Adsorption of Protein by Bentonite in a Model Wine Solution

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The adsorption of bovine serum albumin by bentonite in model wine solutions was studied. The attainment of equilibrium was found to be rapid and complete within 30 seconds of the addition. The extent of adsorption was characterized using both the Langmuir and Freundlich equations. The constants and the goodness of fit were determined by linear regression of the transformed equations. The adsorption was independent of temperature but varied with protein content, pH, and ethanol content. A comparison of various types of bentonite showed that calcium bentonites had lower adsorptive capacities, even though their measured cation exchange capacities were similar.

KEY WORDS: bentonite, adsorption, protein

The protein contents of wines have been determined in several studies (2,8,14,19,25,28). The existence of several fractions was first shown by Koch and Sajak (8), and these have been further resolved by the later studies of Bayly and Berg (2), Yokotsuka *et al.* (28), and Anelli (1). Isoelectric points of these fractions can range from 2.5 to 8.7 (1) in a Malvasia wine, and molecular weights range from 10 000 to 40 000 daltons (28).

Protein contents in a number of California wines were found to be between 20 and 260 mg/L with a mean of 116 mg/L (2,14). Other reports suggest levels of 19 to 94 mg/L in must (1), 7 to 25 mg/L in wine (8), and 80 to 220 mg/L (26) in wine.

The use of bentonite in winemaking dates back to Saywell's report (17) and is now universally used for the adsorption of proteins from wines. It has been used in a number of other applications, such as pharmaceutical, cosmetic, and cleaning preparations. Almost 30 years elapsed between Saywell's finding (17) and a number of studies investigating bentonites and protein stability in wines (7,12,15,27). Since that time, other studies have quantified the effects of bentonite by the use of improved protein analyses (20,26).

The adsorption of proteins and a number of other soluble cationic constituents in wine by bentonites is due primarily to the cation exchange action of these clays. The extent to which exchange is possible is determined by the cation exchange capacity (CEC), and this is dependent on the amount of displacement of aluminum ions by sodium, calcium, or magnesium ions when the clay was formed. These ions also influence the interlayer spacing of the bentonite and its swelling properties (16).

The cationic nature of a protein fraction will be primarily determined by the isoelectric (or isoionic) point (pI) and pH of the juice or wine in which it exists. Proteins with pI values above wine pH will carry a net positive charge and should be readily exchanged onto bentonite. Proteins with effectively no overall positive charge, or perhaps a net negative charge, may be adsorbed due to polar terminal regions which are locally positive, by chelation of other cations, or by a hydrogen-bonding mechanism; however, a strong preference for cationic protein fractions is expected.

Secondary aspects which will effect the adsorption of protein fractions are the competition from other cations in the solution matrix and the solvent properties. In wines, this competition would be from potassium, calcium, magnesium, sodium, and hydrogen ions as simple cations, most amino acids, some peptides, and other cationic protein fractions. The solvent effect of ethanol would be to displace water molecules from the interlattice spacing of the bentonite crystal (22) and to alter the dielectric value of the solutions, thus altering protein conformations and perhaps adsorption.

The role of temperature on protein adsorption by bentonite is open to question, since it has not been addressed in previous studies. Bentonite treatments are actually three distinct physical reactions — dispersion of the agent, adsorption of the solutes, and settling of the complex — and there is some confusion as to whether a temperature effect is related to the adsorption or to the settling phase of the treatment.

The influence of protein concentration on the adsorption may lead to competitive binding and poorer adsorption at relatively high concentrations. This kind of effect is observed in the substrate binding of certain enzymes, but such an effect has not been observed with other fining agents.

The characterization of adsorption reactions in wines by the adsorption equations of Langmuir (9) and Freundlich (6) is limited to two cases. The first is the characterization of phenolic adsorption onto polyvinylpolypyrrollidone (PVPP) using Freundlich's equation (11), and the second is the unsuccessful attempt to quantify proline adsorption by bentonite (4).

The aims of this study were to investigate the adsorption of a single protein in wine-like solutions by bentonites and to characterize the system with the aid of the Langmuir and Freundlich equations. The factors considered were the time to reach equilibrium and the influences of protein content, temperature, ethanol content, pH, and bentonite type on the adsorption.

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Theory

The two most commonly used relationships for the characterization of solid-liquid adsorption systems are the Langmuir and Freundlich equations (23).

The Langmuir equation was developed from a concept that there are a fixed number of adsorption sites on the solid particle and that binding is not influenced by the adsorption at nearby sites. From the rates of adsorption from the solution and desorption from the solid, Langmuir derived an equilibrium expression for the fraction of the surface sites which were occupied at any equilibrium concentration (C^*).

$$\frac{C^*}{K_L + C^*}$$

where K_L is referred to here as Langmuir's constant. The amount of a solute (x) that is adsorbed from solution by an amount of adsorbent (m) is given by Langmuir's equation:

$$\frac{X}{m} = \left(\frac{X}{m}\right)_{max} \cdot \frac{C^*}{K_L + C^*} \qquad \text{Eq. 1}$$

where the $(x/m)_{max}$ is the maximum amount of solute that can be adsorbed per unit of adsorbent. This is the saturation level of solute on the agent.

Langmuir's equation has an obvious similarity to the Michaelis-Henri-Menten equation (12) used in enzyme kinetics:

$$V = V_{max} \cdot \frac{S}{K_m + S} \qquad \text{Eq. 2}$$

where V is the rate of reaction, V_{max} is the maximum rate when the enzyme is saturated with substrate, and K_m is referred to as the 'affinity' of the substrate and the enzyme. Equation 2 is a particular case of an adsorption-controlled reaction in which there is negligible depletion of the substrate concentration (S) by the binding. In the more general case, Langmuir's equation, the solute concentration is depleted when adsorption takes place and there may or may not be a reaction at the surface.

Langmuir's equation has the same properties as the Michaelis-Henri-Menten form (18). At equilibrium concentrations much larger than K_L, the adsorbent is saturated and binding is independent of the solute concentration. At equilibrium concentrations much smaller than K_L, the extent of adsorption is directly proportional to the equilibrium concentration.

The constants $(x/m)_{max}$ and K_L can be obtained from the intercept and slope of a double-reciprocal plot (m/x *vs.* $1/C^*$) using linear regression or directly using non-linear regression.

The Freundlich equation is purely empirical but is widely used to describe adsorption systems in which there does not appear to be a finite number of adsorption sites. Many physical systems exhibit weaker but increasing adsorption as the concentration increases, and these are well described by Freundlich's equation when Langmuir's equation would obviously fail (22,24).

The common form of the Freundlich equation is:

$$C^* = K \cdot (x/m)^n$$

where C^*, x, and m have the same definitions as above. K and n are constants. A more convenient form to express this is:

$$\frac{x}{m} = K_F \cdot C^{*1/n} \qquad \text{Eq. 3}$$

where K_F will now be referred to as the Freundlich constant and (1/n) as the Freundlich index.

For the case n = 1, the amount of solute adsorbed per unit of adsorbent is proportional to the equilibrium concentration (C^*). K_F can be thought of as a distribution or partition coefficient between the solute on the solid (x/m) and that in the liquid at equilibrium (C^*).

For $n > 1$, the adsorption is weakly dependent on the concentration. If n = 2, doubling the equilibrium concentration would only lead to 41% more adsorption. For $n < 1$, the adsorption is strongly dependent on the concentration. If n = 0.5, doubling the equilibrium concentration would lead to four times the amount of adsorption per unit of agent.

The constants K_F and (1/n) can be determined from the intercept and slope of a log-log plot of (x/m) versus C^*, using linear regression.

Materials and Methods

Protein: Bovine serum albumin (BSA), Sigma Chemical Co., was used as the test protein. It has an isoelectric point between 4.3 and 4.6 and a molecular weight of approximately 66 000 daltons.

Model wine solution: The model solution was made up of 2 g/L KHTa, 12% ethanol, and 600 mg/L protein at pH 3.52.

Bentonites: Four types of bentonites were used: a sodium form (Mineral Colloid B.P., Georgia Kaolin Co., Union, NJ), a calcium form (Ca-Granulat™, Erbsloh Co., Geisenheim, FRG), and two sodium-exchanged calcium bentonites (Aktivit™ and NaCalit™, Erbsloh Co.).

Bentonite slurry: The slurries were prepared in deionized water as 0.6% (w/v) for the sodium form and as 2.4% (w/v) for the other forms. Slurries were prepared at least 24 hours prior to use to allow for hydration.

Protein assay: Protein content was assayed by measuring the absorbance at 280 nm on a Gilford 2400 spectrophotometer. The standard curve, linear up to 1000 mg/L, produced an extinction coefficient of 6.7×10^{-4} AU • L/mg.

Procedure: The bentonite slurry was made up to 5 mL containing the desired quantity of bentonite. This was added to 25 mL of model wine solution and thoroughly mixed. After 30 minutes, the sample was centrifuged and membrane-filtered (0.2 μm pore size), and the absorbance at 280 nm was recorded. The levels of protein and ethanol were 16.7% higher in the model solution to account for the dilution by the bentonite slurry. The concentrations reported hereafter are those of the final mixture. All experiments were conducted in triplicate at 25°C, except for the temperature series.

Data analysis: The data were analyzed by a BASIC program called ADSORB. This program calculated the quantity of protein adsorbed per unit of bentonite for each equilibrium replicate. These values were transformed for the double-reciprocal and log-log plots. Linear regression provided the slope, intercept, and correlation coefficient, and in addition, the standard error estimates in the slope and intercept were calculated. Three types of plots were provided: *(1)* a depletion curve of final protein content versus amount of bentonite added; *(2)* a normal curve, a linear plot of (x/m) versus C^* for either the Langmuir or the Freundlich equation; and *(3)* a transformed plot of (x/m) versus C^*, which was used for the linear regression of either of the equations. Further details of these methods can be found in another report (3).

Results

Time to reach equilibrium: A trial with 1 L of model solution (225 mg/L protein, 10% ethanol, pH 3.52, 25°C) and 480 mg/L (4 lb/1000 gal) of bentonite was mixed continuously at approximately 200 rpm.

Samples of approximately 25 mL were withdrawn at 30-second intervals, immediately filtered, and assayed for protein content. The readings at 30 seconds and at longer times were all at approximately 14% of the initial value, indicating that equilibrium was attained in less than 30 seconds.

Effect of protein concentration: The initial protein concentration was adjusted to 250, 500, and 1000 mg/L, and for each, a series of bentonite additions (sodium form) were made. Table 1 summarizes the resultant constants for the Langmuir equation, and Figure 1 shows the depletion curves predicted by the Langmuir equation using these values together with the measured values. Figure 2 shows the normal Langmuir plots.

Effect of temperature: An experiment was conducted −4°C, the coldest temperature that might be used for bentonite fining. The resultant constants for the Langmuir equation are given in Table 2.

Effect of ethanol concentrations: The model solution was adjusted to provide final ethanol concentrations of 7%, 10%, and 13% (v/v), and each of these was then fined with the sodium form of bentonite. The Langmuir constants for these cases are given in Table 3.

Effect of pH: The pH of the model solution was adjusted down to 3.20 and up to 3.80, and fining was conducted with the sodium form of bentonite. Table 4 gives the Langmuir constants, Figure 3 shows the predicted depletion curves with the measured values, and Figure 4 shows the normal curves of the Langmuir equation.

Effect of bentonite type: A calcium form of bentonite and two partly exchanged calcium forms were compared with the sodium form at pH 3.52 and pH 3.20. The constants for both the Langmuir and Freundlich equations are given in Tables 5 (pH 3.52) and 6 (pH 3.20). The predicted depletion curves for the Langmuir equation and the measured values are presented in Figure 5. The relationships between the extent of adsorption and equilibrium concentration for these forms are given in Figure 6 (Langmuir equation) and Figure 7 (Freundlich equation).

Discussion

Current practices with respect to bentonite additions range from in-line additions, with a retention time of

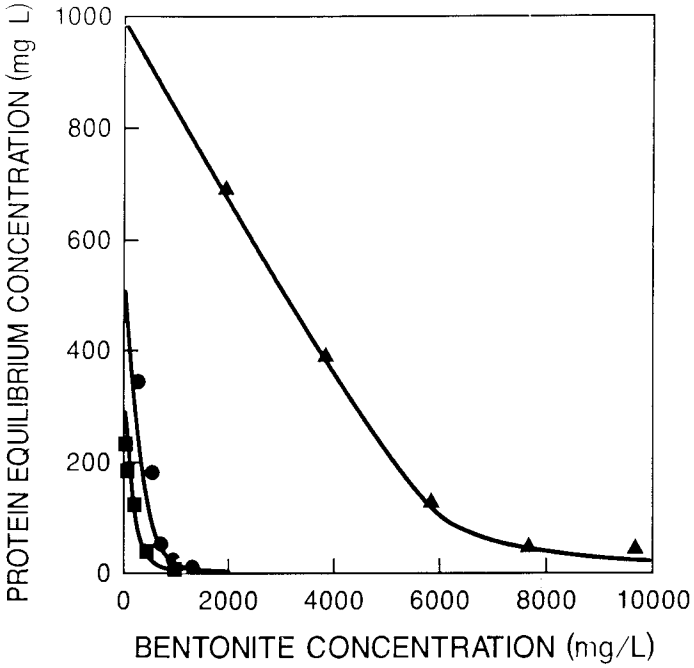

Fig. 1. Effect of initial protein content on the Langmuir depletion curves for initial protein concentrations of 250 mg/L (■), 500 mg/L (•), and 1000 mg/L (▲).

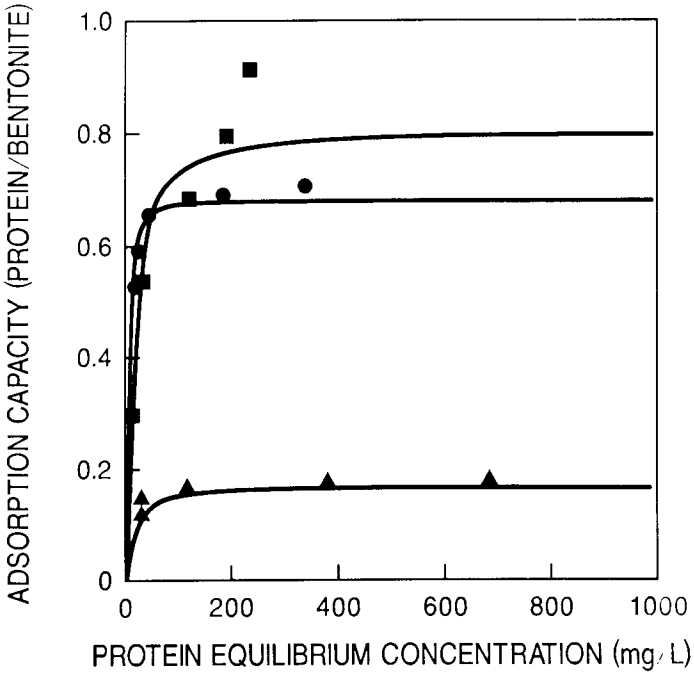

Fig. 2. Normal Langmuir plots for the initial protein concentrations of 250 mg/L (■), 500 mg/L (•), and 1000 mg/L (▲).

approximately two minutes before clarification by centrifuge or filter, to batch additions which are allowed to have contact times of up to 10 days. Some believe that low temperature aids protein adsorption by bentonite, and there are ongoing discussions about the effectiveness of the sodium and calcium forms of bentonites.

The time required for adsorption is less than 30 seconds in an instantly dispersed, well-mixed situation. This corresponds to the shortest interval which could practically be attained, in view of the addition of slurry and the removal of 25 mL through the filter into the syringe. It clearly supports the use of a two-minute contact time and removes the adsorption process from any consideration in longer contact practices.

The experiments of protein concentration were included in an attempt to test the two adsorption equations. The Langmuir description of a finite number of sites would be expected to follow some kind of inhibition to binding either of a competitive nature (increased K_L values) or of a non-competitive nature (reduced $(x/m)_{max}$ values). Figures 1 and 2 and Table 1 indicate that reduced adsorption capacity results at the 1000-mg/L treatment series, and this can be interpreted as non-competitive inhibition of protein adsorption. The adsorptive capacity declines approximately five-fold with a four-fold increase in protein concentration. The binding constants (K_L) are significantly different but show no obvious trend. In this series, the Langmuir equation provided a better description of the adsorption than did the Freundlich equation.

Table 1. Effect of protein concentration on protein adsorption.

Statistic	Protein concentration (mg/L) 250	500	1000
Langmuir constant K_L	11.7	2.14	11.2
Langmuir capacity $(x/m)_{max}$	.819	.689	.163
Langmuir correlation coeff.	.992	.989	.851

The influence of temperature on the adsorption is only minor, leading to a significantly larger binding constant K_L (Table 2). This may be due to a conformational change in the protein, making it less likely to bind at low temperatures. The adsorptive capacity $(x/m)_{max}$ is unaltered by temperature, as would be expected for an ion exchange mechanism.

Table 2. Effect of temperature on protein adsorption.

Statistic	Temperature (°C) −4	25
Langmuir constant K_L	3.90	2.14
Langmuir capacity $(x/m)_{max}$	.686	.689
Langmuir correlation coeff.	.994	.989

Since the effect of the higher K_L value at −4°C would only be seen at protein contents less than 10 mg/L, the actual adsorption at the higher concentrations expected in wines would not be enhanced by fining at low temperatures. Temperature effects which are observed in wines are more likely due to the precipitation of partially soluble colloids and complexes which may interfere with some protein stability tests rather than to enhanced protein adsorption itself. Again the Langmuir equation provided a better description of the adsorption series.

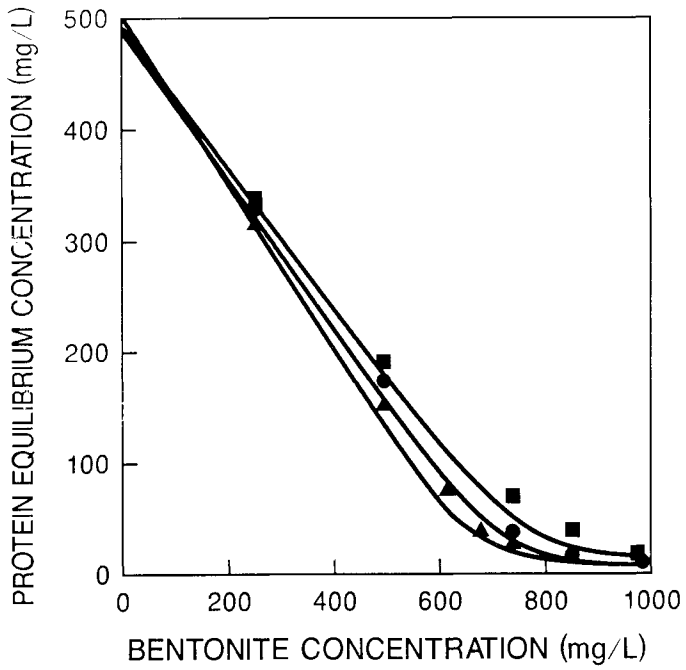

Fig. 3. Effect of solution pH on the Langmuir depletion curves; pH 3.20 (■), pH 3.52 (●), and pH 3.80 (▲).

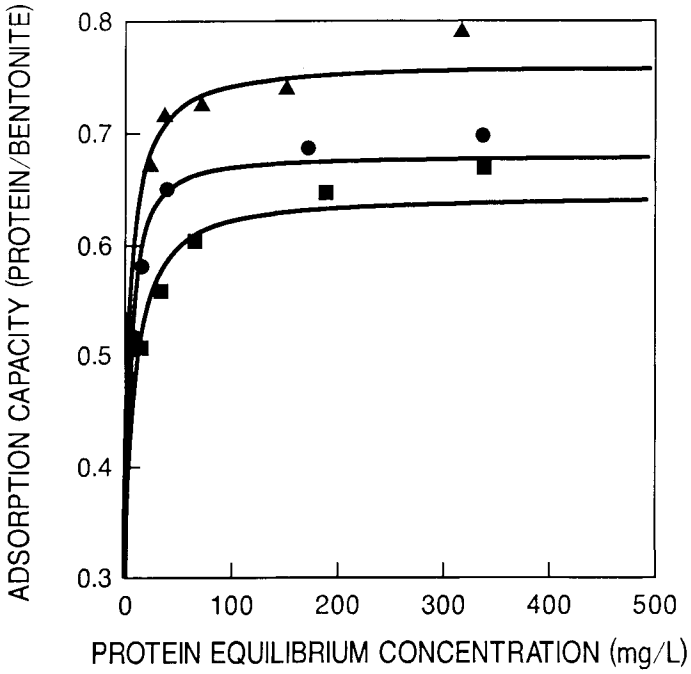

Fig. 4. Normal Langmuir plots for solution pH values of pH 3.20 (■), pH 3.52 (●), and pH 3.80 (▲).

Organic solvents can displace water molecules from the bentonite lattice (22) and lead to extensive swelling (10). The greater swelling may result in additional or perhaps more accessible exchangeable cations, and hence

Table 3. Effect of alcohol concentration on protein adsorption.

Statistic	Alcohol concentration (% v/v) 7.0	10.0	13.0
Langmuir constant K_L	2.55	2.14	6.70
Langmuir capacity $(x/m)_{max}$	.689	.689	.780
Langmuir correlation coeff.	.917	.989	.922

increase the adsorption capacity $(x/m)_{max}$. Table 3 shows no significant changes in either K_L or $(x/m)_{max}$ for the 7% and 10% ethanol cases but significantly higher values in the 13% solution. The adsorption capacity is 13% higher simply due to the influence of ethanol at wine concentration.

The influence of pH on protein adsorption by bentonite has several facets. The protein will be increasingly protonated as the pH is lowered, but without titration curves for either BSA or typical wine proteins, it is not possible to estimate how sensitive their overall charge will be to pH in the range of interest, 3.0 to 4.0. The protons in solution will increase from 100 μM/L at pH 4.0 to 1 mM/L at pH 3.0, and while they are at least an order of magnitude lower in concentration than potassium ions, they are of the same order as the sodium, calcium, and magnesium ions. The extent of sodium (and to a lesser degree, calcium and magnesium) exchange will depend on the cation concentrations in solution and the preference of the bentonite for these cations. While selectivity constants of the ions with bentonites do not appear to have been determined, their relative selectivity might be similar to that of cation exchange resins, *i.e.*, a strong preference for hydrogen over sodium at similar concentrations and a strong preference for very large cations. Thus, the pH should influence both the cationic charge of the protein and the relative exchange of hydrogen, protein, and sodium in the bentonite. There is no significant change in binding affinity (K_L) over the pH range of 3.8 to 3.2 (Table 4). However, there is a significant increase in adsorption capacity at pH 3.8, approximatey 12% higher (Fig. 4, Table 4). Since the protein would be less cationic at the higher pH, the effect seems to be due to less competition between hydrogen ions and the protein in the higher pH solution.

The influence of the bentonite type, in particular the sodium versus calcium forms, has received previous attention (15,20,27). The advantages in the calcium form are the lower increase in the sodium concentration due to treatment and the compactness of settling lees. The disadvantages are the increase in calcium concentrations and the poorer adsorption capacity due to the less extensive swelling. Although the exchange capacities of the calcium forms, as determined by barium displacement or methylene blue adsorption, are often similar to those of the sodium forms, their adsorption of large cations is determined by the interlayer spacing in the swollen state. This has previously been determined for general bentonites (16), and the poor adsorption of protein by the calcium forms is reaffirmed in the present study.

Table 4. Effect of pH on protein adsorption.

Statistic	Solution pH 3.20	3.52	3.80
Langmuir constant K_L	4.31	2.14	3.46
Langmuir capacity $(x/m)_{max}$	.654	.689	.774
Langmuir correlation coeff.	.969	.989	.916

Table 5. Effect of bentonite type on protein adsorption, pH = 3.52.

Statistic	Bentonite type GK (Na)	NaCalit™ (Na-Ca)	Aktivit™ (Ca-Na)	CaGranulat™ (Ca)
Langmuir constant K_L	2.14	8.46	15.3	93.4
Langmuir capacity $(x/m)_{max}$	.689	.404	.303	.389
Langmuir correlation coeff.	.989	.968	.953	.953
Freundlich constant K_F	.471	.194	.161	.0388
Freundlich index 1/n	.0724	.128	.102	.360
Freundlich efficiency x/m @ 200 mg/L	.692	.383	.277	.261
Freundlich correlation coeff.	.950	.933	.917	.965

At pH 3.52, the calcium forms (sodium-calcium, calcium-sodium, and pure calcium) all have significantly poorer binding affinity as reflected by their larger K_L values (Table 5). The lower K_L value of the sodium form, approximately one-fiftieth that of the calcium form, indicated that this bentonite will be saturated by protein levels down to approximately 40 mg/L. By comparison, the three calcium forms would begin to have less than saturated adsorption at protein levels of approximately 100, 100, and 350 mg/L, respectively. Of more importance

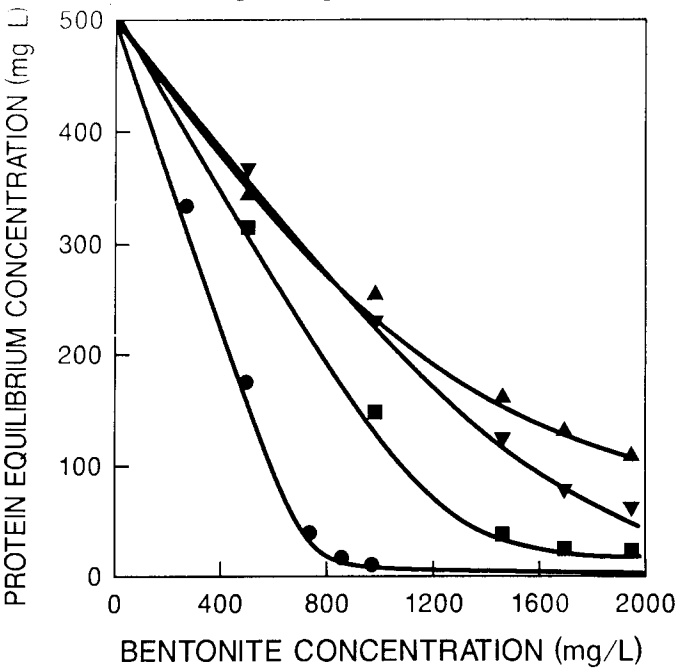

Fig. 5. Effect of bentonite type on the Langmuir depletion curves at pH 3.52; sodium (•), sodium/calcium (■), calcium/sodium (▼) and calcium (▲) forms.

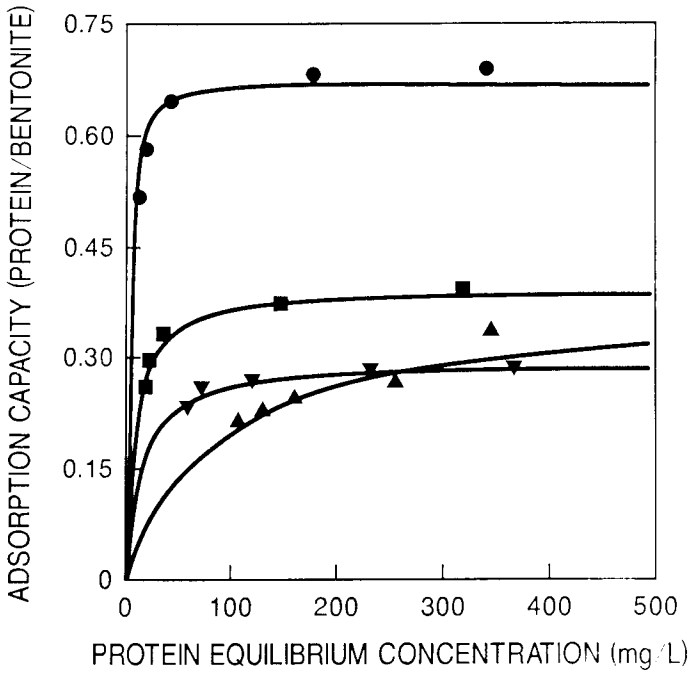

Fig. 6. Normal Langmuir plots for the bentonite types; sodium (•), sodium/calcium (■), calcium/sodium (▼), and calcium (▲) forms.

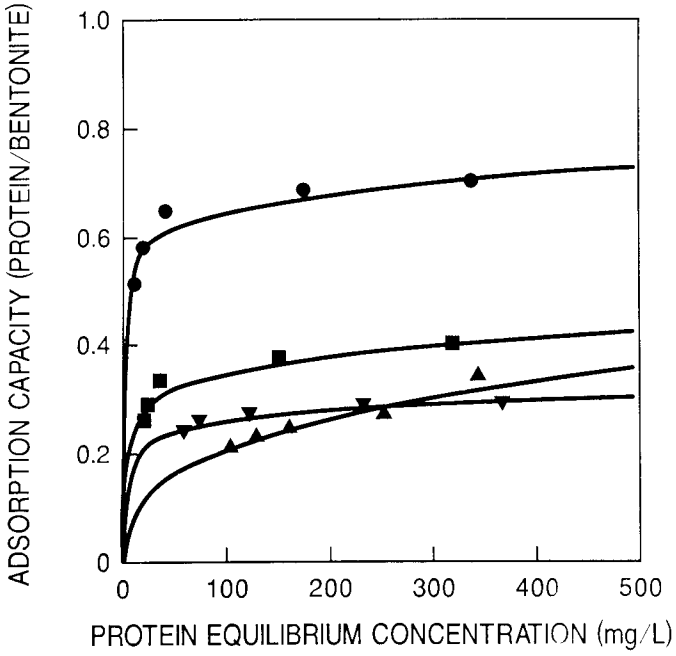

Fig. 7. Normal Freundlich plots for the bentonite types; sodium (•), sodium/calcium (■), calcium/sodium (▼), and calcium (▲) forms.

is the adsorption capacity when saturated $(x/m)_{max}$. The three calcium forms have 59%, 44%, and 56% of the adsorption capacity of the sodium form, respectively, even though their exchange capacities (as measured by barium displacement) are 72, 78, and 81 meq/100 g, respectively (compared to the sodium's 79 meq/100 g). This is dramatically illustrated in Figure 5, where the slope of the depletion curve is a measure at the adsorption capacity when saturated. In Figure 6, this is reflected by the height at which the curve levels off.

The ability of the bentonite forms to adsorb protein at a lower pH (3.20) was also investigated because there have been claims that the calcium forms are more effective at that condition. The binding affinity, reflected by the K_L values, shows significantly poorer attraction to the calcium forms. The adsorption capacities, $(x/m)_{max}$, are also significantly lower, being 54%, 45%, and 72%, respectively, of the sodium form.

In most cases, the Langmuir equation provided the better fit of the experimental data. The results of the Freundlich equation for the bentonite types are presented in Tables 5 and 6 and Figure 7. The Freundlich constant (K_F) is like a distribution coefficient between the solid and liquid phases and shows the fall off in adsorption with calcium forms in both cases. The Freundlich index (tabulated here as 1/n) is the power dependence of the equilibrium concentration term. The sodium form is shown to be far less dependent on the equilibrium concentration than are the calcium forms. Since the Freundlich equation has no saturation value or limit, the expected values of the adsorption capacity (x/m) at 200 mg/L protein have been calculated and are given in Tables 5 and 6. They show a similar pattern of adsorption capacity for the bentonite types as presented above.

The Langmuir equation is the more suitable relation-

Table 6. Effect of bentonite type on protein adsorption, pH = 3.20.

Statistic	Bentonite type			
	GK (Na)	NaCalit™ (Na-Ca)	Aktivit™ (Ca-Na)	CaGranulat™ (Ca)
Langmuir constant K_L	4.31	6.60	31.8	310.0
Langmuir capacity $(x/m)_{max}$	.654	.365	.294	.470
Langmuir correlation coeff.	.969	.981	.733	.800
Freundlich constant K_F	.407	.184	.0894	.00809
Freundlich index 1/n	.0878	.111	.190	.587
Freundlich efficiency x/m @ 200 mg/L	.648	.334	.244	.181
Freundlich correlation coeff.	.990	.989	.799	.750

ship for describing the adsorption of protein by bentonite. This is due to the cation exchange mechanism controlling the adsorption and because only a fixed number of adsorption sites exist. In a previous study (4) of the adsorption of proline by bentonite, the authors were unable to describe the results by either the Langmuir or Freundlich equation, instead they reported the distribution coefficients for sodium and proline. Such a linear relationship is simply a special case of the Langmuir equation at equilibrium concentrations less than the K_L value or that of the Freundlich equation with $n = 1$.

Conclusions

The adsorption of a standard protein by bentonite reaches its equilibrium in less than 30 seconds. The extent of adsorption was characterized by the Langmuir and Freundlich equations. The influence of temperature was essentially non-existent, while slight effects were found due to the protein content, pH, and the ethanol content. The major differences in adsorption were due to bentonite type, with the sodium form capable of adsorbing almost twice the amount of protein per unit of agent than any of three calcium forms.

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