Measuring the Hydrophobicity of Peptides by RPLC

Chih-I Liu, Ying-Chih Chan, Ruoh-Chyu Ruaan

Abstract— **In this study, we have designed a series of 5-residue peptides for evaluating the hydrophobicity of amino acids. The 5-residue peptides owned the sequence of GW-X-WG, where X is substituted by 10 different amino acids, G, A, V, I, L, W, D, E, K, or H. The hydrophobicity of amino acid X can be evaluated by the capacity factors of the 5-residue peptides. The hydrophobicity of four 9-residue peptides were estimated by summating the hydrophobicity of each amino acid residue. Another two 11-residue peptides were synthesized to examine the effect of secondary structure on the peptide hydrophobicity.**

The two 9-residue peptides, owning the same composition but different sequence, were shown to have the similar conformation and the same retention behavior. On the contrary, the two 11-residue peptides, having the same composition but differing in sequence, were shown to have the different conformation and the different retention behavior. By analyzing the thermodynamic parameters, the different retention behaviors of the two 11-residue peptides were caused by the differences in entropy. The different adsorption entropy may be related to their different conformations in solution.

Index Terms—**Hydrophobicity, Reversed phase chromatography, Short chain peptides, Thermodynamic parameters**

I. Introduction

Hydrophobic interaction played an important role in many biological systems and biochemical related applications. For example, hydrophobic interaction was usually a determinant factor in the binding between cell surface receptor and its respective ligand [1]. It was usually a dominant interaction between the antigen-antibody binding [2], [3]. And the interaction between peptides and the cell membrane was also related to hydrophobic interaction [4]. Therefore, the hydrophobic contribution of amino acid had long been evaluated through various means and attempts to establish a hydrophobic scale within a given class of biological substances.

The use of reversed phase chromatography to measure the hydrophobicity has several advantages: it needs short analysis time; it does not need ultra-pure samples; it has high resolution and repeatability. In general, the hydrophobicity of a short chain peptide could be estimated from the individual amino acid's hydrophobicity obtained under identical conditions. However, many studies have reported that the hydrophobicity for peptides change with many factors, such as, the amino acids composition [5] sequence of peptides [6], the length of peptide chain [7], and the conformation of peptides [8].

In the present study the retention behavior of three series peptides of different chain length was investigated. The hydrophobicity of amino acid in peptide (-X-) was determined by measuring the retention time of the peptide GWWG and 5-residue peptides of the sequence GW-X-WG, where X is substituted by 10 amino acids (G, A, V, I, L, W, D, E, K, H) on a C18 column at different acetontrile volume fraction from 0.23 to 0.27 and at various temperatures ranging from 283 to 323K. The four 9-residue peptides were designed to examine the possibility of hydrophobicity estimation by summating the hydrophobicity of each amino acid residue. The two 11-residue peptides were designed to evaluate the effect of peptide conformation. The secondary structures of peptides were determined by circular dichroism (CD). Thermodynamic parameters were calculated to further evaluate the effects of peptide composition, sequence, length, and conformation on the retention behavior.

II. Experimental

A. Materials

HPLC-grade acetonitrile(ACN) was obtained from J. T. Baker (New Jersey, American). Trifluoroacetic acid (TFA) was purchased from Aldrich (Seelze, Germany). Sodium Nitrate was obtained from Shiyaku (Tokyo, Japan). The peptides (amino acid sequences were listed in Table 1) were synthesized from Digital GENE Biosciences (Taipei, Taiwan).

B. Chromatographic operation

Chromatographic operation was carried out through a HPLC system consisting of a Lab Alliance series IV pump (PA, U.S.A), a 150mm × 4.6 mm I. D., Ace 5 C18-300 column (Advanced Chromatography Technologies, Aberdeen, Scotland), a Lab Alliance model 520 UV detector (PA, U.S.A.), and an auto-sampler (Model 816, Spark, Emmen, Holland). The column temperature was controlled in a thermostated water-jacketed cylinder, coupled to a Firstek B402-D (Shinjuang, Taiwan) circulation water bath.

Each of the peptides was dissolved in the mobile phase respectively at a concentration of 0.1mg/mL. The injection volume was controlled at 20μL. The capacity factors were measured for all peptides at various temperatures ranging

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from 283 to 323K and a flow rate of 1mL/min by isocratic elution of 0.1% TFA in acetonitrile-water (23:77, 24:76, 25:75, 26:74, 27:73). Peak profiles were monitored at UV 215nm.

Table 1 Molecule weight, sequence of all the peptides for experiment use

Sequence	MW	Abbreviation
Gly-Trp-Trp-Gly	504.552	GWWG
Gly-Trp-Ala-Trp-Gly	575.62	GWAWG
Gly-Trp-Val-Trp-Gly	603.68	GWVWG
Gly-Trp-Ile-Trp-Gly	617.71	GWIWG
Gly-Trp-Leu-Trp-Gly	617.71	GWLWG
Gly-Trp-Trp-Trp-Gly	699.776	GWWWG
Gly-Trp-Gly-Trp-Gly	561.604	GWGWG
Gly-Trp-Asp-Trp-Gly	619.64	GWDWG
Gly-Trp-Glu-Trp-Gly	633.67	GWEWG
Gly-Trp-Lys-Trp-Gly	632.73	GWKWG
Gly-Trp-His-Trp-Gly	641.693	GWHWG
Gly-Trp-Asp-Trp-Gly-Trp-Asp-Trp-Gly	1164.21	DWDWD
Gly-Trp-Asp-Trp-Asp-Trp-Gly-Trp-Gly	1164.21	DWDWG
Gly-Trp-Asp-Trp-Gly-Trp-His-Trp-Gly	1186.26	DWGWH
Gly-Trp-Asp-Trp-His-Trp-Gly-Trp-Gly	1186.26	DWHWG
Gly-Glu-Leu-Glu-Leu-Lys-Leu-Lys-Leu-Glu-Gly	1228.46	GELE
Gly-Glu-Leu-Lys-Leu-Glu-Leu-Lys-Leu-Glu-Gly	1228.46	GELK

C. Circular dichroism (CD) measurements

Circular dichroism (CD) spectra were measured at different temperatures (varying from 283 to 323K) by a Jasco J-810 spectropolarimeter in a quartz cell of 1mm optical path length between 190-260 nm. The samples were dissolved in various mobile phase at a concentration of 0.1mg/mL.

III. Theoretical Calculations

The relation between capacity factor (k') measured by chromatography and the equilibrium constant (K_{eq}) can therefore be expressed as

$$k' = \frac{t_R - t_0}{t_0} = K_{eq}\phi \quad (1)$$

, where t_R is the retention time and t_0 is the retention time of non-retained molecules and ϕ is the ratio of the volume of the stationary phase to that of the mobile phase. The relation between k' and K_{eq} can be expressed as

$$\ln k' = \ln K_{eq} + \ln \phi \quad (2)$$

Since $\Delta G^0 = \Delta H^0 - T\Delta S^0 = -RT \ln K_{eq}$ (3)

We have $$\ln k' = -\frac{\Delta H^0}{RT} + \frac{\Delta S^0}{R} + \ln \phi \quad (4)$$

Moreover, the dependency of the capacity factor, k', of a peptide on the volume fraction, φ in RP-HPLC under isocratic conditions can be approximated over a limited range of φ values by the empirical relationship:

$$\ln k' = \ln k_0' - s\varphi \quad (5)$$

When enthalpy and entropy changes are temperature invariant, the van't Hoff plots are linear and the enthalpy and entropy of adsorption could be evaluation from (4). The ΔG^0 of adsorption could then be calculated from (3).

When enthalpy and entropy changes are temperature dependent but the change of heat capacity, ΔC_p^0, is invariant to temperature, the dependence of ΔH^0 and ΔS^0 on the experimental temperature will be expressed as.

$$\Delta H^o = \Delta C_p^0 (T - T_H) \quad (6)$$

$$\Delta S^o = \Delta C_p^0 (\ln \frac{T}{T_S}) \quad (7)$$

,where T_H and T_S are temperatures at which ΔH^0 and ΔS^0 are zero. Combining (4), (6), and (7) yields (8), that is, Logarithmic equation.

$$\ln k' = \frac{\Delta C_p^0}{R}(\frac{T_H}{T} - \ln \frac{T_S}{T} - 1) + \ln \phi \quad (8)$$

Equation (8) allows the evaluation of the three thermodynamic parameters from nonlinear Van't Hoff plot by using least-squares regression procedures. The changes of enthalpy and entropy can be evaluated from (6) and (7).

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IV. Results and Discussion

A. Capacity factors of peptides at various acetonitrile concentration

The capacity factors (*k'*) of the 5 residue peptides (GW-X-WG) were measured isocratically at different acetonitrile volume fraction from 0.23 to 0.27 and 283 to 323K. The natural logarithm of k's at 303K were plotted against volume fraction of organic in the mobile phase and were shown in Fig.1. The rank of hydrophobicity of the peptides estimated by lnk' was agreed with that by lnk'_0. This result meant that the order of capacity factors was not affected by solvent composition under experimental conditions. As can be seen from Fig.1, the logarithm of k' decreased linearly with the increase of acetonitrile concentration. The regression parameters were all higher than 0.99. Therefore, the lnk_0' and S of peptides could be calculated from the intercept and slope respectively. Table 2 presented the lnk_0' and S values of peptides GWWG and GW-X-WG. The lnk_0' and s values of each amino acid (-x-) were then determined by subtracting the lnk_0' and s values of the peptide GWWG respectively, which were showed in Table 3.

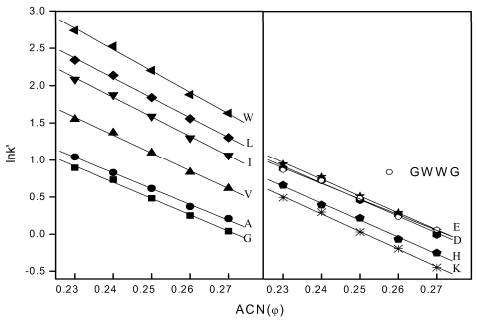

Fig.1 The effect of ACN content on the retention of peptides at temperature 303K

Table 2 The lnk_0' and S values for 5-residue peptides

Sequence	lnk'_0	s
GWWG	-8.7100	-4.5153
GWDWG	-9.4527	-4.8144
GWEWG	-9.5314	-4.8758
GWKWG	-10.4308	-5.0798
GWHWG	-9.9118	-4.9026
GWGWG	-9.1976	-4.6953
GWAWG	-8.8054	-4.5699
GWVWG	-9.5155	-5.1485
GWIWG	-9.9553	-5.5979
GWLWG	-9.8833	-5.6865
GWWWG	-10.4497	-6.1360

Table 3 The lnk_0' and S values of an individual amino acid residue in a peptide

Amino acid	lnk'_0	s
G	-0.4875	0.180
A	-0.0954	0.0545
V	-0.8055	0.6332
I	-1.2453	1.0826
L	-1.1732	1.1711
W	-1.7397	1.6207
D	-0.7427	0.2990
E	-0.8214	0.3605
H	-1.2018	0.3873
K	-1.7208	0.5645
H_3^+N-G~G-COOH	-5.2307	1.2739

B. Prediction of peptide hydrophobicity

Predicted peptide retention was determined by (9).

$$\ln k' = \sum \ln k_{0i}' + (\sum s_i)\varphi \qquad (9)$$

, where lnk' is the predicted capacity factor; lnk_{0i}' denotes the capacity factor in absence of the organic solvent for amino acid i, s_i is the slope of a plot of lnk' against φ, which is volume fraction of organic solvent in the mobile phase.

The retention factors of 9- residue peptides (DWGWD, DWDWG, DWGWH, and DWHWG) and 11-residue peptides (GELE and GELK) were predicted. The predicted values were compared with the experimental values and the results were showed in Fig. 2a, 2b and 2c, respectively.

As shown in Fig. 2a, the predictive values of peptides, DWGWD and DWDWG, were almost identical with experimental values. The peptides, DWGWH and DWHWG, also exhibited similar behaviours and the results were showed in Fig. 2b. However, Fig. 2c showed that there were deviations between experimental and predicted values for the two 11 residue peptides, GELE and GELK. Firstly, the predicted values were higher than the direct measurements. We considered that it might be due to the partial folding of the 11 residue peptides. Only part of the peptide contacted with the stationary phase, which resulted in the decrease of capacity factors. Secondly, the retention times of the two 11 residue peptides, which have the same composition and chain length but different sequence, were different. Krause et al. [9] suggested that the chromatographic retention of a peptide could be correlated to the amino acid composition and peptide chain length. In addition, the conformation of peptide could also be an important factor affecting their retention behaviors. It is possible that the two peptides have different stereo-conformation in solution, which leads to their difference in retention time.

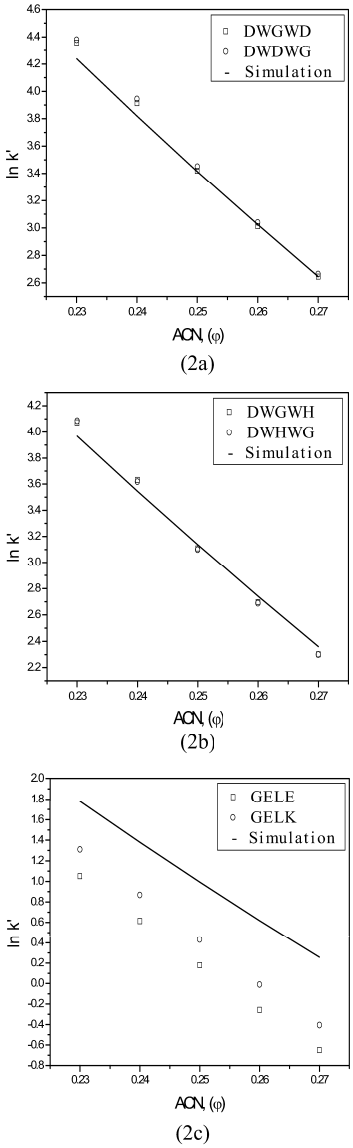

Fig. 2 Correlation among predicted and experimental values of 9 and 11-residue peptides (a) DWGWD and DWDWG; (b) DWGWH and DWHWG; (c) GELE and GELK. The capacity factors were measured under acetonitrile-water mixtures of volume fraction from 0.23 to 0.27 and temperature 303K conditions.

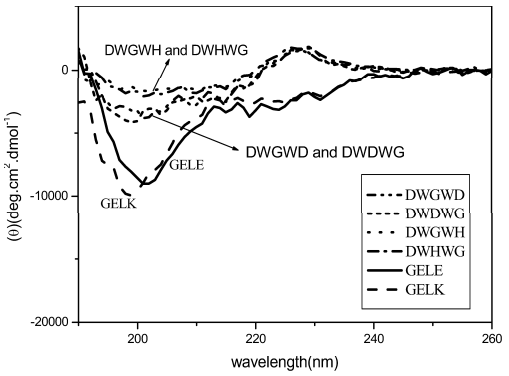

Fig. 3 CD spectra of 9 and 11-residue peptides, were measured under the acetonitrile-water mixtures of volume fraction 0.23 and temperature 303K conditions.

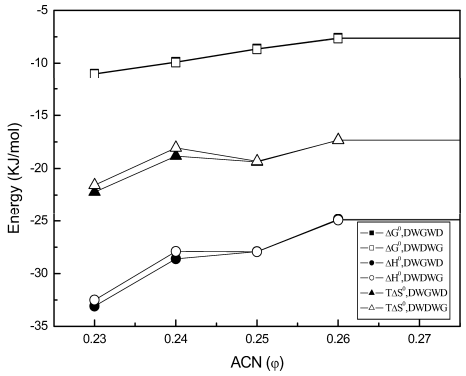

Fig. 4 Comparison of the $\triangle G^0$ (KJ/mol), $\triangle H^0$ (KJ/mol) and $T\triangle S^0$(KJ/mol) of 9-residue peptides, DWGWD and DWDWG onto C18 column under acetonitrile-water mixtures of volume fraction from 0.23 to 0.27 and temperature 303K conditions.

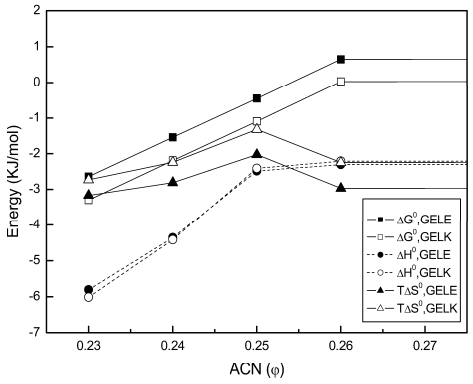

Fig. 5 Comparison of the $\triangle G^0$ (KJ/mol), $\triangle H^0$ (KJ/mol) and $T\triangle S^0$ (KJ/mol) of the 11-residue peptides, GELE and GELK onto C18 column under acetonitrile-water mixtures of volume fraction from 0.23 to 0.27 and temperature 303K conditions

C. Conformation effect on the retention behavior

Circular dichroism measurements revealed that the peptide, DWGWD, had the similar stereo-conformation as DWDWG in the aqueous solution (Fig. 3). A similar phenomenon could also be seen for peptides DWGWH and DWHWG (Fig.3). These results implied that the conformation of 9-residue peptides was not affected by peptide sequence. For the 11- residue peptides, it was found that there was a difference in conformation. Therefore, we supposed that the discrepancy in the retention behavior of 11- residue peptides might be resulted from the difference in conformations.

D. Thermodynamic evaluation of the 9-resudue and 11-residue peptides

As shown in Fig. 4, the ΔG^0, ΔH^0, and $T\Delta S^0$ values of peptide DWGWD increasing as organic solvent content was similar to the peptide DWDWG. In addition, the circular dichroism measurements revealed that the conformation of peptide DWGWD was similar to that of DWDWG (Fig.3). Therefore, we supposed that the peptides had similar conformation in solution. Subsequently, the ΔH^0, ΔS^0, and ΔG^0 values were similar. The phenomena were also observed in peptides DWHWD and DWGWH.

As can be seen in Fig. 5, the ΔH^0 value of GELE was similar to the peptide GELK. The difference in retention behavior (difference in ΔG^0) was primarily due to the difference in entropy change of adsorption (difference in $T\Delta S^0$). Possible explanations were that (i) the peptides of GELE and GELK adopted the same orientation upon binding to the C18 ligands, i.e., both of peptides also interacted with C18 ligands at the same amino acids. Therefore, the adsorption enthalpy (ΔH^0) was similar; and (ii) the 11-residue peptides (GELE and GELK) had different conformations in solution but similar conformation on solid phase, which resulted in different entropy and free energy changes.

V. Conclusion

In this study, we used three series of peptides of different chain length in order to estimate the hydrophobic contribution of each amino acid and the effect of conformation to the retention behaviors. We could also determine the contribution of each amino acid (-X-) by measuring the capacity factors of the 5-residue peptides in reversed-phase chromatography (RPC). For the case of 9-residue peptides, which have same composition but different sequence were shown to have similar conformation and the same retention behavior. However, the 11-residue peptides were shown to have the different conformation and retention behavior. By analyzing the thermodynamic parameters, the different retention behaviors of the two 11-residue peptides were caused by the differences in adsorption entropy. The different adsorption entropy may be related to their different conformations in solution.

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