Peptide Research

Hydrophobicity Indices for Amino Acid Residues as Determined by High-Performance Liquid Chromatography

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ABSTRACT

The retention times of compounds on reversed-phase high-performance liquid chromatography columns are determined by their overall hydrophobicity. This paper exploits this relationship to derive hydrophobicity indices for amino acid residues. Retention times of 20 Z-amino acids and their methyl, ethyl and benzyl esters were determined under standard conditions on reversed-phase high-performance liquid chromatography at pH 3.0 and pH 7.5. Retention times of derivatives of six of the amino acids were used to calculate amino acid residue hydrophobicity values relative to glycine and leucine by a computer-based iterative procedure that used splines to optimize the smoothness of fit. Using these derived curves, values for each derivative of the remaining 14 Z-amino acids were determined and averages calculated. The curves, generated independently for pH 3.0 and pH 7.5 were effectively identical and the determined hydrophobicity values (other than for charged residues) were also similar at the two pHs. The values obtained vary significantly from other published values. Comparisons with some of the more commonly used hydrophobicity/hydropathy tables are presented. The highest correlation was found with constants determined for water/octanol partitioning of N- and C-terminal protected amino acids.

INTRODUCTION

Many scales of amino acid hydrophobicity/hydropathy exist. They have been derived by a variety of methods including: water/solvent partitioning of free (5,7,9,11,12) and N- and C-terminally protected amino acids (16); structural considerations on the degree to which an amino acid side chain is buried inside a protein (7,8) and the retention time of natural and synthetic pepides on reversed-phase high performance liquid chromatography (HPLC) columns under a variety of conditions (3,10,12,14,17). The various scales differ widely, particularly in the values of tyrosine and tryptophan and the polar amino acids.

The scales have been used mainly to compare protein sequences on the basis of hydrophobic similarity (e.g., 15) or to predict structural features from protein sequence data, for example, potential antigenic and transmembrane sites (4). Hopp considers that, in the case of antigenic site prediction, methods based on hydrophobicity were the most successful (4).

As HPLC retention time for a molecule depends on its hydrophobicity, there is a strong physical basis for scales derived from retention time data. We derive here a new HPLC-based scale by using chosen amino acid derivatives. Our strategy was to synthesize a derivative for each amino acid side chain so that the side chain was fully exposed with, as near as possible, a natural hydrophobic/philic profile. Thus we considered it important to use a short derivative (so that the side chain could not be obscured by molecular folding) and derivatives with modified amino acid and carboxyl groups (to neutralize the charge effects). We selected the 20 Z-amino acids and their methyl, ethyl and benzyl esters to meet these requirements.

We report a novel mathematical analysis for our data leading to a new hydrophobic scale at each of two pHs: pH 3.0 and pH 7.5. We include a comparison with a number of other scales derived from a variety of sources. Interestingly, our values show the closest rank correlation with the figures reported by Fauchere and Pliska (in 16), who used water/octanol partitioning of N-acetyl-amino acid amides. Since their method also has a good physical basis, this could be interpreted as a positive indicator for our values.

MATERIALS AND METHODS

Amino Acid Derivatives

Z-amino acids and their methyl, ethyl and benzyl esters were purchased either from Sigma Chemical (St. Louis, MO) or from Novabiochem (Nottingham, UK). Where necessary, methyl and ethyl esters were prepared by the HCl-methanol, HCl-ethanol procedures, respectively (1). Benzyl esters were prepared by the benzyl bromide procedure (13).

HPLC Conditions

The amino acid derivatives were run under standard conditions on Waters HPLC equipment (Waters Chromatography, Div. of Millipore, Milford, MA) on a C18 Novapak column using triethylamine phosphate buffers at pH 3.0 and 7.5 (40 mM with respect to triethylamine). Linear gradients were from 0%–80% acetonitrile over 15 min at 20°C, flow rate 2.0 ml/min. Z-glycine and Z-glycine methyl ester were included as standards in all runs.

Table 1: Matrix of H - H_{ref}

AMINO ACID RESIDUE USED IN ESTABLISHING CURVE	C-TERMINAL DERIVATIVE			
	OMe	OEt	OBzl	COO^-
GLY	0	ΔOEt	ΔOBzl	ΔCOO
LEU	1.8	1.8 + ΔOEt	1.8 + ΔOBzl	1.8 + ΔCOO
ALA	ΔAla	ΔAla + ΔOEt	ΔAla + ΔOBzl	ΔAla + ΔCOO
VAL	ΔVal	ΔVal + ΔOEt	ΔVal + ΔOBzl	ΔVal + ΔCOO
ASN	ΔAsn	ΔAsn + ΔOEt	ΔAsn + ΔOBzl	ΔAsn + ΔCOO
HIS**	ΔHis	ΔHis + ΔOEt	ΔHis + ΔOBzl	ΔHis + ΔCOO

** His at pH 3.0, Asp at pH 7.5

	Solutions							
	ΔAla	ΔVal	ΔAsn	ΔHis	ΔAsp	ΔOEt	ΔOBzl	ΔCOO
pH3.0	0.42	1.34	-1.03	-2.28	-	0.52	1.52	-1.07
pH7.5	0.35	1.32	-0.99	-	-2.15	0.47	1.47	-3.16

Calculation of Hydrophobicity Values from HPLC Retention Times

The method requires the adoption of a reference substance whose retention time forms the baseline for all derivatives used. Our choice was Z-Gly-OMe, with retention time at both pHs of 11 min and 30 s (R_{ref}). Generally, retention times R can be written as

$$R = R_{ref} + f(H - H_{ref}) \qquad \text{(Eq. 1)}$$

where H is the hydrophobicity of the compound on HPLC, H_{ref} is that of our reference and f(•) is a function with two properties. These are (a) f(0) = 0, thus ensuring that R = R_{ref} when the reference is run, and (b) f(•) increases with increasing values of its argument, so that the more hydrophobic the compound, the greater R. We do *not* prejudge f(•) to be linear.

Equation 1 focuses on the difference in hydrophobicities between the compound run and the reference. We have modeled this difference as an average of the hydrophobicity differences for each component of the compound.

$$H - H_{ref} = 1/3\{(H_{NTerminus} - H_Z) + (H_{AAResidue} - H_{Gly}) + (H_{CTerminus} - H_{OMe})\} \qquad \text{(Eq. 2)}$$

To date Z has been the only N-terminal substitution used, so the first term in the sum has always been zero.

We denote the second and third terms by Δ subscripted by the appropriate residue or ester (e.g., Δ_{Ala}, Δ_{OEt}). Note that Δ_{Gly} and Δ_{OMe} equal zero, by definition. Substitution of (Eq. 2) into (Eq. 1) yields

$$R = R_{ref} + f(1/3\{\Delta_{Ala} + \Delta_{OEt}\}) \qquad \text{(Eq. 3)}$$

for the example Z-Ala-OEt, with similar equations for the other derivatives used. R and R_{ref} are known from the data. Our aim, therefore, is to find

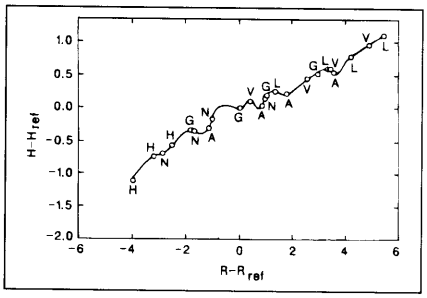

Figure 1. An example of the curve fitting procedure at pH 3 using G, L, A, V, N and H. For a given choice of the 7 Δ-values in the matrix of Table 1, a plot of the relationship between H-H_{ref} and R-R_{Ref} can be made and a spline curve drawn through the points. This figure illustrates a poor choice of Δ_{Ala} which leads to a wiggly curve. A change in Δ_{Ala} moves the block of 4 Ala points in the vertical direction. Clearly a rise in Δ_{Ala} would improve the curve. Similarly a change in an ester value would move a block of 6 points. Iteration on the Δ-values leads to the smooth, increasing curve of Figure 2(a). For technical reasons in the spline fitting, retention times are displayed on the x-axis.

all the Δs and a smooth function f(•) to best satisfy the equations (Eq. 3). The resulting Δ-values for the amino acid residues yield a hydrophobicity index relative to Gly. The index scale needs a zero point; it is convenient to arbitrarily set H_{Gly} = 0 so that Δ = H for each amino acid residue. This choice agrees with Levitt (9), and Hopp and Woods (5). The range of our scale can also be set arbitrarily; we took H_{Leu} = 1.8, again agreeing with these authors (except for a change of sign).

Solving the Δs is an iterative procedure which simultaneously estimates a smooth function f(•) as a "cubic spline" (2). We divided the 20 amino acid residues into two sets: (A) a subset of 6 which provided a computationally manageable group to establish f(•) and the Δ-values of that 6 *via* the above iteration and (B) the remaining 14 whose Δ-values were found using the f(•) of part A. The "establishment" 6 included the range-setters Gly and Leu with four others whose derivatives have the full spread of retention times (A, V, N and D at pH 7.5 and A, V, N and H at pH 3.0).

At any stage in the iteration, the seven Δ-values in the matrix of Table 1 will be specified, enabling the 6 × 4 matrix to have numerical entries for H - H_{ref}. These are plotted against corresponding R - R_{ref} values, for example, in Figure 1. A curve (cubic smoothing spline) is fitted to these points at each iterative stage. During the iteration, changes of a Δ-value move blocks of points. For example, a higher value for Δ_{Ala} would adjust a block of 4 points in Figure 1 and create a less wiggly curve. The final curve is the one which minimizes "wiggliness" (measured by the integrated squared third derivative) while fitting each data point within a close tolerance.

For the remaining 14 residues, each Δ was chosen so that the additional "blocks" of data points lie as close as possible to the established curve. (Δ was selected to minimize the sum of squared discrepancies, this being equivalent to simply selecting the average.)

The exercise was carried out separately at the two pHs used.

Comparison With Other Scales

Other hydrophobicity/hydropathy scales were normalized to Gly = 0, Leu

Table 2. HPLC Retention Time Data

Amino Acid	Z-aa-COO⁻ pH 7.5	Z-aa-COO⁻ pH3.0	Z-aa-OMe pH7.5	Z-aa-OMe pH3.0	Z-aa-OEt pH7.5	Z-aa-OEt pH3.0	Z-aa-OBzl pH7.5	Z-aa-OBzl pH3.0
A	7.91	10.35	12.33	12.33	13.26	13.27	14.99	14.99
C	-	--	13.11	13.09	--	--	--	--
D	6.57	9.26	8.54	10.65	9.00	11.37	10.14	13.23
E	6.77	9.46	8.67	10.87	9.19	11.67	10.34	13.39
F	9.76	12.82	14.76	14.73	15.49	15.52	16.66	16.75
G	7.75	9.67	11.50	11.50	12.46	12.47	14.45	14.44
H	7.58	7.49	10.31	8.32	11.01	8.96	--	--
I	9.29	12.72	14.84	14.83	15.73	15.72	--	--
K	7.45	7.84	9.02	8.75	9.69	9.32	--	--
L	9.31	12.82	14.82	14.82	15.61	15.65	16.81	16.86
M	8.96	11.78	13.77	13.78	14.52	14.53	--	--
N	7.20	8.61	9.77	9.77	10.47	10.45	12.46	12.45
P	7.97	10.91	13.20	13.22	14.09	14.09	15.65	15.71
Q	7.43	8.72	9.93	9.92	10.59	10.61	12.49	12.48
R	7.68	8.21	8.96	9.00	9.87	9.78	--	--
S	7.34	8.95	10.32	10.34	11.21	11.18	13.29	13.32
T	7.75	9.46	11.02	11.01	11.87	11.87	13.89	13.89
V	8.79	11.90	14.08	14.10	14.92	14.92	16.28	16.33
W	9.79	12.62	14.42	14.40	14.98	14.95	16.07	16.16
Y	8.58	10.90	12.67	12.67	13.35	13.35	14.71	14.72

= 1.8, for comparison with the derived values. Rank correlation coefficients were calculated for all pairs.

RESULTS

The retention time of the Z-amino acids and their various esters is shown in Table 2. The "spline" curves established by the six amino acids are shown in Figures 2(a) and 3(a) (pH 3 and pH 7.5, respectively). The values obtained for the hydrophobicity of the four amino acids relative to glycine at 0 and leucine at 1.8 are shown in Table 1 and Tables 3(A) and 4(A). Values for the ethyl and benzyl esters and the free carboxyl group relative to the methyl ester are listed in Table 1.

Hydrophobicity values for the remaining 14 amino acids for each C-terminal derivative and the averages are tabulated in Tables 3(B) and 4(B). In the pH 7.5 results, the values obtained for the Z-amino acids with free carboxyl group were omitted from the calculation of the averages due to obvious differences between many of these values and the values obtained with the ester derivatives.

The average values were then used to calculate values for the derivatives of all 14 amino acids which were then superimposed on the original curves to demonstrate the degree of fit [Figures 2(b) and 3(b)]. The pH 7.5 free carboxyl values are superimposed in Figure 3(c) showing the positively charged

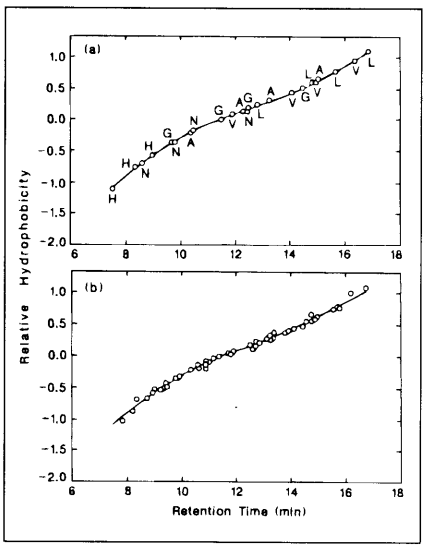

Figure 2. Relative hydrophobicity, defined as H-H$_{ref}$, plotted against retention time at pH 3.0 using the final values of hydrophobicity derived *via* the iterative procedure. (a) The set G, L, A, V, N and H and the smooth curve they establish. (b) The remaining 14 residues plotted with the same curve using the average residue value. (See Materials and Methods)

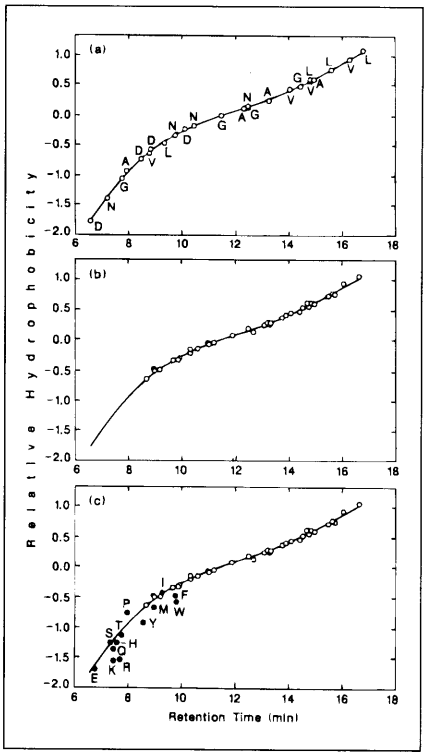

Figure 3. Relative hydrophobicity, defined as H-H$_{ref}$, plotted against retention time at pH 7.5 using the final values of hydrophobicity derived *via* the iterative procedure. (a) The set G, L, A, V, N and D and the smooth curve they establish. (b) The -OMe, -OEt and -OBzl esters of the remaining 14 residues with the same curve. (c) All derivatives of the remaining 14 with the Z-aa-COO⁻ points shown as •.

Table 3. pH 3.0 Hydrophobicity Values

		COO^-	OMe	OEt	OBzl	Average
A.						
G	Gly					0
L	Leu					1.80
A	Ala		Used to Establish			0.42
V	Val		Curve			1.34
N	Asn					-1.03
H	His					-2.28
B.						
C	Cys	--	0.84	--	--	0.84
D	Asp	-0.41	-0.44	-0.55	-0.62	-0.51
E	Glu	-0.23	-0.31	-0.40	-0.54	-0.37
F	Phe	1.77	1.75	1.75	1.67	1.74
I	Ile	1.72	1.81	1.90	--	1.81
K	Lys	-1.80	-2.34	-1.94	--	-2.03
M	Met	1.25	1.19	1.10	--	1.18
P	Pro	0.78	0.90	0.86	0.88	0.86
Q	Gln	-0.90	-0.94	-0.99	-1.00	-0.96
R	Arg	-1.41	-1.71	-1.57	--	-1.56
S	Ser	-0.69	-0.64	-0.66	-0.57	-0.64
T	Thr	-0.23	-0.23	-0.30	-0.27	-0.26
W	Trp	1.67	1.56	1.37	1.22	1.46
Y	Tyr	0.77	0.62	0.44	0.21	0.51

Table 4. pH 7.5 Hydrophobicity Values

		COO^-	OMe	OEt	OBzl	Average
A.						
G	Gly					0
L	Leu					1.80
A	Ala		Used to Establish			0.35
V	Val		Curve			1.32
N	Asn					-0.99
D	Asp					-2.15
B.						
C	Cys	--	0.76	--	--	0.76
E	Glu	-1.73	-1.93	-1.87	-2.06	-1.95
F	Phe	2.17	1.70	1.72	1.65	1.69
H	His	-0.29	-0.61	-0.69	--	-0.65
I	Ile	1.84	1.75	1.90	--	1.83
K	Lys	-0.51	-1.56	-1.51	--	-1.54
M	Met	1.54	1.11	1.08	--	1.10
P	Pro	0.31	0.81	0.85	0.85	0.84
Q	Gln	-0.55	-0.87	-0.91	-1.01	-0.93
R	Arg	-0.13	-1.62	-1.37	--	-1.50
S	Ser	-0.70	-0.61	-0.59	-0.70	-0.63
T	Thr	-0.02	-0.22	-0.29	-0.29	-0.27
W	Trp	2.19	1.51	1.37	1.17	1.35
Y	Tyr	1.13	0.55	0.42	0.20	0.39

residues lysine and arginine and the aromatic amino acids tryptophan, tyrosine and phenylalanine to be the most affected by the proximity of a negatively charged carboxyl; in all cases the residues appear more hydrophobic than expected.

A comparison of the experimental results with normalized hydrophobicity/hydropathy scales of a number of other groups is shown diagrammatically in Figure 4. Rank correlation values for these scales are shown in Table 5.

DISCUSSION

Two assumptions, first, that hydrophobicity of the components of the amino acid derivatives could be averaged and, second, that the curve relating hydrophobicity and retention time was smooth, were used to convert HPLC retention times of a variety of amino acid derivatives into hydrophobicity scales for amino acid residues at different pHs.

The resulting nonlinear curves (Figures 2a and 3a), which were derived independently, are virtually superimposable over their common ranges. We find very similar hydrophobicity values at the two pHs with the exception of the charged amino acids (compare average values in Tables 3 and 4). This is in general agreement with the findings of Guo et al. at pH 2 and 7 (3) and in disagreement with the results of Meek (10) which were determined from the HPLC retention times of a number of test peptides at pH 2.1 and 7.4.

The retention times of derivatives of four nonpolar amino acids (Gly, Leu, Ala, Val) plus two polar amino acids were used to establish the curves. The four nonpolar amino acids were chosen because (a) there is reasonable consensus on their hydrophobicity in various published scales, (b) they would be least likely to show variations due to ionic effects and (c) in the case of Gly and Leu, they fix the range of the scale. The two remaining polar amino acids in each set were chosen, somewhat arbitrarily, to enable full coverage of the range of retention times obtained.

It would be desirable to obtain the fitted curve and the hydrophobicity values by applying the iterative procedure to the derivatives of all 20 amino acids, but this computation is not

Table 5. Rank Correlation Matrix for Various Hydrophobicity Scales

	pH3	pH7.5	F&P*	H&W	K&D	K I	K II	K III	PGH	M7.4	M2.1
pH3	1										
pH7.5	.913	1									
F&P*	.886	.956	1								
H&W	.795	.896	.948	1							
K&D	.830	.863	.839	.749	1						
K I	.760	.770	.763	.780	.511	1					
K II	.836	.938	.944	.977	.749	.820	1				
K III	.816	.883	.917	.853	.941	.615	.831	1			
PGH	.789	.909	.880	.913	.691	.888	.935	.793	1		
M7.4	.705	.801	.705	.728	.513	.712	.805	.507	.774	1	
M2.1	.792	.877	.888	.915	.646	.780	.914	.694	.857	.840	1

*Based on 19 amino acids (proline value absent)

manageable. The procedure with an establishment set of six amino acids involves a nonlinear minimization using seven variables; if the same technique was applied to all 20 amino acids the minimization would involve 21 variables and computational difficulties would be expected.

The validity of the modeling assumptions and of using a set of six to establish the curves for all 20 amino acids is demonstrated in Figures 2b, 3b and 3c where points calculated using the average values determined for the remaining 14 amino acids residues have been superimposed on the derived curves. At pH 3, these points for all four C-terminal groups fall close to the established curve, while at pH 7.5 this is also true, with the exception of the free carboxyl group set (Figure 3c). This deviation is not unexpected in the case of arginine and lysine due to interaction between the negatively charged carboxyl and the positively charged side chains. More interesting is the deviation observed with the aromatic amino acids where Trp, Tyr and Phe all behave more hydrophobically than expected. In the case of Trp and Tyr, altered hydrophobicity is also observed with the methyl, ethyl and benzyl esters (Tables 3(B) and 4(B)) where the hydrophobicity value apparently decreases as the hydrophobicity of the ester group increases. A similar trend may be occurring at a lower level with Phe although it is not statistically significant. This behavior may explain the wide range in the reported hydrophobicity of Trp and Tyr (Figure 4) in published scales which have been determined by a variety of methods and under varying conditions. In the case of these two residues it is probable that our assumption of compound averaging is not valid.

Our scale shows the greatest rank correlation (Figure 4 and Table 5) with the hydrophobicity scale of Fauchere and Pliska (in Ref. 16) determined by the water/octanol partitioning of N-acetyl amino acid amides. The next highest correlation is with the consensus scale II of Kidera et al. (7) based on hydrophobicity properties of free amino acid with the charge contribution included. Poorer correlations are found with the structural-based scales of Kyte and Doolittle (8) and the third (KIII) consensus scale of Kidera et al. (7).

Our scales also vary significantly from those of Parker, Guo and Hodges (14), and Meek (10) although both used reversed-phase HPLC to establish their scales. These differences could be ascribed to the fact that both groups used longer peptides in their determinations; Meek used the retention times of a number of naturally occurring peptides ranging in size from 5–31 residues, while Parker et al. (14) used synthetic octamers containing doublets of the amino acid residue being

investigated. Both studies could be expected to be influenced by the sequence specific variations demonstrated by Houghton and Ostresh (6) although the Parker et al. (14) study [based on Guo et al. (3)] tried to minimize the effect by only varying two residues in the octapeptide. With peptides of this length it is unclear if folding effects encroach upon the natural profile of the tested side chain. When normalized, the scale of Parker et al. (14) is significantly compressed compared with other scales suggesting that the overall nature of the octapeptide significantly affected the mobility of the variant peptides.

The suitability of the derived hydrophobicity values to structural and antigenic site prediction and for other applications remains to be determined. However, we consider that the physical basis of the scale together with the multiple estimates of the hydrophobicity of the individual residues makes it more suitable than many of the scales currently in use. Our study should help to resolve the obvious controversy (Figure 4) surrounding the relative hydrophobicity of amino acid residues.

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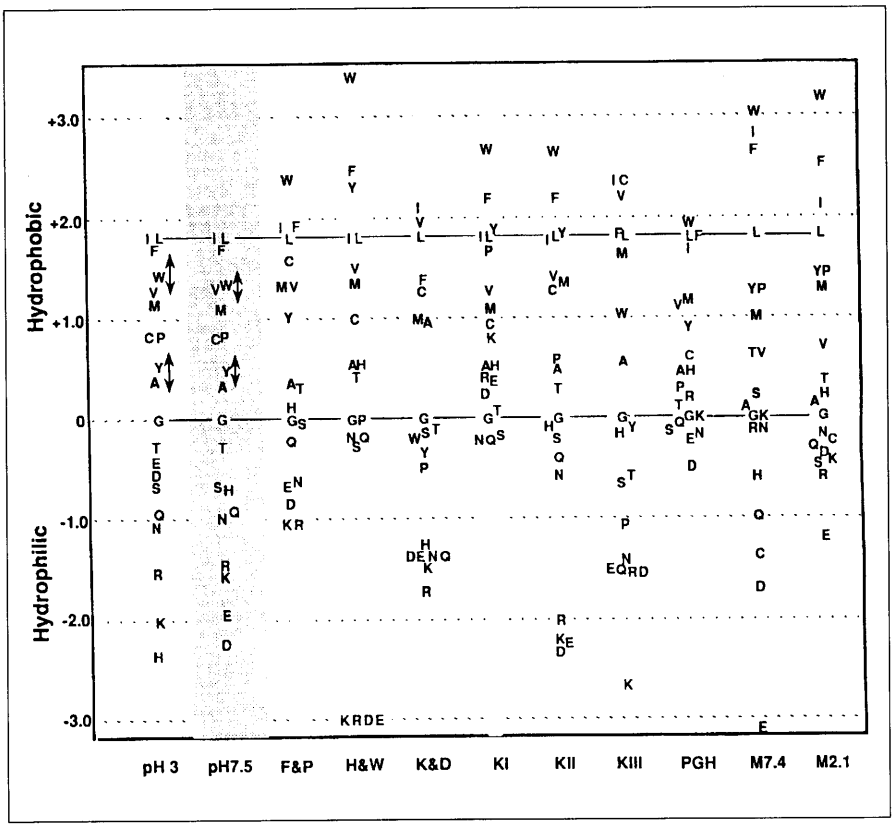

Figure 4. Comparison of hydrophobicity/hydropathy scales. pH 3 & 7.5 this paper; F&P Fauchere and Pliska 1983 (16); H&W Hopp and Woods 1981 (5); K&D Kyte and Doolittle 1982 (8); KI, KII & KIII Kidera et al. 1985 (7); PGH Parker, Guo and Hodges 1986: pH 7 values (14); M7.4 & 2.1 Meek 1980 (10).