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## Separation of small cationic bioactive peptides by strong ion-exchange chromatography

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### Abstract

The high resolving power of a Hyper D cation-exchange column was evaluated to achieve the separation of small cationic bioactive peptides derived from tryptic digest of  $\kappa$ -casein: MAIPPK ( $pI=9.9$ ), MAIPPKK ( $pI=10.5$ ), KNQDK ( $pI=9.6$ ) and NQDK ( $pI=6.35$ ). The influence of pH (1.5–6), gradient slope (2–10 mM sodium chloride/min) and elution of the mixture under isocratic conditions was investigated. Although their physico-chemical properties are very similar, these four peptides were readily resolved with an excellent selectivity and recovery. The selectivity of the exchanger was also expressed toward peptides of the same net positive charge; the most hydrophilic peptide always eluted last. It was also shown that the elution order of these molecules depends on pH. From the observed retention times and the elution order, we have established a simple approach to linearization of peptide retention behaviour on the S-Hyper D support.

**Keywords:** Ion exchangers; Peptides; Casein

### 1. Introduction

Biologically active peptides have been identified in digests of various food proteins, especially milk proteins [1]. Jollès et al. [2] and Maubois et al. [3] have shown that small peptides derived from the C-terminal part of  $\kappa$ -casein, called caseinomacropptide (CMP, residues Met<sub>106</sub>–Val<sub>169</sub>), inhibited platelet aggregation and the binding of fibrinogen to ADP-treated platelets. These antithrombotic peptides were released from CMP by tryptic hydrolysis of the three lysine residues, i.e., Lys<sub>111</sub>, Lys<sub>112</sub> and Lys<sub>116</sub>. Four small cationic peptides were obtained: Met<sub>106</sub>–

Lys<sub>111</sub> (A1); Met<sub>106</sub>–Lys<sub>112</sub> (A2); Lys<sub>112</sub>–Lys<sub>116</sub> (B1) and Asn<sub>113</sub>–Lys<sub>116</sub> (B2). In order to obtain additional insight into the susceptibility of the three cleavage sites to trypsin, and on the other hand to allow for the large-scale preparation of the individual products, an efficient method for their separation and quantitation is required.

Reversed-phase liquid chromatography (RPLC) and ion-exchange liquid chromatography (IELC) are considered as powerful methods for peptide separation in both the analytical and preparative mode. However, RPLC is often inappropriate for separation of highly cationic peptides due to the low hydrophobicity/hydrophilicity ratio of the molecules [4]. Consequently, IELC should be the convenient method for separation of small cationic peptides such as those described above.

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A relatively new and highly hydrophilic ion-exchange packing material, Hyper D, with a superior dynamic capacity, a high resolving power and excellent recovery of proteins has been reported by Horvath et al. [5]. In this study, we evaluated the potential of the Hyper D cation exchanger for the separation of cationic peptides as a function of pH and salt gradient slope. The observed retention behaviour of the four peptides in question was not as predicted from their net positive charge at highly acidic pH. From the results we drew a number of conclusions with respect to the underlying mechanism for the observed behaviour.

## 2. Experimental

### 2.1. Chemicals

The S-Hyper D ion-exchange support (100×4.6 mm I.D., 10 μm particle size) was obtained from BioSeptra (Villeneuve la Garenne, France). A Mono S HR 5/5 ion-exchange matrix (50×5 mm I.D., 10 μm particle size), pre-packed column was purchased from Pharmacia Biotechnology (Orsay, France).

Distilled water was purified using a Milli-Q system (Millipore, Vélizy, France). All salts and products were of analytical-grade. Trifluoroacetic acid (TFA) was from Pierce Chemicals (Rockford, IL, USA). HPLC-grade acetonitrile was from Carlo Erba (Nanterre, France). Buffers were prepared with HPLC-grade water, filtered through a 0.45-μm filter, and degassed by helium bubbling prior to use.

The mixture of cationic peptides used in this study was prepared by tryptic hydrolysis of caseinomacropptide in a membrane reactor as previously reported [6]. The amino acid sequence of individual peptides as well as their properties are listed in Table 1.

### 2.2. Instrumentation

The apparatus used was the Pharmacia Fast Protein Liquid Chromatography (FPLC) system. The equipment consisted of a LCC-500 system controller, two P500 pumps, and a UV-1 detector operated at 214 nm. The system was connected to the type 2600 Chromatography Nelson Analytical Software by

Table 1  
Peptides used in this work

Peptide <sup>a</sup>	Name	<i>M<sub>r</sub></i> (Da)	Sequence <sup>b</sup>
Group A			
M <sub>106</sub> -K <sub>111</sub>	A1	656	M-A-I-P-P-K-OH
M <sub>106</sub> -K <sub>112</sub>	A2	785	M-A-I-P-P-K-K-OH
Group B			
K <sub>112</sub> -K <sub>116</sub>	B1	632	K-N-Q-D-K-OH
N <sub>113</sub> -K <sub>116</sub>	B2	503	N-Q-D-K-OH

<sup>a</sup> Peptides are designated according to their order in the precursor sequence.

<sup>b</sup> Sequences are referred to by the single letter code: M= methionine; A=alanine; I=isoleucine; P=proline; K=lysine; N=asparagine; Q=glutamine; D=aspartic acid.

means of a 900 series ‘‘Intelligent Interface’’ from Nelson Analytical (CA, USA).

### 2.3. Chromatographic procedure

A mixture of 0.1 or 0.3 mg of peptides in 100 μl of buffer A were injected onto a column previously equilibrated in the same buffer A. Three buffer A solutions were used: 10 mM sodium acetate, pH 6; 10 mM sodium acetate, pH 4 and 10 mM KCl/HCl, pH 1.5. Corresponding buffer B was buffer A+1 M sodium chloride. The flow-rate was 0.6 ml/min. After a 4-min initial isocratic run with 100% solution A, elution was performed using a linear gradient from 0 to 1 M sodium chloride. Three gradient slopes of 0.2, 0.4 and 1% solution B/min equivalent to 2, 4 and 10 mM sodium chloride/min were used for each eluent studied. After each run, the column was re-equilibrated during 15 min before the next injection. A continuous detection of conductivity was measured at the outlet of the column to express the results as a real percentage of buffer B. Each experiment was performed at least twice to ensure reproducibility.

### 2.4. Peptide characterisation

The separated peptides were lyophilised and identified by RPLC coupled to electrospray ionisation mass spectrometry (ESI-MS). RPLC was performed at 40°C using a C<sub>18</sub> column (Supersphere RP100 125×2.1 mm I.D.) from Merck (Darmstadt, Germany). The solvent system was: solvent A (0.11%

TFA in water) and solvent B [0.1% TFA in acetonitrile–water (80:20, v/v)]. Elutions were performed at a flow-rate of 0.25 ml/min with linear acetonitrile gradients from 0% to 80% solvent B in 20 min. Samples were delivered to the sprayer by splitter (1/8) of RPLC flux. The electrospray mass spectrometer was a triple quadrupole equipped with an atmospheric-pressure ionisation ion source API-I from Sciex (Thornhill, Ont., Canada).

### 3. Results and discussion

The Hyper D cation exchanger, recently developed for protein separation, is described as a unique stationary phase consisting of two parts: a solid porous skeleton made of a mixture of mineral oxides coated with a thin layer of polystyrene and a soft three-dimensional crosslinked hydrogel located within the pores of the rigid structure [5,7,8]. Acidic sulfonated groups are attached on side chains to the three-dimensional filling gel through a secondary amido bond. The whole organic polymer is very hydrophilic; it contains a high amount of water and behaves like a hydrogel. The advantage of this material for protein separation compared with other available well-known supports has been reported [5].

Fig. 1 shows the separation of these four basic small peptides on the cation exchanger S-Hyper D column. The peptide mixture was subjected to a linear A–B gradient (1% B/min, flow-rate of 0.6 ml/min) at pH 4. The separation obtained was relatively good at low load (100  $\mu$ g injected) considering the lower charge difference between these molecules (Table 2). A higher resolution was obtained between peptides A1 and B1 in relation to the number of lysine residues, i.e. one and two, respectively. When chromatography was performed under the same conditions on a Mono S column, the resolution obtained was lower than that of S-Hyper D (results not shown). The lower resolution is probably due to the hydrophobic interaction of the peptides with the Mono S support as previously reported by Zhu et al. [11]. It is in fact known that hydrophobic interaction with column packing leads to broad, badly skewed peaks [12]. The resolution factor,  $R_s$ , obtained on the Hyper D is given in Table 3.  $R_s$  for each peptide ( $x$ ) is calculated as:

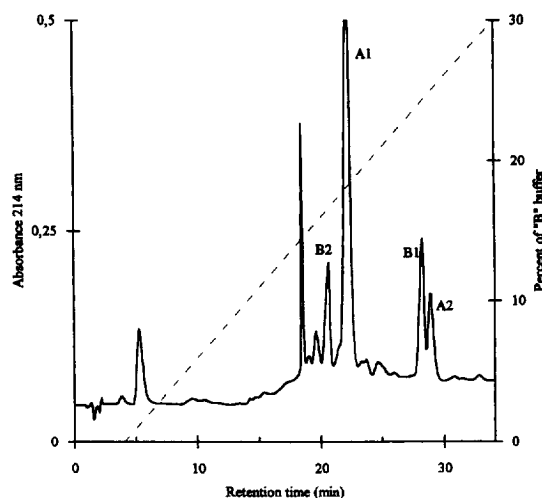


Fig. 1. Strong cation-exchange chromatography of cationic peptides. Conditions: S-Hyper D column (100 $\times$ 4.6 mm I.D., 10  $\mu$ m particle size), linear A–B gradient [10 mM salt per min (1% B/min)], after 4 min isocratic elution with buffer A. Buffer A is 10 mM sodium acetate, pH 4, and buffer B is buffer A plus 1 M NaCl, pH 4. Flow-rate 0.6 ml/min. Properties of the peptides are reported in Table 1.

$$R_s = \frac{2|V_x - V_y|}{W_x + W_y}$$

and refers to its neighbouring peaks ( $y$ );  $V$ =retention volume (ml);  $W$ =peak width at base (ml). The influence of the gradient slope on the resolution among these four peptides is given in Table 3. As expected, a decreasing gradient slope from 1 to 0.2% B/min (from 10 to 2 mM salt/min) leads to a better resolution among peptides. The elution order was as expected on the basis of the nominal net charges at

Table 2  
Physico-chemical properties of the cationic peptides studied

Peptide	Hydrophobicity index <sup>b</sup> (kcal/mol)	pI <sup>a</sup>	Charge at pH <sup>a</sup>		
			1.5	4	6
A1	11.7	9.9	1.83	1.01	0.99
A2	13.2	10.5	2.83	2.01	1.99
B1	3.0	9.6	2.83	2.01	1.07
B2	1.5	6.35	1.83	0.46	0

<sup>a</sup> According to Skoog and Wichman [9].

<sup>b</sup> Calculated according to Bigelow [10].

Table 3  
Influence of pH and gradient slope on the resolution factors

pH	Gradient slope (% B/min)	Resolution factors				
		A1 ↔ B2	A2 ↔ B1	A1 ↔ B	or	A2 ↔ B2
1.5	1	1.2	1.5	–		3.5
	0.4	1.4	2.2	–		5.7
	0.2	1.4	2.5	–		7.9
4	1	1.3	0.6	4.8		–
	0.4	1.9	0.7	6.2		–
	0.2	1.9	1	6.8		–
6	1	7.9	4.3	1.2		–
	0.4	9.3	8.6	2.5		–
	0.2	8.5	9.7	2.2		–

A1 ↔ B1 or A2 ↔ B2 were used according to the elution order at various pH.

pH 4. An excellent recovery of the injected amount (>95%) was always obtained.

The influence of buffer pH on peptide separation is shown in Fig. 2. The resolution ( $R_s$ ) obtained at pH 6 was four times higher than at pH 4 or pH 1.5 (Table 3). Because the net charges of peptides A1 and A2 were not significantly affected by increasing the pH value from 4 to 6 (Table 2), a similar elution time for these molecules was expected at those two pH values. However, as shown in Fig. 2, A1 and A2 retention times were higher at pH 4 than at pH 6. This observation suggested, at least in the case of the peptides studied here, that the S-Hyper D column exhibits a pH effect in such a way that peptides are more strongly retained at acidic pH. An opposite behaviour has been reported in the case of a Synchropak S300 strong cation-exchanger, where peptide retention decreased with decreasing pH values from 6.5 to 3 [12]. The chromatographic behaviour observed in the present work is probably caused by the ionisation state of the COOH terminal groups ( $pK_a=2.2$ ) which decreased at acidic pH. On the other hand, a good separation between A1 and B1 peptides, with a similar net charge (+1) was observed at pH 6. This excellent selectivity of the exchanger is confirmed by the results obtained at pH 1.5 (Fig. 2C). Peptides with a similar net charge (A1, B2=+1.83 and A2, B1=+2.83) were well-resolved. Interestingly, it must also be noted that the elution order of the peptides at pH 1.5 was the reverse of

that observed at the other pH values, at pH 1.5 B2 and B1 being eluted later than A1 and A2, respectively. Consequently, the selectivity of the S-Hyper D column towards peptides of the same net charge observed at pH 1.5 and pH 6, as well as the phenomenon of “retention-time inversion” obtained at pH 1.5 indicate that parameters other than net positive charge were also involved.

The contribution of hydrophobic interactions seems to be negligible because the retention time of these peptides was not reduced in the presence of 20% acetonitrile (results not shown).

A selective separation of peptidic fragments with the same net positive charge has been observed with a poly-(2-sulfo-ethylaspartamide) exchanger by Iadarola et al. [13]. These authors explained the observed selectivity by a positional effect of charged residues in the peptidic sequence: the peptide with cationic amino acid located at the N-terminal end interacts more strongly with the support (more retained) than the peptide having the cationic residue at the C-terminal part of the sequence. Concurrently, the selectivity of such a strong cation-exchanger type towards peptides with the same net charge has been attributed by Zhu et al. [11] to the hydrophilic interactions between peptides and the chromatographic support. With highly hydrophilic supports, retention increases with the polarity of the solute. The elution order obtained at pH 4, where B1 (containing one lysine residue located at the N-

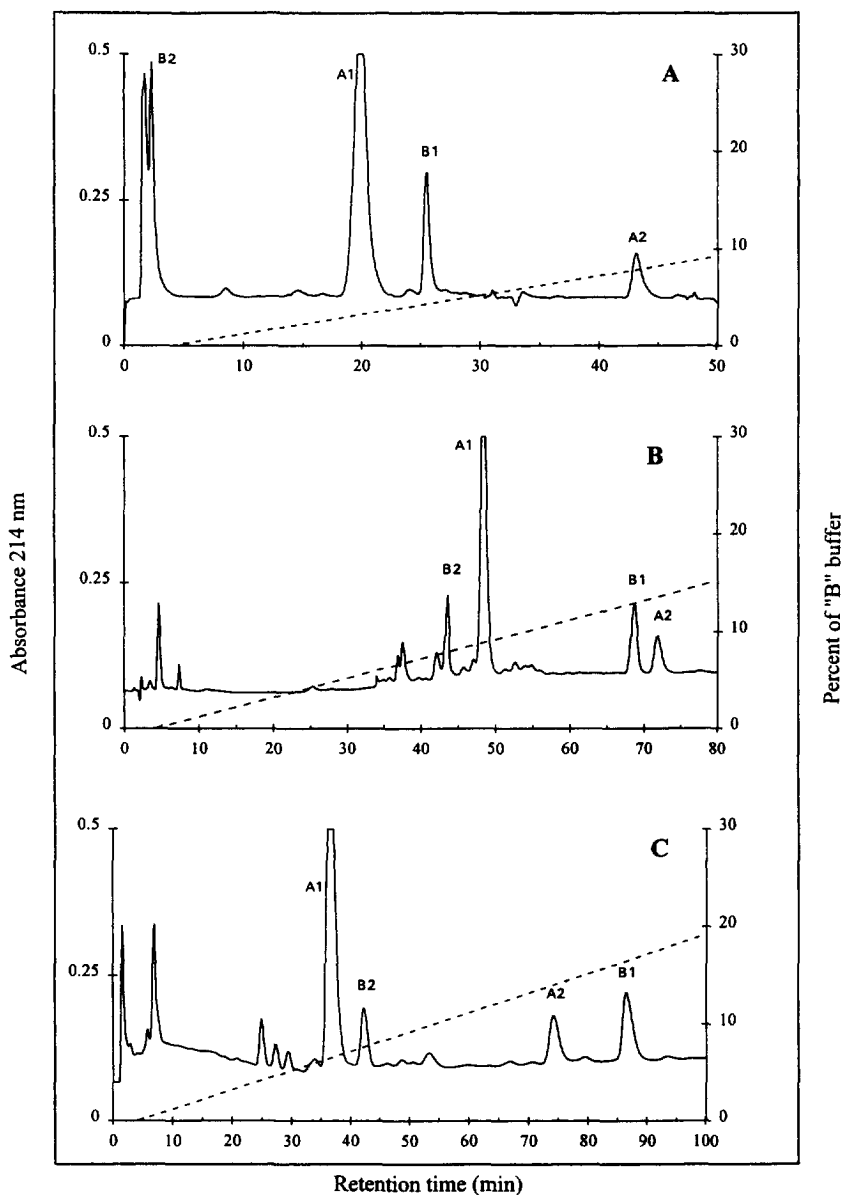


Fig. 2. Separation of the four cationic peptides on a S-Hyper D column ( $100 \times 4.6$  mm I.D.,  $10 \mu\text{m}$  particle size) as a function of buffer pH. (A) pH 6; (B) pH 4; (C) pH 1.5. Linear A–B gradient [ $2 \text{ mM}$  salt per min ( $0.2\% \text{ B/min}$ )] after 4 min isocratic elution with buffer A. Flow-rate  $0.6 \text{ ml/min}$ .

terminal end) was eluted before A2, ruled out the positional effect on the retention behaviour. Hence, the excellent separation observed between peptides with the same net positive charge (Table 2) can be readily explained by the hydrophilic interactions

between the peptides and the Hyper D sorbent. Peptides with the same net charge were eluted in order of increasing hydrophilicity: the least hydrophilic peptide (group A) was eluted earlier than the most hydrophilic one (group B). Peptides of group B

were more hydrophilic (less hydrophobic) than those of group A (Table 1). Selective separation on the basis of hydrophilic interactions was observed for peptides with the same net charge; otherwise the peptides were separated mainly by an ionic mechanism. As a consequence of this mixed-mode separation, the simple well-known linear relationship between retention time and net charge previously reported for several peptide mixtures [14–16] could not be used to predict the elution order of the present particular basic peptides on the Hyper D support.

The importance of charge density on retention behaviour during ion-exchange chromatography was reported by Lorne-Burke et al. [12]. These authors established a linear relationship between retention versus net charges/ $\ln N$  (where  $\ln N$  is the logarithm of the number of amino acid residues of each peptide). However, no correlation was found when the retention time of the four peptides versus the charge density was plotted. Thus, the difference in charge density does not suffice to explain the chromatographic behaviour of the small cationic peptides. However, the observed elution profile could be related to the number of charged groups involved in the adsorption–desorption process. The number of charges interacting with a surface of the support ( $Z$ ) can be estimated, under isocratic conditions, according to the equation reported by Kopaciewicz et al. [17]:

$$\log k' = 2 Z \log \frac{1}{[\text{NaCl}]}$$

where  $k'$  is the capacity factor,  $[\text{NaCl}]$  is the concentration of displacement salt used during the various isocratic elutions. Fig. 3 shows a correlation between this equation and the results from the four peptides studied. As previously mentioned in terms of retention time, the number of sites for each molecule interacting with the support decreased concomitantly with an increase in pH from 1.5 to 6, indicating the increasing influence of negative charges in the case of small peptides. Several values of  $Z$  were lower than the net charge of the molecules. However, a  $Z$  value greater than the net charge was also observed for peptide B2 at pH 4 ( $Z=0.52$ , net charge=0.46). Therefore, as demonstrated in the

case of proteins [17], the number of charges interacting with a surface may either be greater or less than the net charge of the molecule. This result could explain the non-linear relationship between retention time and the net positive charge of actual peptides. Moreover, the elution characteristics of a pair of peptides during gradient elution is also expressed through the similar slopes of this pair of peptides under isocratic conditions. The main difference between these two pairs concerned the number of basic amino acids: 1 lys for A1 and B2 and 2 lys for A2 and B1. Hence, the number of cationic residues of the peptides was taken into account in order to predict the elution order by use of the Hyper D column. From Fig. 4 it can be seen that division of the product between peptide net charge ( $NC$ ) and the number of basic residues ( $B$ ) by the logarithm of the number of residues ( $N$ ):

$$\frac{NC \times B}{\ln N}$$

and plotting this value against the observed elution time, resulted in a single, straight-line plot with a correlation of 0.98, 0.99 and 0.99 at pH 6, 4 and 1.5, respectively. This correlation holds true for all pH values tested, including the inversion of elution order observed at pH 1.5.

#### 4. Conclusion

The efficiency of an S-Hyper D anionic stationary phase in the separation of small closely related peptides was reported. It was shown that this chromatographic support exhibits ionic as well as hydrophilic interactions, according to the charge of the molecules to be separated. This mixed-mode separation mechanism promoted the selective separation between peptides with the same net positive charge, indicating the high discriminating power of the S-Hyper D material. Consequently, the well-known relationship between peptide elution time and net charge could not be used to completely explain the observed elution profile of these particular peptides. Our results suggest that the behaviour of this par-

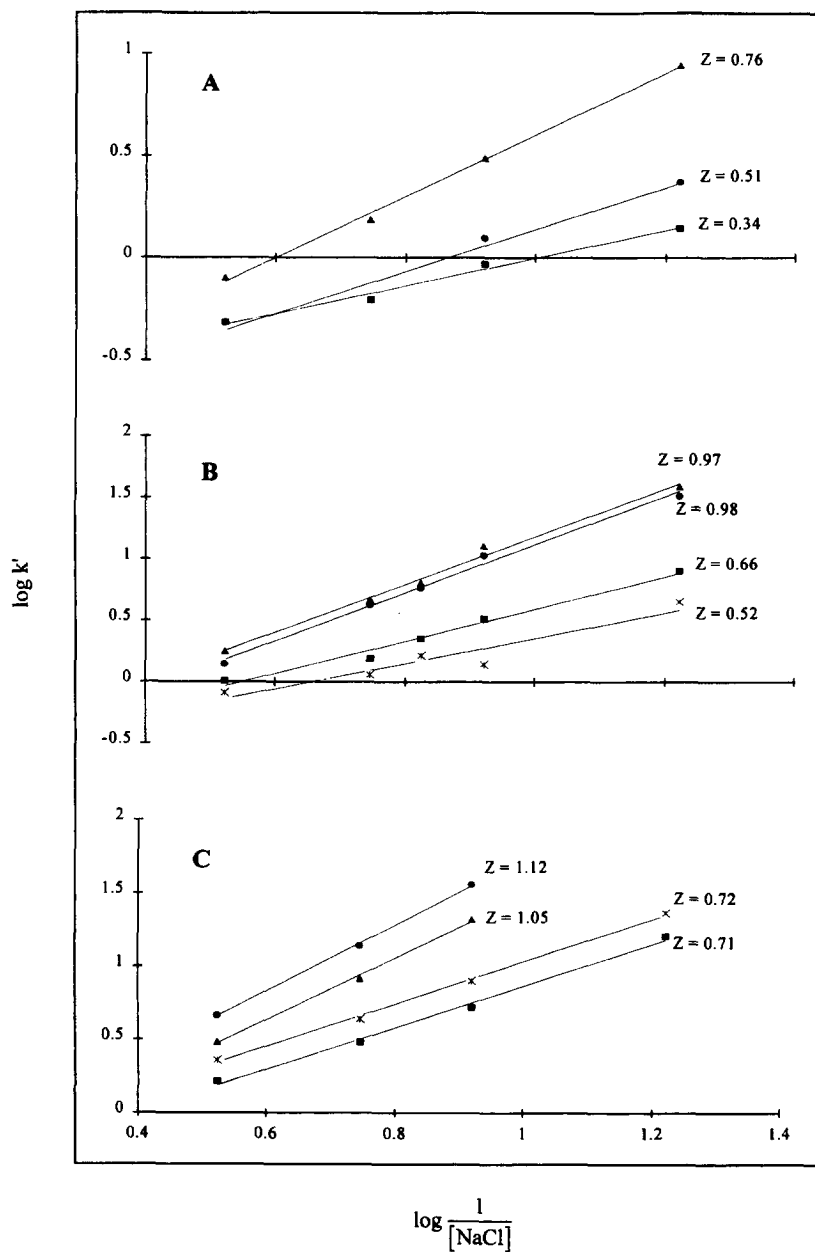


Fig. 3. An estimation of the number of charge interactions between peptides and the surface of a strong cation-exchange support at pH 6 (A), pH 4 (B) and pH 1.5 (C). (■) peptide A1; (▲) peptide A2; (●) peptide B1; (×) peptide B2.

ticular set of four cationic peptides appears to be correlated to the net charge corrected for the peptide chain length and the number of basic residues.

The excellent resolution of individual peptides

could thus be exploited for a preparative scaling-up in order to obtain sufficient quantities of them for subsequent biological as well as pharmacological investigations.

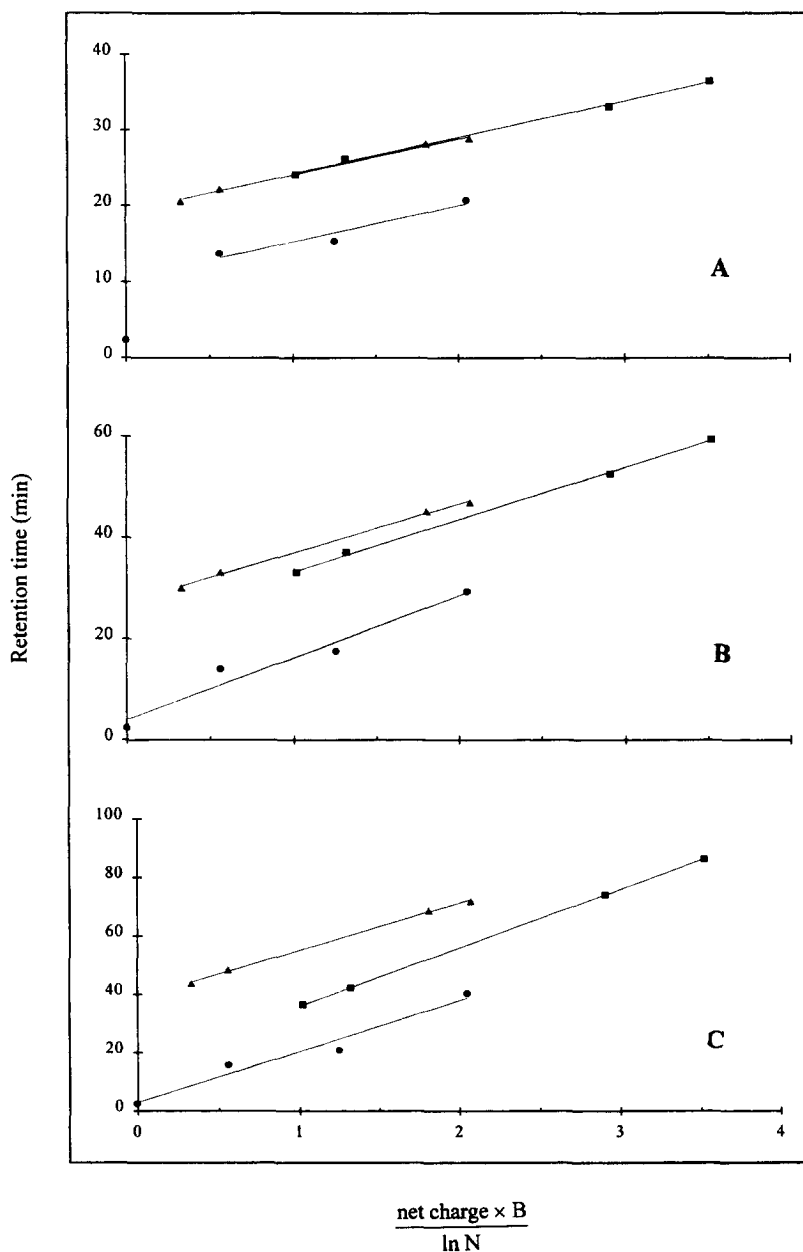


Fig. 4. Plot of peptide elution times with strong cation-exchange chromatography vs. peptide net charge  $\times B / \ln N$  at various pH and gradient slopes. Conditions: (●) pH 6; (▲) pH 4; (■) pH 1.5. (A) 1%/min; (B) 0.4%/min; (C) 0.2%/min.

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