Formulative Analyses of Interactions between Seasoning and Proteins in Comparison

— Comparison using Langmuir Adsorption Equation, Klotz Plot, and Scatchard Plot —

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Selecting acetic acid showing marked interactions with proteins as a low molecular matter out of seasonings, its binding behavior with textured soy protein, a high molecular matter, was investigated. Then the results were analyzed according to three methods, namely, a best fit method to Langmuir adsorption equation (LB), Klotz plot (KP), and Scatchard plot (SP), to obtain the binding number and the binding constant. Values obtained by the respective methods were studied in comparison.

Numbers of binding sites (n) and equilibrium binding constants (K) were different in the three methods. It means that referring to n, it was LB>SP>KP, while referring to K, the order was contrary to that. As far as Scatchard plot is concerned, the multiple correlation coefficient of substituted values for calculation, r/C_{free} and r, was the least among the three methods. However the fluctuation of calculated values to measured values of the bound weight finally obtained was the least among the three methods.

Keywords: Langmuir adsorption equation, Klotz plot, Scatchard plot, Textured soy protein, Acetic acid

Introduction

When interactions between the high and low molecular matters are discussed, it is known that binding is made according to the Langmuir adsorption equation [1], if the following conditions are sufficed:

1) There is only one binding site for the low molecular matter on the high molecular matter,
2) Interactions between binding sites are negligible,
3) Steric changes of high molecular matter upon binding are negligible, and
4) Interactions between the binding matter and free matter are negligible.

Thus the mode of binding can be expressed in general with the absorption isotherm as shown in Fig. 1.

$$r = n \cdot K \cdot C_{free} / 1 - K \cdot C_{free} \qquad [1]$$

wherein:

r: Number of the low molecular matter bound to the high molecular matter
n: Number of binding sites for the low molecular matter on the high molecular matter
K: Equilibrium constant
C_{free}: Equilibrium concentration.

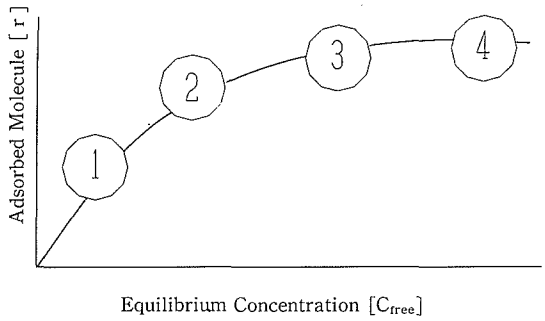

Fig. 1 Langmuir adsorption isotherm

Equation [1] can be replaced with following two formulae:

$$1/r=(1/n \cdot K)\times(1/C_{free})+(1/n) \quad [2]$$

or $$r/C_{free}=n \cdot K-K \cdot r \quad [3]$$

wherein [2] is noted as Klotz plot, and [3] as Scatchard plot. Klotz plot (Fig. 2) shows a relation between 1/r and $1/C_{free}$, while Scatchard plot (Fig. 3) does that between r/C_{free} and r. Since Klotz plot is related to two reciprocals, 1/r and $1/C_{free}$, it is also noted as the double reciprocal plot. In contrast Scatchard plot is known as the single reciprocal plot. In either plot there exists the linearity, $1/(n \cdot K)$ in gradient and 1/n in intercept on y axis in the former, while $(-K)$ in gradient and $n \cdot K$ in intercept on y axis, or n in point of intersection with x axis in the latter. Values of n and K obtained from these two plots are to be equal. However, as reciprocals of C_{free} are plotted in both, it is disadvantageous that measured valued in the range of the greater C_{free} are compressed on the graph. Further, as values obtained from the graph are reciprocals of n and K, a great error might be accompanied occasionally in Klotz plot.

Both equations are used frequently in the field of food science[1~3], biochemistry[4,5] and pharmaceutics[6] for analyses of interactions between high and low molecular matters, but much considerations are required upon the application of the equations[3,5~7].

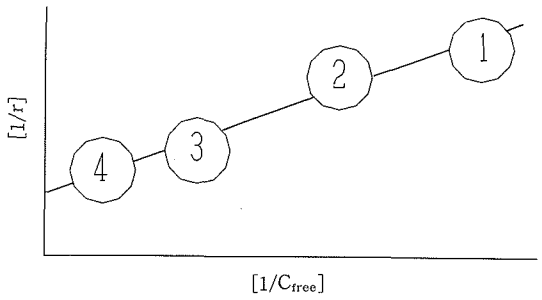

Fig. 2 Klotz plot

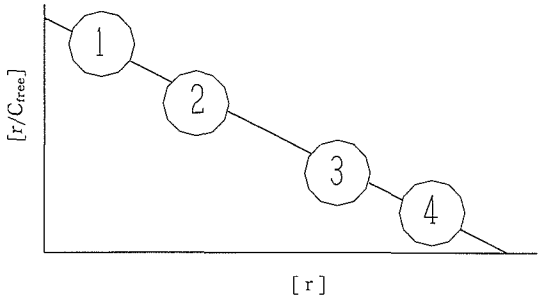

Fig. 3 Scatchard plot

Klotz plot and Scatchard plot are the ways of obtaining number of binding sites and equilibrium binding constants easily by making a linear line on the graph. Accordingly, it was considered as a very useful method when the computer was not available conveniently. At present it is feasible to obtain values of n and K directly by fitting best in the way of the least squares means upon substituting the measured values to Langmuir adsorption equation, the basis of Klotz plot and Scatchard plot, using the computer.

Thus, having acetic acid showing the marked interaction with proteins among seasonings as low molecular matter, its binding behavior with textured soy protein, the high molecular matter, was investigated. The purpose of this study is to obtain numbers of binding sites (n) and equilibrium binding constant (K) upon analyses of binding behaviors between them according to the three methods, i. e., the best-fitted method to Langmuir adsorp-

tion equation (LB), Klotz plot (KP), and Scatchard plot (SP), and the results were compared with each other. Meanwhile, unless otherwise specified, the amount of adsorption based on Langmuir adsorption equation was used as the amount of binding in this study.

Methods

(1) *Samples*

As samples of the high molecular matter, textured soy protein[8,9](TSP), considered to be low in solubility and less degenerative in the course of seasoning, was used. Basic characteristics of TSP are shown in Table 1.

Acetic acid (reagent special class) was used as the low molecular matter. Acetic acid is the major component of vinegar used as the basic seasoning, contained at 3—4%. In this study aqueous solutions of acetic acid prepared at concentrations of 13 steps in the range of 5.0×10^{-3}—1.0M (0.03—6%), were used to obtain the Langmuir's adsorption isotherm.

(2) *Immersing methods*

TSP was immersed in aqueous solutions of acetic acid in the same manner as reported previously[8]. It means that 1 g of TSP was added to 20 ml each of aqueous acetic acid at respective concentrations, and immersed for 72 hours at 20°C after deaeration for 2 minutes.

After immersion, using a centrifuge tube with a flat perforated plate 0.8 mm in pore size inside, it was centrifuged at 2000×g for 2 minutes. Using acetic acid solution dropped on the bottom of the tube as a sample solution after immersion of TSP, changes in the concentration before and after immersion of TSP in each aqueous acetic acid was studied. Measurements were repeated 3 times respectively.

Table 1 Basic properties of TSP

Properties	%
Water	13.5
Protein*	92.0
Lipid*	0.04
Carbohydrate*	7.1
Fiber*	0
Ash*	0.8

* Percent to dry weight

(3) *Concentration of acetic acid*

Concentrations of acetic acid were determined by means of HPLC (Shimadzu LC-4A; Column: Cica-Merck Hiber Lichrosorb RP-18, 4 mm I.D.×25 cm, Column temperature: 38°C; Mobile phase: Phosphate buffer (pH 2), Flow rate: 1 ml/min, Detector: SPD-2AS (210 nm)).

(4) *Calculation of bound acetic acid*

In consideration of moisture content in TSP (measured at 135°C, under the steady state), the amount of acetic acid bound per weight of TSP sample was calculated according to the following equation:

$$A=(X_0 \cdot V_0-X \cdot V)/m \qquad [4]$$

wherein:

A: Bound molecule (mol/g),
m: Solid weight of TSP (g),
V: V_0+moisture content of TSP before immersion (ml)
V_0: Volume of immersion fluid (ml),
X: Acetic acid concentration after immersion (mol/ml), and
X_0: Acetic acid concentration before immersion (mol/ml).

Results and Discussion

(1) *Binding of acetic acid to TSP*

Relation between the bound molecules of acetic acid and the equilibrium concentration upon immersion of TSP in aqueous acetic acid, i.e., the adsorption isotherm, is shown in Fig. 4. Binding behavior of acetic acid to TSP was typical showing an increase of bound molecules as equilibrium concentration increased. In the

experiment, concentration of aqueous acetic acid for immersion was fixed in the range of $5.0\times10^{-3}-1.0$M. At or above such concentration, TSP might be degerated during immersion, thus, it would be out of application conditions to Langmuir's adsorption equation that "steric changes of the high molecular matter upon binding is negligible". Accordingly, it is considered that as shown in Fig. 4, data within the range sufficiently arriving at the equilibrium have not been obtained.

Figures 5 and 6 show the results obtained as above expressed as Klotz plot and Scatchard plot, respectively. Both plots showed the linear relationship between y and x axes, being clearer in Klotz plot.

(2) *Calculated number of binding sites and binding constants in comparison*

Binding behavior of acetic acid to TSP was analyzed in three methods, namely, the best fit method to Langmuir adsorption equation (LB), Klotz plot (KP), and Scatchard plot (SP). Table 2 shows the number of binding sites (n) and the equilibrium binding constant (K).

Either the number of sites or the equilibrium binding constant obtained was not same in the three methods. The number of binding sites n showed LB>SP>KP. The utmost obtained by LB 1.10×10^{-3} was about two times larger than that of the least obtained by KP 0.57×10^{-3}. On the other hand the equilibrium binding constants were noted larger in the order of KP>SP>LB. In binding behaviors of hexanal to the soybean components, the number of binding sites n showed KP > LB under any conditions, the level of the binding constant K being different by conditions of the soybean components[3]. From these results, it can be considered that levels of n and K obtained by the three methods depend on their reaction systems.

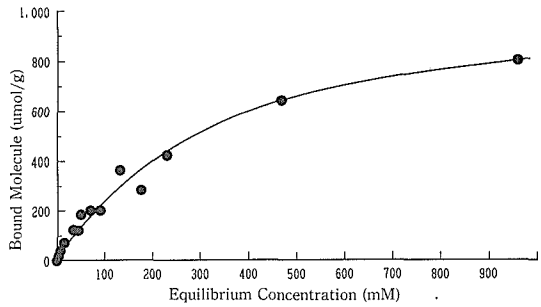

Fig. 4 Langmuir adsorption isotherm for TSP -acetic acid

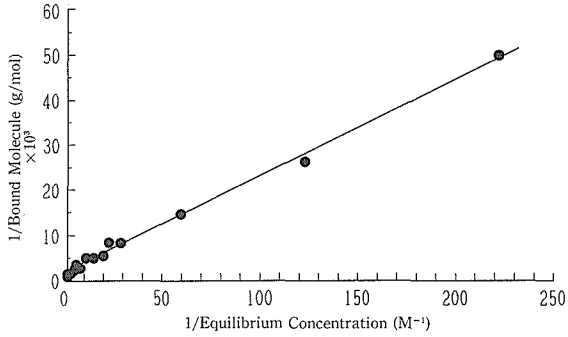

Fig. 5 Klotz plot for TSP-acetic acid

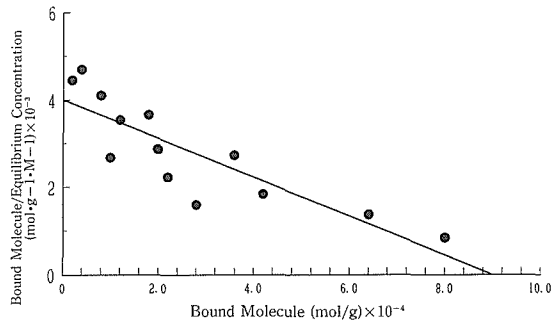

Fig. 6 Scatchard plot for TSP-acetic acid

(3) *Analytical methods compared in calculated values of the bound molecules*

Substituting the obtained values n and K to Eqs. [2] and [3], relation equations were finally obtained. Using these relation equations, we were able to get calculated bound molecule of acetic acid. The relation equations, multiple correlation coefficients between the measured and the calculated values, and their levels of significance are shown in Table 3.

Table 2 Calculated values of n and K using three methods

Method	LB	KP	SP
Number of binding sites [n] (mol)	1.10×10^{-3}	0.57×10^{-3}	0.9×10^{-3}
Equilibrium binding constant [K] (M^{-1})	2.83	8.26	4.45

Table 3 Comparative results in calculating n and K

Method	LB	KP	SP
Relative equation	$r=0.00311\times C_{free}/(1+2.83\times C_{free})$	$1/r=213.6\times(1/C_{free})+1764.3$	$r/C_{free}=-4.45\times(r)+0.004$
Multiple correlation coefficient between V_{mea} & V_{cal}(−)*	0.9786	0.995	0.7398
Significance (P<)	0.0005	0.0005	0.005

* V_{cal}, Calculated value ; V_{mea}, Measured value

Table 4 Calculated values of bound molecules and fluctuation

	measured (umol/g)	calculated					
		LB		KP		SP	
Data No.		V_{cal} (umol/g)	fluctuation (−)	V_{cal} (umol/g)	flactuation (−)	V_{cal} (umol/g)	fluctuation (−)
1	20	14	0.429	20	0	18	0.111
2	38	25	0.520	36	0.055	31	0.226
3	68	49	0.380	68	0	62	0.097
4	120	96	0.250	124	−0.032	118	0.017
5	118	122	−0.033	151	−0.219	147	−0.197
6	182	136	0.338	165	0.103	163	0.123
7	198	180	0.100	206	−0.04	211	−0.060
8	198	221	−0.104	240	−0.175	254	−0.220
9	360	299	0.204	296	0.216	332	0.084
10	280	365	−0.233	336	−0.167	394	−0.289
11	420	432	−0.027	371	0.132	452	−0.071
12	640	626	0.022	450	0.422	606	0.056
13	800	803	−0.004	503	0.590	726	0.101
mean			0.142		0.068		−0.001
S. D.			0.230		0.233		0.155

The multiple correlation coefficients obtained by the three methods are : $R^2=0.9786$ in LB, $R^2=0.7398$ in SP, and $R^2=0.9954$ in KP, respectively, thus difference was noted by methods of the analysis. Even the least value among them, that was in SP, was high in correlation showing the level of significance at P< 0.005.

In order to study fluctuations of these calculated values,

$$\text{Fluctuation}=(V_{mea}-V_{cal})/V_{cal} \quad [5]$$

was obtained, wherein :

V_{cal} : Calculated value,

and V_{mea} : Measured value.

The mean and the standard deviation of the fluctuation in every method was shown in Table 4. Figure 7 shows the values calculated with the equation [5].

Calculated valued obtained by either method showed an increasing trend of the bound molecules as equilibrium concentration increased. However, levels of fluctuation differed by the methods of the calculation. It means that the mean value of fluctuations closest to

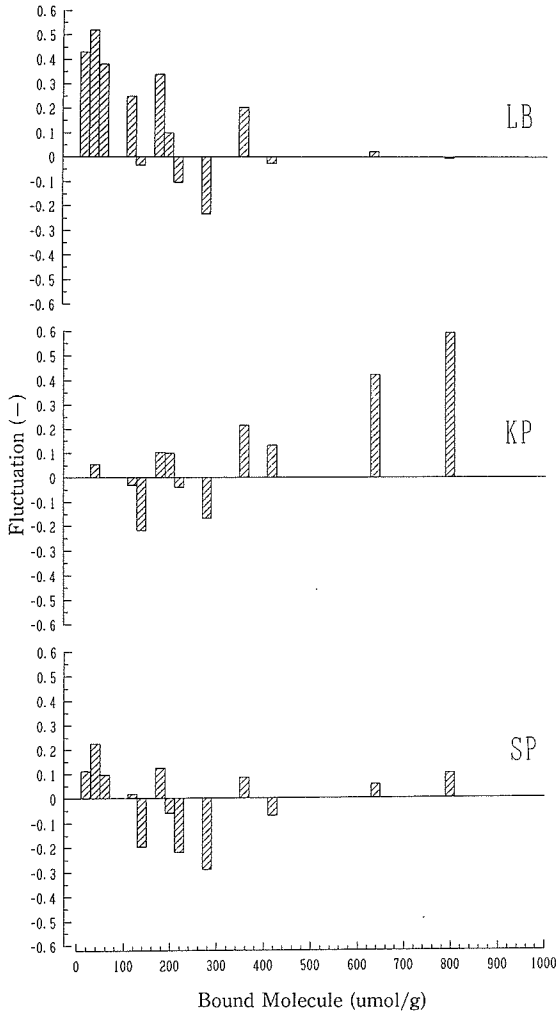

Fig. 7 Fluctuation in each method

nil was shown by SP, followed by LB and KP. Standard deviation was noted as SP<LB=KP. In Fig. 7, it was clarified that the fluctuations of SP were noted in the whole range from low to high bound molecules, while the greater deviations are noted in the range of the lower bound molecules in LB and in the range of higher bound molecules in KP.

Conclusion

Bound molecules of acetic acid to textured soy protein was measured, and interactions of the low molecular matter and the high molecular matter were analyzed according to three methods, namely, the best fit method to Langmuir adsorption equation (LB), Klotz plot (KP), and Scatchard plot (SP). Thus obtained numbers of binding sitesn, equilibrium binding constants K, and fluctuations of calculated values to measured values were studied in comparison, which revealed the following result:

(1) Numbers of binding sites n and equilibrium binding constants K obtained in the three methods differed each other. Referring to the numbers of binding sites n, it was known as LB>SP>KP, while as to the binding constants K, it was in the contrary order.

(2) Either of multiple correlation coefficients between the substituted values upon analyses, namely, r and C_{free} in LB, r and r/C_{free} in SP, and 1/r and $1/C_{free}$ in significantly high at $P<0.005$, in the order of KP>LB>SP.

(3) The standard deviation in fluctuations of calculated values to measured values of the bound molecules was the least in SP among the three methods, and two others were of nearly the same level.

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Nomenclature

A : Bound molecule (mol/g)
C_{free} : Equilibrium concentration
K : Equilibrium constant
m : Solid weight of TSP (g)
n : Number of binding sites for the low molecular matter on the high molecular matter
V : V_0 + moisture content of TSP before immersion (ml)
V_0 : Volume of immersion fluid (ml)
V_{cal} : Calculated value
V_{mea} : Measured valued
X : Acetic acid concentration after immersion (mol/ml)
X_0 : Acetic acid concentration before immersion (mol/ml)

調味料とタンパク質との相互作用における数式的解析方法の比較
―Langmuir吸着式、Klotzプロットおよび Scatchardプロットにおける比較―

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要　旨

調味料の中で、タンパク質との相互作用が顕著にみられる酢酸を低分子物質とし、高分子物質である組織化大豆タンパク質への酢酸の結合挙動を定量測定した。そして、両者間の結合挙動の解析を、Langmuir吸着式へのベストフィット法（LB法)、Klotzプロット法(KP法)、Scatchardプロット法（SP法）の3通りの方法により行い、結合数nおよび結合定数Kを求め、それぞれの方法で得られた値を比較検討した。

3通りの方法で得られた結合数nおよび結合定数Kはいずれも異なっていた。すなわち、結合数nはLB法＞SP法＞KP法であったが、結合定数Kの大小の順序は結合数とは逆であった。Scatchardプロット法の算出に当たっての代入値、r/C_{free}とrとの重相関係数は3方法の中で最も低かったが、Scatchardプロット法によって最終的に得られた結合量計算値の実測値に対するバラツキは、3方法の中で最も小さかった。