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Determination of Intrinsic Hydrophilicity/Hydrophobicity of Amino Acid Side Chains in Peptides in the Absence of Nearest-Neighbor or Conformational Effects

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Abstract

Understanding the hydrophilicity/hydrophobicity of amino acid side chains in peptides/proteins is one the most important aspects of biology. Though many hydrophilicity/hydrophobicity scales have been generated, an “intrinsic” scale has yet to be achieved. “Intrinsic” implies the maximum possible hydrophilicity/hydrophobicity of side chains in the absence of nearest-neighbor or conformational effects that would decrease the full expression of the side-chain hydrophilicity/hydrophobicity when the side chain is in a polypeptide chain. Such a scale is the fundamental starting point for determining the parameters that affect side-chain hydrophobicity and for quantifying such effects in peptides and proteins. A 10-residue peptide sequence, Ac–X–G–A–K–G–A–G–V–G–L-amide, was designed to enable the determination of the intrinsic values, where position X was substituted by all 20 naturally occurring amino acids and norvaline, norleucine, and ornithine. The coefficients were determined by reversed-phase high-performance liquid chromatography using six different mobile phase conditions involving different pH values (2, 5, and 7), ion-pairing reagents, and the presence and absence of different salts. The results show that the intrinsic hydrophilicity/hydrophobicity of amino acid side chains in peptides (proteins) is independent of pH, buffer conditions, or whether C_8 or C_{18} reversed-phase columns were used for 17 side chains (Gly, Ala, Cys, Pro, Val, nVal, Leu, nLeu, Ile, Met, Tyr, Phe, Trp, Ser, Thr, Asn, and Gln) and dependent on pH and buffer conditions, including the type of salt or ion-pairing reagent for potentially charged side chains (Orn, Lys, His, Arg, Asp, and Glu).

Keywords

peptides; hydrophilicity; hydrophobicity; amino acid side chains

INTRODUCTION

The concept of hydrophobicity has been a topic of much study in all aspects of science, particularly in the fields of biology and chemistry.[1] Thus, for example, the hydrophobic effect, as exemplified by the relative hydrophilicity/hydrophobicity of amino acid side chains and how they interact, is considered the most important factor underlying the hierarchical structure and stability of proteins.[2] As noted by Wilce et al.,[3] manifestations of this hydrophobic effect are evident in many facets of protein structure. These include stabilization of protein globular structure in solution, the presence of amphipathic structures induced in peptides or membrane proteins in lipid environments, and protein–protein interactions associated with protein subunit

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assembly, protein–receptor binding, and other intermolecular biorecognition processes. Thus, the quantitative evaluation of the magnitude of the hydrophilic/hydrophobic contribution of a specific amino acid side chain in a polypeptide chain to such processes remains an important challenge.

With a detailed knowledge of the contribution of individual amino acids to the hydrophobic effect, the prediction of the overall three-dimensional structure of a protein from its amino acid sequence alone may one day become a reality. Indeed, hydrophobicity values (also described as coefficients or indices), obtained from a variety of sources and methods, have been used widely for the prediction of protein secondary structure (β-sheet, α-helix, and turns) in globular proteins,[4–7] periodicities in residue distributions,[7] antigenic sites,[8–11] interior–exterior regions,[7] and membrane-associated regions.[12–14] Such indices have also been utilized to simulate the sequence of signal peptides,[15] to study quantitative structure/activity relationships in polypeptide hormones,[16] and to aid the rational de novo design of biologically active peptides, e.g., antimicrobial peptides.[17,18]

The measurement of the amino acid side chain hydrophilicity/hydrophobicity has been carried out by a number of approaches, both chromatographic[19–31] and nonchromatographic,[32–43] as described in an excellent review by Biswas et al.[1] These may be generally divided into five different categories: (1) partitioning (particularly liquid–liquid)[33,35–37,39,41,42]; (2) accessible surface area calculations[9,32,34,37,38,41]; (3) site-directed mutagenesis, where amino acid substitutions were made on the surface or within the core of a protein and, from the change in stability of the mutant proteins, the scales subsequently generated were correlated with a side-chain contribution to protein stability[44–48] (4) physical property measurements, e.g., measurement of surface tension of amino acid solutions,[49] solvation free energy of amino acids, [50] and apparent heat capacity of peptides[51]; and (5) chromatographic techniques, almost invariably reversed-phase high-performance liquid chromatography (RP-HPLC).[19–31] A consensus on the values and ranking of hydrophilicity/hydrophobicity values, however, has still not been obtained based as they are on markedly different techniques and the nature of the solute, i.e., peptides/proteins or amino acids and derivatives thereof. Also, it is very reasonable to assume that if you desire values for side chain hydrophilicity/hydrophobicity in a polypeptide chain, then the polypeptide backbone could significantly affect the observed hydrophilicity/hydrophobicity. Therefore, these values must be determined using polypeptides. Certainly, we believe that despite the general usefulness of hydrophilicity/hydrophobicity scales previously reported, a definitive determination of relative “intrinsic” hydrophilicity/hydrophobicity of side chains has yet to be achieved. “Intrinsic” hydrophobicity implies the maximum possible hydrophilicity/hydrophobicity of side chains in a polypeptide chain in the absence of any nearest-neighbor effects ($i \rightarrow i \pm 1$ side-chain interactions or restriction of conformational freedom from steric hindrance of side chains in positions i and $i \pm 1$) and/or any conformational effects of the poly-peptide chain that prevent full expression of the side-chain hydrophilicity/hydrophobicity. Such a hydrophobicity scale should be the fundamental starting point for truly meaningful predictive applications and understanding the parameters that decrease the “intrinsic” hydrophobicity.

In their review, Bitwas et al.[1] noted that chromatographic methods, particularly RP-HPLC, have shown much promise as generators of amino acid side-chain hydrophilicity/hydrophobicity scales from peptides, based on the premise that the nonpolar stationary phase characteristic of this HPLC mode mimics a biological membrane[52] or hydrophobic interactions involved in the hydrophobic core of proteins and in ligand/receptor interactions. Using this RP-HPLC-based approach, most researchers have carried out regression analysis of a random collection of peptides to relate peptide hydrophobicity to peptide retention behavior.[3,19–24,27,29–31] The preferred approach of our laboratory is to apply RP-HPLC to the separation of mixtures of synthetic model peptides with just single amino acid substitutions in a defined

peptide sequence. We believe that such an approach eliminates such concerns as the relative frequency with which a particular amino acid appears compared to others in a random collection of peptides. In addition to the application of side-chain coefficients generated from such model peptides to the prediction of peptide retention behavior during RP-HPLC (becoming increasingly important for the rational design of separation protocols for complex peptide mixtures characteristic of proteomic applications[53–59]), this approach has enabled the design of a peptide/stationary phase model of ligand/receptor interactions[28,60,61] as well as the ability to predict potential antigenic sites on the surface of proteins.[11]

The present study uses a novel approach to the determination of intrinsic hydrophilicity/hydrophobicity of amino acid side chains using RP-HPLC of synthetic model peptides. Thus, we have applied RP-HPLC to the separation of mixtures of de novo designed model peptides with the sequence Ac–X–GAKGAGVGL–amide, where X is substituted by all 20 naturally occurring amino acids and norvaline, norleucine, and ornithine. From the observed retention behavior of these model peptides, we have obtained intrinsic hydrophilicity/hydrophobicity values of the amino acid side chains at pH 2, 5, and 7 (the latter in the presence and absence of salts).

MATERIALS AND METHODS

Materials

Reagent-grade phosphoric acid (H_3PO_4) was obtained from Caledon Laboratories (Georgetown, Ontario, Canada). Tri-fluoroacetic acid (TFA) was obtained from Hydrocarbon Products (River Edge, NJ, USA); NaCl and $NaClO_4$ were obtained from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Fisher Scientific (Pittsburgh, PA, USA). Fluorenyloxymethylcarbonyl (Fmoc) amino acids and Rink Amide MBHA (methoxy-benzhydrylamine) resin (100–200 mesh) were obtained from Novabiochem (San Diego, CA, USA). De-ionized water was purified by an E-pure water filtration device from Barnstead/Thermolyne (Dubuque, IA, USA).

Instrumentation

RP-HPLC runs were carried out on an Agilent 1100 Series liquid chromatograph from Agilent Technologies (Little Falls, DE, USA).

Columns

RP-HPLC runs at pH 2 were carried out on a Kromasil C_{18} column (150 × 2.1 mm I.D.; 5-μm particle size; 100-Å pore size) from Hichrom, Berkshire, UK); pH 5 and 7 runs were carried out on a Zorbax Eclipse XDB-C_8 column (150 × 2.1 mm I.D.; 5-μm particle size; 80-Å pore size) from Agilent Technologies, this latter column being designed for excellent chemical and thermal stability at neutral pH values.[62,63]

Peptide Synthesis and Purification

Peptide synthesis was carried out by solid-phase peptide synthesis methodology using conventional Fmoc chemistry. Elongation of the peptide chains was carried out in polypropylene reaction vessels. The side-chain protecting groups used were: Arg (Pbf), Lys (Boc), Orn (Boc), Trp (Boc), Asn (Trt), Gln (Trt), Cys (Trt), His (Trt), Asp (OBut), Glu (OBut), Ser (But), Thr (But), and Tyr (But), where Pbf denotes 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl, Boc denotes *tert*-butoxycarbonyl, Trt denotes trityl, OBut denotes butoxy, and But denotes butyl. Following addition of the final amino acid, the peptides were N-terminally acetylated using acetic anhydride. Most peptides were cleaved from the resin using 95% TFA, 2.5% water, and 2.5% trii-sopropylsilane (TIS) for 120 min at

room temperature. Peptides containing Met were cleaved using 95% TFA, 2.5% TIS, and 2.5% methyl sulfide while peptides containing Cys were cleaved using 94.5% TFA, 1.5% TIS, 1.5% water, and 1.5% ethanedithiol (EDT). Crude peptides were washed from the resin with neat TFA. The crude peptides were then precipitated out with cold diethyl ether in a 7-fold excess (v/v) and the precipitate was rinsed twice with cold diethyl ether. Crude peptides were then dissolved in 50% aqueous acetonitrile and lyophilized.

Purification of Crude Peptides

Crude peptides were purified by RP-HPLC on the Kromasil C_{18} column using a linear AB gradient (1% B/min to 30%; 0.25% B/min from 30% to 60%) at a flow rate of 0.3 mL/min, where eluent A is 0.2% aqueous TFA and eluent B is 0.2% TFA in acetonitrile. The correct masses of the peptides were confirmed by electrospray mass spectrometry using a Mariner Biospectrometry Workstation mass spectrometer (PerSeptive Biosystems, Framingham, MA, USA).

Analytical RP-HPLC of Synthetic Model Peptides

Linear AB gradient (0.25% acetonitrile/min) at a flow rate of 0.3 mL/min and a temperature of 25°C.

Mobile phase 1: Eluent A is 20 m*M* aqueous H_3PO_4, pH 2, and eluent B is 20 m*M* H_3PO_4 in acetonitrile; denoted pH 2/H_3PO_4 system.

Mobile phase 2: Eluent A is 20 m*M* aqueous TFA, pH 2, and eluent B is 20 m*M* TFA in acetonitrile; denoted pH 2/TFA system.

Mobile phase 3: Eluent A is 10 m*M* aqueous NaH_2PO_4, pH 5, and eluent B is eluent A containing 50% acetonitrile; denoted pH 5/no salt system.

Mobile phase 4: Eluent A is 10 m*M* aqueous NaH_2PO_4, adjusted to pH 7 with NaOH, and eluent B is eluent A containing 50% acetonitrile; denoted pH 7/no salt system.

Mobile phase 5: Same as mobile phase 4 but both eluents also contain 50 m*M* NaCl; denoted pH 7/NaCl system.

Mobile phase 6: Same as mobile phase 4 but both eluents also contain 50 m*M* $NaClO_4$; denoted pH 7/$NaClO_4$ system.

RESULTS

Design of Model Peptides

In order to determine truly intrinsic hydrophilicity/hydrophobicity values for amino acid side chains in peptides/proteins, several criteria must be met: (1) the model peptide sequence should have no tendency to form any type of secondary structure (α-helix, β-sheet, or β-turn) in any environment (aqueous or hydrophobic) that could restrict the interaction of the substitution site with the hydrophobic matrix during partitioning of the peptide between the mobile phase and stationary phase during RP-HPLC[64]; (2) the peptide should be of sufficient length to ensure multisite binding[64]; (3) the peptide should be of sufficient overall hydrophobicity to allow the substitution of all 20 naturally occurring amino acid side chains while maintaining satisfactory retention behavior; (4) the distribution of amino acid side chains should be such that there is no clustering of hydrophobic side chains that may minimize the contribution of the substituting amino acid side chain; (5) the peptide should be long enough to maintain satisfactory retention behavior on substituting the 20 amino acids but not so long as to diminish the full expression of the hydrophilicity/hydrophobicity of the substituted amino acid due to a chain length effect (generally for peptides >15 residues) on peptide retention times[65]; (6) the substitution site should be next to a residue that has a minimal side chain in terms of size and hydrophobicity,

thus allowing the substituting amino acid to express its true intrinsic hydrophilicity/hydrophobicity; and (7) there should be no nearest neighbor effects (*i* to *i* ± 1 interactions with the substituting residue)—such effects can be eliminated if there is free rotation of the bonds represented by the angles ψ(C^{α}—C) and φ (C^{α}—N) in Figure 1, i.e., there is no streric hindrance between the substituting side chain at position *i* and its nearest-neighbor side chains at position *i* ± 1.

The sequence chosen to reflect the above criteria in determining the intrinsic hydrophilicity/hydrophobicity of 23 amino acid side chains (20 naturally occurring amino acids in peptides/proteins plus norvaline, norleucine, and ornithine) was Ac–X–G–A–K–G–A–G–V–G–L–amide. This sequence contains four Gly residues spread periodically throughout the sequence to ensure that the peptide has no secondary structure tendencies.[66,67] The substitution site (denoted X) is adjacent to a Gly residue to ensure that there is unrestricted rotation on either side of the peptide bond between the substitution site and the residue next to it. In order to demonstrate complete freedom of rotation on either side of this peptide bond, all 23 amino acids were substituted in both the L and D configuration (i.e., X_L and X_D peptides), since the L- and D-diastereomers should have identical retention behavior if there is free rotation. In addition, since the guest site is the N-terminal residue, there is no restriction in its interaction with the reversed-phase matrix. The N-terminus was acetylated and the C-terminus was amidated to eliminate potential effects of a positively charged α-amino group or negatively charged carboxyl group, respectively, on the hydrophilicity/hydrophobicity of the side chains in the peptide. A single Lys residue was incorporated into the model peptide sequence to ensure peptide solubility over a wide pH range. The four hydrophobes in the peptide sequence (2 Ala, 1 Val, 1 Leu) were distributed throughout the peptide sequence to ensure no clustering of hydrophobes and subsequent creation of a preferred hydrophobic binding domain. Finally, the 10-residue length of the model peptide was selected as the minimum size to meet all design requirements (overall hydrophobicity, random coil structure, no clustering of hydrophobic residues, and no chain length effect).

Elimination of Nearest-Neighbor Effects

It was our hypothesis that nearest-neighbor effects (*i* to *i* ± 1 interactions) would affect the full expression of the hydrophilicity/hydrophobicity of an amino acid side chain at position *i* and that this effect would be dependent upon the side chain at position *i* and *i* ± 1. Thus, as noted above, to ensure that there was no steric hindrance about ψ and φ (Figure 1) between the substituting residue at position *i* and the residue at position *i* + 1, Gly was selected for the *i* + 1 position of the peptide sequence shown above (Figure 1, top). It was also our hypothesis that if there is complete rotational freedom on either side of the peptide bond, the difference in retention time between all diastereomeric peptide pairs (X_L and X_D peptides) would approach zero. On the other hand, if there were a restriction in conformational space between the residues at positions *i* and *i* + 1, the difference in retention time of the X_L and X_D peptides would be significantly different from zero. To demonstrate such a situation, 20 L- and D-amino acid substitutions were made in the sequence Ac–$X_{L,D}$–L–G–A–K–G–A–G–V–G–amide, with the substitution site now adjacent (*i* + 1) to a bulky Leu residue (Figure 1, bottom) instead of the Gly residue of our model peptide sequence Ac–$X_{L,D}$–G–A–K–G–A–G–V–G–L–amide (Figure 1, top). It is important to note that the amino acid compositions of these two peptide sequences (i.e., whether the substitution site is next to a Gly or Leu) are identical.

Figure 2 shows representative RP-HPLC elution profiles at pH 2 of the L-Ile/D-Ile peptide pairs of the peptide sequences where the substituted N-terminal Ile residue is adjacent either to a Leu residue (panel A) or a Gly residue (panel B). From Figure 2, when the substitution is made adjacent to a Leu residue (panel A), the L/D peptide pair was well resolved (by 7.8 min) at a gradient rate of 0.25% acetonitrile/min; in contrast, when the substitution is made adjacent

to a Gly residue (panel B), the L- and D-substituted peptides were inseparable, even at this shallow gradient rate. Indeed, from Table I, only 2 out of 23 substitutions of L- and D-amino acids made adjacent to a Gly residue showed even a subtle separation at a gradient rate of 0.25% acetonitrile/min: 0.5 min between the L- and D-Asp peptides and 0.3 min between the L- and D-Trp peptides (Figure 3, top panel). Note that, even though the results presented in Figure 3 were carried out in the pH 2/TFA system, identical separations of these peptide pairs were observed in the pH 2/H_3PO_4 system (Table I). At a more standard gradient rate of 1% acetonitrile/min, these peptide pairs could not be resolved (Figure 3, bottom panel). Thus, based on our criteria, the results presented in Table I and Figures 2 and 3 show that when the substituting residue is adjacent to a Gly residue, there is complete freedom of rotation on either side of the peptide bond between residue X and Gly. In contrast, profound nearest neighbor effects were observed for all 20 amino acid substitutions (L and D) adjacent to a Leu residue, varying by as much as 7.8 min between the Ac–Ile_D–Leu peptide and the Ac–Ile_L–Leu peptide (Figure 2A) (J. M. Kovacs, C. T. Mant, D. J. Osguthorpe, and R. S. Hodges, unpublished results).

RP-HPLC Retention Behavior of Model Peptides at pH 2, 5 and 7

Having clearly shown that when the N-terminal amino acid substitution site is adjacent to a Gly residue, the side-chain is able to express fully its intrinsic hydrophilicity or hydrophobicity on interacting with the hydrophobic reversed-phase matrix, the L-amino acid substituted model peptides were now subjected to RP-HPLC under six mobile phase conditions:20 m*M* H_3PO_4 or 20 m*M* TFA at pH 2; 10 m*M* PO_4 buffer at pH 5; and 10 m*M* PO_4 buffer at pH 7, containing no salt, 50 m*M* NaCl, or 50 m*M* $NaClO_4$. Note that addition of salts (generally 50–100 m*M*) to mobile phases over a pH range of ca. 4–7 has generally been utilized, for silica-based packings, to suppress negatively charged silanol interactions with positively charged solutes.[64,68] The retention data for the 23 peptides are shown in Table II.

From Table II, it can be seen that the observed retention times of the peptides varied depending on mobile phase pH and composition. Thus, the retention times of the 23 peptides are all greater in the pH 2/TFA system compared to the pH 2/H_3PO_4 system due to differences in the properties of the mobile phase anions (phosphate and TFA^-) and their ability to ion-pair with the positively charged groups in the model peptide sequence. Hence, the peptides are retained longer in the presence of the hydrophobic TFA^- anion compared to the hydrophilic phosphate anion.

An interesting observation from Table II lies in the retention behavior of the peptides at pH 5 and 7 in the absence of salt. Thus, while the retention times of 19 of the peptides decrease slightly (average 0.5 min) between pH 5 and 7, the four peptides with substitutions by positively charged residues (Orn, Lys, His, and Arg) show significant retention time increases at pH 7 compared to pH 5 (2.5, 1.9, 7.8, and 6.9 min, respectively). A previous study by Sereda et al.[69] demonstrated that the pKa values of even highly basic side chains were dramatically decreased in the hydrophobic environment characteristic of RP-HPLC, i.e., partial deprotonation (neutralization) of side chains such as Lys and Arg (with pKa values of ~10 and ~12, respectively, in the free amino acid), thus increasing their observed relative hydrophobicity. In the case of His (with a pKa value of ~6 in the free amino acid), the side chain is likely entirely deprotonated at pH 7. In addition, there is an increase in the concentration of the HPO_4^{2-} anion as the pH increases, which could more efficiently neutralize the positively charged side chains in the peptides, thereby increasing their hydrophobicity. The role of this anion in ion-pairing will be discussed later in regard to experiments where the pH was varied from 5 to 8.5.

In contrast to the addition of 50 m*M* NaCl to the mobile phase at pH 7, which had little effect on peptide retention times (average decrease for 23 peptides was 0.3 min), addition of 50

m*M* $NaClO_4$ at this pH value generally significantly increased peptide retention times (Table II). Compared to Cl^-, the perchlorate anion is extremely effective at ion-pairing to positively charged side chains in the peptides with a net positive charge,[68] thus increasing overall peptide hydrophobicity of 18 peptides (including the His-containing peptide) with a net charge of +1 by an average of 7.1 min (t_R $NaClO_4$ minus t_R no salt); increasing hydrophobicity of peptides with a net charge of +2 (Arg, Lys and Orn) by 9.6, 11.6, and 12.8 min, respectively; but only increasing hydrophobicity of peptides with a zero net charge (Asp and Glu) by 0.4 and 0.9 min, respectively (the explanation for the Asp- and Glu-containing peptides is discussed later in the section "Potentially Charged Side-Chains").

From Table II, the difference in retention time between the most hydrophilic and hydrophobic substitutions varies from 33 to 41 min depending on pH and composition of the mobile phase. It should be emphasized that we employed a shallow gradient rate of 0.25% acetonitrile/min to maximize differences between the peptides. The Trp-substituted peptide was the most hydrophobic peptide and the Orn-substituted peptide was the most hydrophilic peptide under five of the six conditions studied. In the case of 10 m*M* NaH_2PO_4 buffer, pH 7, containing 50 m*M* $NaClO_4$, the peptides with positively charged amino acid substitutions (Orn, Lys, and Arg) became much more retentive due to the strong ion-pairing properties of the perchlorate anion; concomitantly, the Asp-substituted peptide was the least retentive under these conditions.

Correlation of RP-HPLC Retention Behavior of Peptides Under Different Mobile Phase Conditions

From Figure 4 (top), when the retention times of 19 of the 23 peptides (with the exception of those with positively charged substitutions Orn, Lys, His, and Arg) in the pH 2/H_3PO_4 system are plotted against those obtained in the pH 2/TFA system, there is an excellent correlation (r = 0.999) of the respective series of data. Such a correlation shows that the change in counterion hydrophobicity from an hydrophilic phosphate anion to an hydrophobic TFA^- anion, despite the increase in overall peptide retention times in the TFA system due to ion-pairing with the single Lys residue in the sequence, does not affect the relative hydrophilicity/hydrophobicity of the 19 uncharged side chains. In contrast, this change in counterion hydrophobicity does affect the hydrophilicity/hydrophobicity of the positively charged residues by making their relative hydrophobicities increase in the presence of TFA compared to H_3PO_4—hence, the increased retention times of the peptides substituted with Orn, Lys, His, or Arg relative to the other 19 peptides (Figure 4, top; Table II).

Figure 4 (bottom) now correlates peptide retention behavior at pH 2 (H_3PO_4 system) and pH 7 (no salt). There is an excellent correlation (r = 0.999) for the 17 peptides substituted at position X with neutral side chains, demonstrating that pH has no effect on the relative hydrophilicity/hydrophobicity of these side chains. In contrast, the increase in pH from 2 to 7 is increasing the relative hydrophobicity of Orn, Lys, His, and Arg, likely (as described above) to partial (Orn, Lys, Arg) or complete (His) deprotonation of these residues at the higher pH value, i.e., the positive charge on these side chains is diminished or eliminated or an increased neutralization of the positive charge by the increased concentration of the anions $H_2PO_4^-$ and HPO_4^{2-} is occurring as the pH is increased from 2 to 7. In contrast, the relative hydrophobicities of the acidic side chains of Asp and Glu are decreasing with an increase in pH, due to deprotonation of these residues at pH 7 (the pKa of these side chains is ~4.0), i.e., they become negatively charged. Although not shown here, a similar plot of retention times at pH 2 vs. pH 5 also indicated deprotonation of these acidic side chains at pH 5.0.

Figure 5 presents elution profiles of the 23 peptides at pH 2 (H_3PO_4) and pH 7 (no salt) for easy visualization of the effect of pH on relative retention behavior of the peptides and hence relative hydrophilicity/hydrophobicity of the side chains of the substituted amino acids. Note

that the elution orders of the peptides substituted with neutral side chains are similar (i.e., correlate well) at these two pH values, albeit small differences are observed; such variations are possibly due to selectivity differences between the Kromasil C_{18} column used at pH 2 and the Eclipse XDB-C_8 column used at pH 7. Clearly, from Figure 5, the major changes in observed relative hydrophilicity/hydrophobicity occur for the peptides substituted with potentially charged side chains—hence, the greater observed hydrophobicity of the peptides substituted with Orn, Lys, His, or Arg with a rise in pH from 2 to 7 (Table II) and the concomitant decrease in peptide hydrophobicity of the peptides substituted with Asp or Glu.

Figure 6 plots peptide retention times at pH 7 vs. pH 5 in the absence of salt. With the exception of peptides substituted with Orn, Lys, His, and Arg, the remainder show an excellent correlation ($r = 0.999$) between these mobile phase systems, demonstrating that the increase in pH of these otherwise identical buffer systems has no effect on the relative hydrophilicity/hydrophobicity of side chains substituted at position X of the model peptides. The noncorrelation of the Orn-, Lys-, His-, and Arg-substituted peptides may be explained by the aforementioned partial (Orn, Lys, Arg) or complete (His) deprotonation and increased ion-pairing from the increased concentration of HPO_4^{2-} anions on raising the pH from 5 to 7, resulting in longer retention times at the higher pH value. Of note here is the correlation of the Asp- and Glu-substituted peptides with the remainder of the peptides substituted with neutral side chains, which may be explained by the side chains of these acidic amino acids being fully deprotonated (i.e., negatively charged) at both pH 5 and 7.

Figure 7 highlights the relative effectiveness of Cl^- ion vs. ClO_4^- ion as ion-pairing reagents at pH 7. Thus, when the retention times of the peptides are correlated at pH 7 in the absence and presence of 50 m*M* NaCl (Figure 7A), there is an excellent correlation ($r = 0.998$) of all 23 amino acids, including the peptides substituted with positively charged amino acids, concomitant with little observable effect of NaCl on peptide retention times (Table II). Such results reflect similar conclusions by Shibue et al.[68] that the chloride ion is relatively ineffective in affecting peptide retention behavior by ion-pairing with positively charged side chains. In contrast, the addition of 50 m*M* $NaClO_4$ to the mobile phase at pH 7 affects the retention behavior of both the peptides substituted with three of the positively charged side chains (Orn, Lys, and Arg), and interestingly, those substituted with negatively charged side chains (Asp and Glu) (Figure 7B). The remainder of the peptides, substituted with neutral side chains (including the His side chain), show an excellent correlation of 0.999, indicating that the relative hydrophilicity/hydrophobicity of these side chains is unaffected by the addition of $NaClO_4$ to the mobile phase. Shibue et al.[68] have demonstrated that the perchlorate anion is a very effective ion-pairing reagent (more effective, indeed, than trifluoroacetate) and thus will interact strongly with positively charged side chains; hence, the increase in retention time of the Orn-, Lys-, and Arg-substituted peptides relative to the remainder (Figure 7B; Table II). Note that, at pH 7, the His side chain is fully deprotonated (i.e., neutral) and thus is unaffected by the addition of $NaClO_4$. The poor correlation of the Asp- and Glu-substituted peptides in the presence of 50 m*M* $NaClO_4$ (Figure 7B) is likely due to the decreased ion-pairing of the perchlo rate anion with the positively charged residues as the net charge on the peptide was reduced to zero (see later discussion under section “Potentially Charged Side Chains”).

Amino Acid Side-Chain Hydrophilicity/Hydrophobicity Coefficients

To determine the hydrophilicity/hydrophobicity of the substituting amino acid side chain at position X of the model peptide sequence, the retention time of the Gly-substituted peptide was used as a reference since Gly has only an hydrogen atom as its side chain. Thus, the hydrophilicity/hydrophobicity coefficients of the 22 side chains (other than Gly) were generated from the RP-HPLC runs carried out under the six mobile phase conditions from the difference in the retention times (Δt_R) of the X-substituted peptide and the Gly-substituted

peptide, i.e., Δt_R = t_R X-substituted peptide minus t_R Gly-substituted peptide (Table III). Thus, from Table III, side chains that are more hydrophobic than Gly have positive Δt_R values and side chains that are more hydrophilic than Gly have negative Δt_R values.

Isosteric Side Chains: n-Leu, Leu, and Ile—Norleucine, leucine, and isoleucine are isosteric but display overall differences in hydrophobicity with coefficients of 24.6, 23.4, and 21.3 min, respectively, in the pH 2/H_3PO_4 system (Table III). Such results are expected since as the side chain is more extended from the polypeptide backbone (*n*-Leu > Leu > Ile), it is more exposed and is thus better able to express its hydrophobicity. Similarly, norvaline is more hydrophobic than valine with coefficients of 15.4 and 13.8 min, respectively, in the pH 2/H_3PO_4 system (Table III). As expected, these differences are independent of pH and mobile phase conditions.

Interestingly, although *n*-Val and Pro have the same number of carbon atoms in their side chains, *n*-Val has a coefficient of 15.4 min compared to just 9.4 min for Pro. This can be explained by the cyclization of the Pro side chain to the polypeptide backbone nitrogen. Thus, the Pro side chain is closer to the backbone and less exposed compared to the side chain of *n*-Val.

Carbon Atom Extension of Side Chains: Nonpolar Groups (Gly/Ala, Val/Ile, n-Val/n-Leu)—From Table III, for the pH 2/H_3PO_4 system, the difference in hydrophobicity between the *n*-Val and *n*-Leu side chains was 9.2 min, compared to 7.5 min for Val and Ile and just 3.6 min for Gly and Ala. Such differences are correlated with the distance of the carbon atom extension from the polypeptide backbone, where the methyl group of Ala is the β-carbon, the methyl group of Ile is the δ-carbon, and the methyl group of *n*-Leu is the ε-carbon, i.e., the greater the distance of the added carbon atom from the polypeptide backbone, the greater its hydrophobicity contribution.

Carbon Atom Extension of Side Chains: Polar and Charged Groups (Asn/Gln, Asp/Glu, and Orn/Lys)—From Table III, under six different mobile phase conditions, the average increase in hydrophobicity between Asn and Gln (i.e., an increase of a CH_2 group) was ~0.6 min. Also, the increase in hydrophobicity between Asp and Glu at pH 5 and pH 7, where the side-chain carboxyl group is negatively charged, showed a similar value of ~0.6 min. On the other hand, when the carboxyl group is protonated (i.e., lacking a charge), the increase in hydrophobicity from Asp to Glu at pH 2 was ~1.3 min. Such results suggest that the less polar the functional group (e.g., protonated Glu > deprotonated, negatively charged Glu), the greater the expression of the hydrophobicity on extension of the side chain by a CH_2 group.

From Table III, the increase in side-chain hydrophobicity on extending the side chain by a CH_2 group from Orn to Lys is very dependent on the mobile phase pH and the ion-pairing properties of the anionic ion-pairing reagent. For example, at pH 2, in the pH 2/H_3PO_4 system, the change is 0.6 min; in contrast, in the pH 2/TFA system (TFA being an hydrophobic ion-pairing reagent), this change is 3.5 min. In the pH 5/no salt vs. pH 7/no salt systems, the increase in hydrophobicity for Orn to Lys was 3.1 and 2.5 min, respectively. On the other hand, on the addition of salt at pH 7, this increase changed dramatically in the presence of 50 m*M* $NaClO_4$ vs. 50 m*M* NaCl. Thus, no separation of the Orn and Lys peptides was achieved in the pH 7/NaCl system while the two peptides were separated by 1.3 min in the pH 7/$NaClO_4$ mobile phase. Such results demonstrate that, for Orn and Lys, not only is the hydrophobicity of the side chain increased by the addition of the CH_2 group, but this increase in hydrophobicity also affects the charge neutralization of the side-chain amino groups, i.e., the properties of the Orn and Lys side-chain amino groups are different (see later discussion).

Carbon Atom Extension of Side Chains: Addition of β-Branch Methyl Group (Ser/Thr)—From Table III, adding a methyl group at a *β*-branch location on the amino acid side chain, in a similar manner to extension of side chains by insertion of a CH_2 group (see above), also increases side-chain hydrophobicity. Thus, in the pH 2/H_3PO_4 system, there is a 2.8 min increase between Ser and Thr. However, interestingly, the addition of a methyl group from Gly to Ala resulted in a greater hydrophobicity increase of 3.6 min. Such a result indicates that, in the case of Thr, the polar hydroxyl group also attached to the *β*-carbon of the side chain is partially shielding the methyl group on this same *β*-carbon and preventing its full hydrophobic expression.

Effect of pH and Mobile Phase Composition on Hydrophilicity/Hydrophobicity of Amino Acid Side Chains

Uncharged Side Chains—A plot of the hydrophilicity/hydrophobicity coefficients [$\Delta t_{R(Gly)}$] of the 17 uncharged (neutral) side chains obtained at pH 7 (no salt) vs. the coefficients obtained at the two pH 2 conditions, the pH 5 mobile phase, and the two pH 7 mobile phases containing either 50 m*M* NaCl or $NaClO_4$ produces a correlation of $r = 0.997$. This excellent correlation, together with the generally negligible variation of these values in all the mobile phase systems employed (Table III), point to the fundamentally vital conclusion that the intrinsic hydrophilicity/hydrophobicity characteristics of uncharged side chains are unaffected by their environment. Also, these results indicate that such RP-HPLC-derived coefficients are independent of differences in the hydrophobic stationary phase (e.g., alkyl chain length, i.e., C_8 vs. C_{18}) used to derive these values, a result that contradicts earlier work[3] where coefficients were generated from a large collection of random peptides rather than the de novo designed model peptides used in the present study.

Potentially Charged Side Chains—As clearly demonstrated in the present study, and emphasized in Table III, any variation in retention behavior of the 23 model peptides under all 6 mobile phase conditions employed is due to the effect of pH and mobile phase composition on the intrinsic hydrophilicity/hydrophobicity of the ionizable side chains Orn, Lys, His, and Arg (potentially positively charged) and Asp and Glu (potentially negatively charged). Figure 8 illustrates the variation of the side-chain coefficients at pH 2, 5, and 7, the latter in the absence and presence of 50 m*M* $NaClO_4$ (the mobile phase containing 50 m*M* NaCl is not included due to the negligible effect of the chloride ion on side-chain hydrophilicity/hydrophobicity of the charged residues relative to perchlorate; Table III; Figure 7A). The general effect on the coefficients for the positively charged residues (O, K, R, H) at pH 2 is a marked decrease in intrinsic hydrophilicity (i.e., increase in hydrophobicity) on substituting the hydrophilic phosphate anion with the hydrophobic TFA^- anion; indeed, Lys would be classed as an extremely hydrophilic amino acid relative to Gly in the pH 2/H_3PO_4 system and a moderately hydrophobic amino acid in the pH 2/TFA mobile phase. In a similar fashion, a pH change from pH 5 to 7, in the same phosphate-based mobile phase, decreases side-chain hydrophilicity (increases hydrophobicity) of these amino acids quite substantially, with Arg and His being classified as very hydrophilic relative to Gly at pH 5 but moderately hydrophobic at pH 7 (the latter pH certainly resulting in deprotonation of His). The addition of perchlorate ion, with its effective ion-pairing properties,[69] further enhances the hydrophobic nature of Arg relative to Gly under these conditions and transforms the hydrophilic nature of the Orn and Lys side chains in the absence of salt at pH 7 to hydrophobic side chains relative to Gly in the presence of 50 m*M* $NaClO_4$. rate; Table III; Figure 7A). The general effect on the coefficients for the positively charged residues (O, K, R, H) at pH 2 is a marked decrease in intrinsic hydrophilicity (i.e., increase in hydrophobicity) on substituting the hydrophilic phosphate anion with the hydrophobic TFA^- anion; indeed, Lys would be classed as an extremely hydrophilic amino acid relative to Gly in the pH 2/H_3PO_4 system and a moderately hydrophobic amino acid in the pH 2/TFA mobile phase. In a similar fashion, a pH change from pH 5 to 7, in the same

phosphate-based mobile phase, decreases side-chain hydrophilicity (increases hydrophobicity) of these amino acids quite substantially, with Arg and His being classified as very hydrophilic relative to Gly at pH 5 but moderately hydrophobic at pH 7 (the latter pH certainly resulting in deprotonation of His). The addition of perchlorate ion, with its effective ion-pairing properties,[69] further enhances the hydrophobic nature of Arg relative to Gly under these conditions and transforms the hydrophilic nature of the Orn and Lys side chains in the absence of salt at pH 7 to hydrophobic side chains relative to Gly in the presence of 50 m*M* $NaClO_4$.

At pH 2, the presence of the hydrophilic phosphate anion or the hydrophobic TFA^- anion has little appreciable effect on the intrinsic hydrophilicity/hydrophobicity of the protonated (i.e., uncharged) side chains of Asp and Glu, being classed as moderately hydrophobic relative to Gly. These side chains become somewhat hydrophilic at pH 5 and 7 (no salt) due to deprotonation of these side chains and hence their negative charge. The moderately hydrophilic characteristics of these two side chains is dramatically affected by the introduction of the negatively charged perchlorate anion in the medium, which appears to transform them into extremely hydrophilic amino acids.

The explanation for the effects of $NaClO_4$ on the retention behavior of the 23 peptides used in this study is shown in Figure 9B, where the change in peptide retention time at pH 7 in the absence and presence of 50 m*M* salt (NaCl or $NaClO_4$) is plotted against peptide net charge. This plot clearly supports the concept that the increase in hydrophobicity of the peptides is due to ion-pairing of the perchlorate anion to the positively charged residues in the peptides. Thus, 18 of the peptides have a single positively charged Lys residue (net charge +1) with an average increase in peptide retention with addition of 50 m*M* $NaClO_4$ of 7.1 min. In contrast, the three peptides with an additional positively charged residue (Lys, Arg, and Orn) have a net charge of +2 and an increase in retention times of 11.6, 9.6, and 12.8 min, respectively. The two peptides with a negatively charged residue (Asp and Glu) and thus a net charge of zero show only a very small increase in peptide retention time in the presence of 50 m*M* $NaClO_4$ (0.4 and 0.9 min, respectively). The latter results suggest that the perchlorate anion is ineffective at ion-pairing when the net charge on the peptide is zero—hence no appreciable increase in retention time on the addition of 50 m*M* $NaClO_4$. The inability of the chloride anion to ion-pair with positively charged groups is shown by the fact that retention behavior in the presence of 50 m*M* NaCl is independent of net charge (zero to +2) (Figure 9B).

Figure 9A illustrates the effect of the trifluoroacetate anion on peptide retention behavior at pH 2. At pH 2, the Asp and Glu side chains are protonated. Thus, while the Lys-, His-, Orn-, and Arg-substituted peptides have a net charge of +2, the remaining 19 peptides have a net charge of +1. The change in peptide retention time between the H_3PO_4 and TFA mobile phases for the +1 peptides averaged 5.6 min, whereas the +2 peptides showed increased retention times for the Lys-, His-, Orn-, and Arg-substituted peptides of 15.7, 12.9, 12.9, and 11.5 min, respectively.

Finally, Figure 10 illustrates the effect of mobile phase pH on intrinsic hydrophilicity/hydrophobicity of potentially positively charged side chains over a range of pH 5–8.5. From Fig. 10, the hydrophilicity of all four side chains decreases (i.e., hydrophobicity increases) with increasing pH, albeit to varying degrees. Thus, Arg is considerably more sensitive to pH changes than Orn, His, or Lys over a range of pH 5–6.5. Interestingly, the effect of pH over this range is essentially identical for the latter side chains, followed by a dramatic change in the His profile between pH 6.5 and 6.75 due to essentially complete deprotonation of its side chain (the pKa of His is ~6.0 in the free amino acid). The profiles for Orn and Lys, similar prior to pH 6.5, diverge somewhat at values greater than pH 6.5. As noted previously, the decrease in hydrophilicity (increase in hydrophobicity) of these side chains is likely due to partial deprotonation with an increase in pH, the extent of this deprotonation being dependent

on the pKa values of these amino acids under the conditions of RP-HPLC or the increasing concentration of HPO_4^{2-} anion as the pH is increased. Thus, the differences in the Orn and Lys profiles suggest the pKa values of these amino acid side chains are different. The dramatic effects observed with the Arg-substituted peptide is most likely due to the more efficient ion-pairing of the HPO_4^{2-} anion with the Arg side chain compared to the alkyl amino groups of Orn and Lys. The resonance-stabilized guanidinium group could lead to easier desolvation and more efficient ion-pairing.

CONCLUSIONS

Intrinsic hydrophilicity/hydrophobicity side-chain coefficients of amino acids have been determined by RP-HPLC of model synthetic peptides over a pH range of pH 2–7 in the absence of any nearest-neighbor or peptide conformational effects. The intrinsic values of neutral side chains were unaffected by pH, mobile phase composition, or functional groups of the reversed-phase matrix. Only potentially charged side chains (Orn, Lys, His, Arg, Asp, Glu) showed a variation in intrinsic hydrophilicity/hydrophobicity with varying mobile phase environments.

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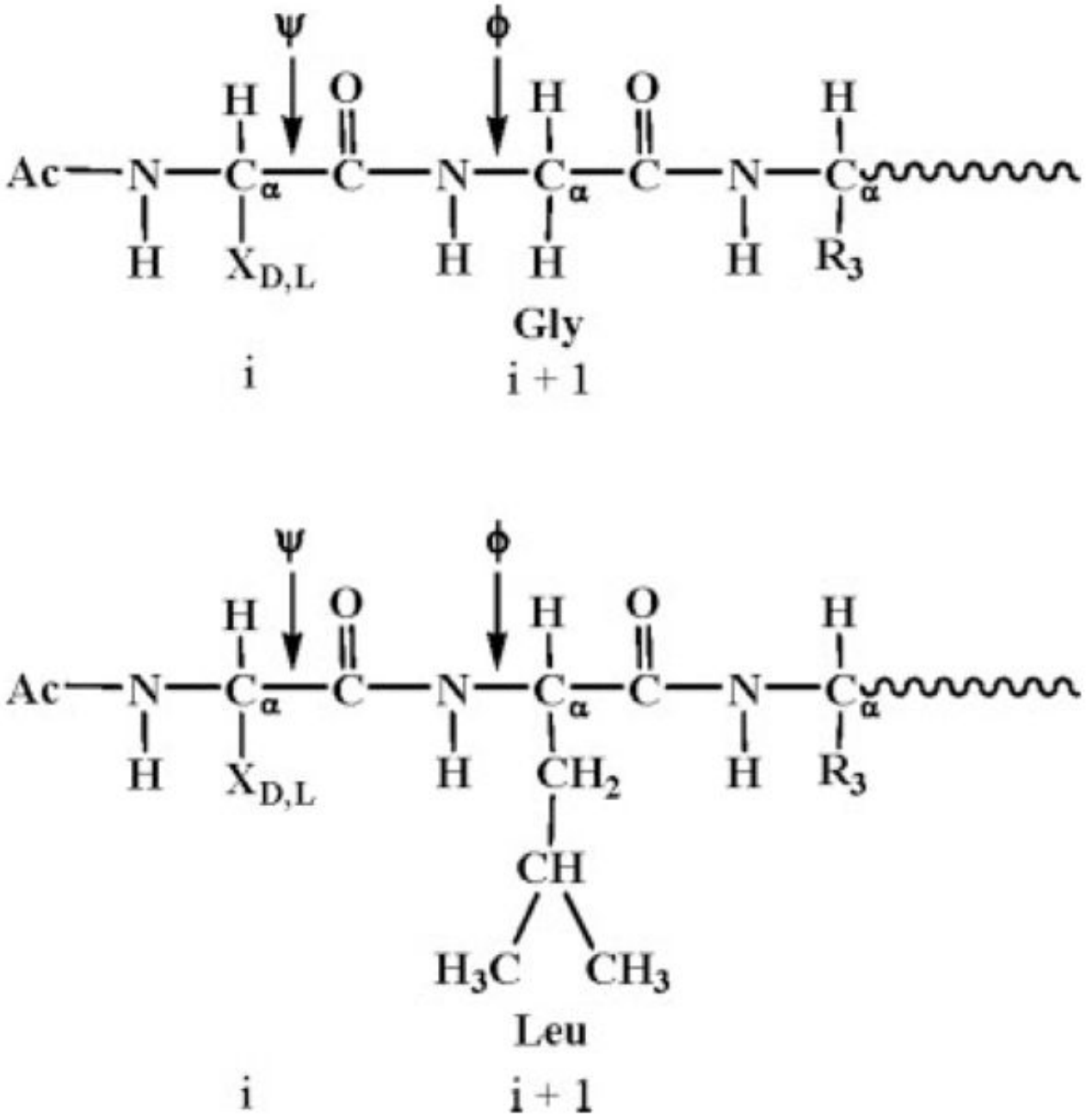

FIGURE 1.

N-terminus of synthetic model peptides used in this study. Top: N-terminus of the peptide sequence Ac–$X_{D,L}$–G–A–K–G–A–G–V–G–L–amide, where L- and D-amino acid substitutions are made at position *i* adjacent to Gly in the *i* + 1 position. Bottom: N-terminus of the peptide sequence Ac–$X_{D,L}$–L–G–A–K–G–A–G–V–G–amide, where L- and D-amino acid substitutions are made at position *i* adjacent to Leu in the *i* + 1 position. The symbols above the C^{α}—C and C^{α}—N bonds denote ψ and φ angles, respectively.

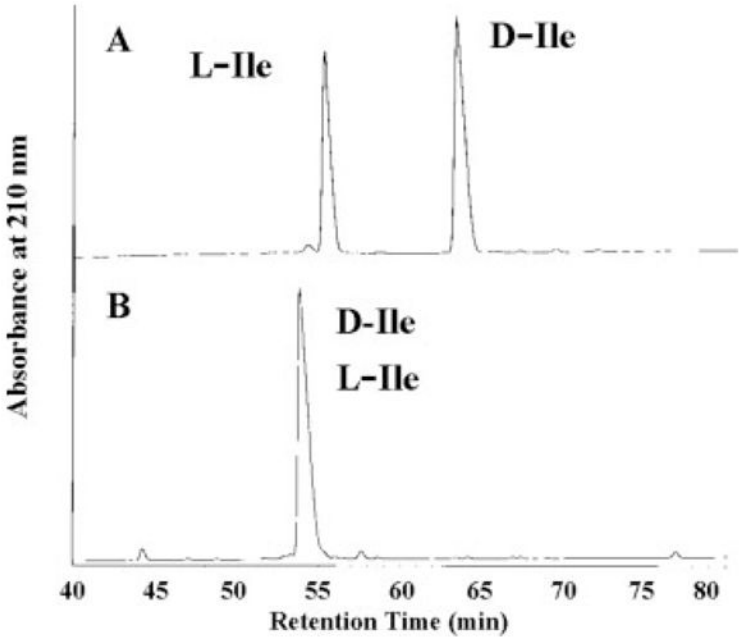

FIGURE 2.

Elimination of nearest-neighbor effects to determine intrinsic hydrophilicity/hydrophobicity coefficients. Column: Kromasil C18. Conditions: linear AB gradient (0.25% CH_3CN/min, starting from 2% CH_3CN) at a flow rate of 0.3 mL/min, where eluent A is 20 m*M* aqueous. TFA and eluent B is 20 m*M* TFA in CH_3CN; temperature, 25°C. Panel A: representative RP-HPLC elution profile at pH 2.0 of two peptides of the same sequence (Ac–X–L–GA–K–G–A–G–V–G–amide), where position X contains L-Ile or D-Ile adjacent to a Leu residue. Panel B: RP-HPLC elution profile of two peptides of the same sequence (Ac–X–G–A–K–G–A–G–V–G–L–amide), where position X contains L-Ile or D-Ile next to a Gly residue.

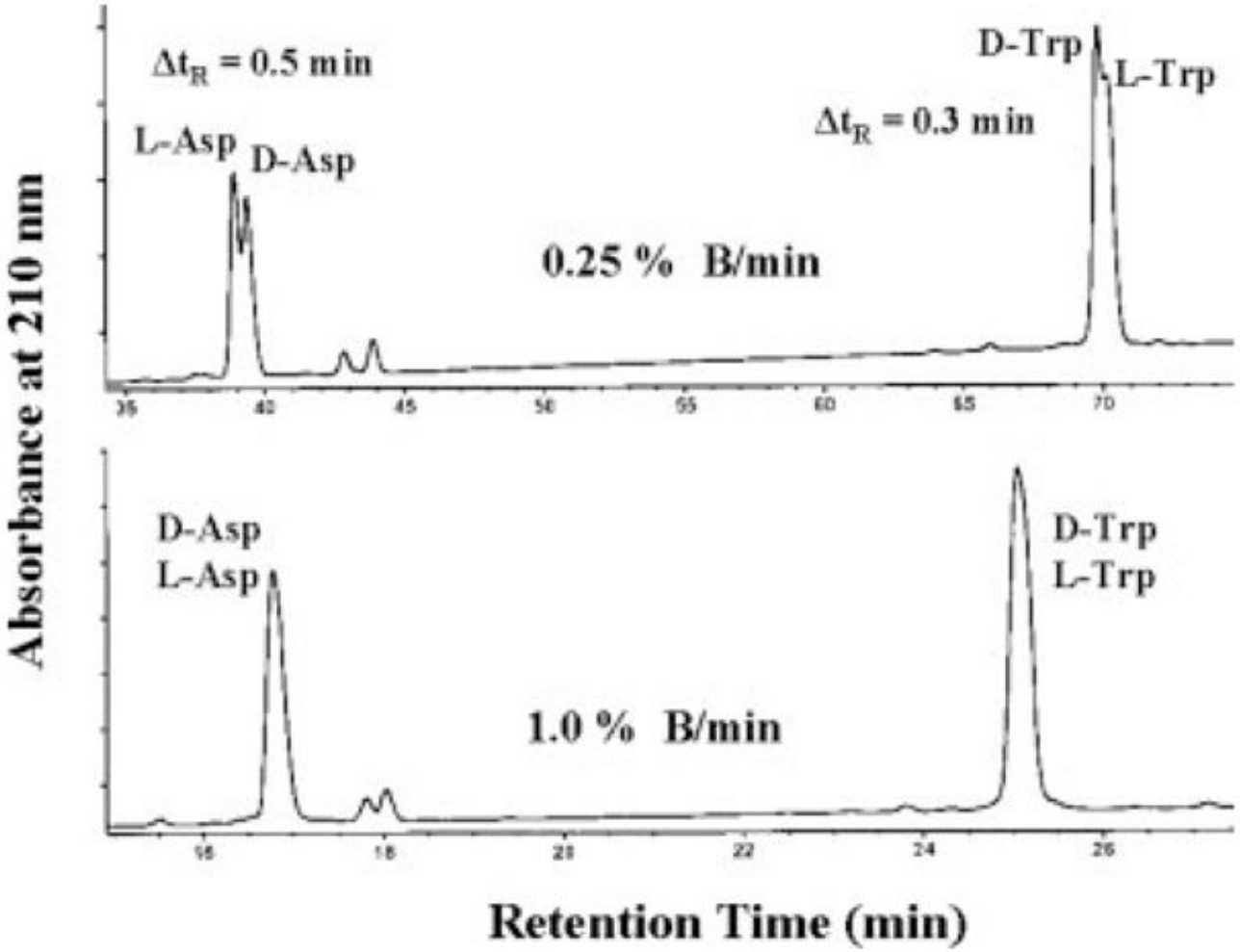

FIGURE 3.

Effect of gradient rate on RP-HPLC elution profile of diastereomeric peptide pairs at pH 2.0. Column: Kromasil C18. Conditions: same as Figure 2, but with gradient rates of 0.25% CH_3CN/min (top) and 1% CH_3CN/min (bottom) starting from 2% CH_3CN. L-Asp, D-Asp, L-Trp, or D-Trp substitutions were made at position X of the peptide sequence Ac–X–G–A–K–G–A–G–V–G–L–amide and were the only substitutions that could be partially resolved when adjacent to a Gly residue.

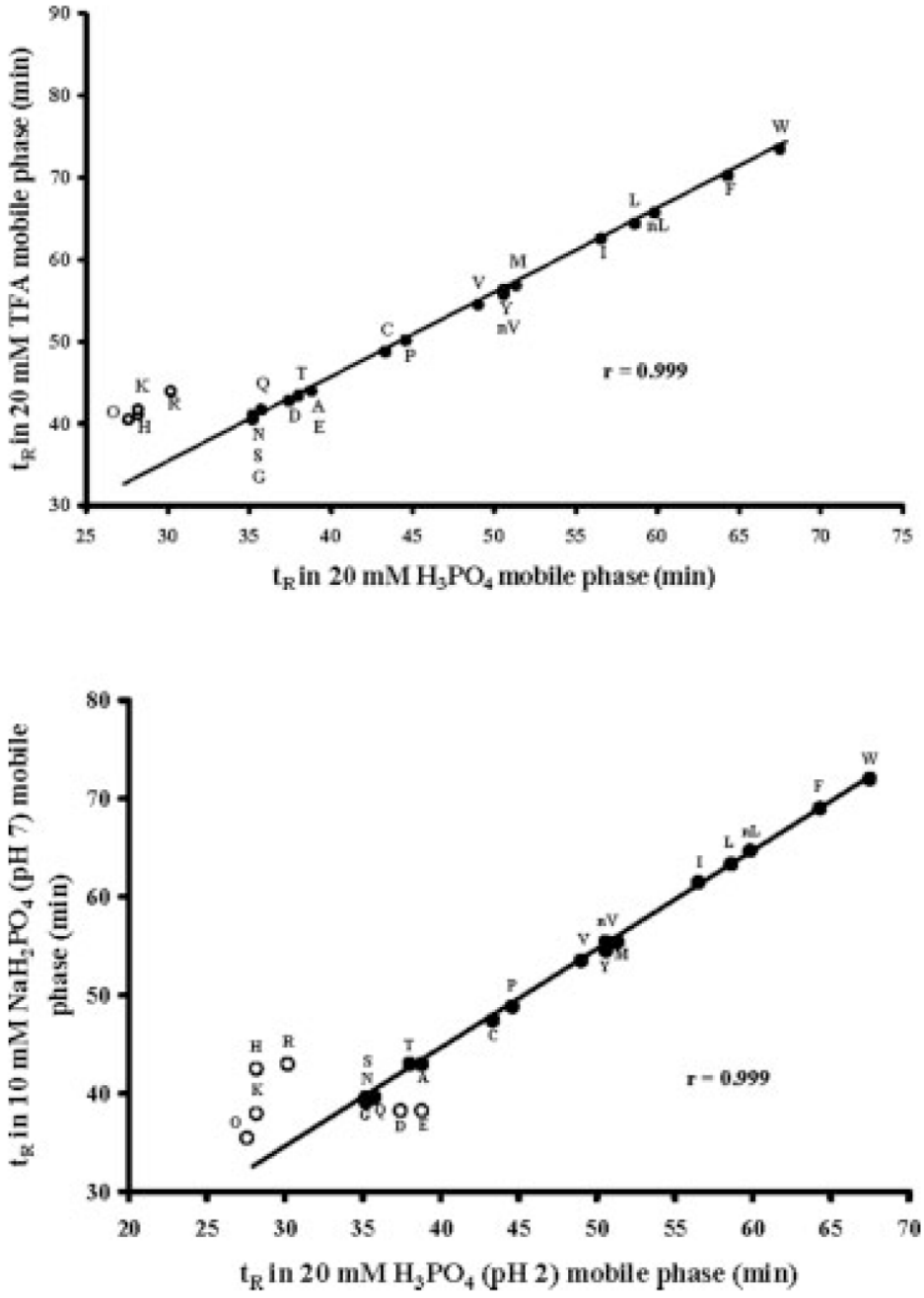

FIGURE 4.

Top: Plot of t_R of peptides in the 20 m*M* TFA mobile phase system vs. t_R in 20 m*M* H_3PO_4 mobile phase system. Column: Kromasil C18. Conditions: linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 20 m*M* aqueous TFA or 20 m*M* aqueous H_3PO_4 and eluent B is 20 m*M* TFA or 20 m*M* H_3PO_4, respectively, in CH_3CN starting from 2% CH_3CN; temperature, 25°C. Bottom: Plot of t_R of peptides in 20 m*M* H_3PO_4 (pH 2) mobile phase system vs. t_R in 10 m*M* NaH_2PO_4 (pH 7; no salt) mobile phase system. Columns: Kromasil C18 (pH 2) and Zorbax XDB C8 (pH 7). Conditions: pH 2, linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 20 m*M* aqueous H_3PO_4 and eluent B is 20 m*M* H_3PO_4 in CH_3CN starting from 2% CH_3CN; pH 7, linear AB gradient (0.25%

CH_3CN/min) at a flow rate of 0.3 mL/min where eluent A is 10 m*M* aqueous NaH_2PO_4, pH 7, and eluent B is eluent A containing 50% CH_3CN; temperature, 25°C. Data are taken from Table II. The single-letter code represents the $_L$-amino acid substitutions at position X of the peptide sequence shown in Figure 3. Closed circles denote data used in the correlation plot (top: $y = 1.0136x + 4.9733$, correlation coefficient $r = 0.999$; bottom: $y = 1.006x - 0.0376$, correlation coefficient $r = 0.999$); open circles represent results from positively charged peptide residues; nL, nV, and O denote norleucine, norvaline, and ornithine, respectively.

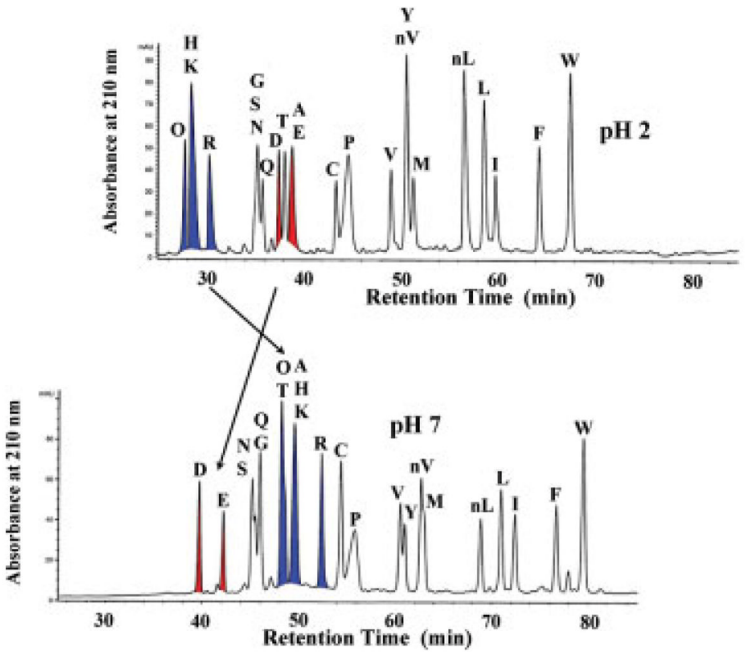

FIGURE 5.

RP-HPLC elution profiles of peptide mixtures at pH 2 (top) and pH 7 (bottom). Columns: pH 2, Kromasil C18; pH 7, Zorbax XDB C8. Conditions: pH 2, linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 20 m*M* aqueous H_3PO_4 and eluent B is 20 m*M* H_3PO_4 in CH_3CN, starting at 2% CH_3CN; pH 7, linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 10 m*M* aqueous NaH_2PO_4 (pH 7) and eluent B is eluent A containing 50% CH_3CN; temperature, 25°C. Peaks are denoted by the one-letter codes of the $_L$-amino acids substituted into position X of the peptide sequence shown in Figure 3. Shaded peaks denote peptides containing potentially positively charged residues (K, R, H, O) or potentially negatively charged residues (D, E). Arrows denote change in relative elution order of the potentially negatively charged and potentially positively charged residues between pH 2 and 7.

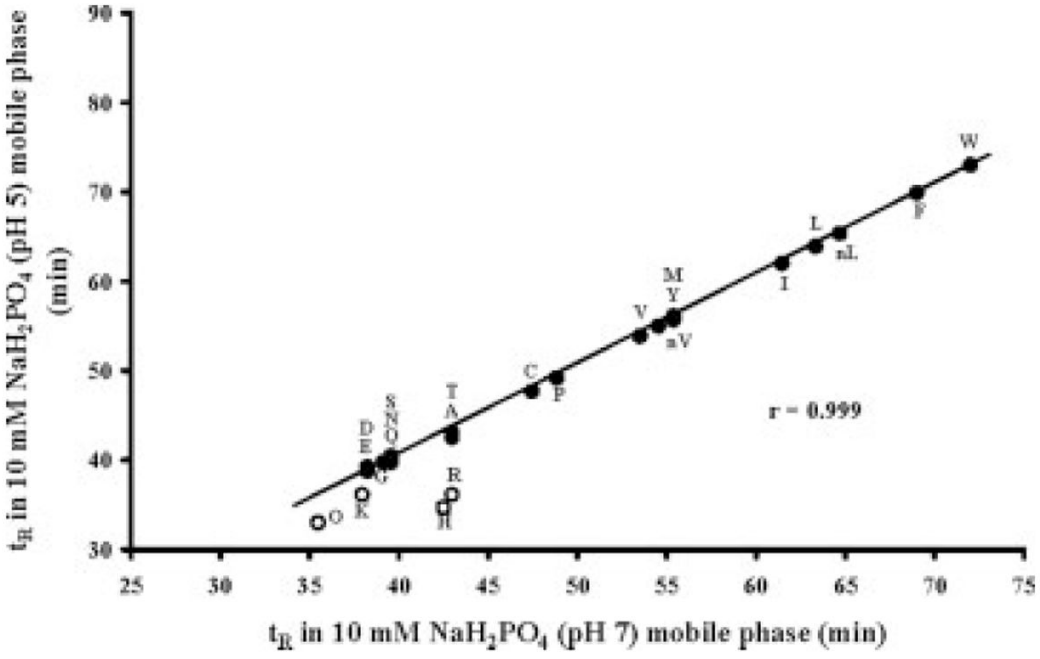

FIGURE 6.

Plot of t_R of peptides in 10 m*M* NaH_2PO_4 (pH 5) mobile phase system vs. 10 m*M* NaH_2PO_4 (pH 7) mobile phase system. Column: Zorbax XDB C8. Conditions: linear gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 10 m*M* aqueous NaH_2PO_4, pH 5 or 7, and eluent B is the respective eluent A containing 50% CH_3CN; temperature, 25°C. Data are taken from Table II. The single-letter code represents the $_L$-amino acid substitutions at position X of the peptide sequence shown in Figure 3. Closed circles denote data used in the correlation plot ($y = 1.011x - 0.0699$, correlation coefficient $r = 0.999$); open circles represent results from positively charged peptide residues; nL, nV, and O denote norleucine, norvaline, and ornithine, respectively.

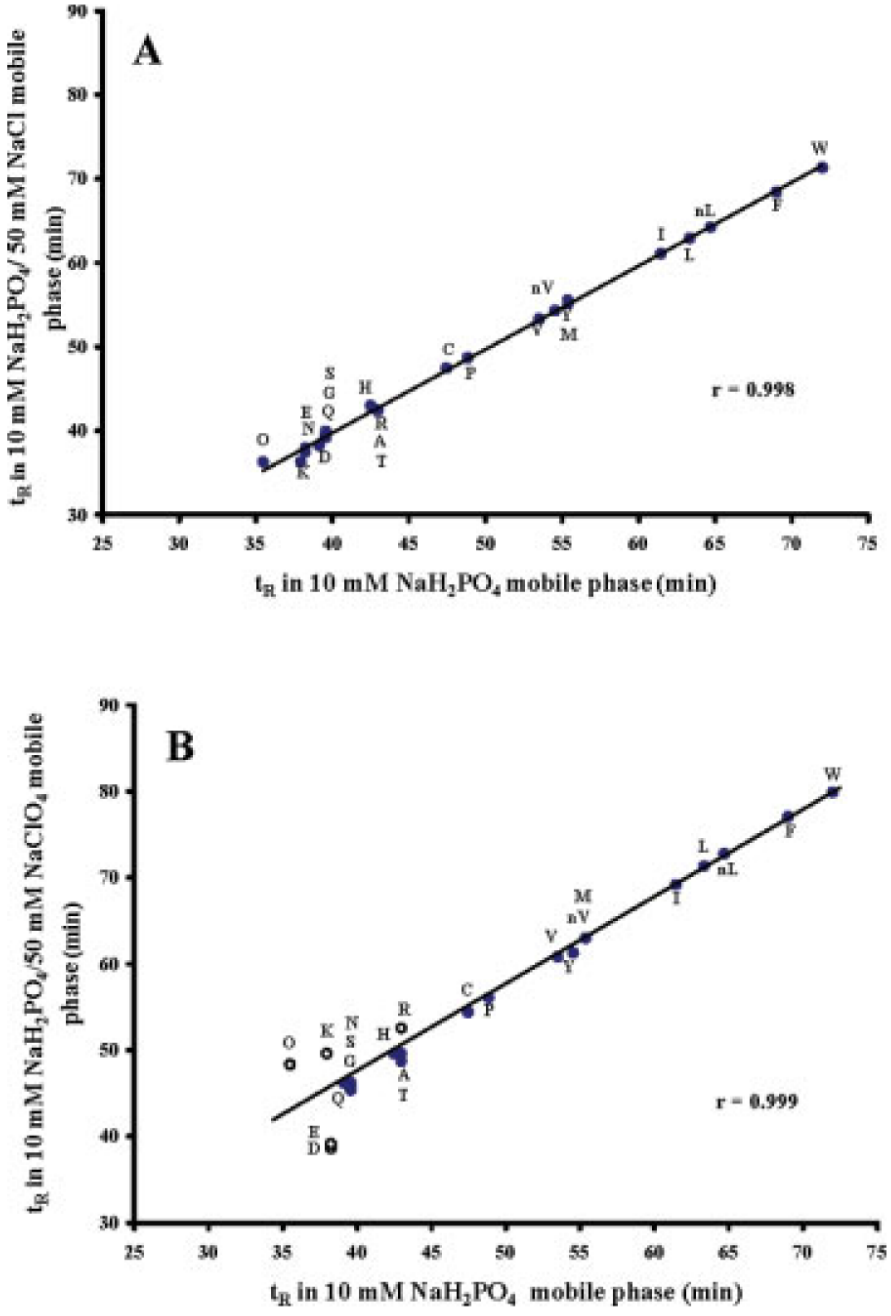

FIGURE 7.

Plot of t_R of peptides in 10 m*M* NaH_2PO_4 (pH7) mobile phase system vs. 10 m*M* NaH_2PO_4 (pH 7) containing 50 m*M* NaCl (A) or 50 m*M* $NaClO_4$ (B) mobile phase systems. Column: Zorbax XDB C8. Conditions: linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 10 m*M* aqueous NaH_2PO_4, pH 7, containing no salt, 50 m*M* NaCl or 50 m*M* $NaClO_4$, and eluent B is the respective eluent A containing 50% CH_3CN; temperature, 25°C. Data are taken from Table II. The single-letter code represents the $_L$-amino acid substitutions at position X of the peptide sequence shown in Figure 3. Closed circles denote data used in the correlation plot ($y = 0.9948x - 0.051$, correlation coefficient $r = 0.998$ and $y = 1.0572x + 4.1618$, correlation coefficient $r = 0.999$ for plots A and B, respectively); open circles

represent results from positively charged peptide residues; nL, nV, and O denote norleucine, norvaline, and ornithine, respectively.

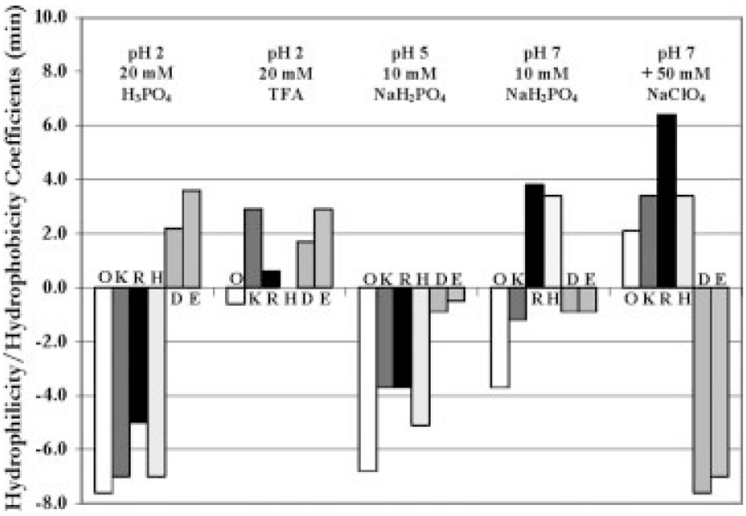

FIGURE 8.

Effect of mobile phase conditions on magnitude of hydrophilicity/hydrophobicity coefficients of potentially charged acidic (D, E) and basic (O, K, R, H) residues. Columns: pH 2, Kromasil C18; pH 5 and 7, Zorbax XDB C8. Conditions: pH 2, shown in Figure 4 (top); pH 5 shown in Figure 6; pH 7 shown in Figures 6 and 7. Data are taken from Table III; O denotes ornithine.

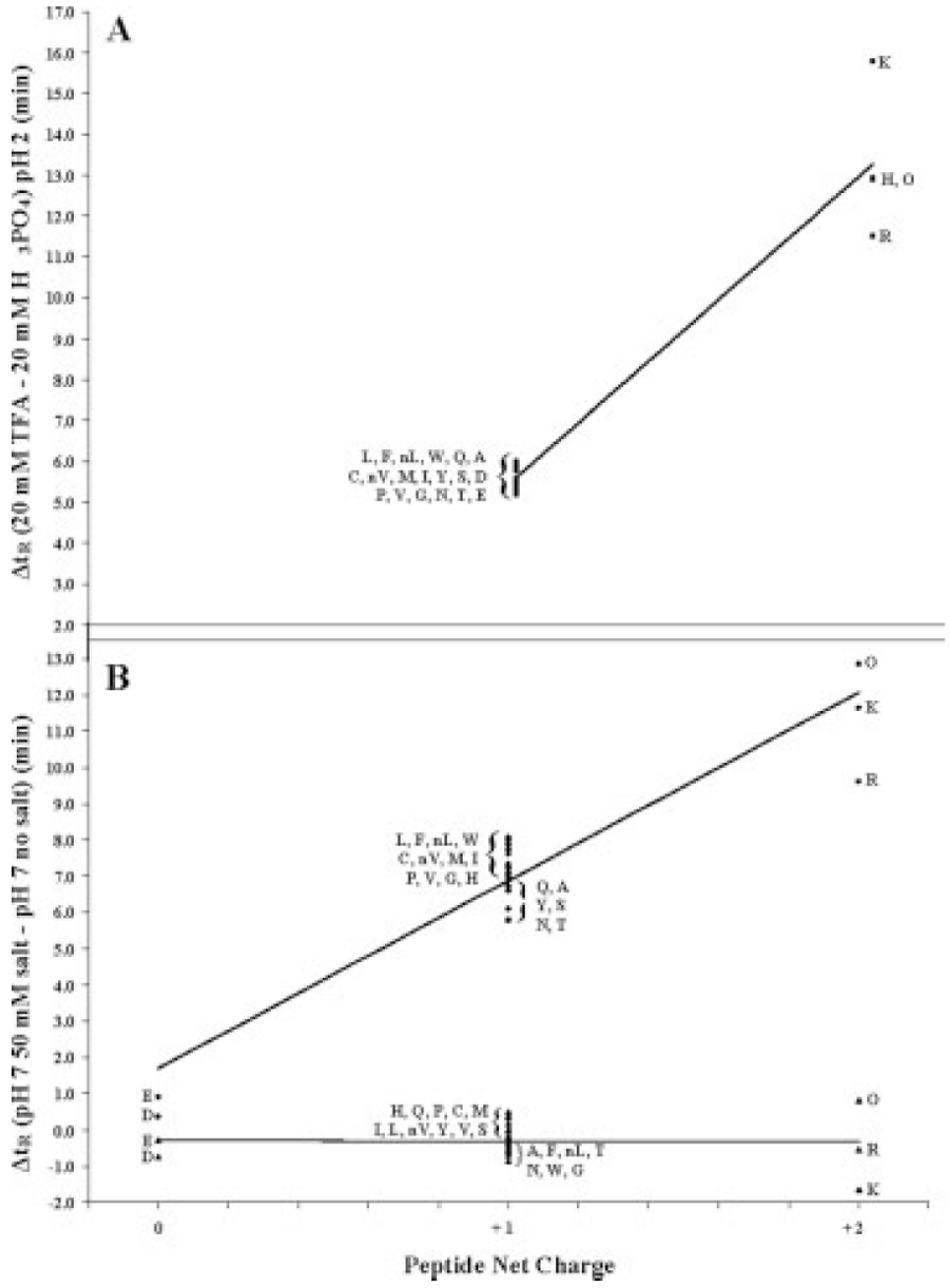

FIGURE 9.

Relationship between peptide net charge and effect of mobile phase counterion at pH 2 (TFA^- anion; panel A) and pH 7 (Cl^-, ClO_4^- anions; panel B). Columns: pH 2, Kromasil C18; pH 7, Zorbax XDB C8. Conditions: pH 2, linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 20 m*M* aqueous H_3PO_4 or TFA and eluent B is 20 m*M* H_3PO_4 or TFA, respectively, in CH_3CN, starting at 2% CH_3CN; pH 7, linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 10 m*M* aqueous NaH_2PO_4, containing no salt or containing 50 m*M* NaCl or 50 m*M* $NaClO_4$ and eluent B is the respective eluent A containing 50% CH_3CN; temperature, 25°C. Δt_R denotes the t_R of the peptide in pH 2/TFA system minus the t_R of the peptide in pH 2/H_3PO_4 (panel A) or the t_R of

the peptide in the pH 7/NaCl or pH 7/$NaClO_4$ system minus t_R of peptide in the pH 7/no salt system (panel B). The horizontal and sloping plots in panel B are for the NaCl and $NaClO_4$ effects, respectively; nL, nV, and O denote norleucine, norvaline, and ornithine, respectively.

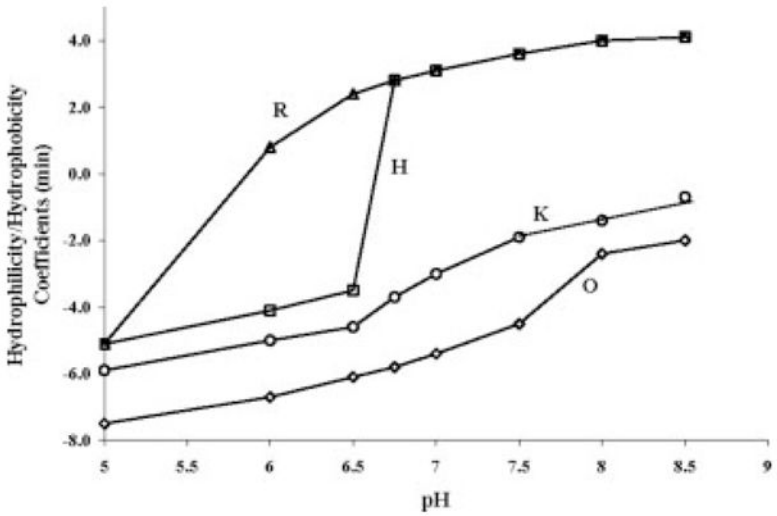

FIGURE 10.

Effect of pH on the magnitude of hydrophilicity/hydrophobicity coefficients of potential positively charged residues. Column: Zorbax XDB C8. Conditions: pH 5–8.5, linear AB gradient (0.25% CH_3CN/min) at a flow rate of 0.3 mL/min, where eluent A is 20 m*M* NaH_2PO_4 and eluent B is 20 m*M* NaH_2PO_4 in 50% CH_3CN; temperature, 25°C. (△) Arginine, (◇) ornithine, (○) lysine, and (□) histidine.

Table I

RP-HPLC Peptide Retention Time Data in 20 mM H_3PO_4, pH 2, at 25°C

Amino Acid Substitution[a]	t_R (min)[b]		$\Delta t_{(D-L)}$[c]
	X_L	X_D	
Trp	67.5	67.8	0.3
Phe	64.3	64.3	0.0
n-Leu	59.8	59.8	0.0
Leu	58.6	58.6	0.0
Ile	56.5	56.5	0.0
Met	51.3	51.3	0.0
n-Val	50.6	50.6	0.0
Tyr	50.6	50.6	0.0
Val	49.0	49.0	0.0
Pro	44.6	44.6	0.0
Cys	43.3	43.3	0.0
Ala	38.8	38.8	0.0
Glu	38.8	38.8	0.0
Thr	38.0	38.0	0.0
Asp	37.4	37.9	0.5
Gln	35.7	35.7	0.0
Ser	35.2	35.2	0.0
Asn	35.2	35.2	0.0
Gly	35.2	35.2	0.0
Arg	30.2	30.2	0.0
His	28.2	28.2	0.0
Lys	28.2	28.2	0.0
Orn	27.6	27.6	0.0

[a] The L- and D-amino acid substitutions at position X in the peptide sequence Ac–X–G–A–K–G–A–G–V–G–L–amide; n-Leu, n-Val, and Orn denote norleucine, norvaline, and ornithine, respectively.

[b] Conditions: shown in Figure 4 (top) for 20 m*M* H_3PO_4 mobile phase system; t_R denotes peptide retention time.

[c] $\Delta t_{(D-L)}$ is the difference in retention time between D- and L-substituted peptides.

Table II

RP-HPLC Peptide Retention Time Data in Various Mobile Phases at 25°C

Amino Acid Substitution[a]	pH 2[b]		pH 5[b] 10 m*M* PO_4 Buffer t_R	pH 7[b], 10 m*M* PO_4 Buffer		
	20 m*M* H_3PO_4 t_R[c]	20 m*M* TFA t_R		No Salt t_R	+50 m*M* NaCl t_R	+50 m*M* $NaClO_4$ t_R
Trp	67.5	73.5	73.0	72.0	71.3	79.9
Phe	64.3	70.2	69.9	69.0	68.4	77.0
n-Leu	59.8	65.7	65.4	64.7	64.2	72.8
Leu	58.6	64.4	63.9	63.3	62.9	71.3
Ile	56.5	62.5	62.0	61.5	61.1	69.2
Met	51.3	56.8	56.2	55.4	55.6	63.0
n-Val	50.6	56.3	55.7	55.4	55.2	63.0
Tyr	50.6	55.8	55.0	54.5	54.3	61.3
Val	49.0	54.5	53.8	53.5	53.3	60.8
Pro	44.6	50.1	49.2	48.8	48.7	56.1
Cys	43.3	48.7	47.7	47.4	47.4	54.4
Ala	38.8	43.9	43.1	43.0	42.4	49.6
Glu	38.8	43.9	39.3	38.2	37.9	39.1
Thr	38.0	43.4	42.6	43.0	42.4	48.7
Asp	37.4	42.7	38.8	38.2	37.5	38.6
Gln	35.7	41.7	40.4	39.6	39.9	46.2
Ser	35.2	41.1	39.8	39.6	39.5	45.7
Asn	35.2	40.5	39.8	39.6	39.3	45.4
Gly	35.2	41.1	39.8	39.1	38.3	46.2
Arg	30.2	41.7	36.1	43.0	42.4	52.6
His	28.2	41.1	34.7	42.5	43.0	49.6
Lys	28.2	43.9	36.1	38.0	36.3	49.6
Orn	27.6	40.5	33.0	35.5	36.3	48.3

[a] The L-amino acid substitutions at position X in the peptide sequence Ac–X–G–A–K–G–A–G–V–G–L–amide; n-Leu, n-Val, and Orn denote norleucine, norvaline, and ornithine, respectively.

[b] Conditions: pH 2, shown in Figure 4 (top); pH 5, shown in Figure 6; pH 7, shown in Figure 6.

[c] The t_R denotes peptide retention time.

Table III

Hydrophilicity/Hydrophobicity Coefficients determined at 25°C by RP-HPLC of Model Peptides

Amino Acid Substitution[a]	pH 2[b]		pH 5[b] 10 m*M* PO_4 Buffer $\Delta t_{R(Gly)}$	pH 7[b], 10 m*M* PO_4 Buffer		
	20 m*M* H_3PO_4 $\Delta t_{R(Gly)}$[c]	20 m*M* TFA $\Delta t_{R(Gly)}$		No Salt $\Delta t_{R(Gly)}$	+50 m*M* NaCl $\Delta t_{R(Gly)}$	+50 m*M* $NaClO_4$ $\Delta t_{R(Gly)}$
Trp	32.3	32.4	33.2	32.9	33.0	33.7
Phe	29.1	29.1	30.1	29.9	30.1	30.8
n-Leu	24.6	24.6	25.6	25.6	25.9	26.6
Leu	23.4	23.3	24.1	24.2	24.6	25.1
Ile	21.3	21.4	22.2	22.4	22.8	23.0
Met	16.1	15.7	16.4	16.3	17.3	16.8
n-Val	15.4	15.2	15.9	16.3	16.9	16.8
Tyr	15.4	14.7	15.2	15.4	16.0	15.1
Val	13.8	13.4	14.0	14.4	15.0	14.6
Pro	9.4	9.0	9.4	9.7	10.4	9.9
Cys	8.1	7.6	7.9	8.3	9.1	8.2
Ala	3.6	2.8	3.3	3.9	4.1	3.4
Glu[d]	**3.6**	**2.8**	**−0.5**	**−0.9**	**−0.4**	**−7.1**
Thr	2.8	2.3	2.8	3.9	4.1	2.5
Asp	**2.2**	**1.6**	**−1.0**	**−0.9**	**−0.8**	**−7.6**
Gln	0.5	0.6	0.6	0.5	1.6	0.0
Ser	0.0	0.0	0.0	0.5	1.2	30.5
Asn	0.0	−0.6	0.0	0.5	1.0	30.8
Gly	0.0	0.0	0.0	0.0	0.0	0.0
Arg	**−5.0**	**0.6**	**−3.7**	**3.9**	**4.1**	**6.4**
His	**−7.0**	**0.0**	**−5.1**	**3.4**	**4.7**	**3.4**
Lys	**−7.0**	**2.8**	**−3.7**	**−1.1**	**−2.0**	**3.4**
Orn	**−7.6**	**−0.6**	**−6.8**	**−3.6**	**−2.0**	**2.1**

[a] The L-amino acid substitutions at position X in the peptide sequence Ac–X–G–A–K–G–A–G–V–G–L–amide; n-Leu, n-Val, and Orn denote norleucine, norvaline, and ornithine, respectively.

[b] Conditions: pH 2, shown in Figure 4 (top); pH 5, shown in Figure 6; pH 7, shown in Figure 6.

[c] The Δt_R(Gly) denotes the change in retention time relative to the Gly, substituted peptide.

[d] The bold values denote the potentially charged residues Asp, Glu, Arg, His, Lys, and Orn.