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Antibiotics as low-molecular-mass displacers in ion-exchange displacement chromatography

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Abstract

While the ability to carry out simultaneous concentration and purification in a single displacement step has significant advantages for downstream processing of pharmaceuticals, a major impediment to the implementation of displacement chromatography has been the lack of suitable displacer compounds. An important recent advance in the state-of-the-art of displacement chromatography has been the discovery that low-molecular-mass dendritic polymers and protected amino acids can be successfully employed as displacers for protein purification in ion-exchange systems. In this paper, the efficacy of aminoglycosidic antibiotic displacers are investigated for protein purification in cation-exchange systems. The displacers neomycin B and streptomycin A are employed with model feed mixtures containing moderately to very strongly retained proteins. These experiments demonstrate that this new class of low-molecular-mass antibiotic displacers can indeed act as efficient protein displacers. In fact, the displacer neomycin B is the first low-molecular-mass displacer reported in the literature which can readily displace very strongly retained proteins such as lysozyme. In addition to the fundamental interest generated by low-molecular-mass displacers, it is likely that these displacers will have significant operational advantages as compared to large polyelectrolyte displacers.

1. Introduction

Preparative ion-exchange chromatography is widely employed in continuous and step gradient modes of operation for the purification of proteins [1,2]. Although, displacement chromatography has been shown to hold significant promise for high resolution preparative separations, a major impediment to the implementation of displacement chromatography has been the lack of suitable displacer compounds.

Operationally displacement chromatography is performed in a manner similar to step-gradient chromatography, where the column is subjected to sequential step changes in the inlet conditions. The column is initially equilibrated with a relatively low ionic strength carrier buffer. The feed mixture is then introduced into the column followed by a constant infusion of the displacer solution. The displacer is selected such that it has a higher affinity for the stationary phase than any of the feed components. Under appropriate conditions, the displacer induces the feed components to develop into adjacent 'square-wave' zones of highly concentrated pure material. After the breakthrough of the displacer from the column effluent, the column is regenerated and re-equilibrated with the carrier buffer.

The displacer, having a higher affinity than any of the feed components, competes effective-

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ly, under non-linear conditions, for the adsorption sites on the stationary phase. An important distinction between displacement and gradient chromatography is that the displacer front always remains *behind* the adjacent feed zones in the displacement train, while desorbents (e.g., salt, organic modifiers) move *through* the feed zones. The implications of this distinction are quite significant in that displacement chromatography can potentially concentrate and purify components from mixtures having low separation factors while in the case of step-gradient chromatography, relatively large separation factors are often required to give satisfactory resolution.

Ion-exchange displacement chromatography of proteins has been studied by several investigators. Peterson and co-workers have used carboxymethyl dextrans as displacers in anion-exchange displacement chromatography [3–5]. Horvath and co-workers have employed chondroitin sulfate to displace β-galactosidase [6], and β-lactoglobulins [7,8]. Ghose and Mattiason [9] have examined the purification of lactate dehydrogenase using a carboxymethyl-starch displacer. Jen and Pinto have employed polyvinyl sulfonic acid [10] and dextran sulfate [11] to separate a mixture of moderately retained proteins, conalbumin and ovalbumin. Cramer and co-workers [12–16] have identified a variety of efficient polyelectrolyte displacers for protein purification including DEAE-dextran, dextran sulfate, protamine, heparin, and pentosan polysulfate. Patrickios et al. [17] have employed block methacrylic polyampholytes as protein displacers in anion-exchange systems.

Until recently, all reports in the literature have employed relatively large polyelectrolytes (>2000 daltons) as displacers for protein purification in ion-exchange systems. An important recent advance in the state-of-the-art of displacement chromatography has been the discovery that low-molecular-mass (<2000 daltons) displacers can be successfully employed for protein purification in ion-exchange systems. Pentaerythritol based dendritic polyelectrolytes ranging in molecular mass from 480–5100 daltons have been shown to be effective displacers for protein purification [18]. Although all three 'generations' of dendrimers had sufficient affinity to displace the proteins, the 480 molecular mass dendrimer produced a higher induced salt gradient as compared to the higher generation dendrimers. Low-molecular-mass protected amino acid displacers have also been shown to be effective displacers of proteins in cation-exchange systems [19]. Furthermore, these displacers have been used for high resolution separations of cytochrome *c* variants [20]. The ability of low-molecular-mass compounds to displace proteins is very significant in that it represents a major departure from the conventional wisdom that large polyelectrolyte polymers are required to displace proteins in ion-exchange systems. In this paper, the efficacy of a new class of low-molecular-mass displacers, aminoglycosidic antibiotics, is investigated for protein purification in cation-exchange systems.

2. Experimental

2.1. Materials

Strong cation-exchange(SCX) (sulfopropyl, 8 μm, 100×5 mm I.D.) columns were donated by Millipore (Waters Chromatography Division, Millipore, Milford, MA, USA). Sodium monobasic phosphate, sodium dibasic phosphate and all proteins were purchased from Sigma (St. Louis, MO, USA). Streptomycin A sulfate and neomycin B sulfate were purchased from Sigma.

2.2. Apparatus

All displacement experiments were carried out using a Model LC 2150 pump (LKB, Bromma, Sweden) connected to the chromatographic columns via a Model C10W 10-port valve (Valco, Houston, TX, USA). Fractions of the column effluent were collected using LKB 2212 Helirac fraction collector (LKB). Protein analysis of the collected fractions was carried out using a Model Waters 590 HPLC pump (Waters), a Model 7125 sampling valve (Rheodyne, Cotati, CA, USA), a spectroflow 757 UV–Vis absorbance detector (Applied Biosystems, Foster City, CA, USA) and a Model C-R3A Chromatographic integrator

(Shimadzu, Kyoto, Japan). Sodium analysis was done using a Perkin-Elmer, Model 3030 (Perkin-Elmer, Norwalk, CT, USA) atomic absorption spectrophotometer. Displacer analysis was carried out on a Model R401 differential refractometer (Waters).

2.3. Procedures

Determination of adsorption isotherms, steric factors, and characteristic charges of displacers

Adsorption isotherms were determined by sequential frontal chromatography according to the method of Jacobson et al. [21]. The steric factor was determined from the breakthrough volume of non-linear frontal chromatographic experiments. The characteristic charge was determined from the induced salt gradients produced during these experiments. A detailed description of these parameter estimation techniques is described elsewhere [22].

Operation of displacement chromatography

In all displacement experiments, the columns were initially equilibrated with the carrier and then sequentially perfused with feed, displacer and regenerant solutions. The feed and the displacer solution were prepared in the same buffer as the carrier. Fractions of the column effluent were collected directly from the column outlet to avoid extra-column dispersion of the purified components.

Displacement chromatography of proteins in cation-exchange systems

Two/Three-Component separations. Feed mixtures of α-chymotrypsinogen A and cytochrome *c* and lysozyme were separated by displacement chromatography using the displacers in a SCX column. The feed load, displacer molecular mass and concentrations employed for each separation are given in the figure legends of the corresponding displacement chromatograms. All displacement experiments were carried out at room temperature (25°C) at a flow-rate of 0.2 ml/min. The pH of the carrier was always 6.0. Fractions of 200 μl (unless otherwise mentioned) were collected for subsequent analysis of the protein, displacer and sodium ion concentrations in the effluent.

Protein analysis by HPLC

Protein analyses of the fractions collected during the displacement experiments were performed by ion-exchange HPLC under isocratic elution conditions. The mobile phase used for the analysis of α-chymotrypsinogen A and cytochrome *c* was 160 m*M* sodium phosphate at a pH of 6.0. Lysozyme was analyzed using a 350 m*M* sodium phosphate, pH 6.0 solution. Displacement fractions were diluted 10–100 fold, with the eluent and 20 μl samples were injected at a flow-rate of 0.5 ml/min. The column effluent was monitored at 280 nm.

Displacer breakthrough volumes

The breakthrough volumes of the aminoglycoside antibiotics were determined from separate frontal chromatographic experiments using an on-line differential refractive index detector.

Analysis of the sodium ion in the mobile phase

Sodium ion analysis was carried out using atomic absorption spectroscopy. Effluent fractions were diluted 3000 fold in plastic tubes in 5 g/l cesium chloride solution (to minimize background noise) and their amounts quantitated against known Na^+ ion standards (10–50 μM).

Evaluation of column regeneration

Various column regeneration protocols were evaluated by comparing the breakthrough volume of a known displacer solution during frontal chromatography both before and after column regeneration. Regeneration protocols are presented in Table 2. (Note: after each regeneration, the column was equilibrated with five column volumes of the carrier buffer.)

3. Results and discussion

As described in the introduction, an important recent advance in the state-of-the-art of displacement chromatography has been the discovery that low-molecular-mass dendritic polymers and

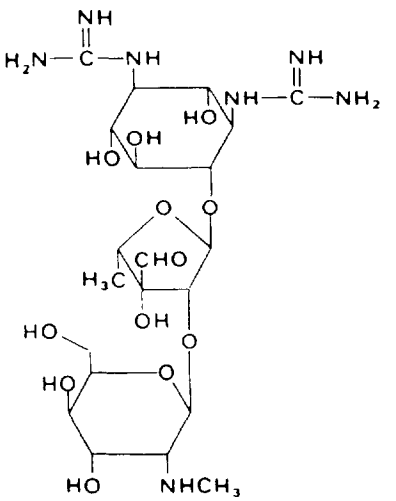

Fig. 1. Structure of streptomycin A.

protected amino acids can be successfully employed as displacers for protein purification in ion-exchange systems. In this paper, the efficacy of a new class of low-molecular-mass displacers, aminoglycosidic antibiotics, is investigated for protein purification in cation-exchange systems. The antibiotics streptomycin A (Fig. 1) and neomycin B (Fig. 2) were employed as displacers with model feed mixtures containing moderately to very strongly retained proteins. As seen in Fig. 1, streptomycin has two closely spaced guanidino functionalities as well as a single secondary amine at the opposite end of the molecule. Neomycin B contains six primary amines, with each glucose ring bearing a pair of primary amines (Fig. 2). The adsorption isotherms of streptomycin A and neomycin B in a mobile phase carrier containing 30 m*M* sodium ions at pH 6.0 are shown in Fig. 3. As seen in the figure, these antibiotics exhibit essentially square adsorption isotherms, indicating high affinity for the stationary phase material.

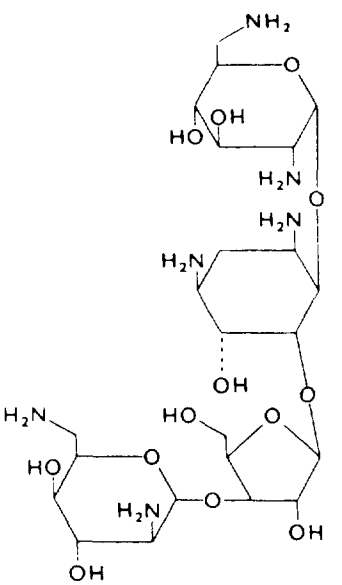

Fig. 2. Structure of neomycin B.

The induced salt gradient produced by the displacer front plays an important role in the behavior of these displacement systems [22,23]. A schematic of an isotachic displacement train is presented in Fig. 4. As seen in the figure, the breakthrough of the displacer front coincides with the reduction of the salt concentration to the carrier conditions. It turns out that the important displacer design parameter for controlling induced salt gradients is the ratio of the steric factor to the characteristic charge for a given displacer. The characteristic charge (ν_i) is the number of salt counterions displaced from the stationary phase by the adsorbed displacer. The steric factor (σ_i) represents the number of salt counterions sterically hindered by the adsorbed displacer and represents the non-linear adsorption behavior of the displacer. These *sterically* hindered salt counterions are *unavailable* for exchange with other solutes in free solution. Table 1 presents the characteristic charge (ν), steric factor (σ), and σ/ν ratios of the antibiotic displacers. In contrast to protected amino acid displacers reported elsewhere [19], these antibiotic displacers had measurable steric factors. The σ/ν ratios, although lower than high-molecular-mass displacers, is comparable to the dendritic polymers [18] and polyelectrolyte displacers such as protamine [14] and pentosan polysulfate [16].

A displacement experiment was carried out using streptomycin A as the displacer and α-chymotrypsinogen A and cytochrome *c* as the feed proteins. As seen in Fig. 5b streptomycin was very effective at displacing both of these proteins. The salt microenvironment in the pro-

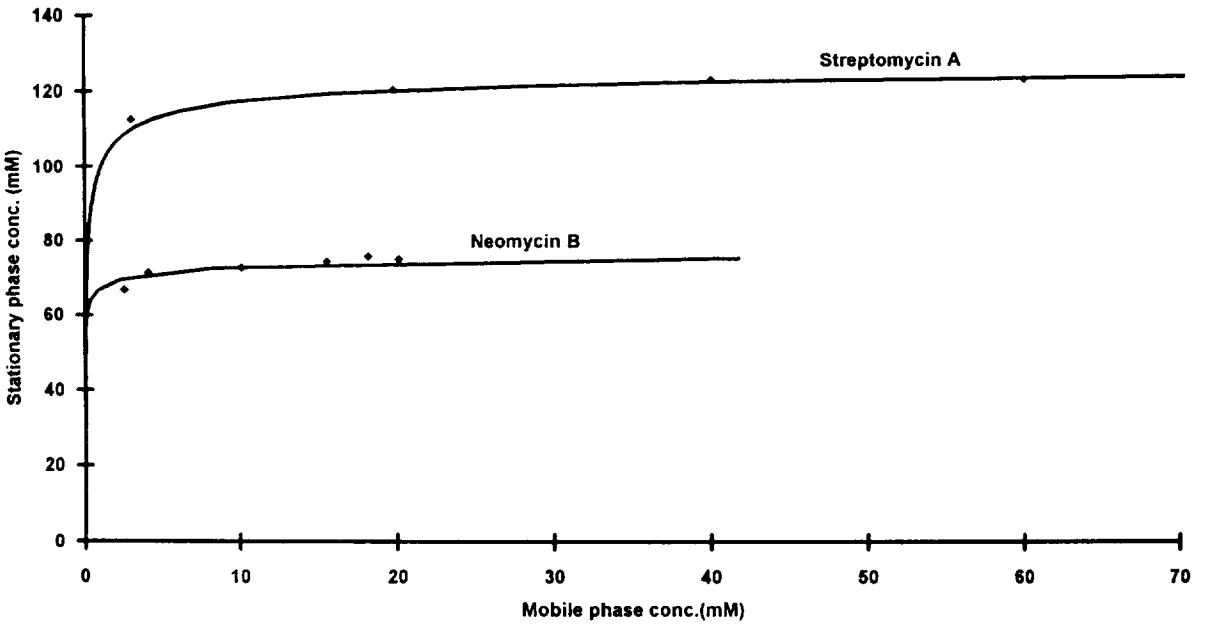

Fig. 3. Adsorption isotherm of neomycin B and streptomycin A. Carrier: 30 m*M* of Na^+, pH 6.0; column: 100 × 5 mm I.D. cation-exchanger (8 μm).

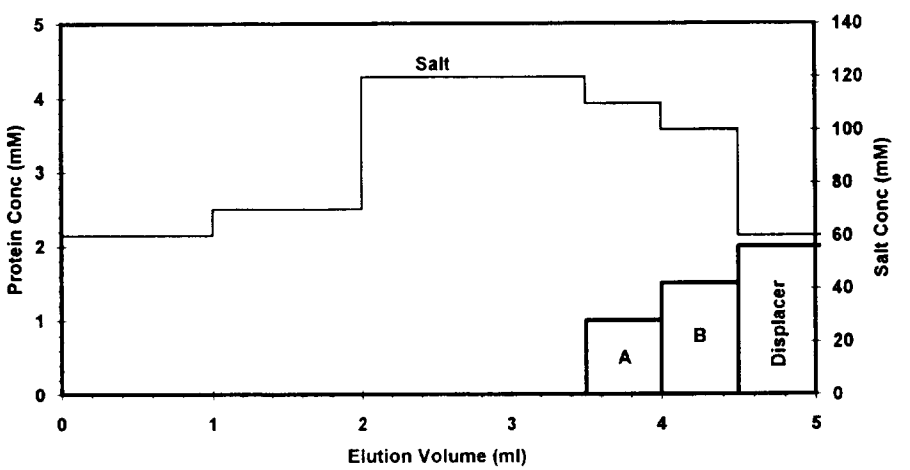

Fig. 4. Ideal isotachic displacement profile and corresponding induced salt gradient.

Table 1
SMA parameters for antibiotic displacers

Displacer	Char. charge (ν)	Steric factor (σ)	σ/ν
Neomycin B	4.5	2.725	0.606
Streptomycin A	2.3	2.281	0.992

tein displacement zones increased to approximately 95 m*M* due to the induced gradients. The displacer front and the cytochrome *c* 'tail' were very sharp, despite the relatively low salt concentration employed in the carrier. A separate frontal chromatographic experiment was carried out using on-line refractive index detection to

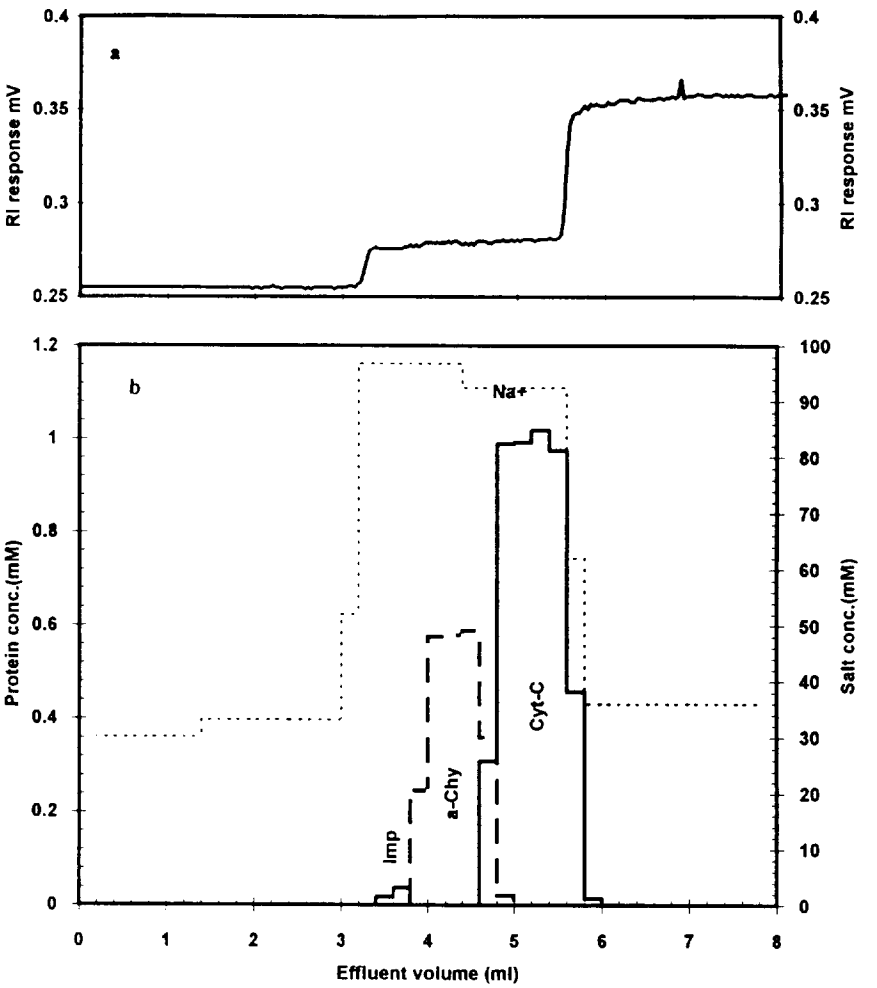

Fig. 5. (a) Breakthrough front of streptomycin A as measured by a refractive index detector. Carrier: 30 m*M* of Na^+, pH 6.0; displacer: 30 m*M* of streptomycin A in carrier; flow-rate: 0.2 ml/min; column: 100 × 5 mm I.D. cation-exchanger (8 μm). (b) Displacement separation of a mixture of two proteins. Carrier: 30 m*M* of Na^+, pH 6.0; feed: 0.275 m*M* of α-chymotrypsinogen A and 0.5536 m*M* of cytochrome *c* in 1.8 ml of the carrier; displacer: 30 m*M* of streptomycin A in carrier; flow-rate: 0.2 ml/min; column: 100 × 5 mm I.D. cation-exchanger (8 μm).

confirm the breakthrough volume of the displacer (Fig. 5a). As seen in the figure, both the induced salt gradient and the breakthrough of the displacer front are in quantitative agreement in the frontal and displacement experiments. Accordingly, the breakthrough volume of the displacer front was determined in all subsequent displacements via a separate frontal experiment and from information on the induced salt profiles. In all cases, the displacer breakthrough volume in the frontal experiment coincided exactly with the induced salt profiles in the displacement experiments.

A displacement experiment was also carried out using neomycin B as the displacer and α-chymotrypsinogen A and cytochrome *c* as the feed proteins (Fig. 6). All conditions were comparable between the displacement experiments shown in Figs. 5 and 6 with the exception of the displacer concentrations which were selected to give comparable breakthrough volumes. As seen in Fig. 6, neomycin B was also a very effective

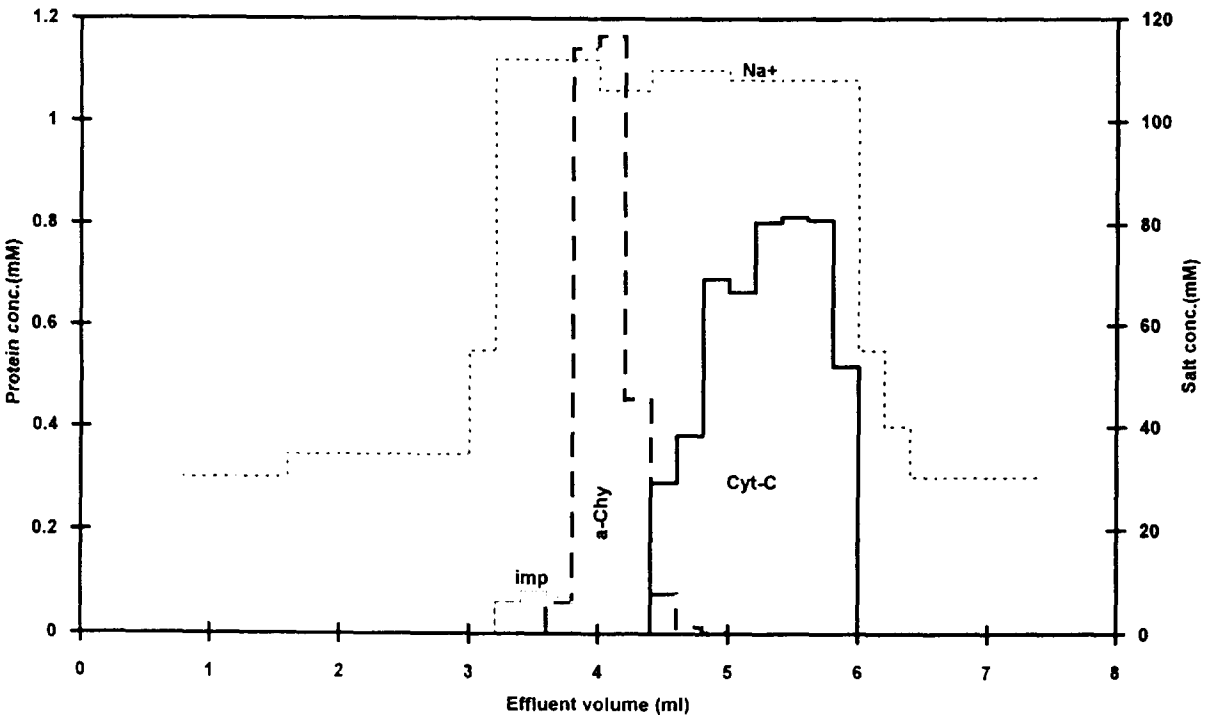

Fig. 6. Displacement separation of a mixture of two proteins. Carrier: 30 mM of Na^+, pH 6.0; feed: 0.32 mM of α-chymotrypsinogen A and 0.57 mM of cytochrome c in 1.8 ml of the carrier; displacer: 18.15 mM of neomycin B in carrier; flow-rate: 0.2 ml/min; column: 100 × 5 mm I.D. cation-exchanger (8 μm).

displacer for these proteins under these low salt, high binding conditions. As described above for a given breakthrough volume, the σ/ν ratio is a key parameter for determining induced salt gradients [23]. Thus, the neomycin B, which has a lower σ/ν ratio as compared to streptomycin A would be expected to produce a higher induced salt gradient. As seen in the figures, the salt microenvironment in the protein displacement zones increased to approximately 110 mM in contrast to 95 mM for the streptomycin A experiment. Although the rear of the cytochrome c zone is well displaced by the neomycin B, this separation was characterized by non-development of the displacement train due to this relatively high induced salt gradient.

Thus, these low-molecular-mass antibiotic displacers are readily able to displace well retained proteins (e.g. cytochrome c) in cation-exchange systems. In fact, the dendritic and protected amino acid displacers [18,19] were also able to displace these proteins under appropriate operating conditions. To date, only large polyelectrolyte displacers (e.g. 10 000 and 40 000 daltons DEAE-dextran) have been shown to be able to displace very strongly retained proteins such as lysozyme in cation-exchange systems.

Displacement experiments were carried out with the antibiotic displacers to determine their efficacy at displacing feed mixtures containing lysozyme. The experimental conditions were selected such that the same salt microenvironments in the protein zones were attained in both displacement experiments. Since the weakly retained protein α-chymotrypsinogen A would elute ahead of the displacement zone at these relatively high induced salt gradient conditions, the experiments were carried out with two component mixtures containing cytochrome c and lysozyme. The experiment carried out with streptomycin A resulted in displacement of cytochrome c and desorption of lysozyme (Fig. 7). In

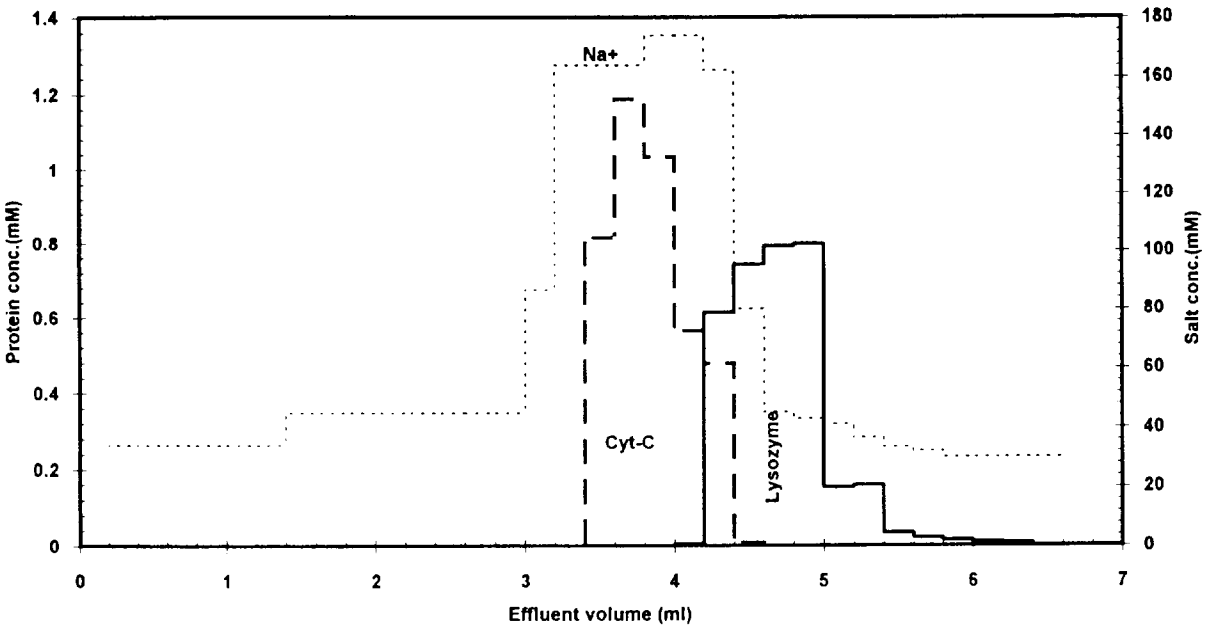

Fig. 7. Displacement chromatogram of a mixture of two proteins. Mobile phase: 30 m*M* of Na^+, pH 6.0; feed: 0.5 m*M* of cytochrome *c* and 0.57 m*M* of lysozyme, in 1.8 ml of the mobile phase; displacer: 60 m*M* of streptomycin A in mobile phase; column: 100 × 5 mm I.D. cation-exchanger (8 μm); flow-rate: 0.2 ml/min.

contrast, the experiment carried out with neomycin B resulted in displacement of both proteins (Fig. 8). Thus, even though the concentration of streptomycin A was two times that of neomycin B, the 'affinity' of the streptomycin was insufficient to displace lysozyme. On the other hand, the neomycin B had sufficient 'affinity' to displace the lysozyme at these relatively low carrier salt conditions. The lack of tailing of the lysozyme into the neomycin B displacer zone is another indication of the relatively high affinity of this low-molecular-mass displacer. In fact, the displacer neomycin B is the first low-molecular-mass displacer reported in the literature which can readily displace very strongly retained proteins such as lysozyme. Although streptomycin A contains two strongly basic guanidino moieties, its affinity is less than neomycin B which contains primary amines. As described above, the characteristic charges of streptomycin and neomycin B are 2.3 and 4.5, respectively. Thus, it is possible to get higher affinities with weaker functionalities, by increasing the number of interactions per molecule with the stationary phase material. A theoretical treatment of affinity in ion-exchange displacement systems is presented elsewhere [23,24].

Neomycin B was employed in a subsequent displacement experiment with the three proteins α-chymotrypsinogen A, cytochrome *c*, and lysozyme. In order to prevent elution of the least retained protein α-chymotrypsinogen A, the displacer concentration was reduced to 15.5 m*M* so that the salt microenvironment in the protein zones would be approximately 95 m*M*. As explained in Fig. 4, the introduction of the displacer results in an induced salt gradient ahead of the displacer front. Thus, higher displacer concentrations would result in higher salt microenvironments and concomitant elution of the α-chymotrypsinogen A. The results of this displacement experiment are shown in Fig. 9. As seen in the figure, even at these relatively low displacer and mobile phase salt concentrations, the neomycin B displacer was readily able to displace lysozyme. The feed load in this experi-

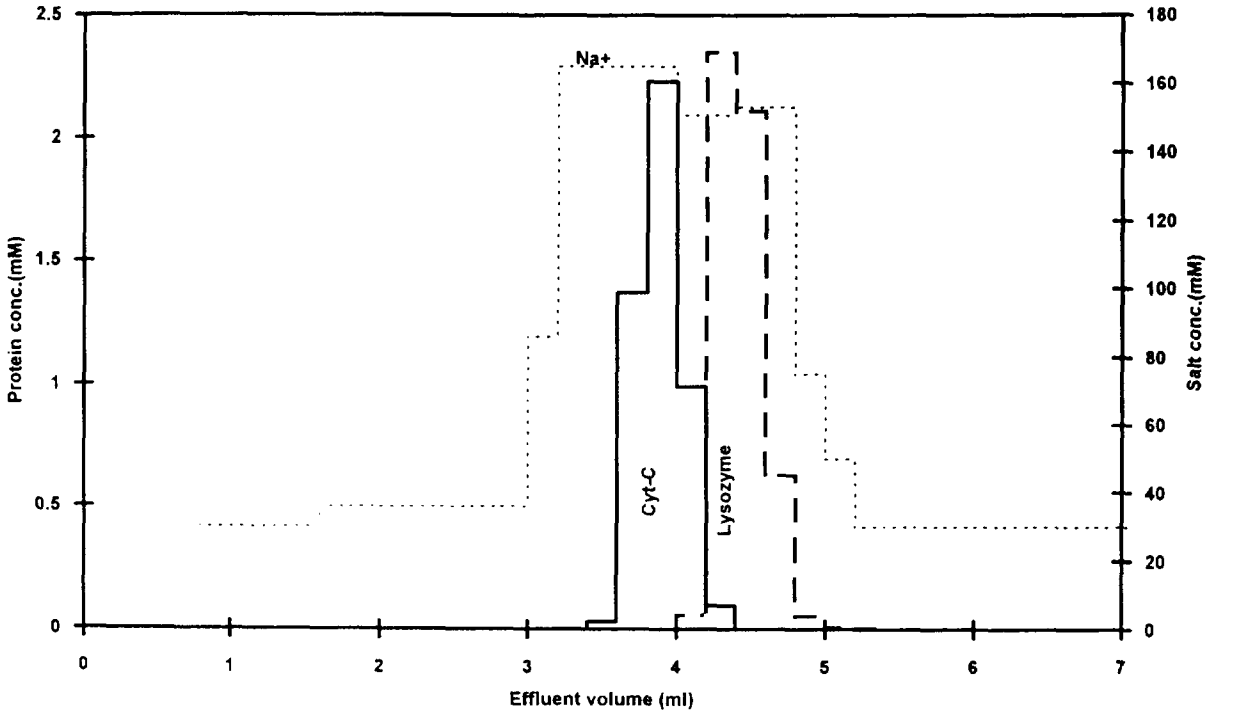

Fig. 8. Displacement chromatogram of a mixture of two proteins. Mobile phase: 30 m*M* of Na^+, pH 6.0; feed: 0.52 m*M* of cytochrome *c* and 0.573 m*M* of lysozyme, in 1.8 ml of the mobile phase; displacer: 30 m*M* of neomycin B in mobile phase; column: 100 × 5 mm I.D. cation-exchanger (8 μm); flow-rate: 0.2 ml/min.

ment was 52 mg of total protein on the 10 cm analytical column. These results are important in that they demonstrate that low-molecular-mass displacers can be employed for the purification of weakly to very strongly retained proteins under high column loadings. The slight degree of tailing of lysozyme into the displacer zone in Fig. 9 is due to the low carrier salt and relatively low displacer concentration employed in the experiment.

The efficacy of various column regeneration protocols were evaluated for the neomycin B displacer. As seen in Table 2, this low-molecular-mass displacer can be readily removed from the column under relatively high pH and salt conditions. In addition, regeneration at the operating pH of 6.0 can be carried out using 10 columns volumes of the 1.5 *M* regenerant solution. While these regenerant conditions are less extreme than those required for large-molecular-mass displacers, they represent a slightly more strenuous regeneration protocol than those required for low-molecular-mass amino acid based displacers [19].

4. Conclusions

In this paper a new class of low-molecular-mass displacers, aminoglycosidic antibiotics, have been shown to be effective displacers for protein purification in cation-exchange systems. While streptomycin A and neomycin B were effective displacers for the proteins α-chymotrypsinogen A and cytochrome *c*, the displacer neomycin B was also able to displace lysozyme. Furthermore, the displacement of lysozyme was carried out at relatively low displacer concentrations, resulting in lower induced salt gradients and concomitant higher protein concentrations. In fact, neomycin B is the first low-molecular-mass displacer reported in the literature which

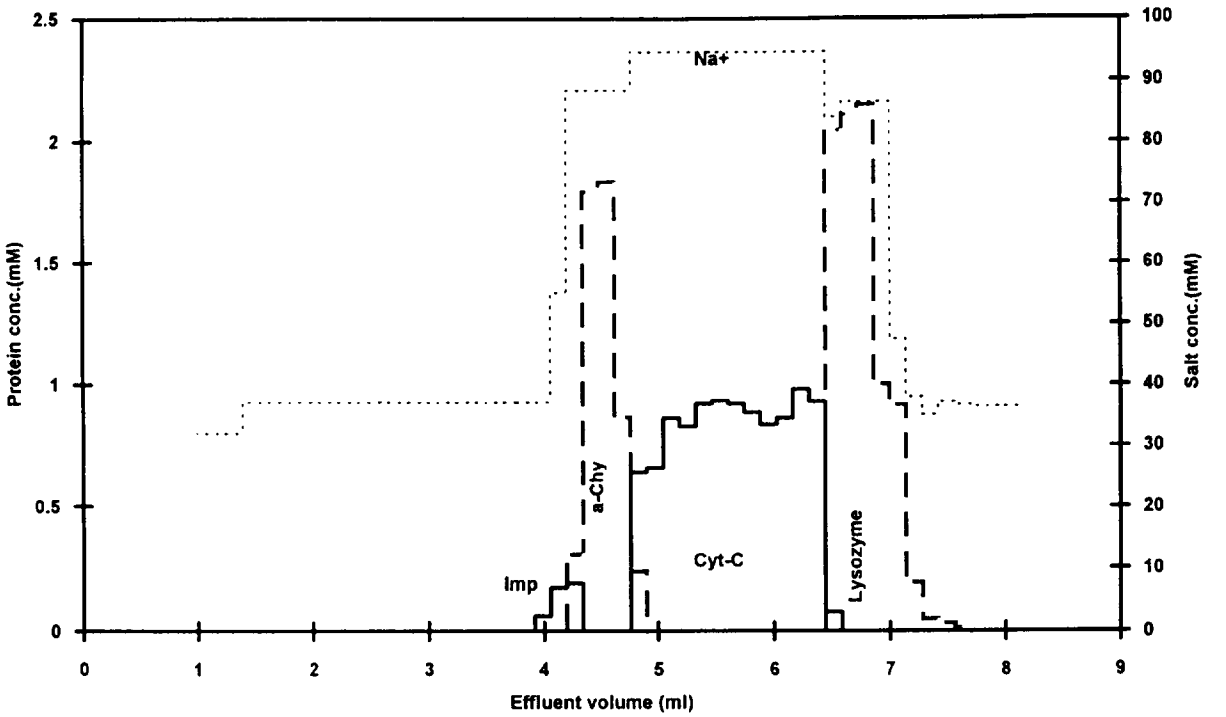

Fig. 9. Displacement separation of a mixture of three proteins. Carrier: 30 m*M* of Na^+ pH 6.0; feed: 0.25 m*M* of α-chymotrypsinogen A and 0.52 m*M* of cytochrome *c* and 0.42 m*M* of lysozyme in 2.8 ml of the carrier; displacer: 15.5 m*M* of neomycin B in carrier; flow-rate: 0.2 ml/min; column: 100 × 5 mm I.D. cation-exchanger (8 μm); fraction collection: 140 μl.

has been shown to displace very strongly retained proteins. The protein concentrations obtained using neomycin B as the displacer were comparable with those obtained using high-molecular-mass displacers. In addition to having sufficiently high affinity for the stationary phase material, these antibiotic displacers are relatively inexpensive and non-toxic which makes them potential candidates for large-scale protein purification. Furthermore, if there is any overlap between the displacer and the protein of interest, these low-molecular-mass displacers can be readily separated from the purified protein zones during post-displacement downstream processing involving size-based purification methods (e.g. size exclusion chromatography, ultrafiltration). A detailed investigation into the mechanisms and efficacy of a wide spectrum of low-molecular-mass displacers is currently underway in our laboratory and will be the subject of future reports.

Table 2
Efficacy of regeneration protocols

No. of column volumes	% regenerated
Regenerant: 1.5 M Na^+, pH 11.00	
2.0	96.6
3.2	97.8
4.8	100
Regenerant: 1.5 M Na^+, pH 6.0	
2.0	95.1
3.2	95.9
4.8	97.0
10	100

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