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Context-dependent effects on the hydrophilicity/hydrophobicity of side-chains during reversed-phase high-performance liquid chromatography: Implications for prediction of peptide retention behaviour

C.T. Mant and R.S. Hodges*

Department of Biochemistry and Molecular Genetics, University of Colorado at Denver and Health Sciences Center, Aurora, CO 80045, USA

Abstract

The present study set out to investigate whether observed relative hydrophilicity/hydrophobicity values of positively charged side-chains (with Lys and Arg as representative side-chains) or hydrophobic side-chains (with Ile as the representative side-chain) were context-dependent, i.e., did such measured values vary depending on characteristics of the peptides within which such side-chains are substituted (overall peptide hydrophobicity, number of positive charges) and/or properties of the mobile phase (anionic counterions of varying hydrophobicity and concentration)? Reversed-phase high-performance liquid chromatography (RP-HPLC) was applied to two series of four synthetic peptide analogues (+1, +2, +3 and +4 net charge), the only difference between the two peptide series being the substitution of one hydrophobic Ile residue for a Gly residue, in the presence of anionic ion-pairing reagents of varying hydrophobicity ($\text{HCOOH} \approx \text{H}_3\text{PO}_4 < \text{TFA} < \text{PFPA} < \text{HFBA}$) and concentration (2–50 mM). RP-HPLC of these peptide series revealed that the relative hydrophilicity of Lys and Arg side-chains in the peptides increased with peptide hydrophobicity. In addition the relative hydrophobicity of Ile decreased dramatically with an increase in the number of positive charges in the peptide, this hydrophobicity decrease being of greater magnitude as the hydrophobicity of the anionic ion-pairing reagent increased. These results have significant implications in the prediction of peptide retention times for proteomic applications.

Keywords

Peptides; Reversed-phase high-performance liquid chromatography; Anionic ion-pairing reagents; Peptide hydrophobicity; Side-chain hydrophilicity; Side-chain hydrophobicity

1. Introduction

The homologous series of volatile perfluorinated acids – trifluoroacetic acid (TFA), pentafluoropropionic acid (PFPA), heptafluorobutyric acid (HFBA) – have proven to be excellent hydrophobic anionic ion-pairing reagents for reversed-phase high-performance liquid chromatography (RP-HPLC) of peptides [1–9]. In addition, phosphoric acid has also seen use as a non-volatile hydrophilic anionic ion-pairing reagent for peptide applications [4, 5,8–15]. Such ion-pairing reagents affect peptide retention behaviour during RP-HPLC by

interaction of the negative anions (i.e., TFA⁻, PFPA⁻, HFBA⁻, phosphate) with positively charged peptide groups (Lys, His and Arg side-chains; free terminal α -amino group), thereby neutralizing these charges and, hence, reducing the hydrophilicity of the peptides. In addition, peptide hydrophobicity is concomitantly enhanced by the hydrophobic anions of the perfluorinated acids in the order TFA < PFPA < HFBA [6,8,9].

In the present study, we applied RP-HPLC in the presence of varying concentrations of formic acid, phosphoric acid, TFA, PFPA and HFBA to a mixture of two series of four model peptides, both series containing peptides of +1, +2, +3 and +4 net charge but with the two series varying in overall peptide hydrophobicity. From the retention behaviour of these peptides, we were able to determine the effect of peptide hydrophobicity, as well as the number of positive charges in a peptide, on RP-HPLC of peptides in the presence of anionic ion-pairing reagents of varying hydrophobicity and concentration.

There is considerable effort to design algorithms to predict accurately peptide retention times in RP-HPLC to aid in peptide assignment from LC/MS/MS data in proteomics applications [16–18]. The use of formic acid or the hydrophobic ion-pairing reagent trifluoroacetic acid is commonplace in the separation by RP-HPLC of tryptic digests of complex protein mixtures during LC/MS or LC/MS/MS. This study clearly addresses some important issues in predicting peptide retention times from amino acid composition in RP-HPLC.

2. Experimental

2.1. Materials

HPLC-grade water was prepared by an E-pure water purification system from Barnstead International (Dubuque, IA, USA). Acetonitrile was obtained from EM Science (Gibbstown, NJ, USA). ACS-grade formic acid (HCOOH) was obtained from Aldrich (Milwaukee, WI, USA); reagent-grade phosphoric acid (H₃PO₄) was obtained from Caledon Laboratories (Georgetown, Ontario, Canada); TFA was obtained from Hydrocarbon Products (River Edge, NJ, USA); PFPA was obtained from Fluka (Buchs, Switzerland); and HFBA was obtained from Pierce Chemical (Rockford, IL, USA).

2.2. Column and HPLC conditions

Analytical RP-HPLC runs were carried out on a Zorbax SB300-C₈ column (150 mm × 2.1 mm I.D.; 5 μ m particle size, 300 Å pore size) from Agilent Technologies (Little Falls, DE, USA), using a linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min and a temperature of 25°C; Eluent A was 2–50 mM aq. HCOOH, H₃PO₄, TFA, PFPA or HFBA and Eluent B was the corresponding concentration of the respective ion-pairing reagent in acetonitrile.

2.3. Instrumentation

Analytical RP-HPLC runs were carried out on an Agilent 1100 Series liquid chromatograph. Preparative RP-HPLC runs were carried out on a System Gold chromatograph from Beckman Coulter (Fullerton, CA, USA). Peptide Synthesis was carried out on an Advanced ChemTech Vantage synthesizer (Louisville, KY, USA). The correct molecular masses of purified peptides were confirmed by matrix assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry using a Voyager DE-PRO mass spectrometer from PerSeptive Biosystems (Foster City, CA, USA) with a high voltage detector employed in linear mode.

2.4. Peptide synthesis

Peptides were synthesized on a Rink Amide 4-methylbenzhydrylamine (MBHA) resin using conventional 9-fluorenylmethoxycarbonyl (Fmoc) chemistry and standard solid-phase synthesis methodology. Each coupling step used eight times excess of amino acid per free

amino group on the resin with 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU)/N-hydroxybenzotriazole (HOBT) activation for the first coupling, then diisopropylcarbodiimide (DIC)/HOBT for the second coupling. Cleavage of the peptide from the resin and deprotection of the side-chain protecting groups was achieved by treating the dried resin with a cleavage cocktail (20 ml/g of resin) consisting of TFA/water/ethanedithiol (EDT)/triisopropylsilane (TIS)/methyl sulfide (94:1.5:1.5:1.5:1.5) for 2 h at room temperature. The resin was removed by filtration under reduced pressure and washed twice with neat TFA. The filtrates were combined and peptide was precipitated with a 10-fold volume of cold ether. The precipitated peptide was then filtered under reduced pressure and washed with cold ether. The peptide was dissolved in a 50:50 mixture of acetonitrile:water and lyophilized.

3. Results and discussion

3.1. Design of synthetic model peptides

From Fig. 1, the two series of model peptides within the peptide mixture exhibit variations in hydrophobicity and the number of positively charged residues. Thus, the peptides of both series ("a" and "b" series) have net charges of +1 (1 Lys), +2 (2 Lys), +3 (2 Lys, 1 Arg) and +4 (2 Lys, 2 Arg); the only difference between the two peptide series is substitution of one hydrophobic Ile residue ("b" series) for a Gly residue ("a" series) in the peptide sequences. The relative intrinsic hydrophobicity of all 20 amino acid side-chains has recently been determined using RP-HPLC of peptides substituted at a single substitution site [19]. The presence of several glycine residues ensures negligible secondary structure for these peptides [20,21], i.e., they have a "random coil" configuration, to avoid potential complications in data interpretation due to selectivity differences in peptide RP-HPLC retention behaviour arising from conformational variations [15,22]. The presence of at least two hydrophobic residues (Leu) in the peptide sequences ensures that the peptides will be retained to a reasonable degree by the stationary phase, even in the presence of hydrophilic ion-pairing reagents HCOOH and H₃PO₄. The 10-residue length of the peptides was chosen to mimic the size of an average peptide fragment from proteolytic digests of proteins since we believe that results obtained with model peptides such as those shown in Fig. 1 can then be extrapolated to peptides as a whole. It is important to note that all model peptides shown in Table 1 exhibited excellent stability in the presence of all acidic ion-pairing reagents and reagent concentrations used in the present study.

3.2. RP-HPLC stationary phase

The Zorbax SB300-C8 column was designed to be highly resistant to siloxane bond hydrolysis at low pH [23–25]. In our hands, this packing has shown excellent stability when employing acidic mobile phases containing up to 60 mM (0.46%) TFA [9] and was therefore the packing of choice for the present study.

It should be noted that the pH values of the aqueous eluents used in the present study ranged from ~1.7 to 2.9, depending on the nature and concentration of the ion-pairing reagent, this pH range generally being referred to as pH 2. Note that this pH range is far enough below the pK_a values of the positively charged side-chains (Lys, Arg) so as not to affect the net positive charge on the peptides; in addition, if any underivatized silanol groups (pK_a~4.0) were on the silica-based packing, they would also remain protonated (i.e., neutral) under the RP-HPLC conditions used in the present study, thus preventing any potential undesirable electrostatic interactions between the positively charged peptides and the hydrophobic stationary phase.

3.3. Effect of anionic ion-pairing reagent hydrophobicity and concentration on peptide retention time

Figs. 2 and 3 present graphical representations of the effect of increasing counterion concentration on peptide retention times. Note that, while the concentration range for the ion-pairing reagents was 2–60 mM for H_3PO_4 , TFA (Fig. 2), PFPA and HFBA (Fig. 3), that of HCOOH was just 10–50 mM, due to poor retention of several of the peptides at HCOOH concentrations <10 mM. From Fig. 2, increasing HCOOH concentration had little or no effect on peptide retention times. A somewhat more marked effect on peptide retention times can be seen with increasing concentrations of H_3PO_4 , TFA (Fig. 2), PFPA and HFBA (Fig. 3) particularly at the lower reagent concentrations (~2–10 mM). In addition, the increase in peptide retention time was more marked both with increasing peptide net charge (+1 < +2 < +3 < +4) and hydrophobicity of the counterion ($\text{HCOO}^- < \text{H}_2\text{PO}_4^- < \text{TFA}^- < \text{PFPA}^- < \text{HFBA}^-$). This relationship between effect of ion-pairing reagent concentration, hydrophobicity and peptide net positive charge is summarized in Table 1 which presents selected peptide retention data.

An interesting observation from Fig. 2 and Table 1 is the complete reversal in elution order of peptides 1a–4a as the TFA concentration is increased. Thus, at low TFA concentration, the elution order is $4a < 3a < 2a < 1a$; in contrast, at higher TFA concentrations (>10–15 mM), the elution order is $1a < 2a < 3a < 4a$. Above this TFA concentration range, the retention profiles of these four peptides are diverging. Note that the elution order of peptides 1b–4b are not reversed over the TFA concentration range employed (Table 1), albeit the profiles of these four peptides are converging with increasing TFA concentration. Elution order reversals within the two peptide series were not observed for either peptide series in the HCOOH and H_3PO_4 systems, although a reversal in elution order of 1a and 4b was observed above 30 mM H_3PO_4 . Indeed, no evidence of any convergence of peptide retention times in these hydrophilic ion-pairing reagent systems was apparent.

In the more hydrophobic PFPA system (Fig. 3, Table 1), reversal of elution order of peptides 1a–4a relative to the hydrophilic HCOOH and H_3PO_4 systems has already occurred at the lowest PFPA concentration employed (2 mM), the retention profiles of these four peptides now diverging as the PFPA concentration is increased. In contrast to TFA (Fig. 2), the elution order of peptides 1b–4b is reversed at PFPA concentrations above ~3mM (Table 1), with concomitant subsequent divergence of the peptide profiles as the PFPA concentration is further increased (Fig. 3).

Finally, in the presence of even the lowest concentration (2 mM) of HFBA, the most hydrophobic ion-pairing reagent of the present study, both series of peptides show an order reversal relative to the hydrophilic HCOOH and H_3PO_4 systems (Fig. 2, Table 1).

3.4. Effect of anionic ion-pairing reagent hydrophobicity and concentration on peptide elution profiles

Fig. 4 illustrates the effect of increasing H_3PO_4 concentration on the elution profile of the eight-peptide mixture. The limited effect of increasing this ion-pairing reagent concentration on the peptide elution profile, save for a small increase in peptide retention times, reflects the graphical representation shown in Fig. 2. Fig. 4 also illustrates the change in elution order of peptides 1a and 4b with increasing H_3PO_4 concentration: $4b < 1a$ at 5 mM H_3PO_4 ; co-elution of 4b and 1a at 30 mM H_3PO_4 ; $1a < 4b$ at 100 mM H_3PO_4 . The elution profile obtained with 30 mM HCOOH is included to illustrate the almost identical profile obtained with just 5 mM H_3PO_4 , indicating that the HCOO^- anion is considerably more hydrophilic and/or less effective as a counterion than H_2PO_4^- . Note that UV absorbance at 215 nm (instead of the more generally used and more sensitive 210 nm wavelength) was employed for peptide bond detection due to

high background absorbance of high concentrations of the perfluorinated acids as well as formic acid.

Fig. 5 now illustrates the effect of TFA concentration on the elution profile of the eight-peptide mixture, previously presented graphically in Fig. 2. Thus, at 2 mM TFA, all eight peptides are well separated, with both groups of peptides showing elution orders of +4 peptides < +3 < +2 < +1. However, at 10 mM TFA, the “a” series peptides are now essentially co-eluted, whilst the “b” series peptides are still separated to baseline in the same elution order, albeit not as well separated as at the lower 2 mM concentration. This essential co-elution of peptides 1a–4a at a concentration of 10 mM TFA was clearly observed in Fig. 2 where the overall elution order of these peptides was in the process of reversing. Finally, at 50 mM TFA, peptides 1a–4a are separated once more to baseline, albeit in the reverse elution order observed at 2 mM TFA. In addition, poor resolution is observed for peptides 1b–4b (reflected in their close convergence in Fig. 2) at 50 mM TFA.

From Fig. 6, at a concentration of 1 mM PFPA, the “a” series peptides are essentially co-eluted while the “b” series peptides are baseline resolved in the order 4b < 3b < 2b < 1b. Raising the concentration to 10 mM PFPA now results in baseline separation of all eight peptides with the peptide elution order now 1a < 2a < 3a < 4a and 1b < 2b < 3b < 4b, such observations already graphically presented in Fig. 3.

The effect of increasing anionic ion-pairing reagent concentration on elution behaviour of positively charged peptides has, of course, been reported previously [4,8,9], i.e., there is a general increase in peptide retention time with increasing reagent concentration, this effect being apparent in Figs. 2, 3, 5 and 6. Furthermore, this increase is related to the number of positively charged residues a peptide contains, i.e., the greater the positive charge on the peptide, the greater the impact of increasing reagent concentration, this effect again being readily apparent in Figs. 5 and 6, where the effect of increasing TFA (Fig. 5) or PFPA (Fig. 6) concentration is more dramatic in the order +4 < +3 < +2 < +1. However, a key observation from Fig. 2 (bottom panel), Figs. 3, 5 and 6 is the disproportionate effect of increasing reagent concentration on the two series of peptides which differ only in the substitution of the hydrophobic Ile residue at position 3 of the peptide sequences (“b” series) in place of Gly (“a” series), i.e., each “b” series peptide is more intrinsically hydrophobic than its “a” series counterpart albeit the net positive charge on such peptide pairs is identical. From the graphical representations shown in Fig. 2 (bottom panel), Fig. 3 and the elution profiles in Fig. 5, the effect of increasing ion-pairing reagent concentration on peptide retention times is greater for the “a” series peptides at a specific reagent concentration compared to their more hydrophobic “b” series counterparts (i.e., 4a > 4b, 3a > 3b, etc.) despite the identical charge on the four peptide pairs (4a/4b, 3a/3b, etc.). Such results indicate that overall peptide hydrophobicity is a parameter to consider when assessing the effect of ion-pairing reagent concentration on peptide retention behaviour and will be discussed later in the manuscript.

The above speculation concerning the role of overall peptide hydrophobicity also applies to the effect of increasing counterion hydrophobicity on relative retention times of the “a” and “b” series peptides. This can be visualized best by comparing the peptide elution profiles in 10 mM TFA (Fig. 5, middle), 10 mM PFPA (Fig. 6, middle) and 10 mM HFBA (Fig. 6, bottom). In a similar manner to the effect of increasing ion-pairing reagent concentration on peptide retention times, it is also known that increasing anionic counterion hydrophobicity results in an increase in retention times of positively charged peptides [1–4,8,9,12]. In addition, the greater the number of positive charges in a peptide, the greater its retention time increase with increasing counterion hydrophobicity. Thus, the retention times of all eight peptides in the present study are increased in the presence of TFA (Fig. 5, middle), PFPA (Fig. 6, middle) and HFBA (Fig. 6, bottom), this increase being more marked with increasing counterion

hydrophobicity ($\text{TFA}^- < \text{PFPA}^- < \text{HFBA}^-$) and increasing peptide net positive charge (+1 < +2 < +3 < +4). However, in a similar manner to the effect of increasing concentration of ion-pairing reagent, the effect of increasing counterion hydrophobicity is different in magnitude for the two peptide series, i.e., the retention times of the “a” series peptides increase more with increasing counterion hydrophobicity relative to their more hydrophobic “b” series counterparts. Thus, in the presence of 10 mM TFA (Fig. 5, middle), peptides 1a–4a are essentially co-eluted whilst the “b” series peptides are baseline resolved in the order $4b < 3b < 2b < 1b$; in 10 mM PFPA (Fig. 6, middle), the “a” series peptides are now completely resolved in the order $1a < 2a < 3a < 4a$, with the “b” series peptides now showing a reversal in elution compared to the results in 10 mM TFA, i.e., $1b < 2b < 3b < 4b$; finally, in the presence of 10 mM HFBA (Fig. 6, bottom), the two groups of peptides now overlap, with 3a and 4a being eluted within the “b” series peptides. Finally, it is worth noting from Figs. 5 and 6 the value of varying the hydrophobicity and/or concentration of these hydrophobic anionic ion-pairing reagents for optimization of separations of positively charged peptides.

3.5. Influence of peptide net positive charge and overall hydrophobicity on peptide retention behaviour when varying anionic ion-pairing reagent concentration and hydrophobicity

Fig. 7 illustrates the relationship between net positive charge and the retention characteristics of the “a” series peptides at different concentrations of TFA, PFPA and HFBA relative to the retention times in the presence of 2 mM H_3PO_4 (Table 2). From Fig. 7, as the net positive charge on the peptide increases, the change in retention time relative to that obtained in 2 mM H_3PO_4 also increases. This change is more dramatic with increasing hydrophobicity of the ion-pairing reagent ($\text{TFA} < \text{PFPA} < \text{HFBA}$; compare 2 mM TFA with 2 mM PFPA and 2 mM HFBA and compare 20 mM TFA with 20 mM PFPA). In addition, this change is also more dramatic with increasing acid concentration (compare 2 mM TFA with 50 mM TFA and 2 mM PFPA with 20 mM PFPA). Note that the effect of increasing the concentration of H_3PO_4 from 2 mM to 50 mM had little effect on peptide retention times, particularly when compared to the considerably more hydrophobic TFA, PFPA and HFBA.

Fig. 8 now compares the relationship between peptide net positive charge and the retention times of the “a” and “b” peptide series in the presence of 2 mM TFA, PFPA and HFBA relative to their retention times in 2 mM H_3PO_4 . From Fig. 8, for both peptide series, as the net positive charge on the peptide increases, the change in retention time relative to that in 2 mM H_3PO_4 (Δt) increases. In addition, the magnitude of the Δt increase is greater with increasing hydrophobicity of ion-pairing reagent: $\text{TFA} < \text{PFPA} < \text{HFBA}$. Of particular note here is that the change in retention time (Δt) relative to the increase in peptide net positive charge is greater for the “a” series peptides than the more hydrophobic “b” series peptides, i.e., as noted previously, overall peptide hydrophobicity appears to be a consideration when delineating the effect of ion-pairing reagent hydrophobicity on retention behaviour of positively charged peptides.

3.6. Relative hydrophilicity/hydrophobicity of Arg, Lys and Ile side-chains in different anionic ion-pairing reagents

From Table 1, factors which influence the response of the peptides to variations in hydrophobicity of ion-pairing reagent clearly would include the number of positively charged residues the peptides contain since it is via interactions with such residues that anionic counterions exert their effect. As noted previously, the relative hydrophilicity/hydrophobicity of positively charged residues such as Lys and Arg is dependent on the type and concentration of hydrophobic ion-pairing reagents such as TFA, PFPA and HFBA, i.e., the relative hydrophobicity of such residues increases with increasing reagent concentration and, particularly, hydrophobicity.

Fig. 9 now examines the relative hydrophilicity/hydrophobicity of the Lys and Arg side-chains in the Gly 3 (“a” series) and Ile 3 (“b” series) peptides in the presence of 10 mM of ion-pairing reagents of increasing hydrophobicity ($\text{HCOOH} < \text{H}_3\text{PO}_4 < \text{TFA} < \text{PFPA} < \text{HFBA}$). Data for Fig. 9 are shown in Table 3. From Fig. 9, the relative hydrophobicity of the Lys and Arg residues within both series of peptides increases with increasing hydrophobicity of the ion-pairing reagent, from a small increase between the hydrophilic reagents HCOOH and H_3PO_4 followed by a substantial increase with the perfluorinated acids. Indeed, the Lys and Arg residues would be classified as hydrophilic (i.e., below the horizontal line) in the presence of certain ion-pairing reagents and moderately hydrophobic (i.e., above the horizontal line) as the hydrophobicity of the reagent increases. The plots for the Gly 3 (“a” series) and Ile 3 (“b” series) peptides, although similar, are of a different magnitude with the relative hydrophobicities of Lys and Arg always being greater in the Gly 3 series compared to the more hydrophobic Ile 3 series. This observation is highlighted by the classification of Lys and Arg in the Ile 3 peptides being moderately hydrophilic in the presence of 10 mM TFA but slightly hydrophobic in the Gly 3 peptides.

Fig. 10 illustrates the relative hydrophobicity of the Ile side-chain within the Ile 3 (“b” series) peptides as a function of the number of positive charges on the peptides as well as the hydrophobicity of the ion-pairing reagent (data shown in Table 3). From Fig. 10, the relative hydrophobicity of the Ile side-chain decreases with an increase in net positive charge of the peptide. Interestingly, the profiles for this decrease are similar for all five ion-pairing reagents, although the overall magnitude of the decrease increases with increasing hydrophobicity of the ion-pairing reagent, i.e., $\text{HCOOH} < \text{H}_3\text{PO}_4 < \text{TFA} < \text{PFPA} < \text{HFBA}$, and increasing net positive charge on the peptide. Thus, the difference in hydrophobicity of Ile in going from the hydrophilic formic acid system to the very hydrophobic HFBA system (expressed as Δt between the two mobile phase systems) is 1.0 min, 1.8 min, 2.1 min and 2.4 min for the +1, +2, +3 and +4 peptides, respectively.

4. Conclusions

The present study set out to investigate whether observed relative hydrophilicity/hydrophobicity values of positively charged side-chains (with Lys and Arg as representative side-chains) or hydrophobic side-chains (with Ile as the representative side-chain) were context-dependent in terms of the environment in which they are measured (peptide and/or mobile phase characteristics). The novel findings from our results were as follows: firstly, the measured relative hydrophilicity/hydrophobicity values of Lys and Arg side-chains was dependent on the hydrophobicity of the peptides within which they are substituted, i.e., relative hydrophilicity of the Lys and Arg side-chains increased with increasing peptide hydrophobicity, i.e., on replacing a Gly residue with a hydrophobic Ile residue; secondly, the relative hydrophobicity of an Ile side-chain decreased dramatically as the number of positive charges in the peptide within which it was substituted increased, regardless of the anionic ion-pairing reagent used; and thirdly, the relative hydrophobicity decrease of the Ile side-chain was of greater magnitude as the hydrophobicity of the anionic ion-pairing reagent increased. These results have significant implications in the prediction of peptide retention times in RP-HPLC in the presence of hydrophobic ion-pairing reagents. Thus, these results show that prediction based on amino acid composition and hydrophobicity coefficients would be significantly in error depending on the positive charge on the peptide. Fortunately, for tryptic digests of proteins, peptide separation in formic acid or TFA has little effect on the hydrophobicity coefficient for Ile in peptides with +1 or +2 charges (the majority of peptides expected from a tryptic digest would be +2 peptides). However, for +3 and +4 peptides, the hydrophobicity of an Ile side-chain changes dramatically. Such results suggest that prediction problems may become significant with the use of other proteolytic enzymes where peptides containing three or more positive charges would be much more likely.

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Peptide Sequences

		1	2	3	4	5	6	7	8	9	10	
1a	Ac	–	G	G	G	G	G	L	G	L	G	(K)– amide
2a	Ac	–	G	G	G	G	(K)	L	G	L	G	(K)– amide
3a	Ac	–	G	(R)	G	G	(K)	L	G	L	G	(K)– amide
4a	Ac	–	(R)	(R)	G	G	(K)	L	G	L	G	(K)– amide
1b	Ac	–	G	G	I	G	G	L	G	L	G	(K)– amide
2b	Ac	–	G	G	I	G	(K)	L	G	L	G	(K)– amide
3b	Ac	–	G	(R)	I	G	(K)	L	G	L	G	(K)– amide
4b	Ac	–	(R)	(R)	I	G	(K)	L	G	L	G	(K)– amide

Fig. 1.

Sequences of synthetic model peptides used in this study. The positively charged residues are circled in each sequence. The “a” series peptides are substituted with glycine at position 3; the “b” series peptides are substituted with isoleucine at position 3 (boxed). The number (e.g., 1a, 3b) denotes the number of positively charged groups in the peptide. Ac- denotes N^α-acetyl and -amide denotes C^α-amide.

Fig. 2.

Effect of anionic ion-pairing reagent concentration on retention behaviour of positively charged peptides. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 10 mM–50 mM aq. HCOOH or 2 mM–50 mM aq. H₃PO₄ or TFA and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25°C. The sequences and denotations of the peptides are shown in Fig. 1. Data taken from Table 1.

Fig. 3. Effect of anionic ion-pairing reagent concentration on retention behaviour of positively charged peptides. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 10 mM–50 mM aq. PFPA or HFBA and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25°C. The sequences and denotations of the peptides are shown in Fig. 1. Data taken from Table 1.

Fig. 4. Effect of H₃PO₄ concentration on peptide elution profiles compared to 30 mM HCOOH. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 5 mM, 30 mM or 100 mM aq. H₃PO₄ or 30 mM aq. HCOOH and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25°C. The sequences and denotations of the peptides are shown in Fig. 1.

Fig. 5. Effect of TFA concentration on peptide elution profiles. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 2 mM, 10 mM or 50 mM aq. TFA and Eluent B is the corresponding TFA concentration in acetonitrile; temperature, 25° C. The sequences and denotations of the peptides are shown in Fig. 1.

Fig. 6. Effect of PFPA concentration on peptide elution profiles compared to 10 mM HFBA. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 1 mM or 10 mM aq. PFPA or 10 mM aq. HFBA and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25°C. The sequences and denotations of the peptides are shown in Fig. 1.

Fig. 7.

Relationship between ion-pairing reagent hydrophobicity and concentration and retention characteristics of “a” series peptides. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 50 mM aq. H_3PO_4 , 2 mM or 50 mM aq. TFA, 2 mM or 20 mM aq. PFPA or 20 mM aq. HFBA and Eluent B is the corresponding ion-pairing reagent concentration in acetonitrile; temperature, 25°C. Δt denotes retention times of the “a” series peptides in a mobile phase containing a particular concentration of ion-pairing reagent minus the retention times of the peptides in a mobile phase containing 2 mM H_3PO_4 . The sequences and denotations of the peptides are shown in Fig. 1.

Fig. 8.

Relationship between ion-pairing reagent hydrophobicity and retention characteristics of “a” and “b” series peptides. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 2 mM aq. TFA, PFPA or HFBA and Eluent B is 2 mM of the corresponding ion-pairing reagent in acetonitrile; temperature, 25°C, Δt denotes the retention times of the peptides in the presence of 2 mM of a particular acid minus the retention times of the peptides in a mobile phase containing 2 mM H_3PO_4 . The sequences and denotations of the peptides are shown in Fig. 1.

Fig. 9.

Relative hydrophilicity/hydrophobicity of Arg and Lys side-chains in Gly 3 ("a" series) and Ile3 ("b" series) peptides in the presence of various anionic ion-pairing reagents. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 10 mM aq. HCOOH, H₃PO₄, TFA, PFPA or HFBA and Eluent B is 10 mM of the corresponding ion-pairing reagent in acetonitrile; temperature, 25°C. The y-axis represents the change in retention time (Δt_R) obtained in each ion-pairing reagent. The closed triangles represent the Δt_R values of "b" series peptides: $t_{R4b}-t_{R3b}$, $t_{R3b}-t_{R2b}$, $t_{R2b}-t_{R1b}$; the open circles represent the Δt_R values of "a" series peptides: $t_{R4a}-t_{R3a}$, $t_{R3a}-t_{R2a}$, $t_{R2a}-t_{R1a}$. The sequences and denotations of the peptides are shown in Fig. 1.

Fig. 10.

Relative hydrophobicity of the Ile side-chain with increasing net positive charge of the peptide in the presence of various anionic ion-pairing reagents. Conditions: linear AB gradient (1% acetonitrile/min) at a flow-rate of 0.25 ml/min, where Eluent A is 10 mM aq. HCOOH, H₃PO₄, TFA, PFPA or HFBA and Eluent B is 10 mM of the corresponding ion-pairing reagent in acetonitrile; temperature, 25°C. Panel A: the y-axis represents the change in relative Ile hydrophobicity (Δt) in 10 mM of each ion-pairing reagent; each data point represents the difference in retention time (Δt) between peptides 1a and 1b (+1), 2a and 2b (+2), 3a and 3b (+3) and 4a and 4b (+4) in the presence of each of the ion-pairing reagents. Panel B: the y-axis represents the change in relative Ile hydrophobicity (Δt) between the +1 and +4 peptides in 10 mM of each ion-pairing reagent; each data point represents Δt (1b–1a) minus Δt (4b–4a) in each of the ion-pairing reagents. Panel C: the y-axis represents the change in relative Ile hydrophobicity (Δt) between the HCOOH and HFBA ion-pairing reagent systems; each data point represents, for the +4 peptides, Δt (4b–4a) in 10 mM HCOOH minus Δt (4b–4a) in 10 mM HFBA, etc. for +1, +2 and +3 peptides. The sequences and denotations of the peptides are shown in Fig. 1.

Table 1
Peptide retention behaviour in different anionic ion-pairing reagents

Peptide ^a	<i>t_R</i> Formic acid (min)		<i>t_R^b</i> H ₃ PO ₄ (min)			<i>t_R</i> TFA ^c (min)			<i>t_R</i> PFPAC ^c (min)			<i>t_R</i> HFBA ^c (min)		
	10mM	30mM	2mM	10mM	50mM	2mM	10mM	50mM	1mM	2mM	10mM	2mM	10mM	10mM
4a	12.34	12.59	11.43	12.67	13.37	16.07	18.92	21.37	19.83	22.04	25.47	29.21	31.95	
3a	13.97	14.17	13.11	14.15	14.74	16.83	18.92	20.75	19.42	21.11	23.94	26.86	29.20	
2a	15.55	15.68	14.74	15.56	15.98	17.49	18.74	19.95	19.42	20.22	22.20	24.24	25.99	
1a	17.54	17.62	16.79	17.46	17.73	18.58	19.07	19.62	19.10	19.83	20.86	21.99	22.86	
4b	16.75	17.00	16.01	17.02	17.94	20.49	23.04	25.35	23.21	25.24	28.53	31.54	34.15	
3b	19.28	19.49	18.56	19.32	20.13	22.02	23.82	25.56	23.94	25.24	27.96	30.18	32.40	
2b	21.87	22.02	21.09	21.68	22.35	23.62	24.68	25.73	24.84	25.71	27.41	28.96	30.50	
1b	24.00	24.09	23.22	23.67	24.19	24.83	25.27	25.73	25.44	25.71	26.64	27.60	28.28	

^aPeptide sequences are shown in Fig. 1.
^b*t_R* is the retention time in minutes at different concentrations of acid in mM.
^cTFA, trifluoroacetic acid; PFPAC, pentafluoropropionic acid; HFBA, heptafluorobutyric acid.

Table 2

Difference in retention behaviour between different anionic ion-pairing reagent concentrations and the retention behaviour in phosphoric acid at 2 mM

Peptide ^d	Δt_R Formic Acid (min)		Δt_R H ₃ PO ₄ (min)		Δt_R TFA ^c (min)		Δt_R PFP ^a (min)		Δt_R HFBA ^c (min)	
	10mM	30mM	10mM	50mM	2mM	10mM	50mM	1mM	2mM	10mM
4a	0.91	1.16	1.24	1.94	4.64	7.49	9.94	8.40	10.61	14.04
3a	0.86	1.06	1.04	1.63	3.72	5.81	7.64	6.31	8.00	10.83
2a	0.81	0.94	0.82	1.24	2.75	4.00	5.21	4.68	5.48	7.46
1a	0.75	0.83	0.67	0.94	1.79	2.28	2.83	2.31	3.04	4.07
4b	0.74	0.99	1.01	1.93	4.48	7.03	9.34	7.20	9.23	12.52
3b	0.72	0.93	0.76	1.57	3.46	5.26	7.00	5.38	6.68	9.40
2b	0.78	0.93	0.59	1.26	2.53	3.59	4.64	3.75	4.62	6.32
1b	0.78	0.87	0.45	0.97	1.61	2.05	2.51	2.22	2.49	3.42

^aPeptide sequences are shown in Fig. 1.

^b Δt_R is the difference in retention time for the acid listed and the retention time in 2 mM H₃PO₄.

^cTFA, trifluoroacetic acid; PFP^a, pentafluoropropionic acid; HFBA, heptafluorobutyric acid.

Table 3
Hydrophilicity/Hydrophobicity of Arg, Lys, and Ile side-chains in different anionic ion-pairing reagents relative to Gly

Peptides ^a	Sequence differences	Δf_R (10 mM)				
		Formic	H ₃ PO ₄	TFA	PFPA	HFBA
4a-3a	Arg1/Gly1	-1.63	-1.48	0.00	+1.53	+2.75
3a-2a	Arg2/Gly2	-1.58	-1.41	+0.18	+1.74	+3.21
2a-1a	Lys5/Gly5	-1.99	-1.90	+0.33	+1.34	+3.13
4b-4a	Ile3/Gly3	+4.41	+4.35	+4.12	+3.06	+2.02
3b-3a	Ile3/Gly3	+5.31	+5.17	+4.90	+4.02	+3.20
2b-2a	Ile3/Gly3	+6.32	+6.12	+5.94	+5.21	+4.51
1b-1a	Ile3/Gly3	+6.46	+6.21	+6.20	+5.78	+5.42
4b-3b	Arg1/Gly1	-2.53	-2.30	-0.78	+0.57	+1.75
3b-2b	Arg2/Gly1	-2.59	-2.36	-0.86	+0.55	+1.90
2b-1b	Lys5/Gly5	-2.13	-1.99	-0.59	+0.77	+2.22

^aThe "a" series peptides have Gly at position 3 and the "b" series peptides have Ile at position 3 (Fig. 1).