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SURFACE-MEDIATED RETENTION EFFECTS OF SUBTILISIN SITE-SPECIFIC VARIANTS IN CATION-EXCHANGE CHROMATOGRAPHY

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SUMMARY

Wild-type subtilisin and several site-specific variants were resolved on a strong cation-exchange column by isocratic elution and a series of sodium chloride concentrations. Changes in primary sequence at the protein surface have an observable effect on the chromatographic behavior of subtilisin. This supports the concept that three-dimensional structure determines which biopolymer surface residues are in position to interact with the stationary phase surface.

The retention data fit the stoichiometric displacement model (SDM) of retention. Plots of ln k' *vs.* ln 1/[NaCl] yield values for the average number of ionic groups (Z) on the protein that interact with the support matrix. Application of the SDM to the chromatographic retention of the variants has uncovered an unusual phenomenon at the protein surface at low ionic strength.

A SDM plot normally provides a linear relatinship between ln k' and ln 1/[NaCl] with the slope corresponding to the Z number. This study revealed two lines differing in slope and intercept, indicating that the Z number of subtilisin changes at some intermediate ionic strength of the eluent. These results are attributed to some salt-induced protein surface event that triggers a change in structure. Chromatographic detection of this occurrence reflects the connection between the surface-mediated event and mobile phase ionic strength.

INTRODUCTION

High-performance ion-exchange chromatography is considered to be a non-denaturing method for the separation of proteins[1]. The electrostatic interaction between surface residues and the stationary phase is utilized to achieve the separation. Depending on both the pI and charge distribution in a protein, cation-exchange chromatography is generally used to separate proteins below their pI and anion-exchange chromatography above their pI. Protein retention is dependent on the number and strength of surface charges accessible to the sorbent matrix. Factors that influence electrostatic interactions include protein conformation, mobile phase pH, and mobile phase ionic strength.

Protein tertiary structure has an essential effect on chromatographic retention,

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due to the access limitations it places on surface residues[2]. Recent studies showed that only a fraction of the total hydrophobic surface residues of lysozyme are able to interact with the sorbent surface in hydrophobic-interaction chromatography[3]. Significant changes in retention were observed only when amino acid substitution occurred in the chromatographic contact region. Hence, the protein structure dictates which amino acids recognize or have access to the column support.

The importance of three-dimensional structure to chromatographic behavior is further reflected in the analysis of retention data on the basis of a non-mechanistic model, the stoichiometric displacement model (SDM)[4]. The central hypothesis of the SDM is that the displacement of a solute from a surface is accompanied by the adsorption of a stoichiometric amount of displacing agent. The process may be described by the equilibrium expression:

$$S_0 + ZD_b \leftrightarrows S_b + ZD_0$$

where S_0 refers to solute capable of being adsorbed on a surface at multiple sites, D_b refers to adsorbed displacing agent, S_b is the solute bound to the stationary phase, D_0 is the free displacing agent, and Z is the number of interaction sites for a particular solute and stationary phase in a given system. Solute retention (expressed by the capacity factor, k') has been related to the displacing salt concentration, $[D_0]$, by eqn. 1.

$$k' = I/[D_0]^Z \tag{1}$$

where I is a constant, proportional to the equilibrium formation constant.

It has been shown in anion-exchange chromatography of oligonucleotides that Z reflects the stoichiometry of ionic displacement of solute from the sorbent surface[5]. The relationship between three-dimensional structure and Z number was examined, using oligonucleotides of varying length as models. With oligonucleotides of less than ten bases, the Z number reflected the total number of anionic positions on the molecule, whereas oligonucleotides larger than ten bases, including tRNA (>70 bases), have a fractional ratio of Z number/total surface charge. Other applications of the SDM to the relationship between three-dimensional structure and protein retention have revealed that only a fraction of the total surface charge on a protein interacts with the sorbent matrix at any one time[4–6]. In a recent retention study, the quaternary structure of lactate dehydrogenase isoenzymes was examined. It showed that Z number values varied, depending on the subunit composition[7]. That study also led to the conclusion that in a multimeric protein there may be equivalent areas on the molecular surface which participate in the adsorption process. The next step in the application of the SDM to chromatographic behavior is the determination of changes in the primary structure which influence the retention process without changing the tertiary or quaternary structure of the protein.

Subtilisin is a model protein meeting the above-stated criteria. This serine protease is well studied. It is a single-chain globular endopeptidase (molecular weight 27 500). The primary sequences of several naturally occurring variants are known[8–10], as well as the three-dimensional structure[11,12]. The *Bacillus amyloliquifaciens* subtilisin gene has been cloned[13], and a number of expressed site-specific variants have

been studied kinetically[14–16]. The structures of this subtilisin and certain variants have been determined to a resolution of 1.8 Å (ref. 17), providing strong evidence that the three-dimensional structure of the wild type is conserved in the engineered subtilisin variants. Subtilisin contains a number of positively charged residues, occupying surface positions, and accounting for protein retention on cation-exchange sorbents. For these reasons, subtilisin was chosen as the model for further retention studies. In this paper, we discuss a protein surface phenomenon, discovered during the initial phase of our data collection.

Normally, according to eqn. 1, the ln k' *versus* ln $1/[D_0]$ plot used in SDM data analysis provides a linear relationship, where the slope corresponds to the Z number. During analysis of the subtilisin retention data, a break was discovered in this line, occurring at specific salt concentrations, depending on the protein variant and the pH of the mobile phase. This observation led to the discovery of two lines, yielding independent Z and I values. We believe this to be the result of a protein surface phenomenon which is dependent on mobile phase ionic strength. Depending on the nature and position of the amino acid substitution, the Z number can either increase or decrease as a result of the protein interaction with salt. Z values from the SDM will be taken to indicate the average number of charges involved in subtilisin binding, while I values represent the affinity or adsorptive equilibrium between the protein and the column. Although not intended for the examination of conformational changes, the SDM is sufficiently sensitive to detect certain structural changes in subtilisin.

EXPERIMENTAL

Proteins and reagents

B. amyloliquefaciens subtilisin wild type and site-directed variants were a generous gift of Genencor (San Francisco, CA, U.S.A.). These proteins were used without further purification. All reagents were of AR grade or comparable quality.

To prevent autolysis, proteins were stored in 50% aq. propylene glycol containing 5 m*M* calcium chloride and 10 m*M* N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.6) at −12°C. Protein concentrations were approximately 10 mg/ml.

Equipment

Protein retention measurements were made with a Varian (Walnut Creek, CA, U.S.A.) 5000 chromatographic system, equipped with a UV-100 detector and a Valco (Houston, TX, U.S.A.) Model C6U injector with a 15-μl sample loop. A 10-μm particle size Mono-S strong cation-exchange column, 5.0 × 0.5 mm I.D. (Pharmacia, Uppsala, Sweden) of 0.13–0.18 mmol/ml ion-exchange capacity was used in all experiments. Protein absorbance was monitored at 280 nm, and eluent conductance was monitored with an Anspec (Ann Arbor, MI, U.S.A.) AN400 ion chromatograph. Data were collected on a Kipp & Zonen (Delft, The Netherlands) BD41 dual-channel chart recorder.

Buffers

Mobile phase buffers were chosen so that the buffer pK_a was within one pH

unit of the desired eluent pH: sodium acetate (Mallinckrodt, Paris, KY, U.S.A.) (pH 5.0 and 5.5); 2-(N-morpholino)ethanesulfonic acid (MES) (Calbiochem-Behring, La-Jolla, CA, U.S.A.) (pH 6.0); N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) (Sigma, St. Louis, MO, U.S.A.) (pH 7.0). Buffer A was prepared to have an ionic strength of 0.01 *M*. Buffer B was a mixture of 0.01 *M* buffer A and either 0.10 *M* or 0.15 *M* sodium chloride, depending on the protein being studied. All buffers were adjusted to the appropriate pH at room temperature with either hydrochloric acid or sodium hydroxide. All solutions were prepared fresh daily and filtered through a Rainin (Woburn, MA, U.S.A.) Nylon-66 (0.45-μm) filter, followed by degassing.

Chromatography

Samples were initially examined by gradient elution with a 20-min linear gradient at a flow-rate of 1 ml/min. Gradient elution was used to estimate the isocratic conditions required for the collection of retention data. Isocratic separations were then performed at salt concentrations both above and below the gradient elution concentration so as to produce k' values in the range 1–15. Between eleven and nineteen salt concentrations were chosen for each analysis. All samples were chromatographed in duplicate with injection volumes varying between 4 and 8 μl (volume was constant for any one analysis). The solvent perturbation peak observed by both the UV and conductance detectors was taken as the retention time for unretained protein (t_0).

Calculations and statistical analysis

Retention times were converted to k' values according to the equation:

$$k' = (t_R - t_0)/t_0$$

where k' is the capacity factor, t_R is the solute retention time, and t_0 is the non-retained solute retention time.

Data analysis was completed by plotting the capacity factor against the eluent salt concentration according to the equation:

$$\ln k' = Z \ln 1/[\mathrm{NaCl}] + \ln I$$

These data were then fitted to a straight line by an iterative least-squares program (on an Apple IIe computer). Values for Z and I were obtained from this statistical analysis. Points outside two standard errors of the linear regression analysis were judged not to be on the line. Based on standard error analysis of sets of data points, it was determined that there were two lines for all subtilisin variants. Further statistical verification was observed when subjecting the data to residuals analysis by standard methods[18]. Data fitted in this manner had a correlation coefficient greater than 0.997.

RESULTS AND DISCUSSION

Subtilisin

The advent of molecular biology has greatly enhanced studies on protein struc-

TABLE I

SUBTILISIN RESIDUE SUBSTITUTIONS

Variant	*Amino acid*	*Substitution*
Wild type		
FQK*	Met-50	Phe
	Glu-156	Gln
	Gly-166	Lys
FQK R27–R43	Lys-27	Arg
	Lys-43	Arg
FQK R43	Lys-43	Arg
FQK R170	Lys-170	Arg
FQK R275	Gln-275	Arg

* FQK denotes all three mutations at positions 50, 156 and 166 present. Hence, this is termed the FQK backbone.

ture and function. Using techniques developed in this field, a number of protein variants can be produced with site-specific changes in primary structure. Coupled with the known structure of the molecule, the effect of a single amino acid on the chromatographic behavior of the molecule can be determined. For this study, we examined a number of subtilisin site-specific variants (Table I). This group of proteins represents differences in overall surface charge (FQK and FQK R275) and local steric effects (FQK R27–R43, FQK R43, and FQK R170). The amino acids selected for substitution are all known to be solvent-accessible surface residues[17]. The cation-exchange retention properties of these subtilisins were examined with respect to mobile-phase ionic strength and pH. All retention experiments were conducted under isocratic conditions to ensure that the column was at equilibrium with the mobile phase. Less than 50 μg of protein was used to minimize the effect of sample load on the capacity factor (k').

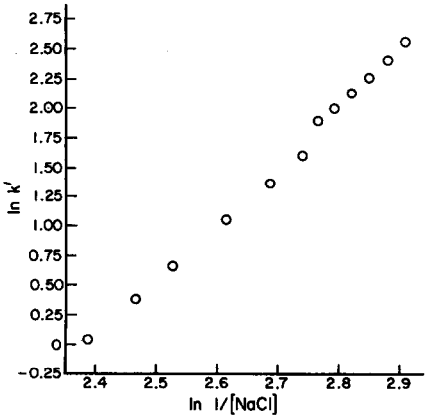

Fig. 1. Plot of ln k' *versus* ln 1/[NaCl] for subtilisin FQK R27–R43 at pH 5.0. Analysis made on retention data with a k' range of 1–15. Two lines of different slopes and intercepts may be determined from this analysis.

TABLE II

SUBTILISIN SDM ANALYSIS: COMPARISON OF Z NUMBERS

pH 5.0; salt, sodium chloride.

Solute	*Z number*	
	Relatively high salt concentration	*Relatively low salt concentration*
Wild type	2.79 ± 0.01	2.32 ± 0.03
FQK	4.08 ± 0.03	3.76 ± 0.02
FQK R275	5.11 ± 0.03	4.96 ± 0.03
FQK R27–R43	4.37 ± 0.01	4.60 ± 0.02
FQK R43	4.24 ± 0.02	4.46 ± 0.03
FQK R170	4.30 ± 0.04	4.45 ± 0.03

Z numbers

The initial purpose of this study was to investigate the direct influence of amino acid substitution on protein retention. It was assumed that every protein examined would have a single Z number in the linear range of the measured k', corresponding to the amino acids interacting with the stationary phase. This was shown not to be the case. In every subtilisin studied, the ln k' *versus* ln 1/[NaCl] plot produced two lines (Fig. 1) of differing slope and intercept. The sharp break in the lines occured at a rather specific ionic strength, depending on the variant and the mobile-phase pH. A change in Z number suggests that contact of the protein with the column is in some way altered. Depending on the variant, the Z number was found to either increase or decrease after reaching a critical ionic strength. Trends observed among the variants studied have interesting implications. This phenomenon has not been described previously. However, in a recent study on cation-exchange chromatography[19] of bovine IgG_2 a similar break in the Z number plot was observed.

Z numbers for subtilisins at pH 5.0 are listed in Table II. It is evident that amino acid substitutions influence the Z number. The FQK subtilisin differs from the wild type by a neutral change at position 50 and two charge substitutions at positions 156 and 166. Even though only one positive charge is introduced (position 166), a negative charge is removed at position 156, giving the net effect of an increase in charge of +2. Comparison of Z numbers for wild type and FQK reveals that the Z number increases by approximately 1.3. The removal of the negative charge at position 156 is reflected in the Z number, even though the Glu-156 should not interact with the column[20]. Addition of another positive charge to the FQK subtilisin (FQK R275) again increases the overall Z number. These three subtilisins do have a common feature. Comparison of the Z numbers before and after the break in the line indicates consistently higher Z numbers at relatively high ionic strength for these proteins.

The remaining variants, FQK R170, FQK R43, and FQK R27–R43 show opposite behavior. At relatively high ionic strength, these variants display a lower Z number than at relatively low ionic strength. These three variants have a common structural feature. Although their net charge is the same as the FQK subtilisin, each differs from the FQK variant by the substitution of a Lys by an Arg residue. The

Lys → Arg substitutions all occur in locations on the back side (with respect to the active-site cleft) of the molecule. Hence, there is a generation of two Z numbers for each subtilisin variant, and a trend in the change in Z with respect to ionic strength.

A mobile phase pH study was performed to determine whether this chromatographic behavior occurred at more alkaline conditions. Retention measurements for all the variants were performed at pH 5.0, 5.5, 6.0, and 7.0. Due to the acid lability of subtilisin[21], experiments were not performed below pH 5.0. Z numbers determinations were independent of temperature between 22°C and 26°C under all pH conditions. Decreases in ionic strength at pH 5.0 resulted in an increase in Z number for the FQK R27–R43, the FQK R43, and the FQK R170 subtilisins. Similar behavior was observed with these variants at pH 5.5, 6.0 and 7.0. The wild-type subtilisin also retained the same relationship between Z number and ionic strength when the pH was varied; however, the FQK and FQK R275 variants behaved like the other FQK variants beyond pH 5.5 and pH 6.0, respectively. The change in Z number with respect to ionic strength (ΔZ) reached a maximum for all proteins studied between pH 5.5 and pH 6.0 (Fig. 2). Although the magnitude varies with different proteins, the curves are similar for those proteins having ΔZ maxima at pH 5.5 and pH 6.0.

The small Z numbers for all of the subtilisins studied required the use of low-

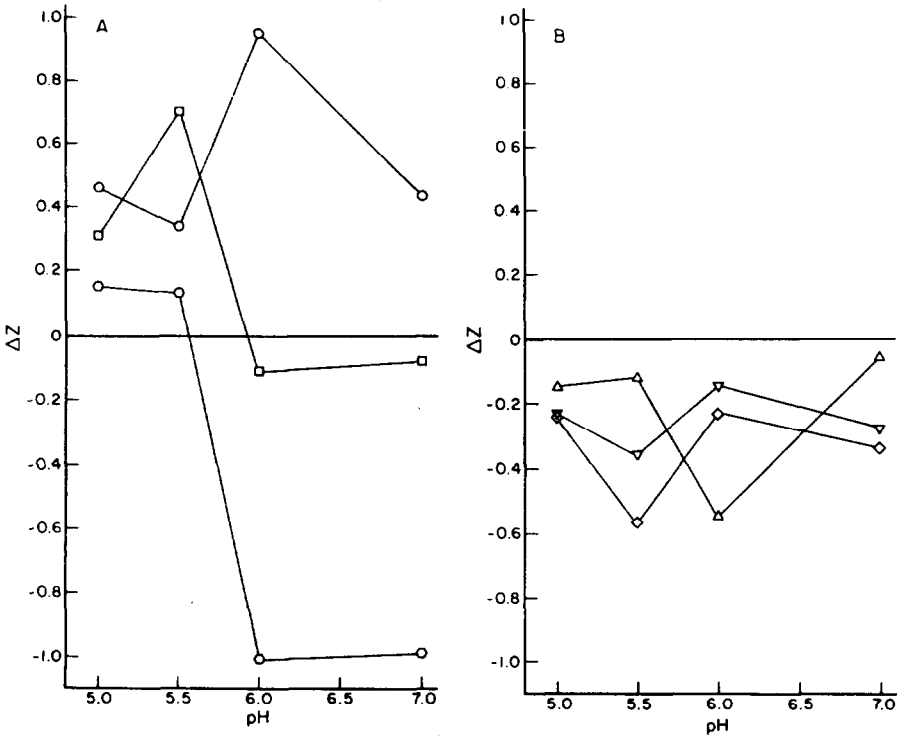

Fig. 2. A plot of the change in Z number between the relatively high-salt eluent and the relatively low-salt eluent ($\Delta Z = Z_{HI} - Z_{LO}$) and the pH of the mobile phase. (A) Subtilisin proteins with the high-salt high-Z-number trend. (○) Wild type; (□) FQK; (○) FQK275. (b) Proteins with the high-salt low-Z-number trend. (◇) FQK R27–R43; (▽) FQK R43; FQK R170.

TABLE III

IONIC STRENGTH DIFFERENCE BETWEEN THE TWO LINES IN *Z* NUMBER PLOT

pH 5.0; salt sodium chloride.

Variant	*Ionic strength at breakpoint in Z number plot (M)*
Wild type	0.020–0.028
FQK	0.060–0.064
FQK R275	0.083–0.087
FQK R27–R43	0.063–0.065
FQK R43	0.061–0.065
FQK R170	0.061–0.065

ionic-strength buffers during isocratic elution. It was recently established that low ionic strength can shift the pK_a of His-64 in engineered subtilisins[22,23]. At sufficiently low ionic strengths (0.005–0.100 *M*), salt shielding is lost at the surface of a protein unmasking a substantial number of charged groups. When more than one charged amino acid is located within 13–15 Å the effects of surface charge on the pK_a of His-64 were found to be cumulative[24].

The sharp transition at intermediate ionic strength (Table III) between the two observed *Z* numbers suggests a protein surface event involving a two-state system. The transition between these states would have to be very fast, because no increase in bandspreading was observed at the transition ionic strength. One possibility is that a salt bridge on the surface of the molecule forms at relatively low ionic strength. This salt-bridged species would have a unique conformation (P_1) with a particular *Z* number. At high ionic strength, this salt bridge would break, and the molecule would assume a second conformational state (P_2), as indicated in Fig. 3. Depending on the amino acid environment around the salt bridge, the *Z* number could either increase or decrease with the breaking of the salt bridge.

A possible explanation for the pH data (Fig. 2) is the ionization of the His imidazole group. The normal pK_a of the His side chain is 6.0. Using Ser and Lys substitutions in subtilisin, Russell and Fersht[24] were able to show that surface amino acids affected the ionization of His-64. Although the variants used in this study utilize substitutions at positions other than those of Russell and Fersht, the positively charged substitutions may still affect the pK_a of the His-64 residue. The His-64 residue

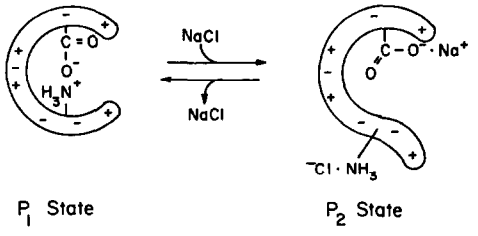

Fig. 3. The proposed structural states of a surface salt bridge at relatively high and low salt conditions. The P_1 state refers to subtilisin at relatively low salt concentration and P_2 reflects the shielded ion pair at relatively high salt concentration.

TABLE IV

SUBTILISIN SDM ANALYSIS: COMPARISON OF *I* VALUES

pH 5.0; salt, sodium chloride.

Variant	*I value*	
	Relative high salt concentration	*Relative low salt concentration*
Wild type	$1.42 \cdot 10^{-4}$	$7.44 \cdot 10^{-4}$
FQK	$5.45 \cdot 10^{-5}$	$1.46 \cdot 10^{-4}$
FQK R275	$2.19 \cdot 10^{-5}$	$2.84 \cdot 10^{-5}$
FQK R27–R43	$3.10 \cdot 10^{-5}$	$1.97 \cdot 10^{-5}$
FQK R43	$2.54 \cdot 10^{-5}$	$1.57 \cdot 10^{-5}$
FQK R170	$2.58 \cdot 10^{-5}$	$1.88 \cdot 10^{-5}$

is part of the active-site triad and is believed to be in the cation-exchange chromatographic contact region, based on an earlier study showing the importance of the active-site Ser in cation-exchange retention[25].

I values

It would seem that chromatographic behavior would be directly related to the number of charges (Z) involved in retention. This is not always the case. The I value in eqn. 1 plays an important role. A summary of the I values (Table IV) shows an inverse relationship between Z number and I value at relatively high and low ionic strengths. Similar relationships, involving the number of charged interactions and the absolute solute retention time, have been observed in ion-exchange chromatography of other proteins[7,26]. Therefore, protein retention is not based solely on the Z number, but on the combined contributions, determined by both the Z number and I value.

The I value contains a complex nest of thermodynamic retention parameters. Whereas Z is the number of interacting charges between protein and stationary phase, I reflects the affinities of each of these interacting charges, the non-specific interactions between solute and sorbent as well as any enthalpic and entropic terms associated with conformational change upon binding. One possible explanation for the inverse relationship between Z and I involves the orientation and distance of the molecule from the support.

CONCLUSION

The three-dimensional structure of subtilisin defines which surface residues contact the column stationary phase. Several kinds of non-covalent interactions between amino acids are involved in preserving the integrity of protein structure. Structural perturbations result in response to competition between these intermolecular interactions. Because proteins are dynamic and can adapt structurally to their surrounding environment, slight conformational changes are possible as a result of changes in mobile phase pH, ionic strength, or even protein–column interactions. In cation-exchange chromatography, the stationary phase ligands compete electrostat-

ically for the positively charged protein surface residues to retain the protein on the surface. Upon adsorption, subtle and reversible structural changes may occur. The bound proteins attempt to retain properties close to those in the solution state and the changes observed have been referred to as native-state "breathing"[27].

This study demonstrates that changes in the primary structure of subtilisin affect the behavior of the molecule in cation-exchange chromatography. The wild-type and variant proteins studied each undergo a conformational change which can be detected by chromatographic application of the SDM. Upon analysis of variant retention the wild-type, FQK, and FQK R275 proteins appear to behave similarly with respect to Z number and ionic strength at pH 5.0. In all cases, the Z number is greater at relatively high ionic strength. The FQK R27–R43, FQK R43, and FQK R170 establish a different trend in which the Z number is larger for relatively low salt concentration. A possible interpretation drawn from these observations is that a surface ion pair is formed in lower-ionic-strength isocratic elution. Depending on the site-specific changes involved, the Z number can either increase or decrease during the formation of the salt bridge. This phenomenon has a ΔZ pH maximum for all species between pH 5.5 and pH 6.0. The wild-type subtilisin conserves the Z number/ionic strength trend, independent of mobile phase pH. Both FQK and FQK R275 behave like the Arg variants after reaching their ΔZ pH maxima. Two explanations for this observation are possible: (1) the FQK substitution common to all the variants studied may have some effect on the ionization state of His-64; (2) the Arg substitutions on the back side of the molecule may allow the molecule to express the FQK substitution effects more freely at the lower pH.

In all cases, there is a reciprocal correlation between Z number and I value. The I value is always larger for the salt concentration giving the lower Z number. This explains why, in some cases, the solute with the higher Z number is eluted earlier than the solute with the lower Z number. Because I encompasses a large amount of information in a single constant, it is difficult to interpret the meaning of this trend. Possible explanations for this observation include changes in cation-exchange binding affinity, non-specific interactions, and protein conformational perturbations. To understand the meaning of I more fully, the thermodynamic parameters (ΔH^0 and ΔS^0) of subtilisin cation-exchange binding should be investigated.

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