Protein variant separations by cation-exchange chromatography on tentacle-type polymeric stationary phases

Michael Weitzhandler*, Dell Farnan, Judit Horvath, Jeffrey S. Rohrer, Rosanne W. Slingsby, Nebojsa Avdalovic, Chris Pohl

Dionex Corporation, Sunnyvale, CA 94088, USA

Abstract

We developed a set of prototype cation-exchange column packings that are based on a hydrophilic coated, pellicular polymeric support with a grafted tentacular surface chemistry that is highly suited to resolving closely related protein variants. These column packings (1) afford minimal band spreading in conjunction with extremely high selectivity, (2) exhibit a very hydrophilic character and (3) have moderate loading capacity. Cytochrome c variants (bovine, horse, rabbit) were baseline-separated, as was native ribonuclease A and its two deamidation products, the Asp^{67} and $isoAsp^{67}$ forms. Humanized monoclonal antibody variants differing in the presence of lysine at the C terminus of the heavy chains were baseline-resolved. Finally, the separation of hemoglobin variants found in a sample containing elevated levels of glycated hemoglobin was also demonstrated.   © 1998 Published by Elsevier Science B.V. All rights reserved.

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1. Introduction

Liquid chromatography is widely used in the quality control analysis of purified, formulated protein pharmaceuticals. High-resolution separations of protein variant forms provide information pertaining to product macro- and micro-heterogeneity that is used in assessing process consistency and product stability. High-resolution ion-exchange chromatography can be used to separate and identify protein variants that may include glycosylated [1], phosphorylated [2], deamidated [3], truncated [4] and oxidized [5] forms. All of these modifications may affect the activity or stability of the final product.

Physico–chemical characteristics associated with proteins that make the chromatographic separations of closely related variants challenging include large molecular masses and a propensity for hydrophobic interaction. The high molecular masses and large Stokes radii associated with proteins imply that they will exhibit very poor mass transfer properties even in free solution [6]. In conjunction with the anfractuous nature of the pore structure found in conventional porous particles, these low free solution diffusivities can lead to excessive band spreading under chromatographic conditions that would be suitable for small eluites. In recent years, an array of supports has been introduced with reported solutions to these problems [7]. The supports fall into three categories: macroporous, gigaporous and gel-filled gigaporous [8].

An alternative solution to the intraparticle mass transfer problem is simply to utilize polymeric particles with fluid-impervious cores in which there would not be any intraparticle mass transfer [9,10]. Such particle types should always result in higher

*Corresponding author.

efficiencies across the practical flow velocity range [11]. With tentacle-type supports, the contact between the protein analyte and the base matrix support surface is markedly reduced, which minimizes undesirable hydrophobic interactions between the analyte and the base matrix surface. Müller [12] has reported interesting properties for tentacle-type ion exchangers including (1) reduced mass transfer resistance, (2) marked changes in selectivity due to the flexibility of the charge arrangement between charged sites on the protein and the functional groups located on the grafted tentacle strands and (3) improved mass recoveries due to diminished non-specific binding.

In this study, we evaluated the performance of a set of pelicular tentacle-type cation exchangers for the separation of proteins. In the described tentacle supports, a rigid and impervious 10 μm 55% cross-linked styrenic substrate particle has been completely coated with a base stable hydrophilic neutral polymer. Tentacles bearing carboxylate (weak cation-exchange) or sulfonic acid (strong cation-exchange) groups were then grafted to the hydrophilic surface. This chemistry offers an alternative selectivity to tentacle supports attached to silica particles where silanol groups can inadvertently contribute to undesirable interactions with the protein analyte. The separation of several different types of protein variants on the new tentacle supports was examined.

2. Experimental

2.1. Apparatus

A DX-500 liquid chromatograph (Dionex, Sunnyvale, CA, USA) consisting of a Model GP40 gradient pump, an AD20 variable-wavelength absorbance detector and an AS3500 autosampler (Thermoseparations, San Jose, CA, USA) equipped with a 50-μl sample loop was used throughout this work. The chromatograph was controlled, and data collected using Dionex PeakNet version 5.01 software running on a Dell[TM] Pentium-based computer.

In order to complete stationary phase binding capacity determinations, we made breakthrough measurements. The AS3500 was replaced with an LC20 chromatographic enclosure (Dionex) in which the injection valve had been fitted with a 5-ml sample loop. Columns used in the frontal chromatography experiments were 50×4 mm and packed with the same stationary phase as was used to pack the larger, 250×4 mm columns used in the elution mode separations.

2.2. Materials

High-quality deionized water was prepared using a Millipore Milli-Q system (Bedford, MA, USA). Analytical-reagent grade crystalline mono- and dibasic sodium phosphate, glacial acetic acid and sodium acetate were purchased from J.T. Baker (Phillipsburg, NJ, USA). Analytical-reagent grade sodium chloride was obtained from Fluka (Ronkonkoma, NY, USA).

Samples of a humanized monoclonal antibody IgG1 were a generous gift from a biotechnology company. Carboxypeptidase B was supplied by Boehringer Mannheim (Indianapolis, IN, USA). The other proteins used in this work were sourced from Sigma (St. Louis, MO, USA).

2.3. Methods

Two types of buffer systems were used during the chromatography, sodium phosphate and sodium acetate. Two solutions were prepared for each system, one with and one without sodium chloride. Sodium phosphate buffer systems were prepared by dissolving appropriate quantities of mono- and dibasic sodium phosphate, to attain a chosen pH, and adding sodium chloride to the appropriate concentration in water. Sodium acetate solutions were prepared by titrating the solutions to the appropriate pH with glacial acetic acid. Protein samples used in this study were prepared by dissolving approximately 0.5 mg of each protein per ml of the mobile phase that was used at the start of the separation.

The separations were made using the prototype columns in the DX-500 chromatograph and operated at a flow-rate of 1 ml min^{-1}. Separations were performed using methods that proportioned various linear gradients of sodium chloride in the presence of indicated sodium phosphate or sodium acetate concentrations at chosen pH values. After the required gradient for the separation was completed, the col-

umn was flushed using 750 mM NaCl for 10 column volumes, then returned to the starting conditions and re-equilibrated for 10 column volumes before starting another analysis.

The column effluent was monitored by an AD20 variable-wavelength absorbance detector at a wavelength of either 254 or 280 nm. Quantification of protein peaks was accomplished using the Optimize module of the PeakNet software.

3. Results and discussion

3.1. Mixtures of proteins with different isoelectric points

Mixtures of commercially available proteins of reasonably different isoelectric points (pI values) were initially used to assess the chromatographic performance of the stationary phases. Fig. 1 is an example separation for a mixture of myoglobin, α-chymotrypsinogen, cytochrome c, ribonuclease A, and lysozyme on the weak cation-exchange (WC) column using a linear sodium chloride gradient in the presence of 10 mM sodium phosphate buffer, pH 6.

Fig. 2. Illustration of the effect of pH, salt gradient and column type on the separation selectivity. Separation of five proteins on ProPac WC and SC columns. Column: 250×4 mm. Sample: 10 µl of a solution containing approximately 0.5 mg ml^{-1} of lysozyme (peak 5), ribonuclease A (peak 4), cytochrome c (peak 3), α-chymotrypsinogen (peak 2) and myoglobin (peak 1). Eluents: A=20 mM sodium phosphate to pH 6.0; B=1 M sodium chloride plus 20 mM sodium phosphate to pH 6.0. C=20 mM sodium acetate to pH 5.0; D=1 M sodium chloride plus 20 mM sodium acetate to pH 5.0. (pH 6.0 gradient: 4–70% B in 30 min at 1 ml min^{-1}; pH 5.0 gradient: 4–60% D in 30 min at 1 ml min^{-1}).

Fig. 2 shows that selectivity changes can be demonstrated by changing either the stationary phase, salt gradient or eluent pH.

3.2. Cytochrome c species variants

Baseline separations of cytochrome c variants (bovine, horse, rabbit) that differ in three amino acid residues between bovine and horse, and in four amino acid residues between bovine and rabbit, were achieved on the strong and weak cation exchangers. Chromatograms obtained on the WC and SC columns are shown in Fig. 3. Differences in selectivity between the strong and weak cation exchangers were apparent from the different retention times of the horse variant. On the WC column, the horse cytochrome c species elutes later and much closer to the rabbit variant than on the SC column.

3.3. Monoclonal (MAb) antibody variants

Processing of C-terminal lysine and arginine residues of proteins isolated from mammalian cell culture has been described [4]. As a result of processing, C-terminal Lys or Arg residues, whose
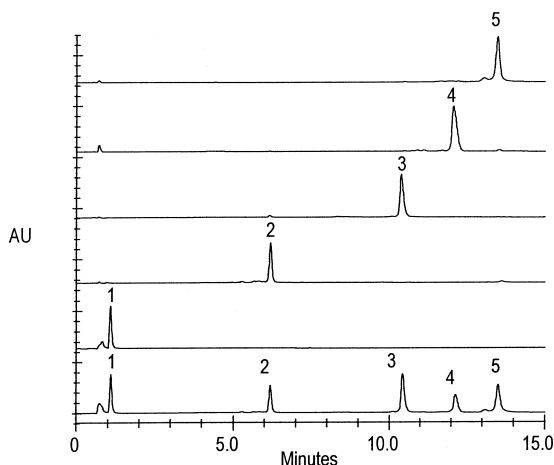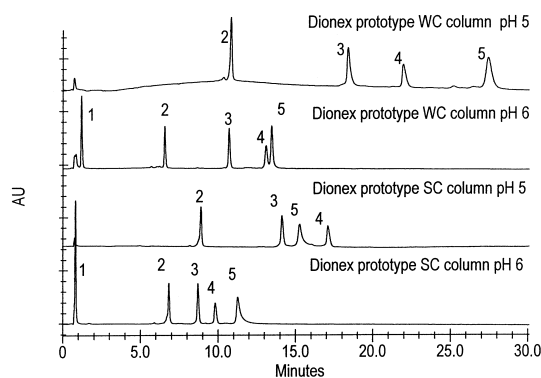

Fig. 1. Separation of five proteins on a prototype ProPac WC column. Column: 250×4 mm. Sample: 10 µl of a solution containing approximately 0.5 mg ml^{-1} of lysozyme (peak 5), ribonuclease A (peak 4), cytochrome c (peak 3), α-chymotrypsinogen (peak 2) and myoglobin (peak 1). Eluents: A=10 mM sodium phosphate to pH 6.0; B=1 M sodium chloride plus 10 mM sodium phosphate to pH 6.0. Gradient: 0–70% B in 30 min at 1 ml min^{-1}.
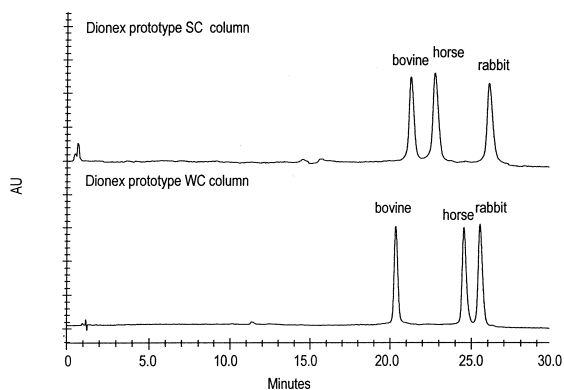
Fig. 3. Separation of three different cytochrome *c* species using either the weak or strong cation-exchange prototype ProPac columns. Eluents: A=20 m*M* sodium phosphate to pH 7.0; B=1 *M* sodium chloride plus 20 m*M* sodium phosphate to pH 7.0. Gradient: 4–15% B in 30 min at 1 ml min^{-1}.
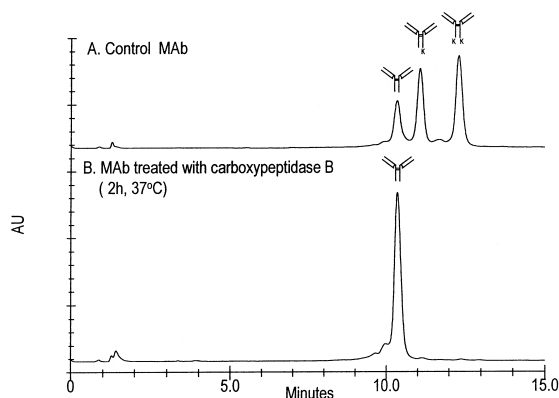

Fig. 4. Overlay of chromatograms obtained for the MAb before and after treatment with carboxypeptidase B for 2 h at 37°C. Column: 250×4 mm WC-10 cation exchanger. Sample: 10 μl of 0.5 mg of IgG per ml of eluent A. Eluents: A=10 m*M* sodium phosphate to pH 7.0; B=1 *M* sodium chloride plus 10 m*M* sodium phosphate to pH 7.0. Gradient: 4–15% B in 30 min at 1 ml min^{-1}.

presence could be expected based on gene sequence information, are often absent in proteins isolated from mammalian cell culture. This discrepancy may result from the activity of one or more basic carboxypeptidases. Charge heterogeneity can result if the processing is incomplete. The resultant charge heterogeneity of the variant forms can be identified by cation-exchange chromatography. C terminal processing of lysine residues from heavy chains of monoclonal antibodies from a variety of sources has been reported [13–17].

We assessed the performance of the tentacular cation-exchange columns for their ability to separate variants of a humanized IgG that was suspected of having variants that differed in the presence of lysine residues at the C-terminal of the heavy chains. As shown in Fig. 4, by using a shallow NaCl gradient (40–150 m*M* NaCl for 30 min) at neutral pH, it was possible to resolve three variant forms differing in the presence of lysine at the C-terminal of the heavy chains (0, 1 or 2 lysine residues). To verify that the reason for the different retention times of the three peaks was the different content of heavy chain C-terminal lysine, the IgG preparation was treated with carboxypeptidase B, an exopeptidase that specifically cleaves C-terminal lysine residues. This treatment of the IgG preparation resulted in the quantitative disappearance of peaks 2 and 3 (containing 1 and 2 terminal lysine residues, respectively, on their heavy

chains). The decreased peak areas in peaks 1 and 2 were accompanied by a corresponding quantitative increase in peak area 1 (variant with no terminal lysine, Fig. 4B), confirming that peaks 2 and 3 differed from peak 1 in that they contained IgG with 1 and 2 terminal heavy chain lysine residues, respectively.

3.4. Ribonuclease A deamidation variants

Deamidation of Asn residues or the isomerization of Asp residues occurs in a variety of protein-based pharmaceuticals including human growth hormone [18], tissue plasminogen activator [19], hirudin [20], monoclonal antibodies [21], acidic fibroblast growth factor [22] and interleukin 1 [23], with varying effects on the activity or stability of the therapeutic protein. Hence, monitoring the deamidation of Asn residues in proteins is of interest to analytical and protein chemists in quality control and process departments at biotechnology and pharmaceutical companies [24]. As described by Donato et al. [25], separation of the Asn^{67} deamidation products of ribonuclease A required cation-exchange on Mono S followed by hydrophobic interaction chromatography to resolve the two deamidation variants (Asp and isoAsp at residue 67). In contrast, using only a tentacle-type weak cation exchanger, deamidation

variant forms having Asp or isoAsp at Asn[67] were baseline separated from each other and from native ribonuclease A in a single chromatographic analysis (Fig. 5). The baseline separation made it possible to quantify the change in amounts of each form within the mixture as a function of time. From the increase in the amount of Asn[67] deamidated forms of ribonuclease A as a function of time, it was observed that the kinetics of deamidation appear to be first order with a $t_{1/2}$ of 159 h (Fig. 6).

3.5. Hemoglobin variants

Clinical laboratories frequently separate and quantify the levels of different hemoglobin variants. For the physician, the determination of glycated hemoglobin levels in the blood of a diabetic serves as an excellent indication of the average glucose level in the patient's blood during the preceding 1–2 months [26,27]. Also, separating and identifying hemoglobins associated with serious haemopathies, including sickle cell, hemoglobin C and Barts disease, is also extremely important in the diagnosis, treatment and counseling of afflicted children. In both cases, the availability of a new, high resolution, cation-exchange chromatographic column can make routine analyses easier and facilitate the identification of new

Time [hours]	Area [mA.s] Peak 1	Peak 2 and 3	A/A0
0	221104	0	0
0	219491	0	0
50	208246	42919	0.19
50	203191	41676	0.19
100	156265	77562	0.35
100	152843	75372	0.34
200	93278	135469	0.61
200	92089	135161	0.61

$$y = 0.003x + 0.0234$$
$$R^2 = 0.9924$$

Fig. 6. Fractional amount of deamidation products formed as a function of time when ribonuclease A (3 mg ml^{-1}) was incubated in 1% ammonium carbonate buffer, pH 8.2, at 37°C.

hemoglobin variants that may be resolved with the enhanced column performance.

In Fig. 7, we have demonstrated the separation of hemoglobin variants found in a sample known to contain elevated levels of glycated hemoglobin. The peaks are labeled in accordance with the conventions specified previously [28]. This sample (Sigma) had glycated hemoglobin at about 10% of the total hemoglobin.

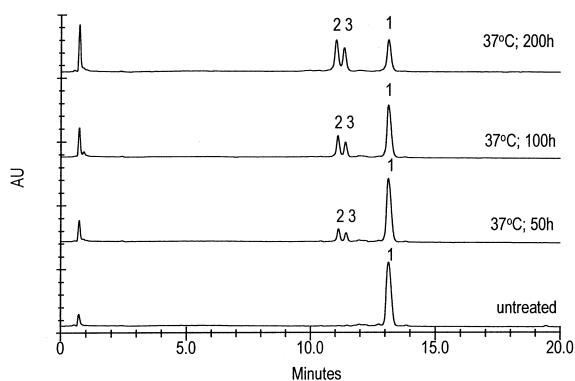The chromatogram reveals the presence of numerous glycated forms of hemoglobin, which is expected

Fig. 5. Chromatograms showing the separation of ribonuclease A and its two deamidation products at several time points during the course of the forced deamidation. Native ribonuclease A is peak 1. The ribonuclease A deamidation products are peaks 2 and 3. Column: 250×4 mm I.D. ProPac WC-10 cation exchanger. Eluents: A=10 mM sodium phosphate to pH 6.0; B=1 M sodium chloride plus 10 mM sodium phosphate to pH 6.0. Gradient: 4–70% B in 30 min at 1 ml min^{-1}.
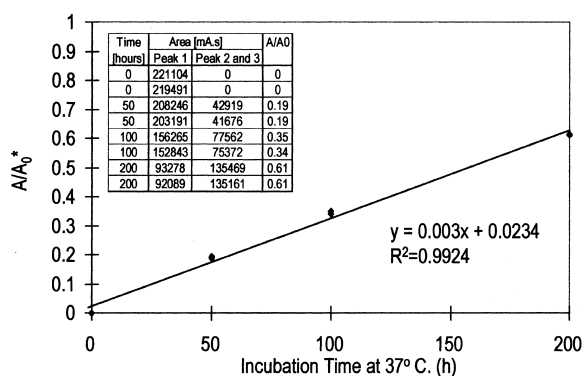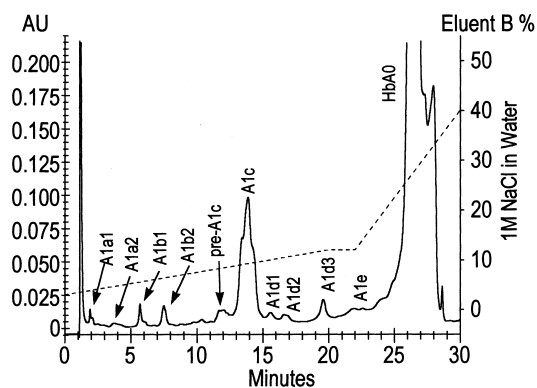

Fig. 7. Separation of hemoglobin variants using a sample known to contain elevated levels of glycated hemoglobins. Column: 250×4 mm ProPac SC-10 cation exchanger. Eluents: A=50 mM sodium phosphate and 2 mM potassium cyanide to pH 6.0; B=0.5 M NaCl, 50 mM sodium phosphate and 2 mM potassium cyanide to pH 6.0. Gradient: at 1 ml min^{-1}: 0 min, 3% B; 20 min, 12% B; 30 min, 40% B.

because such a reaction occurs non-enzymatically between the hemoglobin and sugars in the blood. In principle, non-enymatic glycation can occur with any NH_2 group in the hemoglobin protein (e.g., at the N-terminus of the protein chains or on the side chains of lysine residues) [29]. The major glycated component, HbAc1, is formed when the N-terminals of the protein chains react with glucose, although other forms have been identified and described elsewhere see e.g., Refs. [30,31].

A separation of several hemoglobin sequence variants, including sickle cell hemoglobin, fetal hemoglobin and hemoglobin C, is shown in Fig. 8, where the low mass transfer resistance associated with pellicular particles has resulted in narrow peaks and, consequently, a very high level of resolution. Also, putatively identified on the chromatogram are HbA_2 and $HbA1_c$ which were also apparently present in the sample mixture.

It is apparent from the chromatograms shown in Figs. 7 and 8 that the strong cation-exchange column demonstrates significant selectivity with regard to the hemoglobin variants, and could prove to be a powerful tool in the routine clinical analysis associated with such conditions as well as affording a platform to separate hemoglobin variants from newly discovered haemopathies.

3.6. Stationary phase characterization

To document the hydrophilic nature of the base matrix of the tentacular weak and strong cation exchangers, several proteins (lysozyme, ribonuclease A, and cytochrome c) were chromatographed isocratically in 10 mM sodium phosphate, pH 6.5, at several sodium chloride concentrations. This assay has been used to document the presence or absence of hydrophobic interactions for a variety of proteins on different ion-exchange stationary phases [32,33]. Further, this assay demonstrates that if hydrophobic electrostatic interactions exist between the protein and the stationary phase, a plot of retention factors against salt concentration results in a "u" shaped curve. The increase in retention factor with salt concentration is due to hydrophobic interactions. Because of difficulties in calculating retention factors when the value is less than unity, we plotted the variation in the retention time with NaCl concentration, instead of the retention factor. The constancy of the retention times for the different proteins on both cation exchangers (see Fig. 9) suggests that even at higher salt concentrations, 0.5–2.0 M NaCl, no secondary hydrophobic interactions occur between the stationary phases and the proteins tested. At lower NaCl concentrations, <0.5 M NaCl, the retention times exhibit a strong dependence on the
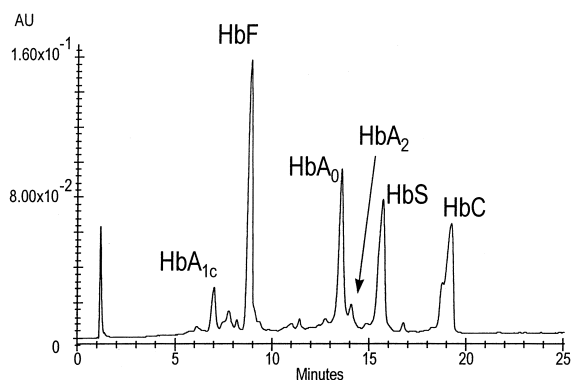

Fig. 8. Cation-exchange chromatographic separation of four hemoglobin (Hb) variants including fetal, sickle cell, normal and C hemoglobins. Column: 250×4 mm ProPac SC-10 cation exchanger. Eluents: A=50 mM sodium phosphate and 2 mM potassium cyanide to pH 6.0; B=0.5 M NaCl, 50 mM sodium phosphate and 2 mM potassium cyanide to pH 6.0. Gradient: at 1 ml min^{-1}: 0–50% B in 30 min.
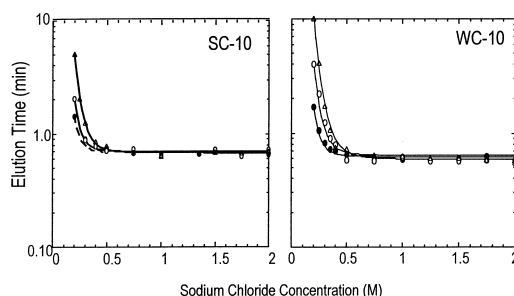

Fig. 9. Dependence of elution times for three proteins as a function of sodium chloride concentration on the prototype weak and strong cation exchangers. Columns: 250×4 mm ProPac SC-10 and WC-10 cation exchangers. Samples: 10 μl of solution containing 0.5 mg of each protein: lysozyme (△), cytochrome c (○) and ribonuclease A (●) in the mobile phase. Isocratic elutions were made using appropriate amounts of sodium chloride plus 10 mM sodium phosphate to pH 6.5.
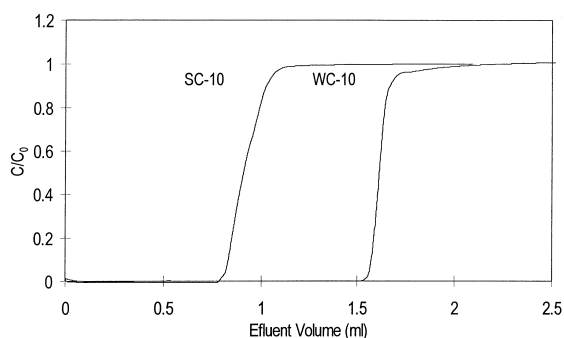
Fig. 10. Breakthrough curves used to estimate the binding capacity of lysozyme on the column packing. Column: 50×4 mm I.D. WC or SC. Feed: 3 mg of lysozyme per ml of 10 mM sodium phosphate to pH 6.5 at 230 cm h^{-1}.

NaCl concentration, as is characteristic in ion-exchange chromatography for proteins [34].

Finally, we evaluated the protein adsorption capacity of the tentacle weak and strong cation exchangers by conducting breakthrough studies with lysozyme at a feed concentration of 3 mg ml^{-1} loaded on the column, using 10 mM sodium phosphate buffer, pH 6.5, at a low flow velocities, <230 cm h^{-1} (see Fig. 10). The binding capacity of the tentacular weak and strong cation exchangers was 6 and 3 mg of lysozyme per ml of column, respectively. Thus, one can comfortably load 30–40 μg of protein on the column without overloading it. Although these capacity values are about a factor of 5–10 lower than normally observed for porous supports, the capacity of the ProPac stationary phase is more than adequate for analytical purposes.

4. Conclusions

Tentacle-type cation exchangers are expected to be most useful for high resolution separation of protein variants usually encountered in assessments of protein macro- and micro-heterogeneity. Determinations of protein micro-heterogeneity are used to assess process consistency and product stability in the production of protein therapeutics. The high-resolution separations of charge variants afforded by the cation-exchange columns, as well as the opportunity of using the cation-exchange columns to isolate fractions for further analysis, offer a convenient and practical alternative to isoelectric focusing gels, a manual technique often described as being tedious and of low preparative value.

Acknowledgements

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