decreases immunogenicity, increases drug stability and the residence time of the conjugates in blood, and reduces proteolysis and renal excretion, thereby allowing a lower dosing frequency [3].

To prepare a *PEGylated* protein, it is desirable that one PEG molecule is attached to a specific site (site-directed *mono-PEGylation*). However, as the *PEGylation* reaction is not completely understood, it is not easy to adjust or optimize the reaction process [4]. The reaction mixture is complex, from which the desired *PEGylated* protein must be highly purified. Purification of *PEGylated* protein is not a trivial task [5].

PEGylation of proteins creates two basic challenges for purification. The first involves separation of *PEGylated* proteins from other reaction products including non-reacted PEG and protein. The second is the sub-fractionation of *PEGylated* proteins on the basis of their degree of *PEGylation* and positional isomerism [4]. While isolation of the unreacted protein from the *PEGylated* species is relatively easy, separation of the various positional isomers of a *PEGylated* protein mixture remains a significant challenge [6]. Individual and combined chromatographic approaches are currently used to purify *PEGylated* proteins [7]. Chromatographic purification of all proteins implies selection of a mode of chromatography that exploits the differences in physicochemical properties [8]. Fee and Van Alstine [4] reviewed separation methods applied to

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downstream processes for *PEGylated* proteins. They have reported that Size Exclusion (SEC) and Ion Exchange Chromatography (IEX) are the predominant methods for purification of the *PEGylated* products. SEC has been widely used for separation of PEG conjugates as an increase in molecular weight is one of the most evident changes caused by *PEGylation* [9]. IEX enables a single step purification of the target PEG-protein conjugate from *unPEGylated* protein, higher *PEGylated* molecules and unreacted PEG; due to charge differences [9]. *PEGylation* should affect protein surface hydrophobicity, increasing or decreasing it depending on the native proteins hydrophobicity [4] and consequently, Hydrophobic Interaction Chromatography (HIC) can be used as an additional method for separation of PEG modified proteins. Even though HIC is used routinely for production-scale purification of proteins, it has not been highlighted for the separation of *PEGylated* species [1,4,10].

Ribonuclease A (RNase A) is a small model enzyme, with a molecular weight of 13,686 Da [11]. RNase A has proven to have several potential therapeutical applications in vivo as an antitumor agent, mainly in its *PEGylated* form [12]. It has been found that the biological activity exhibited by the *PEGylated* conjugates is related to the number of grafted polymer chains as well as their relative position. For most proteins, an excess in the number of grafted polymer chains generates a steric hindrance for its biological receptor, decreasing their specific activity [13]. It has been reported that the *monoPEGylated* RNase A presents the highest biological activity [12].

The purpose of this work is to establish the conditions for purification of *monoPEGylated* RNase A using HIC. The *PEGylation* reaction used by our group [14,15] is a reaction with the N-terminal amino group of the protein. This reaction generates three products: *monoPEGylated* RNase A (monoPEG), *diPEGylated* RNase A (diPEG), and the unmodified protein. In this case, resin type, salt type (ammonium sulphate or sodium chloride), salt concentration and gradient length were the parameters selected to define conditions under which the purification of the *monoPEGylated* protein can be achieved. Yield and purity were calculated using the plate model [16]. The equations used are described below:

$$\text{yield} = \frac{1}{2}\left[1 + \text{erf}\left(\frac{V}{V_0} - \frac{1}{\sqrt{2_*}}\sigma\right)\right] \quad (1)$$

$$\text{purity} = \frac{[y_0(i)_* \,\text{yield}\,(i)]}{[\Sigma_j y_0(j)_* \,\text{yield}\,(j)]} \quad (2)$$

where y_0 is the maximum concentration, V_0 is the volume required to elute the maximum concentration y_0 and $V_0\sigma$ is the standard deviation of the peak [16]. The parameters were determined from the experimental results.

2. Materials and methods

2.1. Materials

Bovine pancreatic ribonuclease A (cat. no. R5000, lot 047K1640) and trizma base (cat. no. T6066, lot 076K54521) were purchased from Sigma–Aldrich (St. Louis, MO). MethoxyPEG-propionaldehyde (Lot ZZ004P225) with a nominal molecular weight of 20 kDa came from Jen Kem Technologies (Allen, TX). Sodium cyanoborohydride (cat. no. 71435, lot 414320) was purchased from Fluka (Switzerland). Phenyl sepharose 6FF HS (cat. no. 17-0973-05, lot 286273), Octyl sepharose (cat. no. 17-0946-05, lot 283600) and Butyl sepharose (cat. no. 17-0980-01, lot 28686) were obtained from GE Healthcare (Uppsala, Sweden). Purification buffers were obtained from J.T. Baker (Toluca, México). Water prepared with Milli-Q water cleaning system (Millipore, Bedford, MA) was used in the preparation of the eluents. Other salts and solvents were of reagent grade.

2.2. Preparation of PEGylated protein

PEGylated RNase A was prepared according to the procedure of Daly et al. [17]. Briefly, a solution of RNase A (5.5 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 75 mg of the nominal weight 20 kDa methoxy poly(ethylene glycol) propionaldehyde. The reaction mixture was stirred vigorously for 17 h at 4 °C. The reaction was stopped by separating the mixture on a size exclusion chromatographic column.

2.3. Separation of PEGylated protein mixture by size exclusion chromatography (SEC)

The reaction (5.5 mL) was analyzed by Size Exclusion Chromatography with an Äkta Explorer system (GE Healthcare, Uppsala, Sweden) equipped with a 5 mL injection loop. A Sephacryl S-300 HP column (2.6 cm ID, 60 cm length, GE Healthcare, Uppsala, Sweden) was used 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 that absorbed at 215 nm were pooled and concentrated by ultrafiltration under nitrogen atmosphere using an Amicon ultrafiltration cell (MA, USA) with a 10 kDa Diaflo ultrafiltration membrane (Amicon Inc., MA, USA). Finally, each *PEGylated* protein were lyophilized and stored at −4 °C [14]. These lyophilized *PEGylated* proteins obtained by SEC were used to perform the studies of HIC separation.

2.4. Separation of PEGylated proteins by hydrophobic interaction chromatography (HIC)

An Äkta Purifier System (GE Healthcare, Uppsala, Sweden) was employed, equipped with a 100 μL injection loop. The chromatographic columns were 1 mL (100 mm × 5 mm ID) packed with Phenyl sepharose 6FF HS, Octyl sepharose or Butyl sepharose 4 FF. The experiments were performed at room temperature, using a flow rate equal to 0.8 mL/min and 25, 35 or 45 column volumes (CVs) [18].

Elution was obtained by a lineal decreasing gradient of ammonium sulphate (AS) or sodium chloride. The initial eluent (solution B) utilized was 20 mM TRIS-HCl, pH 7.0 plus different concentrations of salt (AS or sodium chloride). The final buffer (solution A) used was 20 mM TRIS-HCl, pH 7.0. All buffers were filtered through 0.22-μm Millipore filters after preparation, and degassed with helium for 5 min. The column outlet was monitored at 215 nm. The conductivity was also monitored. Yield and purity were calculated using the plate model [16].

3. Results and discussion

3.1. SEC purification

Initially, the *PEGylation* reaction was monitored by SEC to obtain the *PEGylated* protein standards required for the HIC analysis. The reaction generates two *PEGylated* species: *monoPEGylated* RNase A and *diPEGylated* RNase A. These products were previously characterized [14]. Fig. 1 shows the chromatographic profile of SEC, where in addition to the *PEGylated* proteins, unmodified RNase A can be observed. Despite SEC being the most popular route for separation of *PEGylated* species [4], the resolution obtained is low. The plate

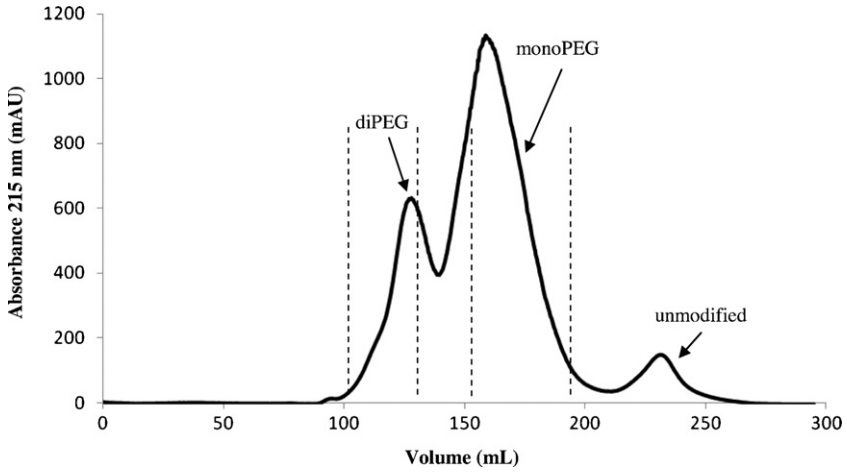

Fig. 1. Size exclusion chromatography profile of *PEGylated* reaction mixture. Column: Sephacryl S-300 HP. Isocratic mobile phase: 10 mM sodium phosphate buffer pH 7.2 + 150 mM KCl. Flow rate: 0.5 mL/min. The vertical lines show where the fractions were collected.

model was used to calculate the yield of the separation of monoPEGylated RNase A and its purity, obtaining values of 65% and 98% respectively. Each *PEGylated* specie was collected, concentrated, lyophilized and stored at −4 °C.

3.2. HIC purification

For HIC purification, three different hydrophobic resins were tested: Butyl sepharose (weakly hydrophobic), Octyl sepharose (moderately hydrophobic) and Phenyl sepharose (strongly hydrophobic). Each *PEGylated* specie and unmodified RNase A was analyzed separately in HIC. Adsorption of proteins to HIC media is favoured by a high salt concentration, but due to differences in the interaction strength between the adsorbent and different proteins, the concentration of salt needed for adsorption can vary considerably [19]. Based on previous studies [14], high concentrations of ammonium sulphate (AS) were used in the initial experiments with the aim that unmodified RNase A was retained by the resins and desorbed in the gradient elution. As shown in Fig. 2, applying a linear gradient (25 CV) of ammonium sulphate resulted in complete separation of unmodified protein from mono and *diPEGylated* RNase A with all resins. The *PEGylation* effect changes the hydrophobicity of the protein. However, it is not sufficient to obtain a high resolution separation of the PEGylated species. Although there exist some differences between retention volumes of *PEGylated* proteins when employing Octyl and Phenyl sepharose (Fig. 1B and C), *diPEGylated* RNase A is completely included into the *monoPEGylated* RNase A. Namely, a stronger hydrophobic resin shows less resolution in the separation of *PEGylated* proteins. Moreover, it is observed that *PEGylated* proteins are not completely soluble at the concentrations of ammonium sulphate used in Fig. 2, while the native protein is completely soluble. Muller et al. [10] conducted a detailed study on the solubility of *PEGylated* conjugates of lysozyme, finding that the solubility in ammonium sulphate decreases with increased length of PEG chains linked to the protein. In our case the solubility decreases as the degree of *PEGylation* increases. When Butyl sepharose is used (Fig. 2A) a slight separation between the *PEGylated* proteins is observed, in fact this is the only profile where it is clear that the *monoPEGylated* protein is not totally pure. For this reason it was decided to refine the parameters of purification using Butyl sepharose.

The effect of AS concentration and gradient length on product separation is depicted in Fig. 3. It is observed that decreasing the concentration of salt did not result in native protein retention on the resin; however, the separation of *PEGylated* proteins is better. When increasing the gradient length the difference was more evident. It is clear that there exists a difference between the hydrophobicity of proteins as a result of the *PEGylation* process, in this case the less hydrophobic protein is the native protein whereas the most hydrophobic is *diPEGylated* RNase A. The best conditions for the separation of *PEGylated* proteins include the use of 1 M AS and gradient elution of 35 CVs (Fig. 3D), under these conditions the native protein it is not retained by the adsorbent and all proteins are completely soluble in 1 M AS. It is not possible to work at a lower concentration than 1 M ammonium sulphate because all proteins are eluted in the dead time.

As mentioned before, the purity and yield were calculated using the plate model for the chromatograms shown in Fig. 3. Calculations were performed focusing on the purity of *monoPEGylated* RNase A. Table 1 shows that whilst the purity remained practically unchanged, the yield increases with decreasing salt concentration (AS). Fig. 4 clearly shows the fractionation of *PEGylated* proteins, using 1 M AS and 35 CVs. The use of these conditions resulted in a yield of up to 84.80% with a purity of 97%. Purity of *monoPEGylated* RNase A is comparable to that obtained when the separation is performed using SEC, but the yield increases from 65% with SEC to ∼85% with HIC. On the other hand, Butyl sepharose is unable to retain the unmodified RNase A using 4 M NaCl (data not shown). The *PEGylated* proteins appear immediately at the start of the elution gradient at the same retention volume (∼10 mL).

Fig. 5 shows the effect of sodium chloride concentration on product separation using Phenyl sepharose as adsorbent. The unmodified protein is not retained by the resin; nevertheless, the *PEGylated* proteins are strongly adsorbed with a slight change in their retention volumes (∼3 mL Fig. 5A and ∼5.5 mL Fig. 5B). The order of elution of the proteins remains equal to that obtained with Butyl sepharose – ammonium sulphate. Since the resolution of the peaks is lower, only two concentrations of NaCl were used with the same length of gradient elution (25 CVs). Unlike their behaviour with ammonium sulphate, in this case all proteins were completely soluble at the two concentrations of NaCl used, but that was not enough to achieve a definite separation between the *PEGylated* conjugates. Table 2 shows the yield and purity obtained from the chromatograms of Fig. 5. With purity similar to that obtained with Butyl sepharose – AS, the yield of *monoPEGylated* RNase A is between 46.9 and 57.5%.

Fig. 6 shows the results obtained for the separation of the products of the *PEGylation* reaction (without using SEC) after selection of the best conditions for the separation of bioconjugates. The mixture

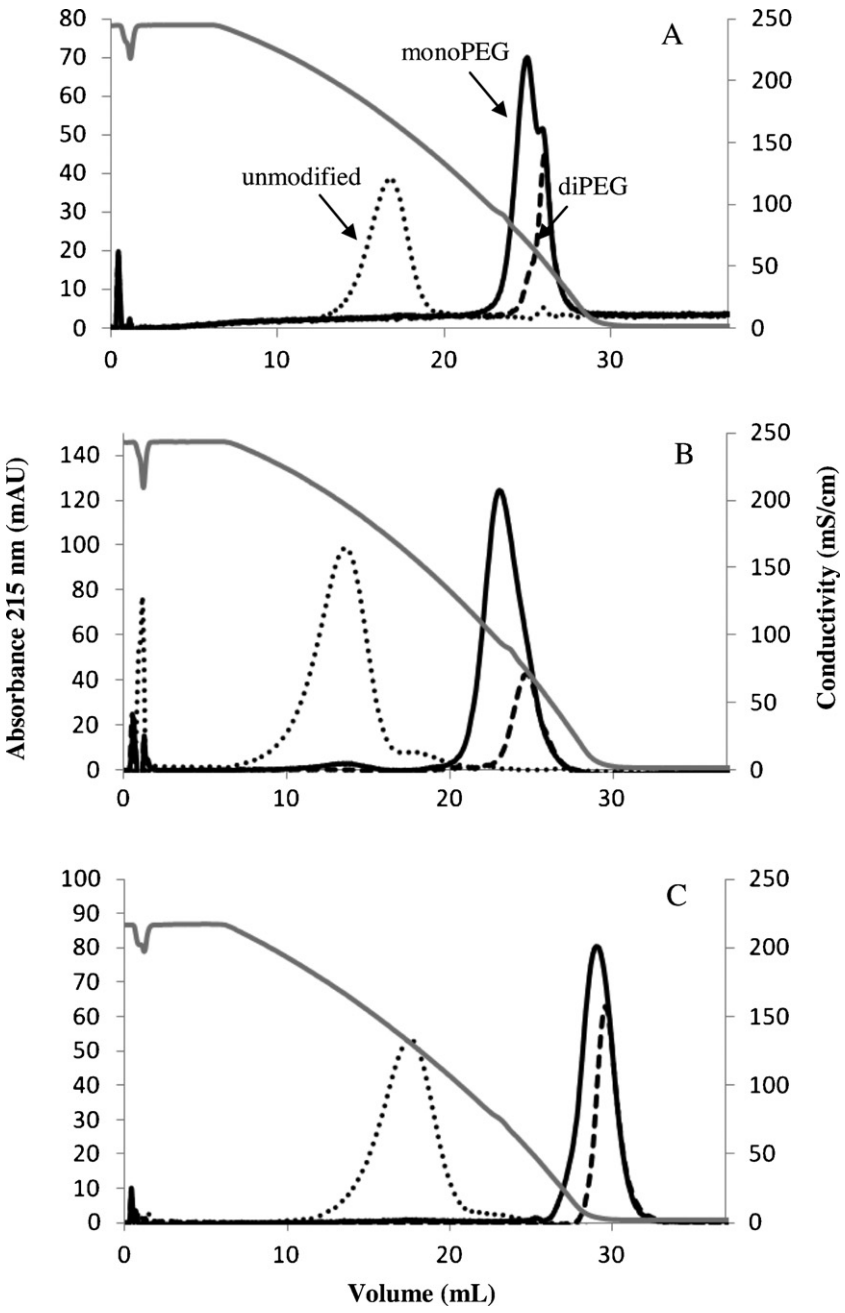

Fig. 2. Effect of type of resin in the purification of *monoPEGylated* RNase A on product separation. Buffer A: Tris–HCl 20 mM pH 7.0 Buffer B: Tris–HCl 20 mM pH 7.0 containing 2 or 2.5 M $(NH_4)_2SO_4$. Loop: 100 μL, flow rate: 0.8 mL/min. CV: 25. Gradient lineal from B to A. (a) Butyl sepharose, 2.5 M $(NH_4)_2SO_4$; (b) octyl sepharose, 2.5 M $(NH_4)_2SO_4$; (c) phenyl sepharose, 2 M $(NH_4)_2SO_4$. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

Table 1
Recovery and purity of *monoPEGylated* RNase A using Butyl sepharose and different concentrations of ammonium sulphate. Data were calculated from the chromatograms of Fig. 3.

	AS (M)	CVs	Volume (mL)	Yield (%)[a]	Purity (%)[a]
Butyl sepharose	2.00	45	33.37	54.66	99.26
	1.75	35	26.80	62.30	98.50
	1.50	35	25.12	66.88	97.80
	1.00	35	17.10	84.80	97.00

AS, ammonium sulphate; CVs, column volumes.
[a] Calculated using the plate model.

Table 2
Recovery and purity of *monoPEGylated* RNase A using phenyl sepharose and sodium chloride. Data were calculated from the chromatograms of Fig. 5.

	NaCl (M)	CVs	Volume (mL)	Yield (%)[a]	Purity (%)[a]
Phenyl sepharose	2.5	25	17.43	46.9	98.52
	2.0	25	15.06	57.5	97.62

CVs, column volumes.
[a] Calculated using the plate model.

was injected directly into the column packed with Butyl sepharose. This validated the results obtained individually for each product of the reaction.

Previously we reported that the separation of the reaction of *PEGylation* of RNase A using a Tris-capped CH Sepharose 4B media

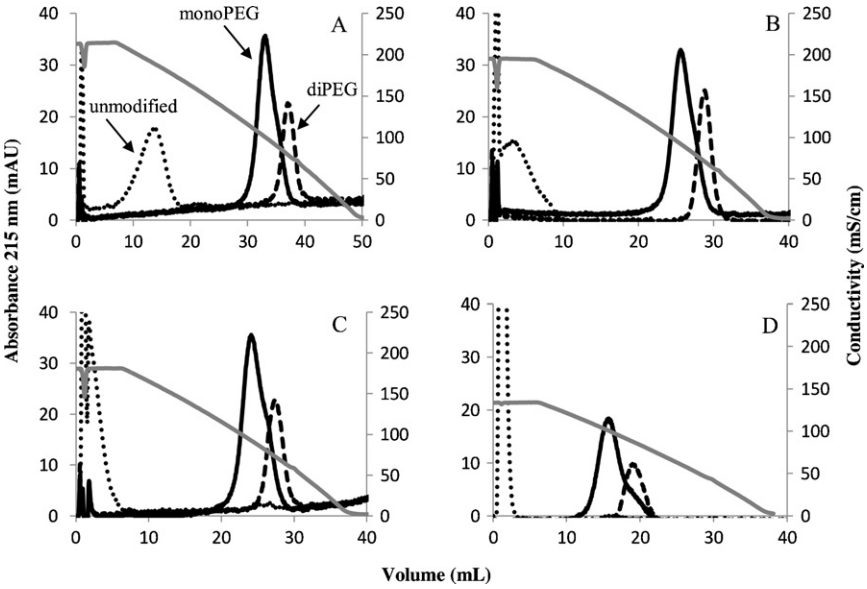

Fig. 3. Effect of ammonium sulphate concentration and gradient length on product separation. Resin: Butyl sepharose. Buffer A: Tris–HCl 20 mM pH 7.0 Buffer B: Tris–HCl 20 mM pH 7.0 containing 2, 1.75, 1.5 or 1 M $(NH_4)_2SO_4$. Loop: 100 μL, Flow rate: 0.8 mL/min. Gradient lineal from B to A. [monoPEG RNase A]: 1.5 mg/mL, [diPEG RNase A]: 0.5 mg/mL, [RNase A]: 0.5 mg/mL. (A) 2 M, 45 CV; (B) 1.75 M, 35 CV; (C) 1.5 M, 35 CV; (D) 1 M, 35 CV. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

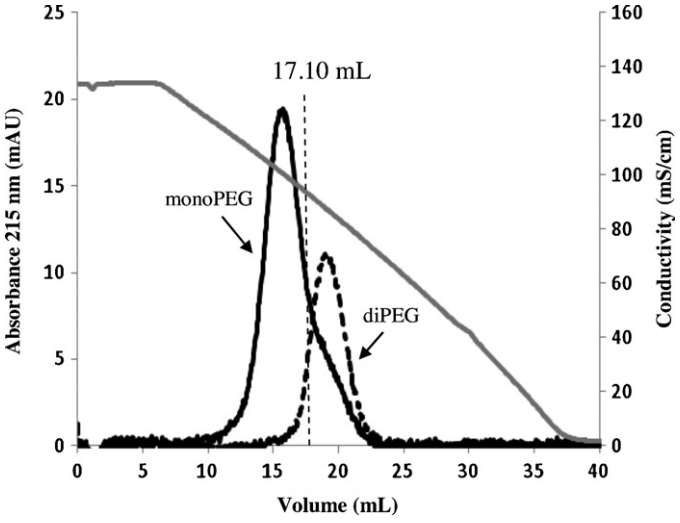

Fig. 4. Fractionation of *monoPEGylated* RNase A highly pure. Resin: butyl sepharose. Buffer A: Tris–HCl 20 mM pH 7.0 Buffer B: Tris–HCl 20 mM pH 7.0 containing 1 M $(NH_4)_2SO_4$, 35 CV. Yield: 84.80%, purity: 97%. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

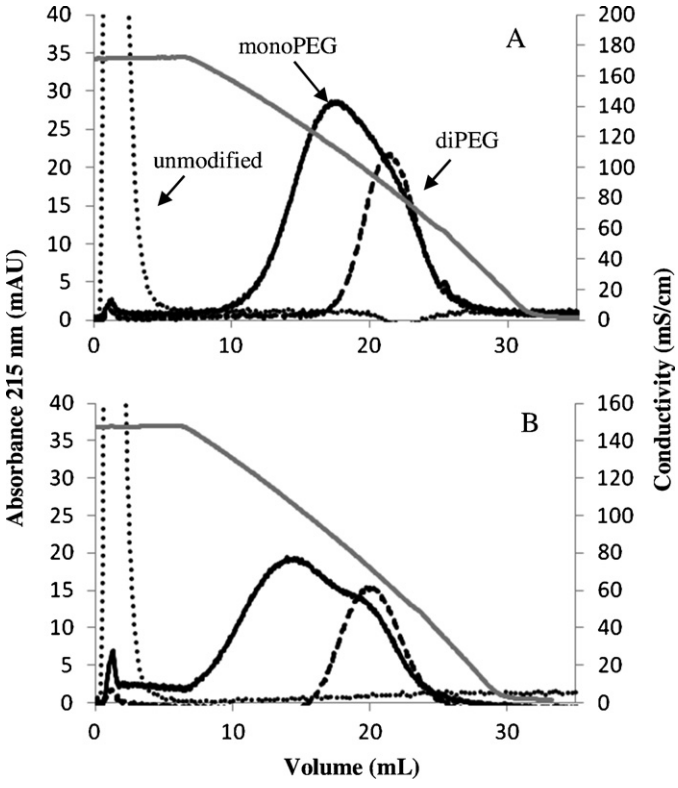

Fig. 5. Effect of sodium chloride concentration on product separation. Resin: phenyl sepharose. Buffer A: Tris–HCl 20 mM pH 7.0 Buffer B: Tris–HCl 20 mM pH 7.0 containing 2 or 2.5 M (NaCl). Loop: 100 μL, flow: 0.8 mL/min. Gradient lineal from B to A. [monPEG RNase A]: 1.5 mg/mL, [diPEG RNase A]: 0.5 mg/mL, [RNase A]: 0.5 mg/mL. (A) 2 M, 25 CV; (B) 2.5 M, 25 CV. Each PEGylated protein and unmodified RNase A was analyzed separately. The chromatograms were superimposed.

with salt gradient elution, resulted in the separation of only unmodified protein and the *PEGylated* conjugates eluted at virtually the same retention time [14]. Using hydrophobic resins such as Butyl and Phenyl sepharose, it is possible to separate *PEGylated* proteins. It is noteworthy that the elution order is the same for the system reported by Cisneros-Ruiz et al. [14] and mentioned here, regardless of the resin and/or salt used.

Muller et al. [10] reported the separation of reaction products of *PEGylation* of lysozyme with 5 kDa PEG, using a TSKgel Butyl-NPR column with 1.2 M ammonium sulphate; under these conditions, they achieved complete separation of the *PEGylated* conjugates from the unmodified lysozyme. Furthermore, contrary to our observations, the elution order changes depending on the type of salt. They inferred that the shift in the elution order can be explained by the different solubilities of the *PEGylated* lysozymes in the two different salt solutions (AS and NaCl). One possible explanation for this difference may be due to the length of the PEG and the intrinsic properties of the proteins.

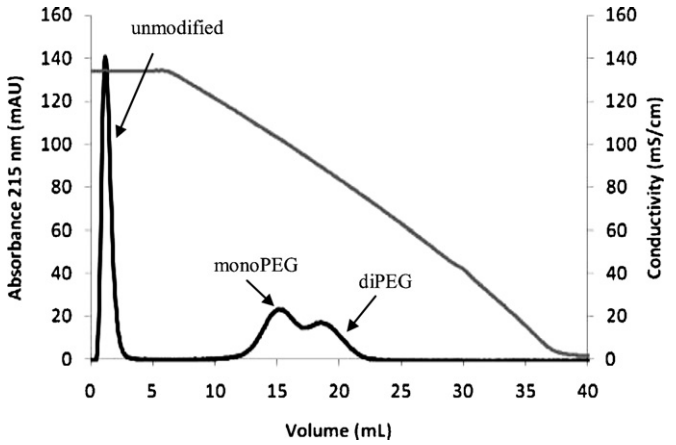

Fig. 6. Separation of products of RNase A *PEGylation* reaction using HIC. Resin: butyl sepharose. Buffer A: Tris–HCl 20 mM pH 7.0 Buffer B: Tris–HCl 20 mM pH 7.0 containing 1 M $(NH_4)_2SO_4$, 35 CV.

The *PEGylated* protein separation using HIC has been little investigated, especially as a first step in the purification process. It has been suggested that SEC followed by IEX and then HIC may form the basis for a general approach for the purification of PEG-protein conjugates [4,20]. The results shown here suggest that HIC can be used as the first stage in the *PEGylated* protein purification; however, in order to propose the general application it is necessary to optimize the process and analyze the separation of other *monoPEGylated* proteins. The results obtained here can serve as a basis for modelling elution curves of *PEGylated* conjugates and to facilitate the optimization of the purification process [18].

4. Conclusions

It is clear that HIC can be used to separate *monoPEGylated* RNase A, *diPEGylated* RNase A and native RNase A. Native RNase A could be separated completely from *PEGylated* proteins under all conditions assayed; demonstrating that the *PEGylation* affected the hydrophobicity of the protein, increasing it with increasing degree of *PEGylation*. The difference between the retention volumes of *PEGylated* proteins is higher when using a weak strength hydrophobic resin such as Butyl sepharose and ammonium sulphate mobile phase. While all proteins were soluble in sodium chloride; the resolution, yield, and purity were very low. The best conditions for the purification of *monoPEGylated* RNase A include the use of Butyl sepharose, 35 CV and 1 M ammonium sulphate; under these conditions, it is possible to obtain *monoPEGylated* RNase A with a yield as high as 85% and 97% purity.

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