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Charged fusions for β-galactosidase retention in anion-exchange chromatography

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Abstract

The retention behavior of a series of negatively charged β-galactosidase fusion proteins carrying an additional 1 (designated BGCD1), 5 (BGCD5), 11 (BGCD11), and 16 (BGCD16) aspartate residues was studied. The added tails promoted protein retention in order of tail length and were most effective closer to the isoelectric point. The two parameters Z and I, obtained from the stoichiometric displacement model, were used to characterize the extent of binding between the protein and the ion-exchange surface. At pH 5.7, the Z number increased with tail length (charge) and was 11.5, 8.5, 6.9 and 5.3 for BGCD11, BGCD5, BGCD1 and wild type β-galactosidase (BGWT), respectively. At these conditions, the fusions had very similar I values which were five times smaller than that of BGWT. However, the increase in Z numbers outweighed the decrease in I values and retention was enhanced. When BGWT was brought to the same net charge (by increasing the mobile phase pH) as each of the fusions, the Z number was similar to that of the corresponding fusion. However, the I value decreased with increasing pH (net charge) and was lower than that of the corresponding fusion by factors of 25–500. Consequently, despite the similar Z numbers, the fusions still had higher retention.

1. Introduction

Protein separation based on ion-exchange adsorption is the most widely used step in downstream processing [1]. In principle, the unique electrostatic interactions of each protein with the stationary phase of the ion-exchange sorbent form the basis for separation. However, the amphoteric nature and three-dimensional structure of the proteins make their interaction with the ion-exchange surface very complex [2–5].

The three-dimensional structure and charge distribution of the protein determine the surface amino acid residues which are in position to interact with the ion-exchange sorbent. The surface area containing the amino acid residues participating in the interaction is referred to as the chromatographic contact region or footprint of retention [5]. A single amino acid variation in the chromatographic contact region can have a marked effect on protein retention [6]. The residues in the contact region may interact directly with the sorbent, or they may influence binding of other residues through electrostatic effects on the dissociation of neighboring residues and steric perturbation of hydrogen bonded water molecules [6]. In addition, the charged density of ion exchanger [7,8] and the type of displacing ions of mobile phase [2,9–11]

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can affect retention, elution, resolution, selectivity and recovery.

Using recombinant DNA technology it is possible to produce genetically engineered proteins with charge characteristics specifically designed to facilitate purification [12–14] and/or to study chromatographic retention mechanisms [6]. Our previous work has taken advantage of negatively charged tails to enhance β-galactosidase recovery [14] and immobilization [15] using ion-exchange membranes. The tails were a series of polyaspartate fusions of the form: Gly–Asp–Pro–Met–Ala–$(Asp)_n$–Tyr adding 1, 5, 11, and 16 negative charges to β-galactosidase designated as BGCD1, BGCD5, BGCD11, and BGCD16, respectively. In this work we use the same series of fusion proteins to investigate the contributions of the high linear charge density region of the fusion tails to protein retention in anion-exchange chromatography. The concept of using fusions to alter charge is illustrative of a larger class of "purification fusions" that have added a wide variety of chromatographic binding regions to proteins [16].

The stoichiometric displacement model [17] based on the mass action law was used to characterize the retention. For this model, the ion-exchange "reaction" is represented by

$$S_o + Z \cdot D_b \Leftrightarrow S_b + Z \cdot D_o \quad (1)$$

The free solute (S_o) will displace multiple (Z) bound ions (D_b) when it is adsorbed (S_b) on the ion-exchange sorbent surface. The stoichiometry of the displacement process is determined by the number (Z) of displacing ions in solution (D_o) required to displace the solute from the sorbent. The equilibrium is expressed as

$$K_{eq} = \frac{[S_b]\,[D_o]^Z}{[S_o]\,[D_b]^Z} \quad (2)$$

where K_{eq} is a binding constant. For low protein coverage, the protein retention (as capacity factor k') can be related to the displacing ion concentration by [2,7]

$$k' = \frac{I}{[D_o]^Z} \quad (3)$$

The capacity factor, k', is related to protein retention by

$$k' = \frac{(t_R - t_O)}{t_O} \quad (4)$$

where t_R and t_O are the retention times of the solute at retained and non-retained conditions, respectively. I is independent of D_o and is given by the equation

$$I = K_{eq}\varphi[D_{bi}]^Z \quad (5)$$

where φ and $[D_{bi}]$ are phase ratio (ratio of stationary and mobile phase volumes) and the ionic capacity of the resin, respectively. Where Z is constant, I can be viewed in terms of K_{eq} which is the ratio of the rate constant for adsorption of protein from the mobile phase to that for desorption of bound protein from the stationary phase. Thus changes in I values have been explained [6,7] in terms of the number of chromatographic contact regions available for effective (i.e. leading to binding) collisions between these regions and the sorbent ($k_{adsorption}$) as well as the ease of solute desorption ($k_{desorption}$).

The two parameters, Z and I, are obtained using the linear form of Eq. (3),

$$\log k' = \log I + Z \log \frac{1}{[D_o]} \quad (6)$$

By measuring t_R for isocratic elution at a series of D_o levels, k' can be calculated from Eq. (4) and Z and I from the slope and intercept of the data plotted according to Eq. (6).

The model has been widely used to describe ion-exchange retention of nucleic acids [18] and proteins [2,6–8,19]. For example, Kopaciewicz et al. [2] found that increased protein retention was due to an increase in Z number. Others [6,7] reported that even proteins with similar Z numbers can have different retentions based on their I values. These latter studies found that the probability of an effective collision and, hence, the rate of adsorption of a solute is proportional to the number of chromatographic regions available for interaction with the ion-exchange sorbent.

The model has also been successfully applied to hydrophobic [20] and reversed-phase [5,21,22] chromatography of proteins. Mazsaroff et al. [19] have derived a similar model for preparative ion-exchange chromatography to investigate the effects of protein concentration on Z numbers.

The Z numbers obtained using this model provide some insight into the role of three-dimensional structure in ion-exchange chromatography. For small molecules such as oligonucleotides with less than 10 residues, Z is equivalent to the number of negative charges in the molecule [20]. For larger molecules such as proteins and oligonucleotides with more than 10 residues, due to steric limitations and charge asymmetry, the Z numbers are substantially lower than their net charges [2,10].

2. Experimental

2.1. Materials

All the BGCD fusions were purified from *Escherichia coli* cell extracts using ammonium sulphate precipitation followed by binding to an affinity matrix [14]. After elution from the affinity matrix, the protein was dialyzed against 100 mM, pH 7.0 potassium phosphate buffer containing 5 mM $MgCl_2.6H_2O$ and 1 mM 2-mercaptoethanol, freeze dried and stored desiccated at 4°C. Before use, the protein was redissolved in deionized water, filtered (0.2 μm cut-off) and checked for DNA content using the ratio of absorbance at 280 nm to 260 nm. In cases where high amounts of DNA ($>10\%$) were detected, the proteins were further purified using ion-exchange (ActiDisk with quaternary amine functionality, FMC Corp., Pine Brook, NJ, USA). For the ion exchange step, each fusion was first dialyzed against 25 mM, pH 5.7 bis-Tris buffer and loaded by syringe onto a new membrane preequilibrated with the same buffer. The effluent was collected and recirculated through the membrane (five times). The loaded membrane was then washed with 10 ml of the bis-Tris buffer followed by step gradient elution using NaCl steps (0.1 M, 0.2 M, ..., 0.7 M, 1 M, 2 M) in the same buffer. Samples from each step were analyzed for A280/A260 nm ratio. Fractions (0.4 M and 0.5 M for BGCD1 and BGCD5; and 0.5 M and 0.6 M for BGCD11 and BGCD16) with ratios higher than 0.7 ($<10\%$ DNA) were pooled, dialyzed against 25 mM, pH 5.7 bis-Tris buffer and stored at 4°C until use. The wild type β-galactosidase (BGWT) and all reagents were purchased from Sigma Chemical Company, St. Louis, MO, USA. All solutions were prepared with deionized water (resistivity >16 M$\Omega \cdot$cm; NANOpure II, Barnstead, Boston, MA, USA) adjusted to the desired pH using HCl, and filtered (0.2 μm cut-off).

2.2. Chromatography

All chromatographic experiments were performed on a strong anion-exchange perfusion column with quaternized polyethyleneimine functionality (POROS Q, M Series, 30 mm $\times$ 2.1 mm I.D.; PerSeptive Biosystems, Cambridge, MA, USA). The chromatographic system consisted of a gradient mixer (Model 2360 Gradient Programmer, ISCO, Lincoln, NE, USA), pump (Model 2350 HPLC Pump, ISCO) and a variable wavelength detector (Beckman 165, Beckman Instruments, Berkeley, CA, USA). Data were collected and retention times determined using Dionex Advanced Interface and Software (Dionex Corp., Sunnyvale, CA, USA).

Retention data were obtained using gradient elution at a flow rate of 1 ml/min at room temperature (21°C). A 10-min linear gradient from 25 mM bis-Tris buffer (buffer A) to 2 M NaCl in buffer A (buffer B) at various pHs was used. At the end of the gradient, buffer B was held for 3 min before switching to buffer A to reequilibrate (at least 7 min) the column for subsequent injection. All samples were injected using a 20-μl sample loop.

Isocratic analyses were carried out at the same conditions: 1 ml/min, 21°C and 20-μl sample. Initial values of NaCl concentration for isocratic elution were obtained from the gradient elution. The NaCl concentration was adjusted using buffers A and B in the desired ratio. After each isocratic experiment the column was cleaned

using buffer B (5 min) and reequilibrated (at least 7 min). The retention time for the 2 *M* NaCl experiment was taken to be t_0.

3. Results and discussions

3.1. Retention maps

Fig. 1 shows the protein retention (as NaCl concentration required for elution, μ_{elute}) as a function of mobile phase pH for BGWT and all the BGCD fusions. For BGWT, BGCD1, and BGCD5, protein retention increased with increasing pH. This is expected of anion-exchange chromatography; retention generally increases as the surrounding environment becomes more basic and the protein becomes more negatively charged. However, BGCD11 and BGCD16 did not show significant variation in retention over the pH range studied. One possible explanation is that even at low pH, the fusion tail has provided sufficient charge for maximum retention and further increases in charge (by increasing pH) did not increase retention. At all the pH values, the fusion proteins are retained longer than BGWT and the order of retention increased with increasing tail length (charge).

In order to better understand the effect of the high linear charge density region on β-galactosidase retention, the retention data in Fig. 1 were replotted as a function of estimated net charge [14]. Fig. 2 shows that the protein retention increased with increasing net charge for BGWT (up to $Z_P \approx -75$), BGCD1 and BGCD5, confirming the previous observation that μ_{elute} increased with increasing pH is a direct consequence of the increase in net charge. On the other hand, the retentions of BGCD11 and BGCD16 did not vary significantly with the net charges. Fig. 2 also shows that at a given net charge, all the fusions show higher retention than the BGWT. This clearly demonstrates that the added tails promote protein retention and the increase parallels the tail length.

It is general practice in ion-exchange chromatography to vary the mobile phase pH and, hence, the protein net charge to obtain optimum

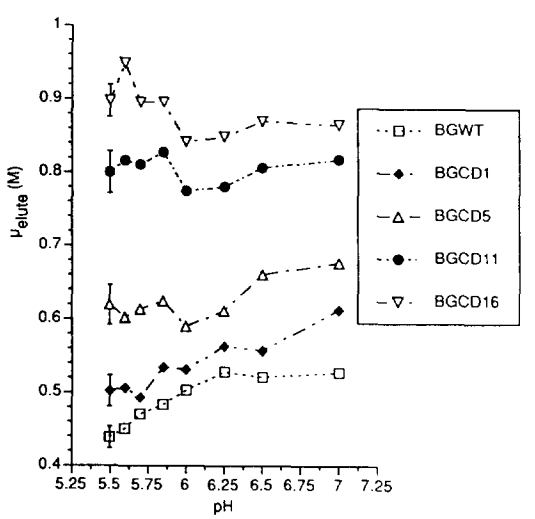

Fig. 1. Protein retention (measured as the NaCl concentration required to elute the protein, μ_{elute}) as function of pH for BGWT and BGCD fusions at 25 m*M* bis-Tris, 1 ml/min and 21°C. Each data point is the mean of at least three replicates. Error bar indicates the average standard deviation (pooled estimates for each protein over all pHs studied).

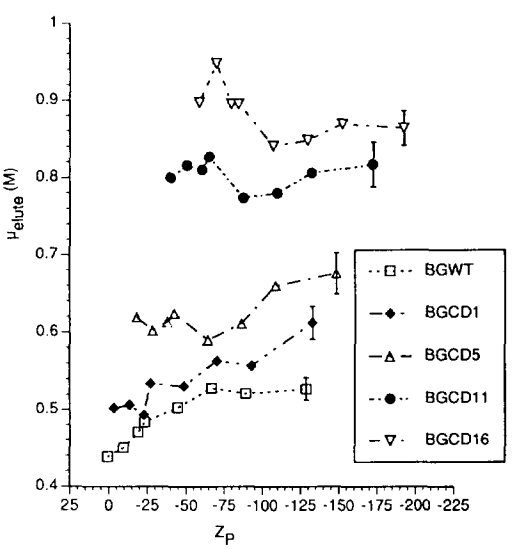

Fig. 2. Protein retention (measured as the NaCl concentration required to elute the protein, μ_{elute}) as function of estimated net charge (Z_P) for BGWT and BGCD fusions at 25 m*M* bis-Tris, 1 ml/min and 21°C. Each data point is the mean of at least three replicates. Error bar indicates the average standard deviation (pooled estimates for each protein over all pHs studied).

separation. Here protein net charge has been altered by adding charged tails to the protein. In order to correlate the dependence of μ_{elute} on the additional charge due to the added tail, linear regression plots of μ_{elute} versus the protein net charge at a various pHs were constructed and Fig. 3 shows a typical plot obtained at pH 5.5. The absolute value of the slope thus obtained, $|\Delta\mu_{elute}/\Delta Z_P|$, is a measure of the effectiveness of the additional charge due to the added tail in enhancing the β-galactosidase retention. Fig. 4 shows such slopes as a function of pH. The $|\Delta\mu_{elute}/\Delta Z_P|$ generally decreased until pH 6.0 and then remained relatively constant, indicating that the additional charges carried by the tails were best utilized (to enhance retention) at the low pH conditions. One possible explanation is that as the pH increases, the protein net charge also increases and the tail represents only a small portion of the total charge.

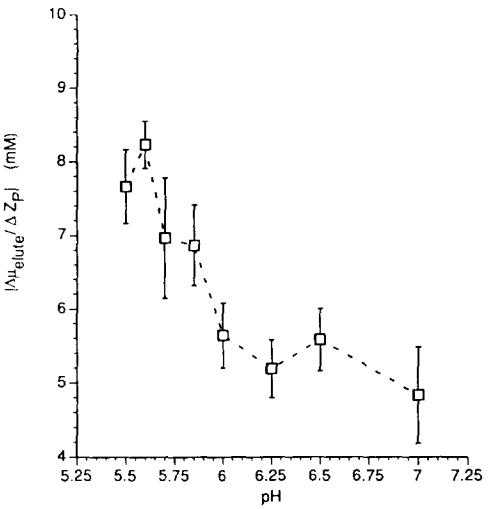

Fig. 4. Effectiveness of added tails for enhancing retention as a function of pH. The $|\Delta\mu_{elute}/\Delta Z_P|$ values were obtained from the slope of the plot in Fig. 3 at various pHs.

3.2. Stoichiometric displacement model

Eq. (6) was fitted using linear least-square regression (JMP version 2, Software for Statistical Visualization on the Apple Macintosh, SAS Institute, Inc.). The correlation coefficients (r) for all proteins, except for the BGCD1 (r = 0.94), are greater than 0.98. The Z numbers and I values obtained were used to characterize the strength of binding between the protein and the ion-exchange surface.

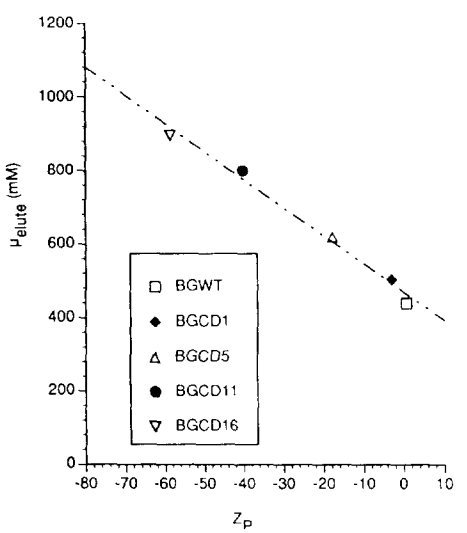

Fig. 3. NaCl concentration required for elution (μ_{elute}) versus estimated protein net charges (Z_P) at pH 5.5 with 25 mM, pH 5.5 bis-Tris buffer, 1 ml/min flow and 21°C. Results for other pHs are similar and all have linear correlation coefficients (r) greater than 0.98.

Effects of adding charged tails at constant pH

Fig. 5 shows the plots of log k′ at pH 5.7 versus log $1/[D_0]$ for BGWT, BGCD1, BGCD5, and BGCD11. The values of Z (i.e. the slope) increased with increasing tail length. They were 11.5, 8.5, 6.9 and 5.3 for BGCD11, BGCD5, BGCD1 and BGWT, respectively (see Fig. 6). The additional charges carried by the fusion tails result in a greater direct electrostatic contact between the protein and the ion exchanger. The Z number of each protein represents only a small fraction of the protein net charge: 0.28, 0.32, 0.23, and 0.19 for BGWT, BGCD1, BGCD5, and BGCD11, respectively. This is consistent with many early reports [2,6,7], which suggest that due to steric limitations only a small portion of the protein is participating in the interaction. It is particularly understandable for this large (M_r 460 000) tetrameric protein, where simulta-

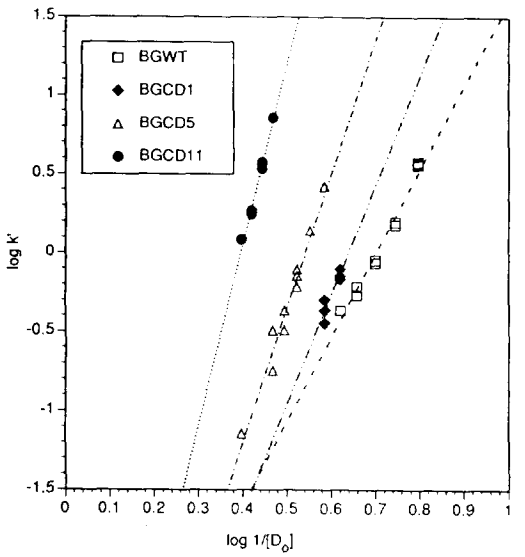

Fig. 5. Log k' versus log $1/[D_o]$ for BGWT and BGCD fusions at 25 mM, pH 5.7 bis-Tris buffer, 1 ml/min and 21°C. $[D_o]$ is the NaCl concentration used in each isocratic experiment.

neous interaction of the resin with all four fusion tails would seem precluded.

The I values for all the fusions were of the same order of magnitude and were approximately 5 times smaller than those for BGWT (Table 1, column 3). However, the overall enhanced retention seen earlier (Fig. 1) indicates that the increase in the number of direct electrostatic interactions (Z) outweighed the decrease in I value.

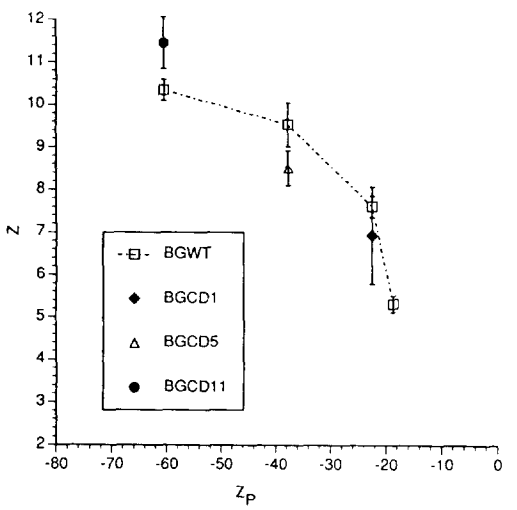

Fig. 6. Comparison of binding numbers (Z) for BGCD fusions at pH 5.7 and BGWT brought to the same estimated net charge (Z_p) by varying the mobile phase pH (to pH 5.74, 5.92, and 6.18 to match BGCD1, BGCD5, and BGCD11, respectively).

Table 1
Comparison of I values for β-galactosidase fusions at pH 5.7 and BGWT brought to the same net charge by varying the mobile phase pH

Protein	Estimated net charge at pH 5.7	I at pH 5.7	I for BGWT at same net charge as fusion[a]
BGWT	−18.67	$1.85 \cdot 10^{-4}$	–
BGCD1	−22.48	$3.73 \cdot 10^{-5}$	$1.45 \cdot 10^{-6}$
BGCD5	−37.72	$2.46 \cdot 10^{-5}$	$1.04 \cdot 10^{-7}$
BGCD11	−60.57	$2.95 \cdot 10^{-5}$	$6.80 \cdot 10^{-8}$

The I values are obtained at 25 mM bis-Tris buffer at 1 ml/min, 21°C

[a] Achieved by varying the mobile phase pH to 5.74, 5.92 and 6.18 to obtain the same net charge as BGCD1, BGCD5 and BGCD11, respectively.

Distributed charges versus high linear charge density region

In order to compare the contributions of a high linear charge density region to those of distributed charges, the Z and I values of BGWT having the same net charge (by varying the mobile phase pH) as each of BGCD1, BGCD5, and BGCD11 were determined as before. Fig. 6 shows that changing pH was effective in shifting the Z values for BGWT to values comparable to those of each of the fusions at pH 5.7. However, the I value for BGWT decreased with increasing pH (Table 1, column 4) and was lower than that of the corresponding fusion (Table 1, column 3 versus 4).

Position on the Z_p axis of Fig. 6 required an estimation of Z_p. Experimental determination of charge via titration was not possible for β-galactosidase because of insolubility near the isoelectric point (pI) and denaturation below the pI. In the estimate plotted [14], all the charged amino acid residues were assumed to be fully ionized.

After completion of this work, the BGWT net charge was estimated by using titration data above pH 6 and a pI value (4.8) approximated from the solubility data [23]. Based on these new charge estimates, the data of Fig. 6 would shift to lower values of Z_p with the BGWT points spanning a smaller range of Z_p. However, the above comparison of I values at similar Z number does not change as Z is independent of the estimate of Z_p. Overall then, despite the similar (or lower) Z numbers, each of the fusions studied has a higher I value and, hence, a higher μ_{elute} (Fig. 2).

There are two possible ways in which the added tails can enhance binding relative to the untailed protein of the same binding number (i.e. increase I at the same Z). First, the tails may orient or steer the protein into a position such that the chromatographic contact region can interact more favorably with the ion-exchange surface [2]. The more likely alternative, given the correspondence of Z and tail length, is that the tails serve as the chromatographic contact region. The flexibility and accessibility of the tails, relative to the more distributed surface contact region of the BGWT, would increase the probability of an effective interaction between the protein and the ion-exchange surface. In other words, the collisional efficiency of the binding step would be increased. Additionally, one could speculate that the rate of desorption would also be lower for the tailed case where displaced sections of the tail are kept positioned for resorption by those sections not yet displaced. Hence, desorption would require nearly simultaneous displacement of all the tail sites. Either effect would increase K_{eq} and, hence, I. At a given pH, the high linear charged density region fused to the β-galactosidase provided an effective way to enhance protein retention by increasing the Z numbers without greatly reducing the I values.

4. Conclusions

It was demonstrated that a high linear charge density tail fused to β-galactosidase can enhance protein retention in anion-exchange chromatography. The additional charges carried by the tails enhanced retention in the following order: BGCD16 > BGCD11 > BGCD5 > BGCD1 > BGWT. The added tails were most effective in enhancing retention at low pHs.

Two parameters, Z and I, obtained from the stoichiometric displacement model, were used to characterize the extent of binding between the protein and the ion-exchange surface. At pH 5.7, the Z number increased with tail length (charge) and was 11.5, 8.5, 6.9 and 5.3 for BGCD11, BGCD5, BGCD1 and BGWT, respectively. At these conditions, the fusions had very similar I values and those were five times smaller than that of BGWT. However, the increase in Z numbers outweighed the decrease in I values and an overall enhanced retention was observed. Similar Z numbers were obtained for the BGWT brought to the same approximate net charge (by varying the mobile phase pH) as each of the fusions. The I values of BGWT decreased with increasing pH (net charge) and were lower than that of the corresponding fusion. Consequently, despite the similar Z numbers, the fusions had a higher retention than the corresponding BGWT, demonstrating the effectiveness of the high charge density tail.

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Symbols and abbreviations

BGCD	β-galactosidase with carboxyl aspartate fusion peptide
BGWT	wild-type β-galactosidase
[D]	displacing ion concentration
$[D_{bi}]$	ionic capacity of ion-exchange resin
I	parameter in Eq. (5)
k'	capacity factor
K_{eq}	binding constant

[S]	solute (protein) concentration
t_R	retention time
t_o	retention time of solute at non-retained condition
Z	number of charge interactions
Z_P	protein estimated net charge
μ_{elute}	NaCl concentration required for elution
φ	phase ratio (ratio of stationary and mobile phase volumes)

subscripts for D and S

b	bound state
o	free state (in solution)

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