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Effect of pH on Binding of Mutagenic Heterocyclic Amines by the Natural Biopolymer Poly(γ-glutamic acid)

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Poly(γ-glutamic acid) (γ-PGA), a nontoxic and biodegradable macropolymer, was evaluated for its efficiency in binding three mutagenic heterocyclic amines (HAs), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-p-2), as affected by pH in a batch mode. The maximum HA sorption was attained for pH 3−7 and decreased sharply for pH less than 3. Binding isotherms obtained at pH 2.5 and 5.5 showed different isotherm shapes that belong to S and L types, respectively. The isotherm data at pH 2.5 were well described by a linear form of the Langmuir equation, while at pH 5.5 it showed two distinct curves, which were precisely fitted as multiple Langmuir curves. The deviation of linearity in Scatchard plot proved the multisite HA sorption. The Brunauer−Emmett−Teller equation also fitted better to isotherm data at pH 5.5, suggesting a multisite sorption caused by multimolecular HA layers on γ-PGA. High HA sorption levels of 1250, 667, and 1429 mg/g at pH 2.5 and 1429, 909, and 1667 mg/g at pH 5.5 were observed for MeIQ, 4,8-DiMeIQx, and Trp-p-2, respectively. Among the HAs studied, the sorption capacity correlated directly with hydrophobicity of HAs and inversely with the number of methyl groups in HA molecules. The plausible binding mechanism of HAs on γ-PGA may include a combination of hydrophobic, hydrogen-bonding, ionic, and dipole−dipole interactions.

KEYWORDS: Adsorption; poly(γ-glutamic acid); heterocyclic amines; isotherms; Langmuir isotherm; Scatchard plot; multimolecular adsorption

INTRODUCTION

Heterocyclic amines (HAs), a class of mutagenic/carcinogenic compounds, were produced during pyrolysis of creatine or creatinine, amino acids, and sugars in cooked fish and meat products (*1*−*4*). The optimal conditions for HA formation are longer cooking time (*2*, *5*), internal temperatures between 150 and 200 °C (*2*, *6*), and greater external charring (*7*). It was postulated that a nitrenium ion (an electrophilic derivative) formed from amines after a series of reactions binds covalently with DNA to cause damage, and cytochrome P450 may play an important role in this conversion (*8*). Quantitative structure−activity relationships (QSAR) for 80 aromatic and heterocyclic amines affecting mutagenic potency have been thoroughly investigated (*8*). Common HAs in human diets include 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo-[4,5-*f*]quinoxaline (MeIQx), 2-amino-3,4,8-trimethylimidazo[4,5-*f*]quinoxaline (4,8-DiMeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) (*4*, *9*). Several epidemiological reports have shown a positive correlation between HA intake and occurrence of cancer in human (*7*−*10*).

Different varieties of dietary fiber have been investigated for adsorption of HAs, as dietary fiber was reported to protect against development of colorectal cancer (*11*−*16*). Hydrophobicity of HAs is one of the crucial factors responsible for their binding on dietary fibers. Harris et al. (*14*) have demonstrated that HA binding on dietary fibers increased with increasing hydrophobicity of HAs. In another study, Ryden and Robertson (*15*) showed that the affinity of MeIQx on dietary fibers varied significantly over the physiological pH range, and bile salts in solution did not appreciably affect the HA binding. While a considerable number of reports studying the binding of HAs by dietary fibers are available, the effect on sorption of HAs by some other compounds that could serve as nutritional supplement or drug carrier remains unknown.

Poly(γ-glutamic acid) (γ-PGA) is a natural and biodegradable macropolymer produced by *Bacillus* species through a fermentation process, and the enzyme PGA synthetase plays a key role in its biosynthesis (*17*). It is a polypeptide made up of numerous glutamic acid units linked by the connection of α-amino and γ-carboxyl groups of each glutamic acid (**Figure 1**). The

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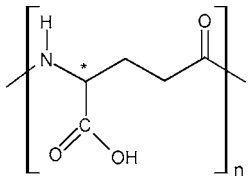

Figure 1. Structure of γ-PGA.

Table 1. Some Physicochemical Characteristics of γ-PGA

physical appearance	white, granulate, free-flow powders
purity	95% (HPLC)
molecular mass	990 kDa (HPLC)
pH	2∼2.5
clarity, OD_{400}	0.18
moisture content	5% (automatic infrared analyzer)
bulk density	0.32 g/mL
decolorizing power (methylene blue dye)	135 mg/g
heavy metals	
total (as Pb)	15 mg/L
Pb	5 mg/L
Cd	2 mg/L
As_2O_3	2 mg/L
E. coli	ND[a]/1 g
Salmonella	ND/1 g
particle size	100% through 100 mesh

[a] Not detectable.

industrial production of γ-PGA has been successful as it can be produced extracellularly in high yield by culturing bacteria in a fermentor. Since it can be produced from sustainable resources in different ionic forms (Ca, Na, H forms) and in varying molecular masses (10 000 to 2 million Da), γ-PGA finds wide application in various industrial fields such as food, cosmetics, medicine, and wastewater treatment (*18*). However, only limited reports are available on its use as an adsorbent for toxic compounds, an antioxidant, or an encapsulation agent (*19*, *20*). Recently the ability of γ-PGA for removal of some basic dyes from an environmental viewpoint was evaluated and was proved to be efficient (*21*). But the feasibility of removing HAs by use of γ-PGA remains unknown. The objectives of the present study were to determine the sorption efficacy of HAs by γ-PGA, with equilibrium study carried out by a batch mode as a function of pH, since the physiological pH varies widely between 2.5 and 7.5 in the gastrointestinal tract.

MATERIALS AND METHODS

Materials. The γ-PGA (H-form), produced from L-glutamic acid with *Bacillus subtilis* var. natto, was provided by Vedan Enterprise Corp. (Taichung, Taiwan). The physicochemical characteristics of γ-PGA as provided by the manufacturer are summarized in **Table 1**. The HA standards MeIQ, 4,8-DiMeIQx, and Trp-p-2 and the internal standard (IS) 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-p-1) were purchased from Aldrich Co. (Steinheim, Germany). The structures of HAs used as adsorbates are shown in **Figure 2**. A TSK-gel ODS C18 column (250 × 4.6 mm i.d., particle size 5 μm) bought from Tosoh Co. (Tokyo, Japan), was used for separation of HAs by high-performance liquid chromatography (HPLC). The HPLC-grade acetonitrile and methanol were from Merck Co. (Darmstadt, Germany). Ammonium acetate was obtained from Merck Co. (Darmstadt, Germany), hydrochloric acid (HCl) was from Nacalai Tesque Inc. (Kyoto, Japan), and sodium hydroxide (NaOH) was from, Riedel-de Haën Co. (Seelze, Germany). The mobile-phase solvents acetonitrile and ammonium acetate solution were degassed by sonication and filtered through a 0.2-μm membrane filter prior to use. Deionized water was obtained from a Milli-Q water purification system (Millipore Co., Bedford, MA).

Binding Experiments. The HA binding experiments were performed with 1 mL HA solutions of desired concentration taken in 4-mL brown vials and agitated at 120 rpm with a known weight of γ-PGA for predetermined equilibration time (30 min) in a Firstek B601 reciprocating water bath shaker (Firstek Scientific Co., Tau-Yen, Taiwan) maintained at 37 °C. After equilibration, the samples were withdrawn from the shaker and filtered through a 0.2-μm membrane filter, and the filtrate was analyzed for residual HA concentration by HPLC.

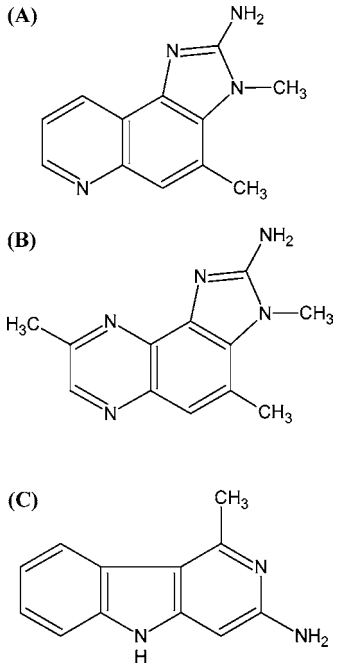

Figure 2. Structure of HAs used as adsorbates: (**A**) MeIQ, (**B**) 4,8-DiMeIQx, and (**C**) Trp-p-2.

Initially, 10 mg of each HA standard was dissolved in 10 mL of methanol. A stock solution of 5000 mg/L was prepared by taking 5 mL each in two 10 mL brown vials, evaporating to dryness, and reconstituting in 1 mL of methanol. The influence of pH on HA binding was studied by equilibrating 500 mg/L HA solutions (1 mL) adjusted to different pH values (1, 2, 3, 4, 5, 6, and 7) with 5 mg of γ-PGA. For isotherm study, all HA solutions (1 mL each) of nine different concentrations, 100, 200, 300, 400, 600, 800, 1000, 1500, and 2000 mg/L, prepared at pH 2.5 and 5.5 were agitated separately with 1 mg of γ-PGA. The pH of each HA solution was adjusted with dilute HCl or NaOH. The γ-PGA was not soluble in the working pH range (1−7); however, a notable solubility occurred at pH above 8.5. Each batch experiment was carried out in duplicate and mean values were used for calculation. Standard deviation and analytical errors were calculated and the maximum deviation was ±5%.

HPLC Analysis of HAs. The HA concentrations before and after equilibration with γ-PGA were analyzed on a HPLC system consisting of an Agilent G1312A binary pump (Agilent 1100 series, Agilent Technology, Palo Alto, CA), a Rheodyne model 7161 injector (Rheodyne, Rohnert Park, CA), an Agilent G1314A model UV/VIS detector, and a Chemstation computer software for LC (Agilent Technologies). A HPLC method developed by Chen and Yang (*22*) was followed. Briefly, a binary solvent system of acetonitrile as solvent A and 0.05 M ammonium acetate solution (pH 3.6) as solvent B was programmed for gradient elution: 9% A and 91% B in the beginning, 15% A in 8 min, 27% A in 18 min, 55% A in 28 min, and 100% A in 30 min, with a flow rate of 1.0 mL/min and UV detection at 258 nm. **Figure 3** shows the HPLC chromatogram of three HA standards, MeIQ, 4,8-DiMeIQx, and Trp-p-2, along with the internal standard Glu-p-1.

Quantification and Uptake Calculation. The quantification of HA in each sample after HPLC analysis was carried out by an internal standard method. A fixed concentration of Glu-p-1 (IS) (20 mg/L) was added to various concentrations of MeIQ, 4,8-DiMeIQx, and Trp-p-2 (3, 6, 10, 20, 30, 40, 50, and 60 mg/L) separately, and the standard curve for each HA was prepared by plotting area ratio against concentration ratio. The regression analysis was performed in Microsoft Excel XP software, and a high correlation coefficient ($r^2 > 0.99$) was

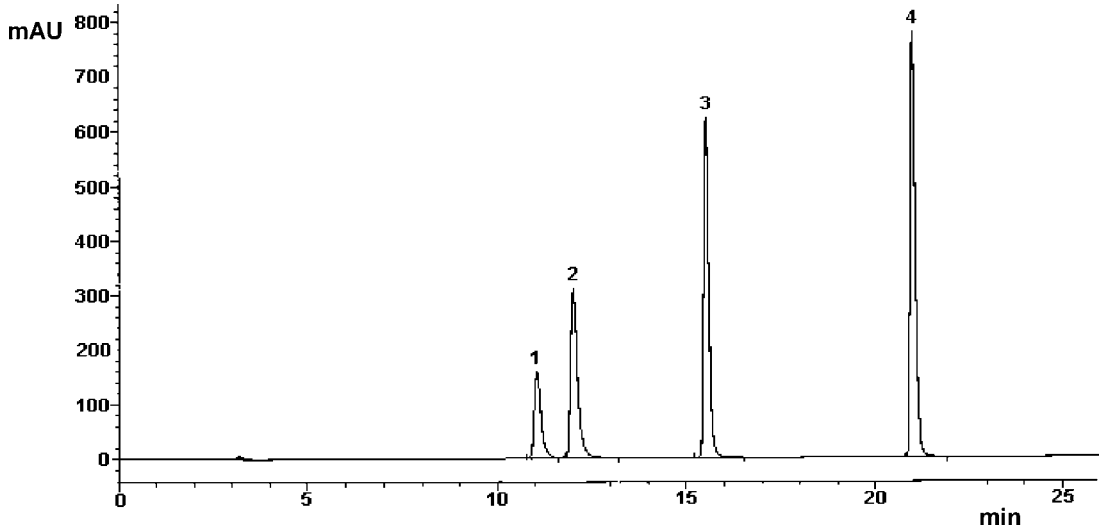

Figure 3. HPLC chromatogram of four heterocyclic amines. Peaks: (1) Glu-p-1 (internal standard), (2) MeIQ, (3) 4,8-DiMeIQx, and (4) Trp-p-2.

obtained for all the HA standard curves. The linear regression equations obtained were $y = 2.4356x + 0.3234$ for MeIQ, $y = 4.0739x + 0.2548$ for 4,8-DiMeIQx, and $y = 6.7446x + 0.3530$ for Trp-p-2. The HA concentrations in the unknown sample were determined by use ofeq 1 by substituting the slope and intercept of the respective straight-line equation:

$$C = \left(\frac{(A/A_i) - \text{intercept}}{\text{slope}}\right)C_i \quad (1)$$

where C = concentration of HA in unknown sample (milligrams/liter), C_i = concentration of internal standard (milligrams/liter), A = area of HA in unknown sample, and A_i = area of the internal standard.

The amount of HA adsorbed at equilibrium (q_e, milligrams/gram) was calculated from the mass balance equation (eq 2):

$$q_e = (C_0 - C_e)\frac{V}{m} \quad (2)$$

where C_0 and C_e (milligrams/liter) are the initial and final HA concentrations, respectively, V (liters) is the volume of HA solution, and m (grams) is the mass of γ-PGA.

RESULTS AND DISCUSSION

Influence of pH on Binding of HAs. The gastrointestinal environment contains a complex of many different minerals and secretions, with pH varying from 2.5 in the stomach to 7.5 in the small intestine. Hence, the study on influence of pH on HA binding by γ-PGA is important to determine the maximum sorption at each pH and the underlying uptake mechanism. **Figure 4** illustrates the binding trend of HAs on γ-PGA as affected by pH. A small amount of HA was removed at pH 1, followed by a steep rise for maximum sorption at pH 3. The amount of HA binding increased by 344.47, 355.97, and 248.05 mg/g for MeIQ, 4,8-DiMeIQx, and Trp-p-2, respectively, when the pH was raised from 1 to 3 and remained consistent thereafter. This tendency leads one to anticipate an exchange mechanism predominantly involved in binding. However, the nature of the solute and the adsorbent material should also be considered before any conclusion is drawn on the nature of binding. At low pH there is a possibility for the primary amino group ($-NH_2$) in HA and the secondary amino group (>NH) in γ-PGA to get protonated, creating an electrostatic repulsion that may prevent HA molecules from interacting with γ-PGA (*23*). However, this repulsive force decreased along with increasing pH, allowing some plausible interactions to occur between them eventually.

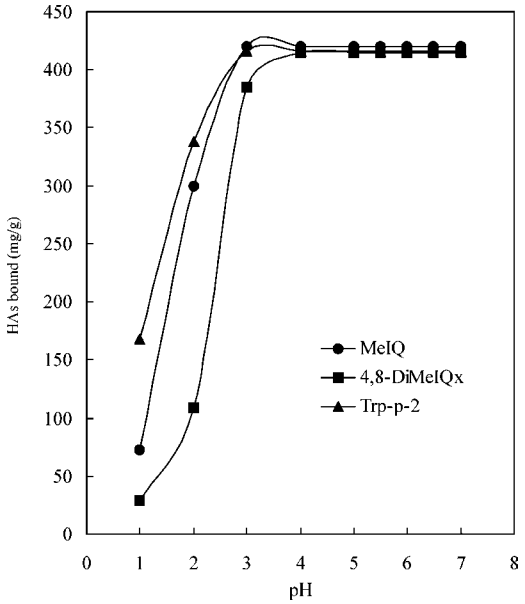

Figure 4. Influence of HA sorption by γ-PGA at different pH.

Heterocyclic amines may predominantly undergo hydrophobic interaction with γ-PGA because of its nonpolar nature (*24*, *25*). Several reports attributed the capability of HA binding on various dietary fibers to the hydrophobicity of HAs (*12*−*14*). In addition, interaction through hydrogen bonding may also have occurred, as it is likely that the organic solutes with amines and heterocyclic nitrogen are capable of forming hydrogen bonds (*26*). Since γ-PGA tends to ionize completely due to dissociation of carboxylic acid groups above pH 4 (pK_a of glutamic acid is 4.29), ionic interactions may also play a role in HA binding (*21*). Thus, sorption of HAs on γ-PGA might involve complex interactions, that is, hydrophobic, hydrogen−bonding, and ionic forces. Delval et al. (*27*) proposed a complex mechanism involving hydrogen bonding, hydrophobic interaction, complexation, and acid−base interactions for removal of phenol derivatives by cross-linked starch polymers.

It was observed that the affinity of HAs on γ-PGA was appreciable throughout the physiological pH range of gastrointestinal tract, typically being 2.5, 7.5, 5.5, and 6.8 in stomach, small intestine, cecum/ascending colon, and distal colon, respectively. As different mechanisms may occur for HA sorption, the isotherm curves obtained at two representative pHs

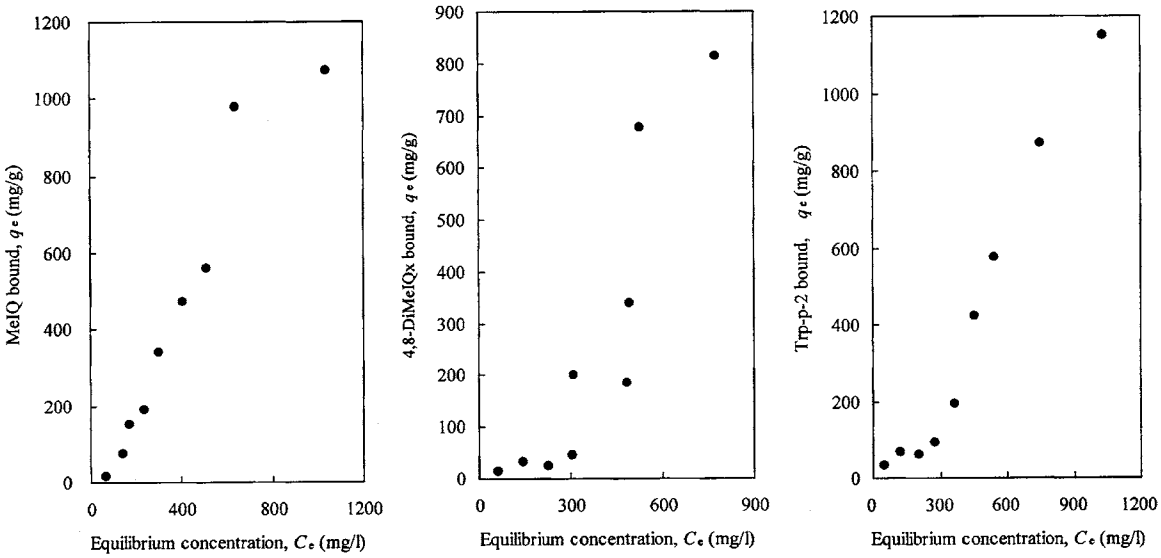

Figure 5. Isotherm data for HA sorption by γ-PGA at pH 2.5.

(2.5 and 5.5) should provide useful information on the nature of binding under physiological conditions.

Binding Isotherm Curves at pH 2.5. The isotherm curves for sorption of MeIQ, 4,8-DiMeIQx, and Trp-p-2 on γ-PGA at pH 2.5 are shown in **Figure 5**. The shape of isotherms closely resembles that of S-type, according to the classification of isotherms for adsorption in solution by Giles et al. (*28*). Three distinct portions showing characteristics of an S-type curve (*29*) were evident from **Figure 5**. Initially, a concave portion signifying less or no sorption was observed at low HA concentration, which may be caused by clustering or agglomeration of HA molecules that is preferred over their sorption on γ-PGA. Conversely, at high HA concentration, the interaction between HA and γ-PGA tends to become stronger and sorption becomes significant for the convex portion. Finally, the sorption slows down to reach a plateau. The repulsive force between primary amino group ($-NH_2$) group of HA and secondary amino group ($>$NH) of γ-PGA caused by protonation at low pH may also account for this effect. However, the repulsive behavior became insignificant at high HA concentration due to multiple physical interactions. The S-type adsorption, also called cooperative adsorption, may be associated with the physicochemical properties of the adsorbates. The interaction between HA molecules tends to be greater at low concentration, and several cooperative physical interactions are induced following the increase of HA concentration for sorption on γ-PGA, resulting in a S-type curve (*25*, *28*, *30*). Theoretically, S-shaped isotherms are obtained when lateral interaction between adsorbed molecules has a major impact on sorption thermodynamics (*31*). The S-type adsorption has been reported for removal of phenol and phenol derivatives by silica and alumina (*28*) and petroleum asphaltenes (*30*).

The fitting of adsorption isotherm equation to experimental data is important for data analysis. Several isotherm equations such as Freundlich, Langmuir, Redlich−Peterson, Sips, Flory−Huggins, Tempkin, and Toth models (*32*, *33*) were tested, of which only the Langmuir equation (eq 3) fitted well the experimental data at pH 2.5, while the others fitted poorly or resulted in a negative value of isotherm constants. Such negative isotherm constants indicated the inadequacy of these models to explain the sorption process (*34*). The noncompliance of equilibrium data with other isotherm models suggests the complex nature of adsorption, which may involve several interactions taking place simultaneously. The linear form of Langmuir model employed is given by

$$\frac{1}{q_e} = \frac{1}{q_m b C_e} + \frac{1}{q_m} \tag{3}$$

where q_m (milligrams/gram) and b (liters/milligram) are Langmuir constants denoting maximum adsorption capacity and energy of sorption, respectively. **Figure 6** shows the linear plots of Langmuir model, and the sorption parameters are reported in **Table 2**. The difference in q_m values between HAs may be due to variation in their hydrophobic character, which follows the order Trp-p-2 > MeIQ > 4,8-DiMeIQx (*13*). Ferguson and Harris (*13*) demonstrated that the adsorption of nine HAs on dietary fiber α-cellulose increased with increasing hydrophobicity. Also, the number of bulky methyl groups of HA may be responsible for the degree of their interaction with γ-PGA. The greater the number of methyl groups in HA, the greater the steric hindrance, which should prevent more HA molecules from interacting with γ-PGA. Accordingly, 4,8-DiMeIQx, containing three methyl groups, showed the lowest q_m value (666.67 mg/g), followed by MeIQ (1250.00 mg/g) and Trp-p-2 (1428.57 mg/g). Furthermore, a relatively large difference in q_m value between 4,8-DiMeIQx and the other two HAs should be due to the third methyl group in the first ring of the quinoxaline moiety, preventing more 4,8-DiMeIQx molecules from lateral binding on γ-PGA when compared to MeIQ and Trp-p-2 (**Figure 2**). The foregoing discussion implied the interaction between HAs and γ-PGA to be largely dependent on physical forces.

Binding Isotherm Curves at pH 5.5. Figure 7 illustrates the binding curves for HA sorption by γ-PGA at pH 5.5. The q_e versus C_e plots were quite different from those obtained at pH 2.5. Two distinct curve portions (designated as curve I and curve II) were characterized by a curve shift appearing between the same C_0 values (600 and 800 mg/L) for all three HAs. Curve I covers the initial five experimental points, whereas curve II encompasses the final four points. This phenomenon suggests the presence of more than one adsorption site with varied affinities and binding energies for HA molecules, that is, curve I should possess higher binding strength than curve II. The isotherm portion in curve I, which corresponded to the less or no sorption region (concave portion) in the S-type isotherm at pH 2.5, showed significant sorption at pH 5.5. This improved sorption at pH 5.5 may be due to absence of the repulsive force, which might have occurred in the corresponding isotherm

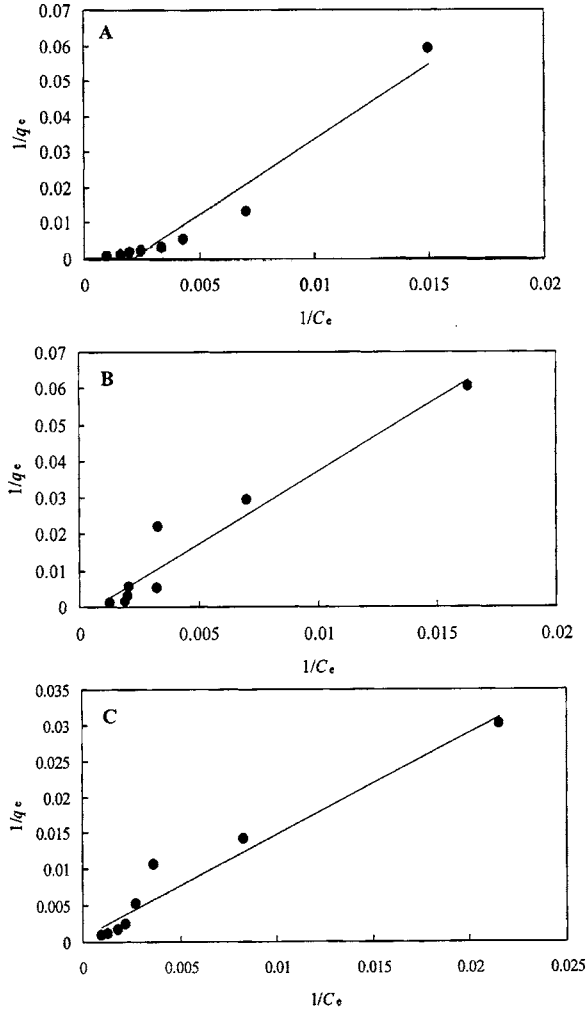

Figure 6. Linear Langmuir fits for HA sorption by γ-PGA at pH 2.5: (**A**) MeIQ, (**B**) 4,8-DiMeIQx, and (**C**) Trp-p-2.

Table 2. Langmuir Isotherm Parameters for HA Sorption by γ-PGA at pH 2.5 and 5.5

	Langmuir model parameters					
	pH 2.5			pH 5.5		
HA	q_m (mg/g)	b (L/mg)	r^2	q_m (mg/g)	b (L/mg)	r^2
MeIQ	1250.00	0.0020	0.948	1428.57	0.0173	0.859
4,8-DiMeIQx	666.67	0.0004	0.933	909.09	0.0156	0.924
Trp-p-2	1428.57	0.0005	0.952	1666.67	0.0319	0.980

portion at pH 2.5. The linear form of Langmuir model used for isotherms at pH 2.5 did not fit the experimental data at pH 5.5. Hence, the isotherms at pH 5.5 were assessed by using another linear form of the Langmuir model (*32*, *35*):

$$\frac{C_e}{q_e} = \frac{C_e}{q_m} + \frac{1}{bq_m} \quad (4)$$

The Langmuir parameters derived from the fit (plots not given) are shown in **Table 2**. Although the Langmuir model provided a better fit of the whole experimental data, a significant deviation of linearity still existed. Therefore, as both curves I and II resemble an L-type curve (*28*), the Langmuir equation (eq 4) was applied separately to both by assuming that the two monolayer curves were formed by saturation of binding sites with different energies. A high correlation ($r^2 > 0.98$) was

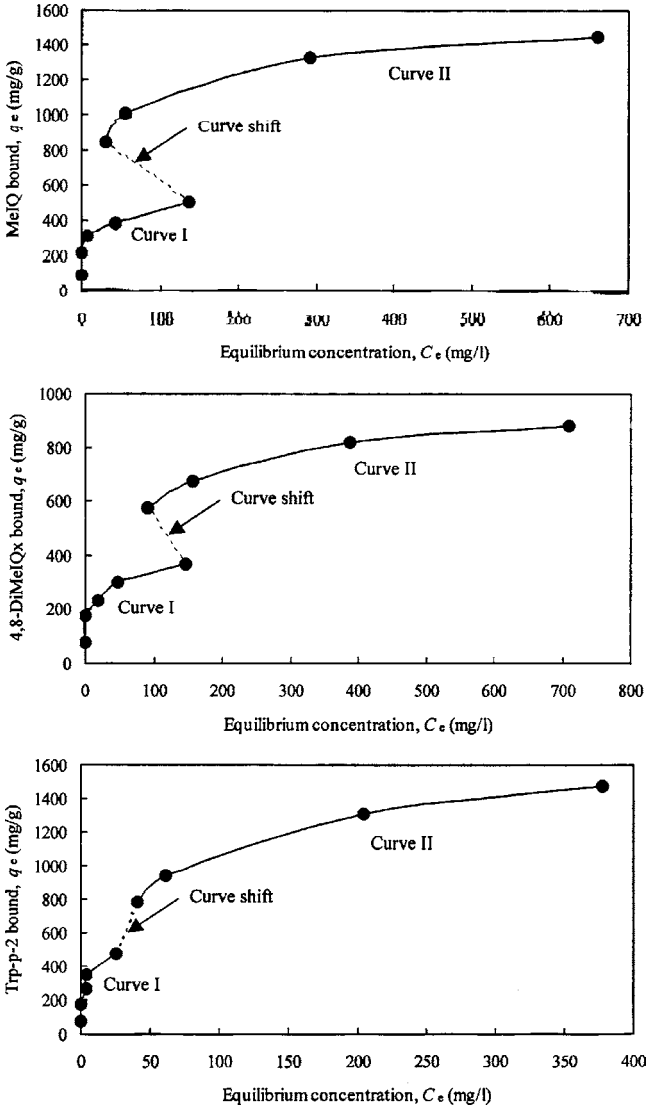

Figure 7. Isotherm data for HA sorption by γ-PGA at pH 5.5.

observed for curves I and II when fitted individually (plots not shown), revealing HA sorption on γ-PGA involved saturation of two sites of varying binding strength. The linear fitting of curve I yielded the isotherm parameters for initial monolayer saturation, while that of curve II gave the relevant parameters corresponding to the overall HA sorption on γ-PGA (**Table 3**).

The two-site HA sorption was confirmed by subjecting the data to Scatchard plot analysis, which is widely used to study the characteristics of adsorption isotherms (*36*, *37*). The Scatchard equation is represented as

$$\frac{q_e}{C_e} = q_m b - q_e b \quad (5)$$

The linearity of the Scatchard plot indicates the binding sites to be identical and independent, while a deviation from linearity signifies the presence of more than one type of binding site and a negative slope is related to the Langmuir type of interaction (*38*). A significant deviation from linearity (**Figure 8**) was observed in each HA sorption, demonstrating the presence of more than one type of binding site on γ-PGA. Nevertheless, when the Scatchard equation was applied to curves I and II separately, a good correlation ($r^2 > 0.91$) was shown

Table 3. Langmuir Isotherm and Scatchard Plot Parameters for HA Sorption by γ-PGA at pH 5.5 for Curve I and Curve II Portions

	curve I			curve II		
HA	q_m (mg/g)	b (L/mg)	r^2	q_m (mg/ g)	b (L/mg)	r^2
Langmuir model parameters						
MeIQ	500.00	0.2632	0.989	1428.57	0.0374	0.999
4,8-diMeIQx	370.37	0.2030	0.991	1000.00	0.0152	0.999
Trp-p-2	500.00	0.6897	0.982	1666.67	0.0211	0.999
Scatchard plot parameters						
MeIQ	499.16	0.1805	0.910	1465.09	0.0432	0.983
4,8-diMeIQx	397.79	0.0706	0.994	944.68	0.0169	0.991
Trp-p-2	454.30	1.5903	0.913	1619.74	0.0228	0.995

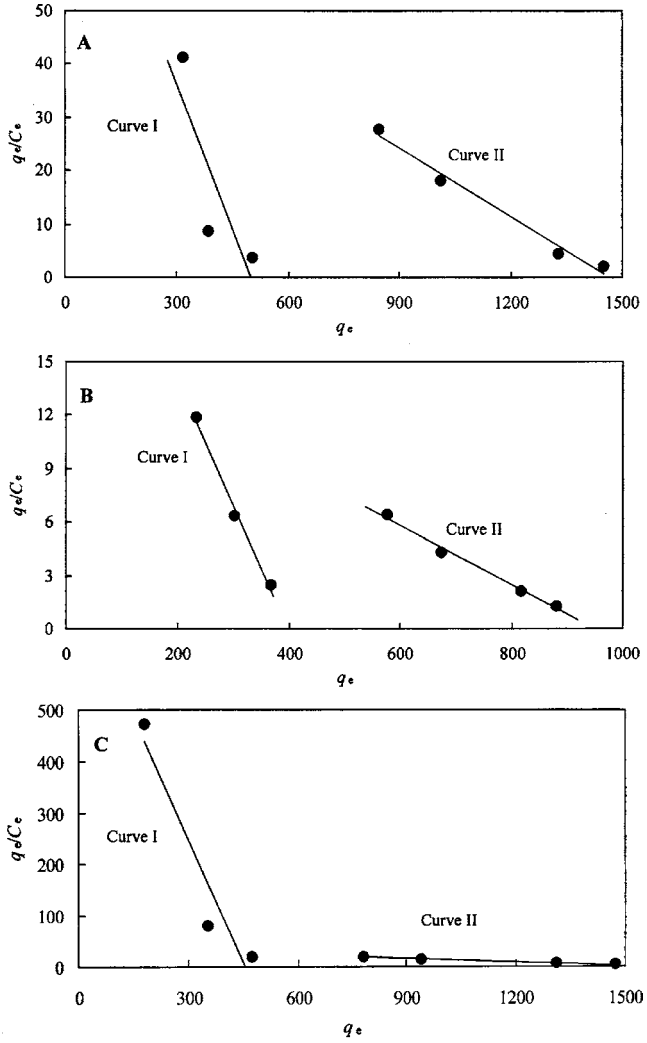

Figure 8. Scatchard plots for HA sorption by γ-PGA at pH 5.5: (**A**) MeIQ, (**B**) 4,8-DiMeIQx, and (**C**) Trp-p-2.

for all the curves, confirming the presence of at least two different types of binding. The Scatchard plot parameters for curves I and II are given in **Table 3**.

The multisite HA sorption involving different energy sites can also be explained on the basis of a multimolecular sorption by applying an isotherm equation developed by Brunauer et al. (*39*) (BET equation). The BET equation, which was derived with a generalization of a Langmuir's treatment of unimolecular layer, is given by

$$\frac{C_e}{(C_s - C_e)} = \frac{1}{aq_m} + \left(\frac{a-1}{aq_m}\right)\left(\frac{C_e}{C_s}\right) \quad (6)$$

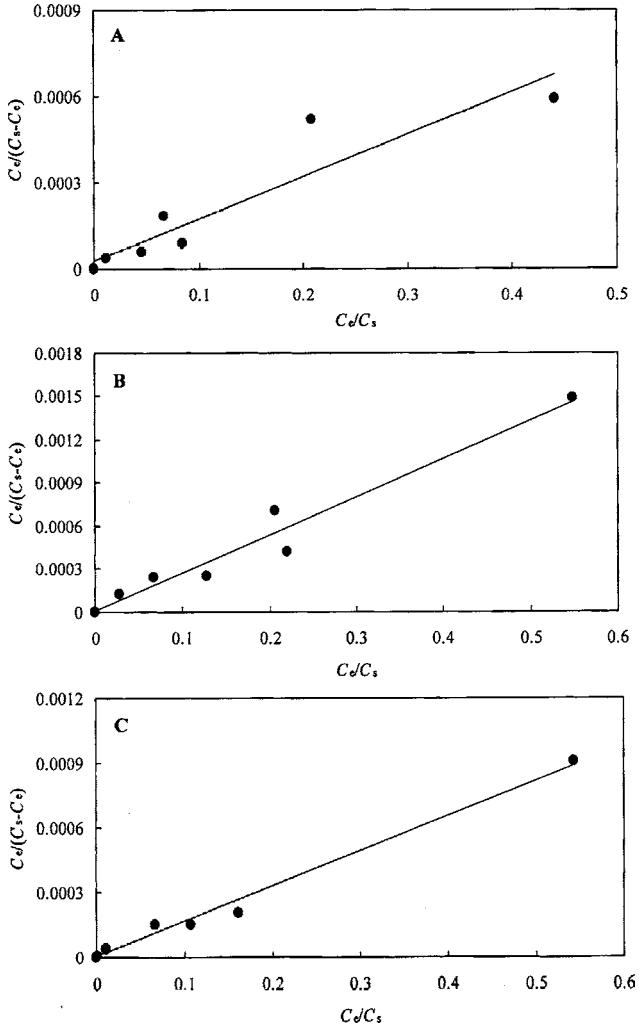

Figure 9. BET isotherm fits for HA sorption by γ-PGA at pH 5.5: (**A**) MeIQ, (**B**) 4,8-DiMeIQx, and (**C**) Trp-p-2.

Table 4. BET Isotherm Parameters for HA Sorption by γ-PGA at pH 5.5

	BET model parameters		
HA	q_m (mg/g)	a	r^2
MeIQ	653.60	51.00	0.867
4,8-DiMeIQx	370.37	451.00	0.961
Trp-p-2	623.44	401.00	0.989

where C_s (milligrams/liter) is the HA concentration at the saturation of all layers, q_m (milligrams/gram) is the amount of HA required to form a unimolecular layer, and a is a BET constant representing average heat of adsorption in the first layer. Linear plots of $C_e/(C_s - C_e)$ versus C_e/C_s (**Figure 9**) gave high correlation for 4,8-DiMeIQx and Trp-p-2 ($r^2 > 0.96$) but not for MeIQ ($r^2 > 0.87$), and the fitted parameters (q_m and a) are presented in **Table 4**. The maximum sorption capacity (q_m) value for the first layer (or unimolecular layer) is about ~2.5 times higher than the q_m value obtained by the Langmuir equation for saturation of all layers (**Tables 2** and **4**). This suggests that the multimolecular HA sorption may have occurred at least for two layers on γ-PGA. Though the basic BET theory (*39*) assumed similar forces causing condensation to be involved in multimolecular adsorption, the nature of binding forces was interpreted in a different way in the literature for multimolecular adsorption in solution. For instance, for multimolecular adsorption of methylene blue dye on waste dogrose seed carbon,

Table 5. Separation Factor (R_L) for HA Sorption by γ-PGA at pH 2.5 and 5.5

	R_L values					
	MeIQ		4,8-DiMeIQx		Trp-p-2	
C_0[a]	pH 2.5	pH 5.5	pH 2.5	pH 5.5	pH 2.5	pH 5.5
100	0.8567	0.4088	0.9711	0.4516	0.9637	0.2915
200	0.6970	0.2101	0.9368	0.2663	0.9187	0.1491
300	0.5410	0.1523	0.9066	0.2028	0.8910	0.1045
400	0.4382	0.1195	0.8824	0.1552	0.8504	0.0811
500	0.3632	0.0827	0.8366	0.1114	0.8021	0.0592
800	0.3188	0.0619	0.7968	0.0876	0.7111	0.0368
1000	0.2362	0.0513	0.7593	0.0717	0.6688	0.0304
1500	0.1916	0.0345	0.6849	0.0505	0.5714	0.0203
2000	0.4382	0.0267	0.6222	0.0388	0.5219	0.0167

[a] C_0 = initial HA concentration (milligrams/liter).

Gürses et al. (*40*) postulated that first and second layers were formed by hydrophobic force and weak dipole−dipole interaction, respectively. DeBoer and Zwicker (*41*) generalized a polarization theory for adsorption of nonpolar molecules on ionic adsorbents, stating that the adsorbent may induce dipoles in the first layer of adsorbed molecules, which in turn induce dipoles in the next layer, and so on. However, invariably all the reports suggest the first layer to possess greater binding energy than the subsequent layers of adsorbed molecules. The surface of anionic γ-PGA may also induce dipoles on the first layer, which subsequently stimulate dipoles in the next layer and thus favor multilayer adsorption. Therefore, for sorption of HA molecules on γ-PGA, the first layer may be formed mainly due to hydrogen-bonding, hydrophobic, and ionic interactions, while the subsequent layer(s) may result from the weak dipole−dipole and hydrophobic interactions between the HA molecules of the preceding layer and the fresh HA molecules. Similar multimolecular adsorption was reported for uptake of amphiphilic drugs on human serum albumin (*42*) and disperse red 1 dye on peat (*34*).

Binding Favorability. Hall et al. (*43*) proposed four idealized types of equilibrium behavior, namely, irreversible (type 1), favorable (type 2), linear (type 3), and unfavorable (type 4). The behavior of each equilibrium type was explained by a dimensionless constant separation factor or equilibrium parameter R_L, represented as

$$R_L = \frac{1}{(1 + bC_0)} \quad (7)$$

where b (liters/milligram) is the Langmuir constant. The value of R_L indicates the shape of isotherm to be either unfavorable ($R_L > 1$) or linear ($R_L = 1$) or favorable ($0 < R_L < 1$) or irreversible ($R_L = 0$). Accordingly, the favorability of HA sorption by γ-PGA can be predicted by calculating R_L values for each HA concentration. **Table 5** summarizes the R_L values obtained by use of the b values derived from Langmuir isotherms at pH 2.5 and 5.5 given in **Table 2**. The R_L values ranged from 0 to 1, indicating favorable sorption of HAs on γ-PGA in the concentration range (100−2000 mg/L) studied. The HA sorption was more favorable at high HA concentration than at low concentration, since the R_L values decreased with increasing HA concentration.

On the basis of the nature and modeling of isotherm curves and interpretation of experimental data, it may be inferred that the adsorption mechanism of HA molecules on γ-PGA is different at varying pH, as evident from an S-type curve at pH 2.5 and a two-site L-type curve at pH 5.5. A fit of the BET model for isotherm data at pH 5.5 suggested the two different sites of varying energies may result from a multimolecular HA adsorption on γ-PGA, involving hydrophobic, hydrogen-bonding, ionic, and dipole−dipole interactions. Further research is warranted on studying the effect of bile salts and bile pigments on HA sorption by γ-PGA.

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