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Deamination of aspartic acid by aspartase–Ca-montmorillonite complex

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Abstract

Aspartase was adsorbed by Ca-montmorillonite at an initial pH of 7.0 and 25°C; the equilibrium adsorption isotherm was of L (Langmuir) type with a maximum adsorption of 0.22 mg aspartase protein/mg clay. The d_{001} spacing of the aspartase–Ca-montmorillonite complex was 26.5 Å when 0.20 mg aspartase protein was adsorbed per mg clay, indicating the intercalation of the aspartase in the clay structure. After washing, the amount of aspartase protein remaining immobilized on the clay surfaces was 0.0073 mg/mg clay. The activity of free and immobilized aspartase in the deamination of aspartic acid was studied in unbuffered systems at pH of 7.0 to avoid the effect of the buffer on the surface reactivity of montmorillonite. During the first 2 h of the reaction no ammonium was released from aspartic acid in the presence of aspartase–Ca-montmorillonite complex, probably because there was insufficient time for the expansion and swelling of the mineral and/or the diffusion of the aspartase molecules to the interlayer edges and external surfaces of montmorillonite or the infusion of the substrate (aspartic acid). After 3 days, the specific activity of the aspartase immobilized on Ca-montmorillonite was slightly higher than that of free aspartase in homogenous solution. This was evidently due to the preservation of the activity of the adsorbed aspartase, the contribution of the catalytic power of the montmorillonite to the deamination of aspartic acid, and the complementary effects of the enzymatic and abiotic catalysis. The purification treatment of the montmorillonite did not show a significant effect on either the adsorption or the activity of the immobilized aspartase.

Keywords: Activity; Adsorption; Immobilization; Aspartase; Montmorillonite; Enzyme–clay complex

1. Introduction

Enzymes accumulating in soil have a biological significance as they participate in the biological cycles of elements [1] and play an important role in the transformations of organic and mineral compounds. According to Burns [2] enzymes are to be immobilized by clay minerals and humic colloids in soil environments. Some aspects of interactions of proteins, including enzymes, with clays have been reviewed by Theng [3] and Boyd and Mortland [4]. The persistence and stability of enzymes and other proteins in soil are generally attributed to their association with clay and humus [5,6]. Expandable clays such as smectites have a high affinity for proteins [7,8] and they evidently protect such proteins from proteolytic hydrolysis [9] and/or microbial decomposition [5]. On the other hand, clay minerals exert some catalytic

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activity. Mortland [10] suggested that smectite lattice could play the role of 'pseudo-enzyme' in the deamination of glutamic acid. Naidja and Siffert [11,12] compared the activity of homoionic montmorillonite saturated with different cations to that of pure enzymes in the deamination of glutamic acid and decarboxylation of citric acid. In soils and plants, deaminating enzymes play a paramount role in the fate of nitrogen in the form of ammonium. Studies of the activity of some deaminases in soil have been carried out, including those of Frankenberger [13] on the kinetic properties of L-histidine ammonia–lyase (EC 4.3.1.3) in different soils. Burton and McGill [14] employed a kinetic analysis to examine the components of L-histidine ammonia–lyase activity in soil, their stability, and the implications for the control of this enzyme. More recently, Frankenberger and Tabatabai [15,16] determined the kinetic parameters of asparginase in soil and investigated the effect of pesticides on the activity of this enzyme. Aspartase is present in higher plants [17] and its activity has been observed in soil micro-organisms [18,19] such as bacteroids, the specific symbiotic forms of *Rhizobium* in plant root nodules [20]. Also, aspartase is one of the two key enzymes known to mediate aspartate catabolism in bacterial production of Krebs (TCA) cycle intermediates [21]. Furthermore, according to Kretovich et al. [18], the biosynthesis of aspartate catalyzed by aspartase plays a very important role in the assimilation of ammonia in nodules. Therefore, the activity of aspartase (EC 4.3.1.1) immobilized on clay minerals in the deamination of aspartic acid which is an important source of nitrogen in soil, and one of the most common amino acids in nature [22,23], deserves attention.

The objective of the present study was to examine the deamination of aspartic acid by aspartase–Ca-montmorillonite complex. The adsorption of aspartase on Ca-montmorillonite was investigated, the aspartase–clay complex formed was characterized and the rate of ammonia formation from aspartic acid in the presence of aspartase–Ca-montmorillonite complex was studied.

2. Experimental

2.1. Montmorillonite preparation

The montmorillonite sample (SWy-1 Crook County, WY, USA) was obtained from the Source Clay Repository of the Clay Mineral Society. The purification treatments were previously described by Naidja and Huang [24].

2.2. Biochemical compounds

Aspartic acid ($HOOC-CH_2-CH(NH_2)-COOH$) of high purity grade and aspartase containing 40% protein were supplied by the Sigma Chemical Company. The aspartase (EC 4. 1. 3. 1), which was produced from *Hafnia alvei (Bacterium cadaveris)* ATC 9760, was stored in a desiccated state at $-5°C$.

2.3. Reagents

All reagents (organic and inorganic compounds) of analytical grade were obtained from Sigma or BDH.

2.4. Adsorption experiments

Ca-montmorillonite (natural and/or purified clay) was suspended in boiled–deionized–distilled (bdd) water (2 mg/ml). After ultrasonification (3 min), 1 ml of toluene per liter [25] was added to prevent microbial growth, and the pH of the suspension was adjusted to 7.0 by addition of 0.1 M NaOH. The aspartase solution (1.6 mg aspartase protein/ml) was prepared in phosphate buffer at pH 7.0 and stored in an ice bath during use. One ml (2 mg) portions of clay suspension were pipetted into a series of polysulfone flasks, and different amounts of the aspartase protein (from 0.04 to 1.6 mg) were added. The suspension was shaken until equilib-

rium was reached (for 2 h at 25°C). After centrifugation at 19,000 g, the amount of the aspartase protein remaining in solution was determined by the method of Bradford [26] using bovine serum albumin as the standard. The amount of protein adsorbed was obtained using the formula of Thomas et al. [27]:

$$Q_a = (C_0 - C_e)V/W$$

where Q_a is the amount of protein adsorbed per unit weight of adsorbent (mg/mg), C_0 is the initial concentration of protein (mg/ml), C_e is the equilibrium concentration of protein (mg/ml), V is the solution volume (ml) and W is the adsorbent weight (mg).

2.5. Preparation of aspartic acid solution and adjustment of the pH

Aspartic acid in the amount of 0.8874 g was suspended in 40 ml of bdd water. The solution, which had an initial pH of 2.9, was stirred at 25°C. NaOH solution (0.1 M) was added dropwise until the pH approached 7.0, whereupon the turbidity of the solution disappeared, indicating complete dissolution of the aspartic acid. The final volume was then brought to 100 ml by addition of bdd water, and the pH adjusted to 7.0. The final concentration of the aspartic acid solution was 66.7 mM. At pH 7.0, aspartic acid has a net negative charge ($^-OOC–CH_2–CH(NH_3^+)–COO^-$) [24]. Aspartase solution (1 mg/ml) was prepared in 50 mM phosphate buffer at pH 7.0 and maintained in an ice bath until used. The pH of the Ca-montmorillonite suspension was adjusted to 7.0 within 24 h by addition of 0.1 M HCl or 0.1 M NaOH. The initial pH of the aspartic acid solution in the presence of aspartase, Ca-montmorillonite or aspartase–Ca-montmorillonite complex was 7.0 $\pm$ 0.1, and the final pH after reaction was 6.9 $\pm$ 0.1.

2.6. X-ray diffraction analysis

X-ray diffraction analysis (XRD) was carried out with a Rigaku D/MAX-RBX diffractometer (Rigaku Company, Tokyo, Japan), with CuKα radiation filtered by a graphite monochromator at settings of 50 kV and 150 mA. The enzyme–clay complexes, before and after the reaction with aspartic acid, were smeared on glass slides and maintained at a relative humidity (RH) of 50% at room temperature. Replicate portions of the complexes were heated at different temperatures (40–250°C) and kept in a desiccator containing silica gel until they were examined.

2.7. Infrared analysis

Fourier transform infrared (FTIR) absorption spectra of the free aspartase and the aspartase–Ca-montmorillonite complexes (samples were washed with bdd water and air dried) were recorded on KBr disks, which contained 1% of the sample by weight, using Biorad 3240 SPS spectrophotometer (Cambridge, MA, USA). The spectra were referenced against a single beam spectrum of pure KBr and expressed in absorbance units. The spectrum of the Ca-montmorillonite was subtracted from that of the aspartase–Ca-montmorillonite complex to obtain the differential FTIR spectrum of adsorbed aspartase.

2.8. Ammonium formation as a function of time

The different systems were treated with toluene as described above.

2.8.1. Free enzyme

The assay procedure of Williams and Lartigue [28] to study the activity of aspartase was used. 0.3 ml of $MgSO_4 \cdot 7H_2O$ (60 mM), 0.3 ml of EDTA (3 mM), and 0.3 ml of aspartic acid (66.7 mM) were pipetted in a series of flasks. The solution was adjusted to pH 7.0 as described above. Then 0.3 ml of the aspartase solution (1 mg/ml phosphate buffer) containing 0.12 mg of protein, was added. The final volume was brought to 10 ml by addition of bdd water adjusted to pH 7.0.

2.8.2. Immobilized enzyme

0.3 ml of the aspartase solution, containing 0.12 mg of protein, was added to 2 ml of clay suspension (4 mg of montmorillonite), the enzyme–clay suspension was shaken for 2 h at pH 7.0 and 25°C. After centrifugation at 19,000g, the supernatant of the aspartase–clay suspension was decanted. The sediment was washed with bdd water until no protein was detected in the washings, using the method of Bradford as mentioned above. According to Guibault [29], adsorption is one of the immobilization procedures, thus, aspartase remaining bound to the clay after washing is considered to be immobilized. Aspartic acid solution was added to the aspartase–clay complex in the presence of $MgSO_4 \cdot 7H_2O$ and EDTA as described above.

After a series of a short and/or long reaction periods in a water bath shaker at 25°C, the ammonia released, which is converted to ammonium at the pH of the reaction, was determined by a steam distillation technique as described by Keeney and Nelson [30], using aspartic acid in the absence of the catalysts as the blank. The absence of dissolved gaseous ammonia in a boric acid solution used as a trap during the reaction indicated that ammonia release from the reaction was totally converted to ammonium. The absence of ammonium in the aspartic acid–Ca-montmorillonite system during a short reaction period (2 h) also indicated that no ammonium was present as an impurity in the Ca-montmorillonite. The activity of free and immobilized aspartase was quenched by addition of 25% trichloroacetic acid. The measurements are the averages of two replicates.

2.9. Examination of microbial growth

The systems studied were examined for any microbial growth during the reaction period of 3 days, using the method of Koch [31]. From duplicate portions of the aspartic acid solution in the presence of free or immobilized aspartase as described above, 0.1 ml was taken and poured, after dilution, onto the agar surface of the Petri plate. The spread plate technique was used for total counts of bacteria.

3. Results

3.1. Adsorption isotherm of aspartase on Ca-montmorillonite

To estimate the adsorption capacity (X_m) of the clay, the adsorption data were fitted to the Langmuir equation using a least square regression ($r^2 = 0.979$ and $p < 0.010$);

$$X = X_m KC/(1 + KC)$$

where X is the amount of protein adsorbed/unit mass of clay (mg/mg), X_m is the adsorption capacity (maximum amount of protein adsorbed/unit mass of clay), K is the adsorption/desorption equilibrium constant related to the bonding energy and C is the equilibrium concentration of the aspartase protein in solution after adsorption. The adsorption isotherm at pH 7.0 (Fig. 1) belongs to the L (Langmuir type) as classified by Giles et al. [32]. At low concentrations (up to 0.013 mg/ml), the affinity of the clay surfaces for the aspartase protein molecules is clearly shown by the initial steep slope, and almost all of the protein added was retained by the montmorillonite surfaces. With increasing equilibrium concentration of aspartase, the adsorption tapered off as the adsorption sites were filled, and the maximum amount of the protein adsorbed reached 0.22 mg/mg clay. Aspartase is a tetramer with a molecular weight (MW) of 180,000 and an isoelectric point (pI) of 4.8 [28]. At pH 7.0, the net charge of the enzyme is negative, and the mineral surface is also negatively charged. However, adsorption of aspartase on Ca-montmorillonite evidently took place. Also similarly, several other enzymes and proteins are reported to have been adsorbed by clay minerals at pH values above their pI [33,34]. Thus, Harter and Stotzky [35] showed that catalase (MW $\sim$ 238,000; pI = 5.7) and α-casein (MW = 121,000; pI = 4.0) were ad-

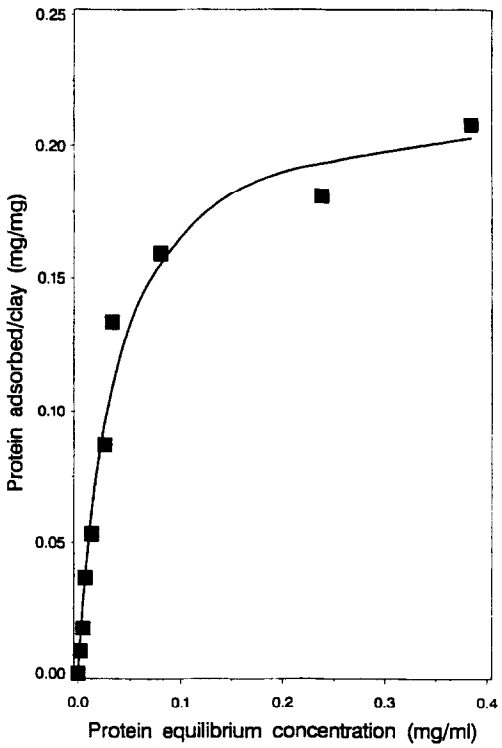


Fig. 1. Adsorption isotherm of aspartase on purified Ca-montmorillonite at an initial pH = 7.0 and 25°C.

sorbed by Ca-montmorillonite at pH 7.0. They offered two possible reasons for these observations: (1) the surface acidity of smectite is higher than the pH measured in suspension [36]; and (2) pI indicates only the pH at which the net charge of the molecule is zero, but proteins may have a positive charge at some 'loci' at pH above the pI. They assumed that other factors (such as the saturating cations and steric hindrance) have stronger effects on the adsorption of proteins than does the pH–pI relationship.

3.2. X-ray diffraction

X-ray diffraction analysis of aspartase–Ca-montmorillonite complexes showed an expansion of the mineral structure to $d_{001} = 26.5$ Å after adsorption of the enzyme, the natural and purified montmorillonite expanding to nearly the same extent (Fig. 2). The data show that the large molecule of aspartase (MW = 180,000) was intercalated between the montmorillonite layers. Garwood et al. [37] observed an expansion of 35 Å for the complex glucose oxidase (MW = 153,000)–Na-montmorillonite. They suggested a model of intercalation of the enzyme, assuming that the enzyme shape is more oblate in the intercalated state than in homogeneous solution. On the other hand, Harter and Stotzky [38] reported that the adsorption of a considerable amount of catalase (MW ~ 238,000) on Ca-montmorillonite did not result in expansion of the mineral structure. They concluded that the adsorption is entirely external. Recently, however, Fusi et al. [34] showed that catalase was intercalated by Ca-montmorillonite with an expansion of 19.8 Å (29.4–9.6 Å). This apparent contradiction may have arisen because Harter and Stotzky [38] used protein and clay from different sources and hence with different properties. Aspartase, besides being adsorbed in the interlayers, may also be adsorbed on the external surfaces. Considering the molecular weight, our results are in accord with those of Fusi et al. [34]. In view of the large molecular sizes of aspartase and catalase, the

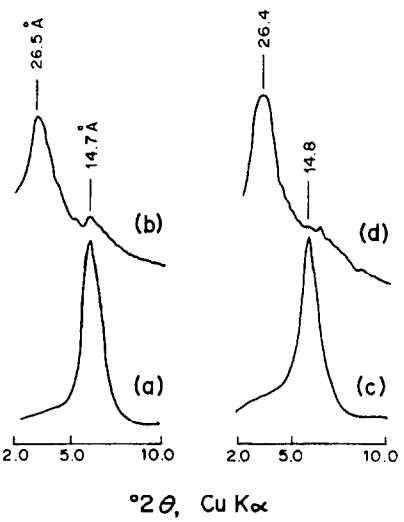


Fig. 2. X-ray diffraction patterns of the aspartase–Ca-montmorillonite complex (0.20 mg aspartase protein adsorbed/mg clay) at 50.0% RH and 25°C. (a) natural montmorillonite, (b) aspartase–natural montmorillonite complex, (c) purified montmorillonite and (d) aspartase-purified montmorillonite complex.

Table 1
Effect of temperature on the d_{001} spacing of the aspartase–Ca-montmorillonite complex

Temperature (°C) [a]	d_{001} (Å)	
	(a) [b]	(b) [c]
25 (50.0% RH)	14.9	26.5
60	14.9	26.5
110	11.5	26.2
150	10.9	26.0
170	10.2	25.1
200	10.1	24.7
250	9.8	22.5

[a] Except for 25°C, the sample was heated for 2 h.
[b] Ca-montmorillonite (natural).
[c] Aspartase–Ca-montmorillonite complex.

molecules of these enzymes might be unfolded on the interlayer space as proposed by Lora [39]. After heating at increasing temperatures for 2 h, the d_{001} spacing of the aspartase–Ca-montmorillonite complex gradually decreased to 25.1 Å at 170°C and 22.5 Å at 250°C (Table 1). Fusi et al. [34] showed that when catalase–Ca-montmorillonite complex was heated at 110°C for 12 h, the d_{001} spacing decreased from 29.4 Å to 27.6 Å. Presumably, the enzymes broke down to some extent, because denatured owing to destruction of quaternary and tertiary structures.

3.3. Infrared spectroscopy

Before contact with natural Ca-montmorillonite, aspartase was prepared in phosphate buffer (pH 7.0) at 2 mg/ml. To eliminate the absorption bands due to the buffer, the differential spectrum of aspartase adsorbed on Ca-montmorillonite (Fig. 3) was obtained by subtracting the spectrum of Ca-montmorillonite after reaction with phosphate buffer in the absence of aspartase from the spectrum of Ca-montmorillonite after reaction with phosphate buffer in the presence of aspartase. The assignment of different bands of the free and adsorbed aspartase is summarized in Table 2. The amide A band (N–H stretching) at 3301 cm^{-1} characteristic of protein in solid crystalline form [40]

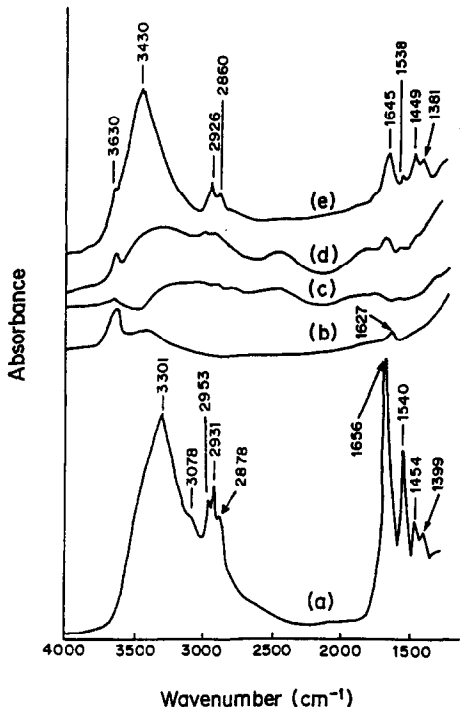


Fig. 3. Infrared spectra of aspartase bound on Ca-montmorillonite (0.029 mg aspartase protein/4 mg clay). (a) aspartase, (b) Ca-montmorillonite, (c) Ca-montmorillonite after reaction with the phosphate buffer solution in the absence of aspartase, (d) Ca-montmorillonite after reaction with the phosphate buffer containing aspartase and (e) immobilized aspartase, a differential spectrum of (d) minus (c).

Table 2
Fourier transform infrared absorption bands of the free aspartase and the bound aspartase on Ca-montmorillonite (natural clay)

Wavenumber (cm^{-1})		Assignment
(a) [a]	(b) [b]	
n.o. [c]	3630	νOH [d]
3301	3430	Amide A [e]
3078	NO	Amide B
2953	NO	νCH
2931	2926	νCH
2878	2860	νCH
1656	1645	Amide I
1540	1538	Amide II
1454	1449	δCH
1399	1381	δCH

[a] Free aspartase.
[b] Immobilized aspartase.
[c] n.o. = not observed.
[d] νOH band from the montmorillonite due to the incomplete subtraction in the differential spectrum.
[e] Amide A and B, strong and weak NH stretching vibration; Amide I, C=O stretching vibration; and amide II, NH deformation.

Table 3
Examination of microbial growth in the systems during a 3-day reaction period

System	Growth of microorganisms
	No treatment with toluene
aspartic acid	– [a]
aspartic acid–aspartase	+ [b]
aspartic acid–Ca-Mte	+
aspartic acid–aspartase–Ca-Mte	+
	Treatment with toluene
aspartic acid	–
aspartic acid–aspartase	–
aspartic acid–Ca-Mte	–
aspartic acid–aspartase–Ca-Mte	–

[a] No microbial growth detected (0 CFU/ml).
[b] Microbial growth evident ($\geq 2\times 10^3$ CFU/ml).

was shifted to 3430 cm^{-1} after adsorption. It was shown that the NH amide stretching frequency of acetamide was shifted from 3330 cm^{-1} to 3490 and 3500 cm^{-1}, respectively, after adsorption on Cu- and Ca-montmorillonite [41]. Whereas the amide B band (weak N–H stretching) at 3078 cm^{-1} was not evident after adsorption. Amide I band (C=O stretching) at 1656 cm^{-1} [42,43] in native protein was also shifted to 1645 cm^{-1} and amide II band (N–H deformation) at 1540 cm^{-1} [44] to 1538 cm^{-1}. The shifts to higher and lower absorption frequencies indicated a slight modification in protein structure when adsorbed or bound to clay surfaces [34].

3.4. Ammonium formation as a function of time

Table 3 shows that in the systems treated with toluene, growth of microorganisms was not evident during the reaction period. This ensures the absence of microbial activity in the deamination of aspartic acid.

3.4.1. Short period

Different factors (pH, temperature, inhibitors, cofactors) affecting free aspartase activity have already been studied [45–47]. Fig. 4 shows that, in contrast to the free aspartase, when aspartase was immobilized on Ca-montmorillonite (0.029 mg aspartase protein/4 mg clay) the ammonium release was undetectable during a short period of time (2 h). This observation can be explained in two ways. Firstly, the enzyme was deactivated when adsorbed on clay mineral surfaces. Secondly, a certain time was required for the expansion and swelling of the mineral, the diffusion of the enzyme to the interlayer edges and the external surfaces of the montmorillonite and the infusion of the substrate, aspartic acid, to the catalytic sites to take place. According to McLaren and Packer [48], the diffusion rate of an enzyme towards the surface of the adsorbent is slow, but the enzyme has a measurable mobility. To ascertain whether or not the protein molecule was deactivated by the montmorillonite, ammonium release should be measured during a longer reaction period.

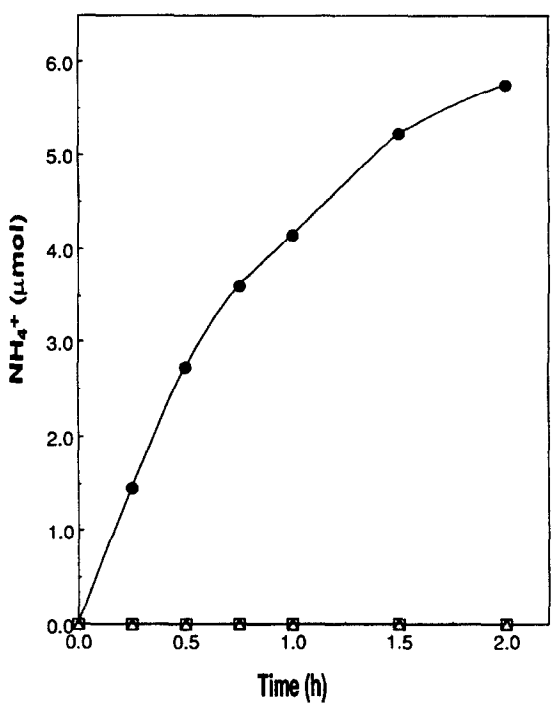


Fig. 4. Time function (short period) of ammonium release from aspartic acid in the presence of free or bound aspartase at pH 7.0 and 25°C. ● free aspartase (0.12 mg aspartase protein), □ immobilized aspartase (0.029 mg aspartase protein/4 mg clay), △ Ca-montmorillonite (4 mg).

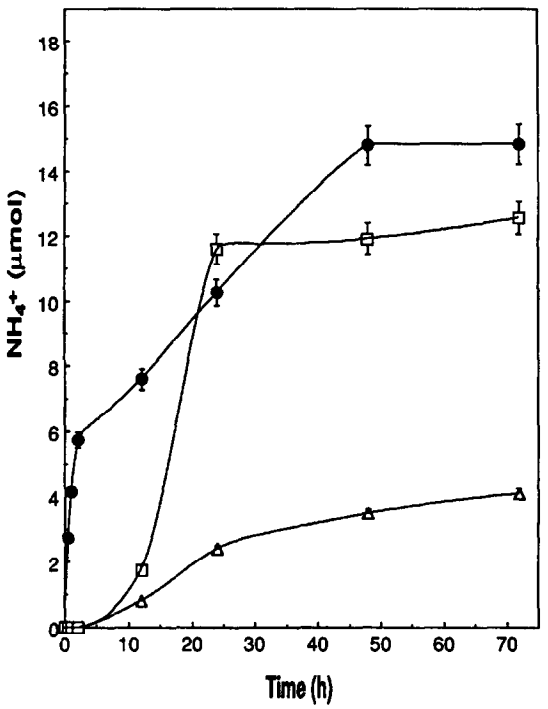


Fig. 5. Time function (long period) of ammonium release from aspartic acid in the presence of free or immobilized aspartase at pH 7.0 and 25°C. ● free aspartase (0.12 mg aspartase protein), □ immobilized aspartase (0.029 mg aspartase protein/4 mg clay), △ Ca-montmorillonite (4 mg).

3.4.2. Long period

The release of ammonium from aspartic acid during the time course (Fig. 5) showed a striking difference in the catalysis of the aspartic acid deamination by aspartase free in solution and the aspartase–Ca-montmorillonite complex. Compared with the immobilized enzyme, the ammonium release in the presence of free aspartase increased more rapidly initially and reached a plateau at a higher NH_4^+ level after 48 h. In the presence of the immobilized aspartase on Ca-montmorillonite, the ammonium release abruptly increased from 12 to 24 h, exceeding that caused by the free aspartase in solution at 24 h and then reached a plateau, below the free enzyme plateau. This fast increase of the amount of ammonium released from 12 to 24 h in the presence of aspartase–Ca-montmorillonite system suggests that there was a 12-h induction period for catalysis of deamination of aspartic acid by the immobilized enzyme. The amount of ammonium released in the presence of Ca-montmorillonite alone slowly increased but was much smaller than that released in the presence of aspartase, indicating a weak catalytic ability of the montmorillonite surfaces [10,11,49] in the abiotic deamination of aspartic acid. Compared to the free aspartase which was present in the amount of 0.12 mg aspartase protein in solution, the amount of aspartase protein bound to the clay was 0.029 mg/4 mg clay. Fig. 6 shows that the ammonium released per mg protein in the presence of aspartase–Ca-montmorillonite complex increased abruptly after 12 h, and then reached a maximum at 24 h. Despite the decline after 24 h (Fig. 6) due to the subtraction of the increasing amount of ammonium released through the abiotic catalysis of the montmoril-

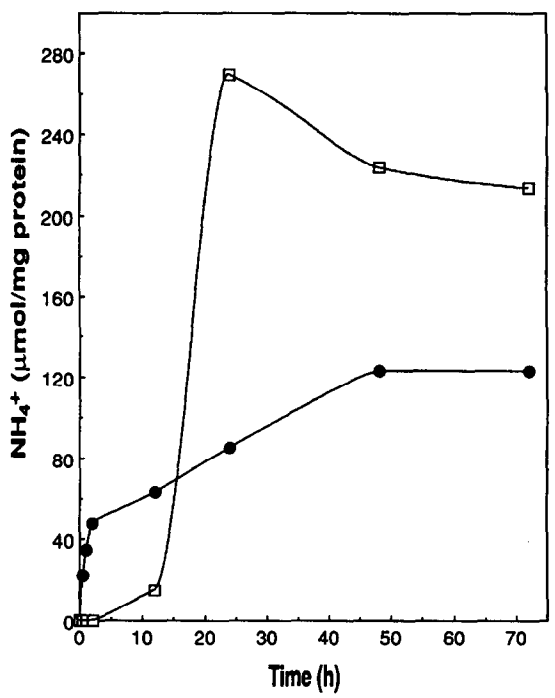


Fig. 6. Time function of ammonium release from aspartic acid per mg of the aspartase protein in the presence of free or bound aspartase at pH 7.0 and 25°C. ● free aspartase (0.12 mg aspartase protein) and □ immobilized aspartase (0.029 mg aspartase protein/4 mg clay).

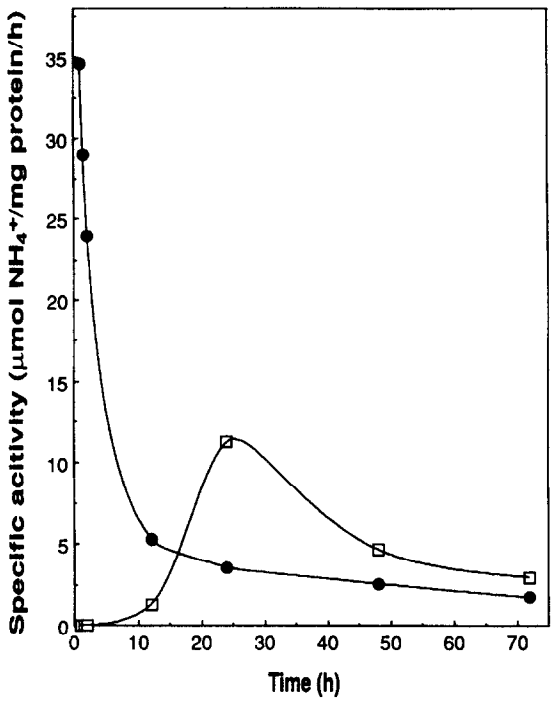


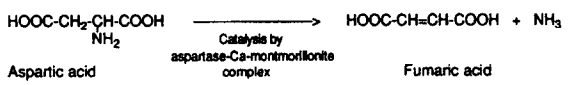
Fig. 7. Time function of the specific activity of free and the bound aspartase at pH 7.0 and 25°C. ● free aspartase (0.12 mg aspartase protein) and □ immobilized aspartase (0.029 mg aspartase protein/4 mg clay).

lonite (Fig. 5) from the total ammonium released, the amount of ammonium released per mg protein in the aspartase–Ca-montmorillonite system remained much higher than that released by the catalysis of free aspartase. As frequently observed in enzymatic reactions, the specific activity of free aspartase exhibited a decay curve versus time (Fig. 7). In contrast, the specific activity of the bound aspartase showed an initial lag period, indicating the retardation of the reaction caused by the clay mineral for the same reasons as discussed above. However the specific activity of the immobilized aspartase increased with increasing time up to 24 h (Fig. 7). Even after 24 h, the specific activity of the bound aspartase remained higher than that of free aspartase in solution.

Purification treatments of the clay had no effect on the deamination of aspartic acid (data not shown).

4. Discussion

In the present study, we examined the enzymatic deamination of aspartic acid by aspartase as influenced by Ca-saturated montmorillonite.



Enzyme properties could be modified after immobilization [50] which is carried out by different methods [29] including: (a) adsorption and (b) covalent cross-linking by bifunctional reagents. In the present study, the immobilization of aspartase by Ca-montmorillonite was carried out by adsorption to avoid the alteration of the surface and structural properties of montmorillonite by using bifunctional reagents. The aspartase bound to the clay surfaces by adsorption even after washing is considered immobilized.

The behavior of immobilized enzymes towards their substrates could be different from that in homogeneous solutions [3]. The retention of enzyme activity after immobilization depends on the characteristics of the protein molecule, the support and the type of attachment [8]. When clay minerals are used as supports for enzymes, different types of binding mechanisms are possible, including ion exchange, Van der Waals interactions, hydrogen bonding, and ion–dipole interactions with metal exchange ions on the clay surfaces [4,51]. Nevertheless, the mechanisms of the reactions of the immobilized enzyme with the substrate remained unclear.

The enzymatic activity of catalase has been shown to be greater when adsorbed by montmorillonite than in free state [52], and the retention of its activity was higher when adsorbed on bentonite than in solution [8]. Immobilized glucose oxidase [37] and immobilized urease [25] by hydrophobic binding to smectite were as active as in free state. In contrast, Makboul and

Table 4
Basal spacing d_{001} of the aspartase–Ca-montmorillonite complex before and after reaction with aspartic acid at pH 7.0 and 25°C

Sample	Reaction period (h)	d_{001} [a] (Å)
Ca-montmorillonite	n.a. [b]	14.9
aspartase–Ca-montmorillonite	n.a.	26.5
aspartase–Ca-montmorillonite–aspartic acid [c]	24	11.9
aspartase–Ca-montmorillonite–aspartic acid	72	11.9

[a] At 25°C and 50% RH.
[b] n.a. = not applicable.
[c] Aspartic acid was added to the aspartase–clay complex.

Ottow [53] reported an inhibitory effect of Ca-montmorillonite, -illite and -kaolinite on alkaline phosphatase activity, and Hughes and Simpson [54] hypothesized that arylsulphatase intercalated by montmorillonite was inactivated by the attractive force of adsorption. In the case of the aspartase, our present results show the complementary effect of both enzymatic and abiotic catalysis in the deamination of aspartic acid after a lag period (Figs. 5 and 6). If the clay mineral surface only acts as an inert support for the enzyme, the specific activity of the immobilized aspartase would be basically the same as that of free aspartase. According to Burns [55], the activity of any particular enzyme in soil is a composite of activities associated with various biotic and abiotic components including proliferating cells, cell debris, clay minerals and humic colloids. Our data clearly show the effect of the clay surface; the lag period (the initial retardation of the ammonium formation) was followed by an abrupt increase of the activity of the immobilized aspartase which exceeded that of free aspartase in solution after an induction period (Figs. 6 and 7). The increase of aspartase specific activity after immobilization by montmorillonite is attributed to the stabilization of the enzyme by adsorption which does not affect the active sites of the enzyme. The deamination of aspartic acid in the presence of the immobilized aspartase on Ca-montmorillonite occurred in two steps: In the first step, a very small amount of ammonia was released in the first 12 h of the reaction (Fig. 5). This is attributed to the induction period required for the catalysis to take place as discussed above. In the second step, a high amount of ammonia was released after 24 h (Fig. 5), which was apparently caused by the accessibility of aspartase to aspartic acid after swelling of montmorillonite and diffusion of the enzyme molecules to the interlayer edges and the external surfaces. The catalysis apparently took place at the interlayer edges and the external clay surfaces. This interpretation is substantiated by the X-ray diffraction data (Table 4). After 24 h, the d_{001} spacing of the aspartase–Ca-montmorillonite complex decreased from 26.5 Å before the reaction with aspartic acid to 11.9 Å after the reaction with aspartic acid.

In conclusion, the adsorption of aspartase on clay is a natural process resulting in immobilization of the enzyme in soil and sediments. The data in the present study show that after a lag period, the aspartase in the aspartase–montmorillonite complex behaved as a stronger catalyst than the free aspartase in the deamination of aspartic acid. Therefore, the role of the clay minerals such as montmorillonite in protecting aspartase from inactivation and denaturation and in influencing its activity in soil and associated environments merits increasing attention.

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