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ADSORPTION OF PROTAMINE AND PAPAIN PROTEINS ON SAPONITE

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Abstract—Due to the increased importance of bionanocomposites, protamine and papain proteins were adsorbed on Na^+- and on Cs^+-exchanged saponite from aqueous solution. Protein analysis of equilibrium solutions and thermogravimetric analyses of biocomposites were used to prepare adsorption isotherms. Based on the isotherm shape, and on the amounts of protein adsorbed and the amounts of Na^+ and Cs^+ released, the initial protein sorption apparently was due to ion exchange. Additional sorbed protein was weakly retained and could be removed by washing with water. From ion exchange, the average charge of the protamine adsorbed was estimated to be +13.1 to +13.5. Similar papain measurements could not be made due to partial decomposition. Quantitatively, protamine was adsorbed at levels up to 400 mg/g on Na^+-saponite and 200 mg/g on Cs^+-saponite. The maximum protamine adsorption was 650 to 700 mg/g for Na^+-saponite and 350−400 mg/g for Cs^+-saponite. Protamine was sorbed to edge surfaces and the basal spacing of the interlamellar region of saponite was 1.75 nm. Protamine displaced only 36% of the Cs^+ in Cs^+-saponite and expanded the interlamellar region by 36% for a basal spacing of 1.6 nm. Papain sorption to Na^+-saponite occurred by a two-step process: (1) adsorption to saponite particle external surfaces followed, (2) by partial intercalation. Quantitatively, Papain was adsorbed up to 100 mg/g for Na^+- and Cs^+-saponite. Greater initial papain concentrations resulted in a 450 mg/g maximum for Na^+-saponite, but no increase above 100 mg/g for Cs^+-saponite. Papain apparently only sorbed to external Cs^+-saponite surfaces that were estimated to be 33−40 m^2/g. Step-wise thermal decomposition of the saponite-protein composites occurred between 300 and 800ºC.

Key Words—Intercalation, Ion Exchange, Papain, Protamine, Saponite.

INTRODUCTION

The adsorption of proteins on surfaces is an active research area. It has significant scientific and technological implications for: (1) the development of biomaterials for integration with tissue; (2) nanotechnology for the development of sensors, activators, and biological-electronic junctions; (3) drug delivery systems; and (4) biocatalysis and optimization of immobilized enzyme systems (Gray, 2004; Zhao *et al.*, 2006; Rasor, 2000; Hudson *et al.*, 2005; Ruiz-Hitzky *et al.*, 2008).

Both organic (polymeric) and inorganic surfaces are under investigation. Among the latter, clay minerals have a unique position as they play a significant role in the adsorption of proteins in soils and contribute to the regulation of biological life in soils (Brack, 2006). Despite the importance of such studies, only a limited amount of fundamental research has been done on clay-protein interactions. Early research was reviewed by Theng (1974) and ion exchange was found to be the main adsorption mechanism. The amount of protein sorbed commonly can exceed the cation exchange capacity (CEC) of the clay mineral. When this occurs, other adsorption forces come into play. In their study of the adsorption of bovine serum albumin (BSA) on Wyoming montmorillonite, Quiquampoix *et al.* (1993) found that adsorption of BSA was governed by electrostatic (ion exchange) and hydrophobic interactions. The former were dominant when the pH of the aqueous suspension was below the isoelectric point (IEP) of BSA (4.8). Maximum adsorption occurred near the IEP of BSA (*i.e.* at a pH of 4.8). The surface area per adsorbed BSA molecule was calculated to be 60 nm^2 from an assumed Wyoming montmorillonite total surface area of 700 m^2/g (Quiquampoix *et al.*, 1993).

Monolayer coverage of irreversibly adsorbed lysozyme (LYS) on mica (muscovite) was reached in 2 h (Kim *et al.*, 2002). After a mica surface was exposed to a dilute LYS solution, clusters of 5 LYS molecules first formed and with more concentrated solutions a monolayer grew uniformly (Kim *et al.*, 2002). Blomberg *et al.* (1994) found that the thickness of this monolayer was 3 nm. Based on the crystal structure, the dimensions of the LYS molecule are 4.5 nm × 3 nm × 3 nm. This means that LYS was adsorbed with the 4.5 nm × 3 nm side to the mica surface. In other words, maximum contact or maximum interaction of the protein with the mica surface was achieved.

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On the basis of these and many other studies, proteins are divided into three classes (Blomberg *et al.* 1994): (1) globular, compact proteins or hard proteins, which undergo little if any conformational change upon adsorption (LYS belongs to this category); (2) soft proteins, which can undergo significant geometrical and conformational changes upon adsorption, such as BSA; (3) large molecular weight, complex, flexible proteins, which can adopt many different conformations on surfaces. Such is the case of fibrinogen (FIB) (Wertz and Santore, 2002) and mucin (Blomberg *et al.*, 1994). These adsorption-induced conformational changes depend on the strength of the protein-surface interaction. Therefore, the type of interaction, the type of protein, and the type of surface (hydrophilic, hydrophobic) are all important parameters to consider (Wertz and Santore, 2001, 2002a, 2002b).

The neutral portion of the siloxane surface of clay minerals is hydrophobic (Nulens *et al.*, 1996). This type of surface is found on the basal surface of talc and pyrophyllite and on the siloxane surface of kaolinite. Hydrophilicity is introduced by isomorphous substitution in the octahedral or tetrahedral sheets. The resulting negative charge of the clay mineral structure, together with the presence of hydrated exchangeable cations in the interlayer space, give the clay minerals their hydrophilic properties. However, regions of the surface between the exchangeable cations still retain their hydrophobic character as shown by Jaynes and Boyd (1991) and Laird *et al.* (1992). Aside from this hydrophilic-hydrophobic balance, the following properties make smectites or swelling clays interesting materials for adsorption of proteins: (1) swelling and, therefore, the possibility of intercalation of proteins in the interlamellar space; (2) ion exchange; and (3) the presence of a non-negligible edge surface, which can make up to 10% of the total theoretical surface area of 750 m^2/g. Thus, adsorption can occur at pH-dependent, charged-edge sites and to interlamellar surface sites with pH-independent charge sites.

A study of the adsorption of protamine (PROT) and papain (PAP) on saponite is presented here. The relevant properties of these proteins are given in Table 1.

Table 1. Properties of protamine and papain.

	Protamine	Papain
Amino acid residues	32	212
Molecular weight (Da)	4500	23000
Isoelectric point	10–12	9.6
DLS size (nm)[1]	2.4	3.9
Hard sphere diameter (nm)[2]	2.18	3.78
Crystal dimensions (nm)[2]	–	$3 \times 3 \times 4.5$

[1] The dynamic light scattering (DLS) experiments were performed in 5 mM NaCl at pH = 4
[2] Harpaz *et al.* (1994)

Saponite is a trioctahedral smectite with Al^{3+} substituting for Si^{4+} in the tetrahedral sheets. This is considered to give localized negative charges in the elementary clay mineral layers and a more pronounced hydrophilic character. Adsorption isotherms are obtained by analysis of the amount of residual proteins in the equilibrium solutions and by thermogravimetric weight loss analysis of the washed and air-dried clay-protein composites. In the latter case, the amount of Na^+ and Cs^+ released in the equilibrium solution were also measured to assess the ion-exchange mechanism. Intercalation of PAP and PROT in Na^+- and Cs^+-saponite was studied by X-ray diffraction (XRD).

EXPERIMENTAL

Materials and chemicals

Saponite (SAPCa-1, SAPCa-2) was obtained from the Source Clays Repository of The Clay Minerals Society (based at Purdue University). It was saturated with Na^+ by repeated exchange with 1 M NaCl solutions followed by dialysis with water until the water was Cl^--free (test with $AgNO_3$). The 0.5–2.0 μm particle-size fraction was separated by centrifugation (6 min at 3200 rpm). Finally, the saponite dispersion was freeze-dried and stored as a powder at room temperature. Cs^+-saponite was obtained by threefold exchange of 10 g of freeze-dried Na^+-saponite with 0.1 M CsCl solutions, followed by washing and freeze-drying. In separate experiments, the cation exchange capacity (CEC) of different size fractions of saponite was determined by exchange overnight with CsCl (110 mg of air-dried saponite dispersed in 500 mL of 2 mM CsCl solution). The Cs^+ in the equilibrium solution was determined by atomic absorption spectrometry (AAS). The results were: <2 μm: 1.28 meq/g; 0.5–2 μm: 1.06 meq/g; and <0.5 μm: 1.17 meq/g. All numbers are the averages of three measurements.

Protamine sulfate from herring (grade III) and papain were obtained from Sigma-Aldrich (St. Louis, Missouri) and used as received. They were stored in a refrigerator at 4ºC. Papain was stored in the presence of silica gel desiccating agent. The BCA™ protein assay kit and the protein assay procedure were obtained from Pierce (Rockford, Illinois).

Adsorption isotherms

A stock dispersion of 100 mg (freeze-dried) of Na^+-saponite in 100 mL of water was prepared and allowed to equilibrate for 24 h under continuous stirring. The dry weight of the clay in the dispersion was determined by drying a 10 mL aliquot at 120ºC. Stock solutions of 50 mg of protamine or 50 mg of papain in 50 mL of H_2O were prepared immediately before use.

For each isotherm, seven 50 mL centrifuge tubes were each loaded with 10 mL of the saponite (10 mg) dispersion and 0 mL (no protein) to 15 mL (15 mg

protein) of the protein solution. The volume in the centrifuge tubes was then brought to 25 mL with water. The tubes were closed and equilibrated on an end-over-end shaker overnight at room temperature. The centrifuge tube with no protein was used to determine the dry weight of the saponite dispersion. The other dispersions were passed through 0.2 μm micropore filters. The liquid was used to determine the pH and the residual protein was measured using the BCA™ protein assay kit. The saponite-protein biocomposites retained on the filters were washed three more times with water and dried at room temperature in the dark. The adsorption isotherms of Cs^+-saponite were obtained in exactly the same way.

Adsorption isotherms were also determined on 200 mg (freeze-dried weight) of Na^+- and Cs^+-saponite. Seven 50 mL centrifuge tubes were each loaded with 200 mg (freeze-dried weight) saponite and increasing amounts of protein (between 0 and 640 mg) in 25 mL aqueous solution. These dispersions were allowed to equilibrate overnight on an end-over-end shaker. Then, the solid was separated from the liquid by centrifugation. The pH as well as the $Na^+$and Cs^+ contents were measured in the liquid. The solid was washed three times with 30 mL of water and freeze dried. The amount of protein adsorbed was determined by thermogravimetry. Na^+ and Cs^+ in the exchange solution were determined by a Solaar 969AA flame spectrometer (Thermo Elemental, UK). The amounts of C and N in the proteins adsorbed to saponite were measured using a Vario Max C/N analyzer (RomA, Germany).

Physicochemical measurements

The XRD patterns of the saponite-protein biocomposites were collected at room temperature using a Stadi P Combi diffractometer (STOE & Cie GmbH, Germany) with CuKα radiation (λ = 0.15417 nm) and a focusing monochromator. The XRD patterns were collected in transmission mode in 0.25º2θ steps at 200 s per step. Thermogravimetric analysis was performed using a Q500 Thermogravimetric Analyzer (TGA, TA Instruments, USA) in O_2 atmosphere with heating ramp of 5ºC/min.

RESULTS

Adsorption isotherms

The protamine adsorption isotherms (BCA™ analysis) for Na^+- and Cs^+-saponite have similar shapes (Figure 1a). Up to a loading of 400 mg/g on Na^+-saponite and 200 mg/g on Cs^+-saponite, protamine was quantitatively adsorbed. Protamine concentrations in the equilibrium solutions were below the detection limit. Quantitative adsorption was followed by a continuous, non-linear increase in adsorbed protamine, which tended towards maximum values in the 650 to 700 mg/g range for Na^+-saponite and 350−400 mg/g for Cs^+-saponite.

Papain was quantitatively (100 mg/g) adsorbed on Na^+- and Cs^+-saponite (Figure 1b). Greater initial concentrations of papain yielded a linear increase in adsorbed papain. The isotherm slopes were 5848 mL/g for Na^+-saponite and 1143 mL/g for Cs^+-saponite.

The protamine adsorption isotherms were measured using thermogravimetry (Figure 2a). The weight losses of the washed and freeze-dried samples (see thermogravimetry section) above 200ºC were corrected for saponite dehydroxylation and these corrected values were used as the amount of protamine adsorbed. Quantitative adsorption of protamine to Na^+-saponite up to ~400 mg/g was followed by a slight but continuous decrease in adsorption. This confirms quantitative protamine adsorption of 400 mg/g that was measured using BCA™ analysis as shown in Figure 1. A Langmuir-type adsorption of

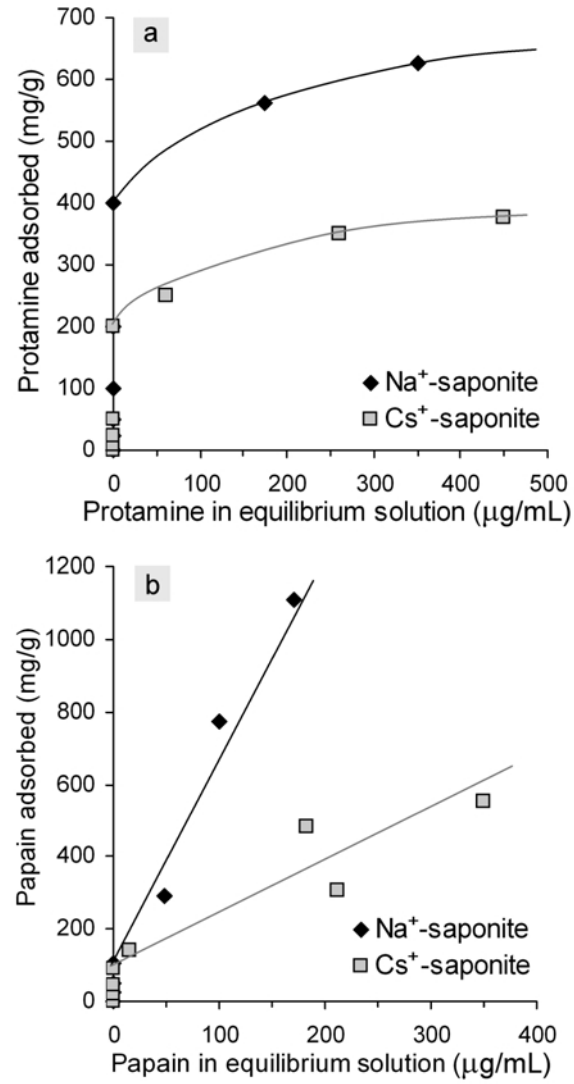

Figure 1. Protein adsorption isotherms on saponites measured using the BCA™ method: (a) protamine adsorption on Na^+- and Cs^+-saponites; (b) papain adsorption on Na^+- and Cs^+-saponites.

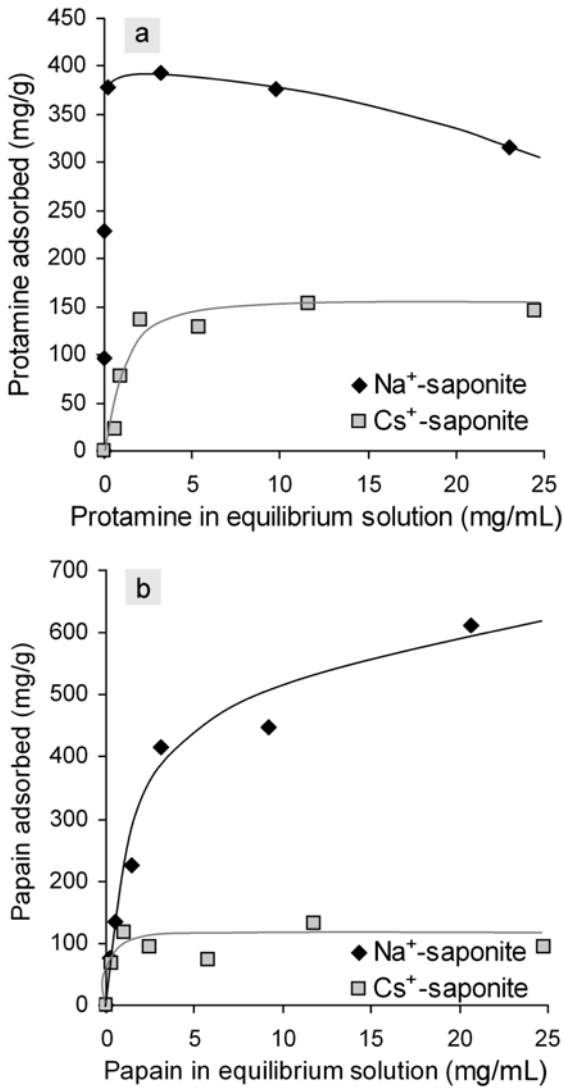

Figure 2. Protein adsorption isotherms on saponites measured using thermogravimetric analysis: (a) protamine adsorption on Na^+- and Cs^+-saponites; (b) papain adsorption on Na^+- and Cs^+-saponites.

protamine to Cs^+-saponite was observed up to a maximum adsorption of 145 mg/g. This 145 mg/g thermogravimetry-measured adsorption was less than the 200 mg/g quantitative adsorption measured using BCA™.

The papain adsorption isotherms determined by thermogravimetry displayed an almost vertical or quantitative adsorption up to ~100 mg/g (Figure 2b). Afterward, papain adsorption to Cs^+-saponite leveled off and did not increase with increases in papain equilibrium concentration. The amount of papain adsorbed to Na^+-saponite increased linearly up to ~420 mg/g and then leveled off at ~450 mg/g.

In experiments that used thermogravimetric analysis to measure protein adsorption, the C:N ratio was measured on the adsorbed proteins and the concentrations of Na^+ and Cs^+ and pH were measured on the equilibrium solutions (Tables 2–5).

In the case of protamine adsorption, the maximum amount of Na^+ released into solution averaged 1.15 mmol/g, which is close to the 1.06 meq/g CEC value determined using Cs^+ (Table 2). This maximum Na^+ release value was obtained before maximum protamine adsorption was reached and is also evident in the molar ratios of Na^+ released to protamine adsorbed (Table 2). The molar ratios were very high (>20) at low protamine loadings and quickly reached a constant value of ~14 at higher loadings. Excess Na^+ released at low protamine loadings was probably due to the low ionic strength of the exchange solutions. The C:N molar ratio of adsorbed protamine averaged 1.84. The significantly larger and smaller C:N molar ratios of 1.95 and 1.76 might have been caused by traces of carbonaceous material in Na^+-saponite or protamine decomposition, respectively. The pH of the equilibrium solution was not constant but steadily decreased from the slightly basic (8.9) values for Na^+-saponite to slightly acidic (6.0) values with greater equilibrium protamine concentrations.

Similar data collected for protamine adsorption to Cs^+-saponite yielded an average molar C:N ratio for adsorbed protamine of 1.53 (Table 3). Use of a different batch of protamine and residual C adsorbed to saponite might explain the lower adsorbed protamine C:N ratio (1.53) for Cs^+-saponite in comparison to that for Na^+-saponite C:N ratio (1.84). The variation in pH with protamine loading was similar for Na^+- and Cs^+-saponite. Released Cs^+ was only ~36% of Na^+ released and suggests incomplete ion exchange for Cs^+-saponite. Maximum protamine adsorption to Cs^+-saponite (141 mg/g) was only 35% of maximum protamine adsorption to Na^+-saponite (394 mg/g). The ratio of Cs^+ released from Cs^+-saponite to Na^+ released from Na^+-saponite after protamine adsorption was similar to the adsorbed protamine ratio and suggests that similar ion-exchange mechanisms operated with incomplete exchange of Cs^+.

The C:N ratios, Na^+ and Cs^+ release, and pH changes that accompanied papain adsorption (Figure 2b) to Na^+- and Cs^+-saponite were measured (Tables 4 and 5). The pH changes after papain adsorption were similar to pH changes after protamine adsorption. As was noted for protamine adsorption, all the Na^+ from Na^+-saponite but only a fraction of the Cs^+ from the Cs^+-saponite was transferred to the solution phase. The maximum amount of Cs^+ released into the solution was 0.27 mmol/g, which is only 17% of the maximum Na^+ release. The molar C:N ratio of adsorbed papain decreased with increased papain loading, but the C:N ratio remained fairly constant for protamine adsorption. Partial decomposition of papain might have occurred during the experiments and might explain the excessively large and somewhat erratic Na^+/adsorbed papain ratios.

Table 2. Adsorption of protamine on Na^+-saponite.

Protamine adsorbed (mg/g clay)	C:N molar ratio	pH equilibrium	Na^+ content (mmol/g clay)	Molar ratio of Na^+ released to adsorbed protamine
0	–	8.9	0.23	–
97.1	1.95	8.4	0.57	26.4
228.0	1.86	7.8	1.24	24.5
378.6	1.84	7.3	1.13	13.4
394.2	1.82	6.9	1.11	12.7
376.0	1.82	6.5	1.10	13.2
316.0	1.76	6.0	1.19	16.9

Table 3. Adsorption of protamine on Cs^+-saponite.

Protamine adsorbed (mg/g clay)	C:N molar ratio	pH equilibrium	Cs^+ content (mmol/g clay)	Molar ratio of Cs^+ released to adsorbed protamine
0	–	8.7	0.04	–
22.2	2.09	7.9	0.11	22.3
77.5	1.53	7.6	0.40	23.2
136.7	1.50	7.0	0.46	15.1
128.3	1.54	6.7	0.43	15.1
153.0	1.56	6.4	0.40	11.8
145.1	1.53	5.9	0.38	11.8

Table 4. Adsorption of papain on Na^+-saponite.

Papain adsorbed (mg/g clay)	C:N molar ratio	pH equilibrium	Na^+ content (mmol/g clay)	Molar ratio of Na^+ released to adsorbed papain
0	–	8.8	0.31	–
77.4	4.86	8.4	0.33	98.1
135.6	4.85	8.1	0.40	67.8
224.1	4.76	7.9	0.74	75.9
415.6	4.16	7.4	0.69	38.2
448.5	3.81	6.8	1.10	56.4
612.0	3.60	6.0	1.57	59.0

Table 5. Adsorption of papain on Cs^+-saponite.

Papain adsorbed (mg/g clay)	C:N molar ratio	pH equilibrium	Cs^+ content (mmol/g clay)	Molar ratio of released Cs^+ to adsorbed papain
0	–	8.5	0.05	–
66.5	5.85	7.9	0.07	24.2
115.7	5.09	7.3	0.13	25.8
94.7	5.09	7.0	0.13	31.6
72.0	5.39	6.6	0.18	57.5
132.3	4.89	5.9	0.20	34.8
94.8	3.61	5.2	0.27	65.5

Characterization

X-ray diffraction. The washed and freeze-dried saponite-protein complexes corresponding to the isotherms shown in Figure 1 were investigated using XRD (Figures 3–5). The Na^+-saponite XRD pattern (Figure 3) has a d_{001} reflection with a maximum at 1.27 nm which indicates a monolayer hydrate. After loading with protamine, the 1.27 nm peak decreased in intensity and a new peak appeared at 1.57 nm which shifted to 1.75 nm for loadings of 100 mg/g and greater. The 1.22 nm peak of freeze-dried Cs^+-saponite (Figure 4) also decreased in intensity with increased protamine loading, but did not disappear completely and a new, broad d_{001} peak appeared at 1.50–1.61 nm.

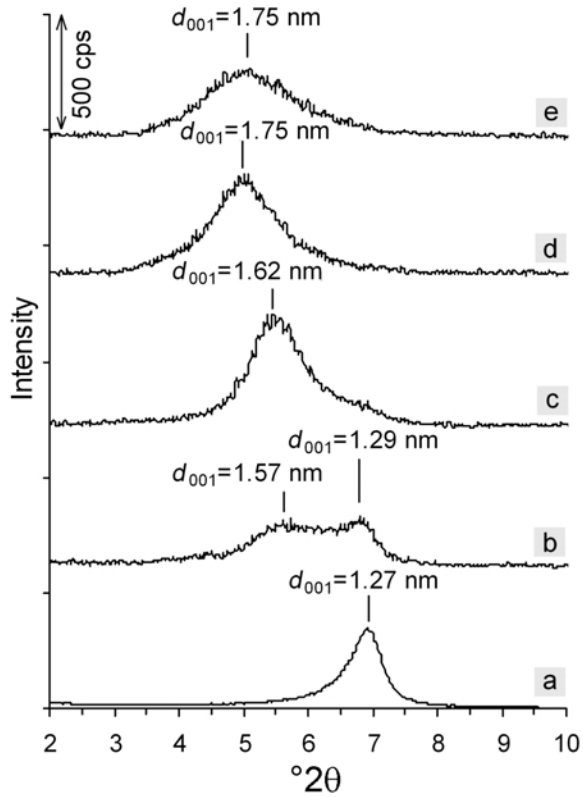

Figure 3. XRD patterns of (a) Na^+-saponite, and Na^+-saponite/protamine biocomposites with protamine loadings of (b) 25 mg/g, (c) 50 mg/g, (d) 100 mg/g, and (e) 400 mg/g. The patterns are offset by 500 cps and the intensity of (a) was divided by ten.

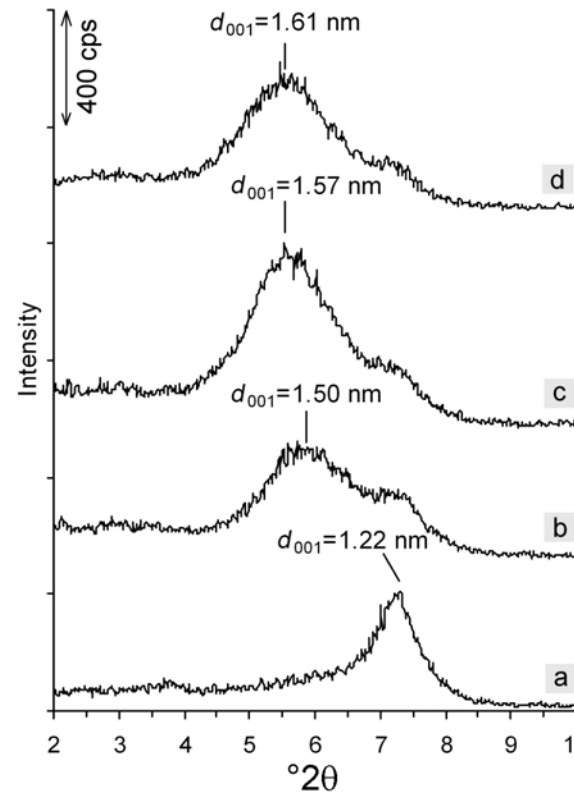

Figure 4. XRD patterns of Cs^+-saponite/protamine biocomposites with protamine loadings of (a) 25 mg/g, (b) 50 mg/g, (c) 200 mg/g, and (d) 250 mg/g. The patterns are offset by 500 or 600 cps.

The XRD profiles of papain-saponite composites are shown in Figure 5. The original freeze-dried Na^+-saponite has a d_{001} spacing of 1.27 nm of a monolayer hydrate. Upon adsorption of papain, this d_{001} line broadens at its small 2θ side, then shifts to 1.46 nm and 1.70 nm while broadening. At the greatest papain loadings, a supplementary peak at 2.94–2.54 nm became clearly visible, but Cs^+-saponite (not shown) did not expand upon adsorption of papain.

Thermogravimetry. Figure 6 shows the weight-loss curves and the corresponding first derivative curves of Na^+-saponite, protamine, and papain, respectively.

For Na^+-saponite (Figure 6), three weight-loss regions are identified: (1) desorption of physisorbed water below 120ºC, amounting to 12.7 wt.%; (2) a continuous weight loss between 300ºC and 750ºC of 1.5 wt.% (the present authors suggest that it is H_2O and CO_2; the water comes from some dehydroxylation, the CO_2 from the burning of the residual carbonaceous material on the saponite); and (3) dehydroxylation of structural OH groups at 820ºC, giving a weight loss of 3.3%.

Protamine (Figure 6) lost 10 wt.% below 175ºC. This is ascribed here to loss of physisorbed water. The molecule decomposes in O_2 in two steps. In the first step a sharp weight loss is observed with maximum of the first derivative at 300ºC. The second step is a continuous weight loss up to 650ºC. At 650ºC, all protamine was burned away.

Papain (Figure 6) contains 5.8 wt.% of physisorbed water, which is desorbed between room temperature and 150ºC. Between 175ºC and 600ºC, the molecule decomposes in four steps. The corresponding maxima in the first derivative curve are at 190ºC, 300ºC, 440ºC, and 560ºC. A residual fraction of 8.3% of papain could not be burned and the chemical composition of that fraction was not determined.

Figure 7a shows typical weight-loss traces of protamine-saponite and papain-saponite biocomposites. Weight loss for the protamine-saponite biocomposite below 200ºC was typically ~5 wt.%, which is significantly less than the 12.7 wt.% weight loss of Na^+-saponite in the same temperature range. Weight loss in this temperature range is ascribed to physically sorbed water on saponite and protamine. Above 200ºC, weight loss maxima occurred at 306, 620, and 775ºC. The 306ºC weight loss corresponds to the sharp weight loss of pure protamine observed at 299ºC (Figure 6). At higher temperatures, the weight loss profile of adsorbed

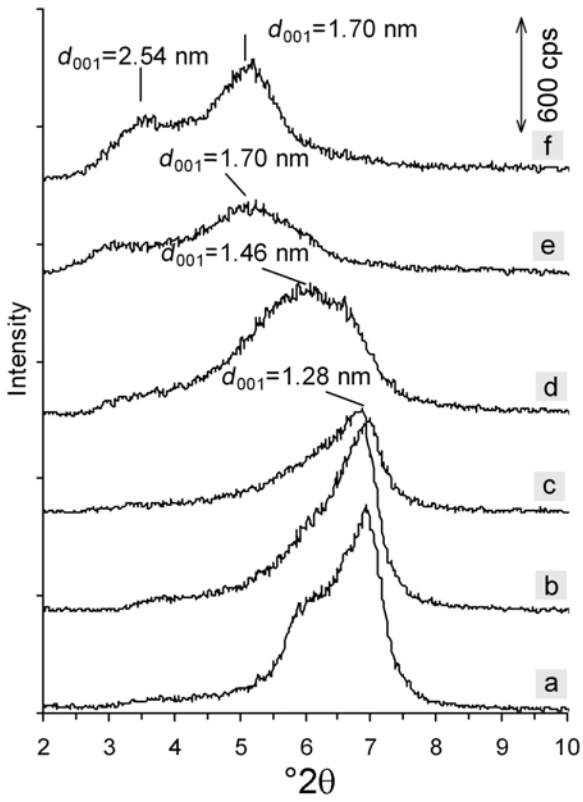

Figure 5. XRD patterns of (a) Na^+-saponite, and Na^+-saponite/papain biocomposites with papain loadings of (b) 25 mg/g, (c) 50 mg/g, (d) 100 mg/g, (e) 400 mg/g, and (f) 550 mg/g. The patterns are offset by 500 cps.

protamine was significantly different from that of the pure compound. In the latter case, a continuous weight loss was observed up to 650ºC, at which temperature all protamine was burned away without residue. In the biocomposite, a two-step weight loss was observed with maxima in the derivative curve at ~620ºC and 775ºC. The sharp dehydroxylation peak of the original Na^+-saponite at 820ºC broadens with increasing protamine loading to disappear almost completely at the greatest loading (Figure 7). This peak may have broadened to such an extent that it became invisible or it shifted to a lower temperature and overlapped with the 780ºC weight loss. Whatever the case, the amount of adsorbed protamine was calculated from the total weight loss above 200ºC, taking into account the loss due to dehydroxylation of saponite. These calculated amounts were used to construct the isotherms in Figure 2.

A representative weight-loss curve and its derivative for papain-saponite biocomposites are given in Figure 7b; those of papain are given in Figure 6. Weight loss occurred at low temperature (<200ºC), which is ascribed to desorption of water, similar to the biocomposite with protamine. As for papain, the weight loss above 200ºC is ascribed to decomposition of papain and desorption of the decomposition products and to the dehydroxylation of saponite. The difference with protamine is that four desorption peaks are observed instead of three: 305–310ºC, 508–530ºC, 670–700ºC, and 774–784ºC. Papain was also decomposed in four steps (Figure 6), but the temperatures were quite different: 190ºC, 300ºC, 440ºC, and 560ºC. The weight-loss curve of papain also shows a residue of 8.3 wt.%. The weight losses of the biocomposites above 200ºC were used to construct the isotherms of Figure 2, taking into account the dehydroxylation of saponite, but not the residue, which might remain on the surface of saponite. The absence of the 190ºC loss peak in the biocomposite is an indication that the adsorbed papain is not the same as the original papain, *i.e.* a chemical transformation of papain occurred in the course of the experiments.

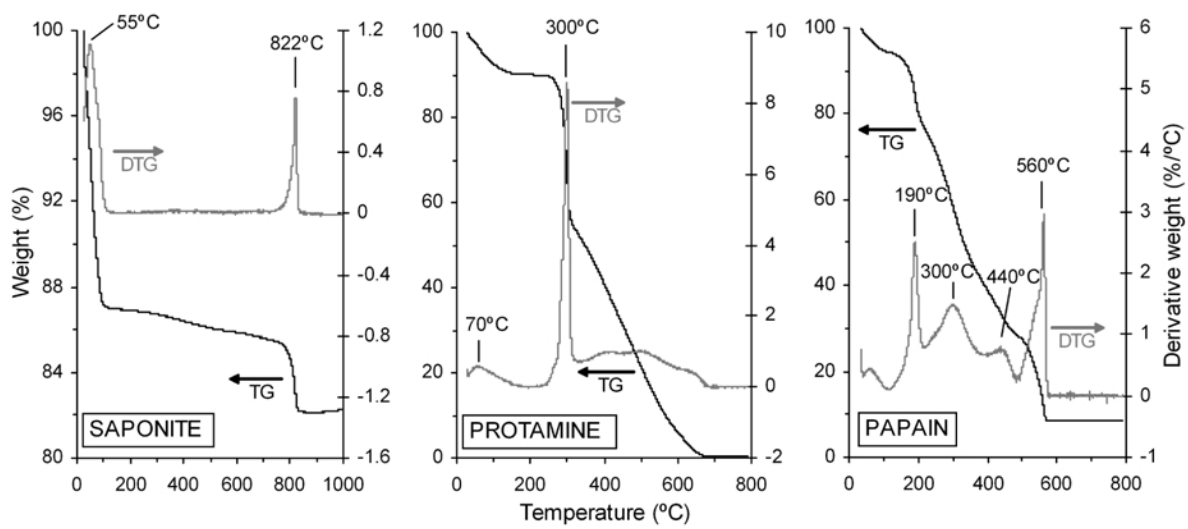

Figure 6. Weight-loss curves (TG) and the corresponding first derivative curves (DTG) of Na^+-saponite, protamine, and papain.

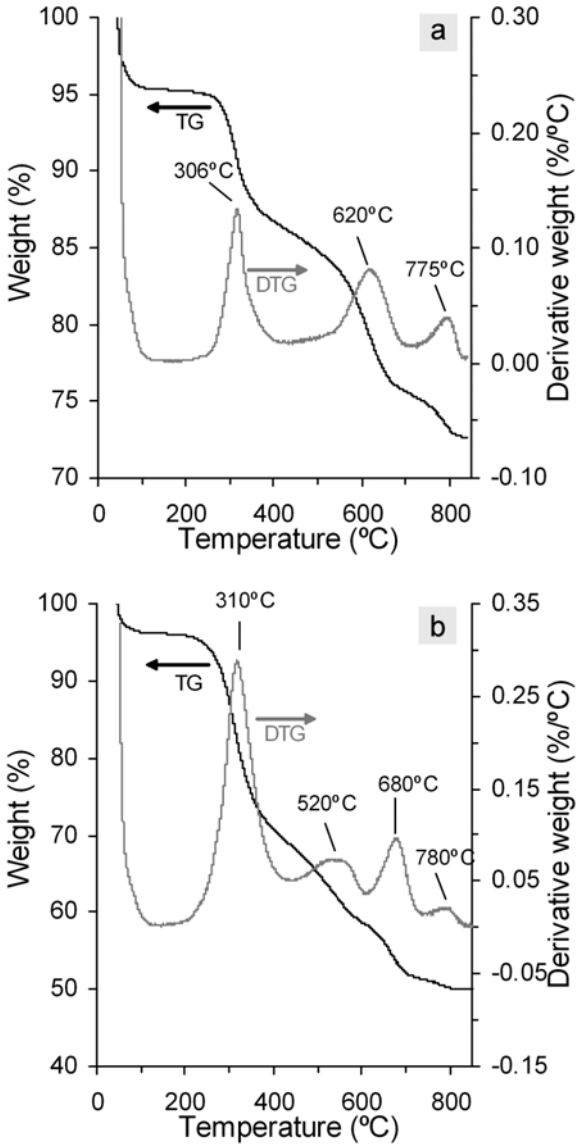

Figure 7. Thermogravimetric weight-loss and first derivative plots for (a) Na-saponite/protamine biocomposite with 398 mg protamine/g; (b) Na-saponite/papain biocomposite with 876 mg of papain/g.

DISCUSSION

Several phenomena have been observed in the adsorption of protamine and papain on saponite: ion-exchange adsorption, intercalation, adsorption on external surfaces, and decomposition of proteins.

Ion-exchange adsorption

The adsorption of protamine is clearly an ion-exchange process. The amount of Na^+ released is, within experimental accuracy, equal to the CEC of Na^+-saponite. Thus, 100% exchange has been achieved. Three interesting features of this ion-exchange reaction are noted:

(1) Na^+ release was complete, *i.e.* equals the CEC, before the maximum amount of adsorbed protamine was reached. This is due to the weak ionic strength of the exchange solutions with small protamine contents. In water, the equilibrium

$$Na^+\text{-saponite} \rightleftharpoons Na^+(1-x)-\text{saponite} + xNa^+$$

is established with x the fraction of the CEC, which is released in solution. In the present case, 0.23 mmol/g Na^+ or $x = 0.22$ in the absence of protamine, *i.e.* in pure water. For Cs^+-saponite, an analogous equilibrium can be written but the amount of Cs^+ released is only 0.04 mmol/g. This indicates that Cs^+ was preferred over Na^+ on the surface of saponite, a well established fact in ion exchange of smectites (Maes and Cremers, 1978).

(2) The ion exchange of protamine was partial in the case of Cs^+-saponite. Only 36% of the Cs^+ was exchangeable for protamine. This number was obtained from a comparison of the release of Na^+ and Cs^+ in solution and from the adsorbed amounts of protamine. This leads to the conclusion that Cs^+-saponite consists of 36% expandable clay layers and 64% non-expandable clay layers.

(3) The average charge per protamine molecule is 13.1−13.5, both for exchange of Na^+-saponite and Cs^+-saponite. This is smaller than +20, calculated from the amino acid composition of protamine and from the pK values of typical acidic and basic groups of proteins in aqueous solution (http://www.chemie.fu-berlin.de/chemistry/bio/amino-acids_en.html). This charge difference may be caused by one or more of the following: (1) the amino acid composition of the protamine used is different from that of the standard protamine; (2) incomplete dissociation of protamine sulfate into sulfate anions and protamine cations; and (3) the pK values of the amino acids of adsorbed protamine are different from those in aqueous solution. The second is documented in the literature (Trojanek *et al.*, 2007; Amemiya *et al.*, 2003). The following ion-exchange reaction can be envisioned:

$$[\text{PROT-}(SO_4)_{10-n}]^{2n+} + Na^+-\text{SAP} \rightleftharpoons \text{PROT}-(SO_4)_{10-n-m} - \text{SAP} + (n+m)SO_4^{2-} + 2(n+m)Na^+$$

Where PROT stands for protamine; n is the number of sulfate anions in equilibrium with protamine-sulfate in aqueous solution, and m is the amount of sulfate released upon ion exchange. A charge of +13.1−13.5 would indicate then that 3.2−3.5 sulfate ions are co-adsorbed with each protamine molecule. However, in a separate study of layer-by-layer thin-film formation of protamine and Na^+-saponite, no sulfate was detected in the films by FTIR (Szabó *et al.*, 2007). Clearly a more detailed study is necessary to establish the stoichiometry of the exchange reaction.

The third reason is reminiscent of the classical research of Mortland *et al.* (1963). As the water content of the smectites decreases, ammonia is increasingly

converted to ammonium. This is attributed to the increased acidity of the residual water in the interlamellar space. The same might occur in the biocomposites, which have a residual water content of 5 wt.% or less, considerably less than 12.7 wt.% of the monolayer hydrate of an air-dry Na^+-saponite. One can also look at the problem from a different viewpoint. In dilute solution, the activity of water is taken as 1. The biocomposites contain no large excess of water so the activity of water must be taken into account explicitly. The following is an example of a basic group:

$$=NH_2^+ + H_2O \rightleftharpoons -NH + H_3O^+ \text{ with } \quad K_A^N = \frac{a_{NH} \times a_{H^+}}{a_{NH_2^+} \times a_{H_2O}}$$

and this is an example of a carboxylic group:

$$=COOH + H_2O \rightleftharpoons -COO^- + H_3O^+ \text{ with } \quad K_A^C = \frac{a_{COO^-} \times a_{H^+}}{a_{COOH} \times a_{H_2O}}$$

In the papain-adsorption experiments, ion exchange is also operative. However, several data support the suggestion that papain is partially decomposed in the course of the experiments: (1) the (C:N) ratios decrease with increasing loading; (2) the ratio (Na^+ released/papain adsorbed) is erratic and exceeds the expected charge of papain by a factor of 5–10; (3) the low-temperature decomposition peak of papain at ~190ºC is absent in the case of adsorbed papain. A supplementary analysis of decomposition products in the exchange solutions before and after the adsorption experiments is necessary to obtain details of the decomposition and adsorption processes going on. In any case, the decrease in the C/N ratio with increasing loading is indicative of decomposition of papain in solution and preferential adsorption of N-containing fragments, because they are probably positively charged.

Finally, the difference between the adsorption isotherms obtained with BCA™ analysis and those obtained with thermogravimetry needs to be commented upon, certainly in the case of protamine. With the BCA™ method the amount of protamine in the equilibrium solution is measured. In order to construct the thermogravimetric isotherms, the amount of adsorbed protamine is measured from weight losses after washing and freeze-drying of the biocomposites. The difference between both series of experiments was the washing and freeze-drying of the samples prior to thermogravimetric measurement. As a consequence, only the quantitatively adsorbed fraction in the vertical parts of Figure 1 was measured thermogravimetrically. The remaining fraction was weakly adsorbed protamine, which was a significant fraction with 230–250 mg/g both on Na^+- and Cs^+-saponite.

Interpretation of the results was not so clear for papain sorption due to partial decomposition. The first 100 mg/g of adsorbed papain can be ascribed to the external surfaces of saponite. Once the external surface was covered, papain and papain decomposition products were distributed between liquid and solid phases. Partial intercalation of Na^+-saponite occurred and the adsorbed papain could not be washed away, but no intercalation of Cs^+-saponite occurred and all papain in excess of 100 mg/g could be washed away.

Intercalation

The protamine-saponite biocomposite XRD patterns indicate protamine intercalation. The basal spacing of Na^+-saponite reached 1.75 nm for a loading of 394 mg/g. This basal spacing corresponds to an interlayer expansion of 0.79 nm for a saponite single layer thickness of 0.96 nm. This interlayer expansion is interpreted to indicate a monolayer of densely packed protamine molecules on external and internal saponite surfaces. The surface areas occupied by a densely-packed monolayer were calculated by assuming the adsorption of 400 mg/g of protamine molecules with the spherical diameters listed in Table 1 (Table 6). Surface areas are calculated for a hexagonal close packing (hcp), where the space-filling factor is 0.89, and for cubic close packing (ccp) where the space-filling factor is 0.79. The two numbers for each surface area correspond with the two diameters of Table 1 (DLS and hard-sphere diameters). The interlamellar surface area occupied by adsorbed protein was multiplied by two because both upper and lower saponite interlayer surfaces are covered. The numbers in Table 6 indicate that protamine occupies 440–612 m^2/g, the exact number depending on the diameter assumed and on the packing. Thus, in the case of Na^+-saponite, 59–82% of the theoretical surface area of 750 m^2/g was occupied by protamine molecules.

However, the spacing of 0.8 nm is smaller than the diameters of protamine listed in Table 1. Adsorbed protamine molecules might not be spherical but rod-like with rod diameters of 0.8 nm, due to the low water content, strong surface-protein interactions, and intercalation. The dark-field electron microscopy study by Ottensmeyer *et al.* (1975) describes fish protamines as rod-like molecules consisting of loose helices with turns of various sizes.

The basal spacing of Cs^+-saponite reached 1.61 nm, which is 0.14 nm less than Na^+-saponite. However, the XRD peak was broad and encompassed a range of

Table 6. Estimated surface areas occupied by protein molecules.

	Protamine	Papain
Amount adsorbed (mg/g)	400	100
Molecules/g	5.35×10^{19}	2.62×10^{18}
Surface area hcp (m^2/g)	440–544	33–35
Surface area ccp (m^2/g)	504–612	37–40

hcp: hexagonal close packing
ccp: cubic close packing

spacings. A residual peak of Na^+-saponite at 1.21−1.24 nm was evident at all protamine loadings. These data indicate that the expansion of Cs^+-saponite is incomplete. The same conclusion was obtained from the ion-exchange data. Cs^+-saponite consists of two fractions: an expandable fraction, which undergoes ion exchange and interlamellar expansion due to protamine adsorption, and an inexpandable fraction. If the amount of Cs^+ released (0.41 mmol/g), expressed as a fraction of the amount of Na^+ released (1.15 mmol/g), is taken as a measure of the expandable fraction, the value obtained is 0.36 or 36%. This is the same as the ratio of strongly bound protamine on Cs^+-saponite to strongly bound protamine on Na^+-saponite, or 145 mg/394 mg = 0.37. Thus, in the expandable fraction of Cs^+-saponite, a monolayer of protamine molecules was formed in exactly the same way as in Na^+-saponite.

Interlayer expansion by papain adsorption was only observed for Na^+-saponite; Cs^+-saponite did not expand. The external surfaces of both Na^+- and Cs^+-saponite adsorbed 100 mg of papain/g. Expansion of Na^+-saponite started for loadings that exceeded 100 mg/g. A loading of 100 mg of papain/g corresponds to 2.62×10^{18} molecules. The diameter of papain (Table 1) and the number of molecules adsorbed were used to calculate the surface areas (Table 6), which are comparable to typical smectite (external) surface areas measured by N_2 adsorption (Van Olphen and Fripiat, 1979).

The XRD patterns of protamine and papain biocomposites give indications of weak broad lines, corresponding to *d* spacings in the 2.5−3.15 nm range. For papain they are smaller than expected, on the basis of hard-sphere or DLS diameters listed in Table 1. Possible reasons for the discrepancy between molecular size and interlayer space are as follows: (1) the adsorbed molecule environment is significantly different from that in aqueous solution or in single crystals; in particular there is much less water; (2) the proteins maximize surface contact and might undergo conformational changes; (3) the effect of partial decomposition of papain adsorbed to saponite is unknown.

Thermogravimetry

Because the protein decomposition products have not been analyzed, a detailed discussion of the thermogravimetric data is beyond the scope of this paper. The first decomposition step of the protamine-saponite biocomposite at 306ºC corresponds to the 299ºC decomposition step of protamine. Weight losses from the protamine-saponite biocomposite decomposition products occurred at greater temperatures than for protamine without saponite. Clearly, carbonaceous residues were strongly adsorbed to saponite and required higher temperatures (770−780 *vs.* 650ºC) for complete decomposition and emission as gases (*e.g.* CO_2). These carbonaceous residues strongly affect the dehydroxylation of saponite as shown by the disappearance of the sharp weight loss at 820ºC for both protamine and papain biocomposites. Dehydroxylation still occurred but was either smeared out over a broad temperature range or was shifted to 780ºC and merged with losses from carbon residues that were burned away at that temperature.

CONCLUSIONS

Many parameters must be considered in the adsorption of proteins to smectites such as saponite. First, the protein molecular weight, charge, shape, size, and stability must be considered. Second, the CEC, the nature of the exchangeable cation, and the interlayer and external surfaces of saponite must be considered. Some of these parameters have been examined in this study. Positively charged protein was ion-exchanged and the exchange process was complete as indicated by Na^+ release after protamine and papain adsorption. Intercalation occurred and was measured by the increased *d* spacings. Complete intercalation occurred for protamine, but papain was only partially intercalated. A similar ion-exchange reaction occurred for Cs^+-saponite. However, only 36% of Cs^+ was accessible for protamine ion exchange and protamine was able to expand only 36% of Cs^+-saponite. Ion exchange of papain with Cs^+-saponite was only ~20% of the CEC and no intercalation occurred. However, the number of papain molecules adsorbed cannot be determined because papain partially decomposes during the experiments. When ion-exchange isotherms were constructed from an analysis of protamine concentrations in the equilibrium solution, ion-exchange adsorption was followed by weak physical adsorption. The physically adsorbed protamine was not intercalated and could be largely washed off. Papain adsorbed first to external surfaces and, with greater equilibrium papain concentrations, was partially intercalated into Na^+-saponite. At even greater papain concentrations, papain molecules were distributed between the solid and liquid phases. No papain intercalation into Cs^+-saponite was observed and papain adsorption in excess of external surface adsorption could be washed off. The papain adsorption data need to be confirmed by studies using proteins of similar molecular weight and charge, but chemically stable in solution and in the presence of saponite or other clay minerals.

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