Influence of Clay Minerals on Sorption of Bacteriolytic Enzymes

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Abstract

Myxobacteria presumably produce extracellular bacteriolytic enzymes when they are growing in soil. In order to study their ecological significance, adsorption experiments were performed with lytic enzymes produced by *Myxococcus virescens* in casitone media. Different soils as well as montmorillonite and kaolinite can rapidly adsorb the bacteriolytic but not the proteolytic enzymes. About 1 gm of montmorillonite per liter of cell-free culture solution is enough for the adsorption of 97% of the bacteriolytic enzymes. The adsorption per unit weight is about 100 times greater on montmorillonite than on kaolinite. About 40% of the adsorbed enzymes can be eluted with solutions of high pH or high ionic strength. The only desorbed bacteriolytic enzyme is the alanyl-ϵ-*N*-lysine endopeptidase.

Introduction

Bacteriolytic myxobacteria are common in soil [5] but there are no reports on their ability to lyse other bacteria in their natural habitat. A program has been started to investigate the ability of myxobacteria to lyse and feed on other bacteria in nature. As a first attempt to study this ability, cell-free culture solutions from *Myxococcus virescens* were chromatographed on columns of different soils. The extracellular bacteriolytic enzymes, in contrast to the proteolytic enzymes, were completely adsorbed and they could later be eluted from the column by solutions with increased pH or ionic strength. In order to simplify the adsorption systems, clay minerals were used for further adsorption experiments. This paper deals with the adsorption and desorption of the bacteriolytic enzymes onto montmorillonite and kaolinite.

Materials and Methods

Enzyme Solution. Myxococcus virescens strain B2-S1H was grown in casitone medium at 30°C [10]. When the enzyme activity was estimated to be maximal the culture solutions were centrifuged and the cell-free solutions were stored at −25°C until used. The

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cell-free solution contained at least three bacteriolytic enzymes, all with an isoelectric point near or above 10 [11].

Bacteriolytic activity was assayed as the ability to decrease the turbidity in suspensions of *Micrococcus lysodeikticus* cells. One bacteriolytic enzyme unit (BEU) was defined as the amount of enzyme which gives a decrease of absorbancy (540 nm) = 0.001 per minute in 0.05 *M* Tris-HCl buffer, pH 7.5 at 37°C [13].

Proteolytic activity was determined by the release of trichloro acetic acid soluble tyrosine residues from casein [13].

Clay Minerals. The montmorillonite (bentonite) and kaolinite used were commercial samples obtained from Kebo AB, Stockholm, Sweden. Clay suspensions were fractionated by sedimentation to give an equivalent spherical diameter of $\leq 2\ \mu m$ [4]. The fractionated materials were washed three times with an equal volume of NaCl (1 *M*, pH 3) and twice with NaCl (1 *M*, pH 5.7). The clay minerals were finally washed in distilled water until their conductivity was of the same order as that of distilled water. During the washing procedure the clay was recovered by centrifugation at 45,000 *g* in a Sorvall RC-2B centrifuge.

The cation exchange capacities of the clays were determined with ammonium saturation (1 *M* NH_4-OAc, pH 7). The ammonium-saturated clays were leached with acidified NaCl and the leachates were distilled according to Kjeldahl [3]. The cation exchange capacities were 0.72 and 0.045 mEq/gm of montmorillonite and kaolinite, respectively.

Universal Buffer. For some experiments the universal buffer described by Östling and Virtama [21] was utilized. It contains approximately 0.07 *M* Na^+, 0.01 *M* phosphate, 0.01 *M* borate, and 0.007 *M* citrate with varying amounts of HCl and has a nearly constant ionic strength (μ = 0.07-0.10) over a wide range of pH.

Adsorption Studies. To an 0.5 ml clay suspension in screw cap vials (1.5 × 16.0 cm) there were first added 2.5 ml of a universal buffer 4 × normal concentration which was followed by the addition of distilled water and enzyme solution to get a total volume of 10 ml. The capped vials were placed radially on a disk and rotated at 2 rpm at 22°C. This speed was high enough to prevent settling of the clay suspensions, but slow enough to prevent foaming. After rotation, the clay was sedimented in a high speed centrifuge at 48,000 *g* for 10 min and the enzyme activity in the solution was assayed immediately.

Desorption. A 7 ml (120 mg) suspension of montmorillonite was added to a 100 ml enzyme solution (290 BEU/ml). The suspension was mixed for half an hour at 22°C by a magnetic stirrer and then centrifuged (48,000 *g* for 10 min). Three percent of the initial bacteriolytic activity remained in the solution. The clay pellet was washed twice with 10 ml portions of a universal buffer pH 6.0. The washing solutions had no enzymatic activity. For desorption of the enzymes the pellet was washed with 10 ml of universal buffer (pH 7.0). The suspension was stirred for half an hour and then centrifuged. The pellet was resuspended in 10 ml of the same buffer and stirring and centrifugation were repeated. The two supernatants were mixed and the enzyme activity was assayed immediately. After that the same pellet was treated as described with universal buffer of increasing pH (8.0, 9.0, 10.0, 10.8, and 11.8). In a similar experiment with the same amount of montmorillonite and enzyme solution the adsorbed enzymes were eluted with universal buffer (pH 6.0) containing increasing concentrations of NaCl.

For desorption experiments with kaolinite, 12 gm of kaolinite per 100 ml of enzyme solution were added.

Gel Chromatography. Gel chromatography experiments were run on a Sephadex G-100 column (55 × 0.9 cm) equilibrated with 0.05 *M* Tris-HCl buffer pH 7.5 + 0.4 *M* NaCl. In each experiment 0.25-0.50 ml of enzyme solution were applied and fractions of 1 ml were collected. The elution volume of the enzymes (= V_e) is expressed in relation to the elution volume of Blue Dextran (= V_0) (Fig. 5).

Analytical methods. The procedures for determination of hexosamines, reducing sugars, and free amino groups and for the identification of N-terminal amino acids were principally those described by Ghuysen *et al.* [7].

Results

Adsorption: Effect of Time. Four series of tubes containing buffers of different pH (4, 6, 8, and 10) were used to investigate the binding of bacteriolytic enzymes to clay minerals as a function of time. In each series of 21 tubes, montmorillonite was added to seven tubes and kaolinite to another seven tubes at a final concentration of 0.25 and 20 mg/ml, respectively. Seven tubes were used as controls. To all the tubes 7.0 ml of enzyme solution (170 BEU/ml) were added and the unadsorbed enzymatic activity was assayed after various periods of rotation (5 min to 7 h).

As can be seen in Fig. 1 the bacteriolytic activity in the solutions containing clay rapidly decreased at pH 4, 6, and 8 to a level where it was almost constant. At pH 10 the initial reduction of activity was followed by a slower decrease owing to the instability of the enzymes at high pH (cf. Ref. [11]). The maximal adsorption capacity was considerably larger for montmorillonite (about 300,000 BEU/gm) than for kaolinite (about 3000 BEU/gm). The proteolytic activity was not changed during the incubation, which indicates that these enzymes cannot be adsorbed to montmorillonite and kaolinite under the specified conditions.

Adsorption: Effect of pH. The effect of the pH on the adsorption of bacteriolytic enzymes on montmorillonite and kaolinite was studied by using universal buffers with a pH range from 3 to 11. The amounts of clay minerals and enzyme solutions were the same as in the adsorption experiment described above. The mean values (duplicate) of bacteriolytic activity remaining after 2 hr rotation are shown in Fig. 2. The controls, where no clay minerals had been added, showed that the enzymes were somewhat inactivated at pH values in excess of 6. The adsorption of the enzymes on kaolinite increased progressively with a decrease in pH, while the adsorption on montmorillonite seemed to have a maximum at pH 5-6.

Adsorption Isotherms. To investigate the maximal ability of clay minerals to adsorb bacteriolytic enzymes at pH 6, a fixed concentration of montmorillonite and kaolinite was used (final concentrations: 0.265 and 15.7 mg/ml, respectively) together with initial volumes of 1-7 ml enzyme solutions. After 2 hr rota-

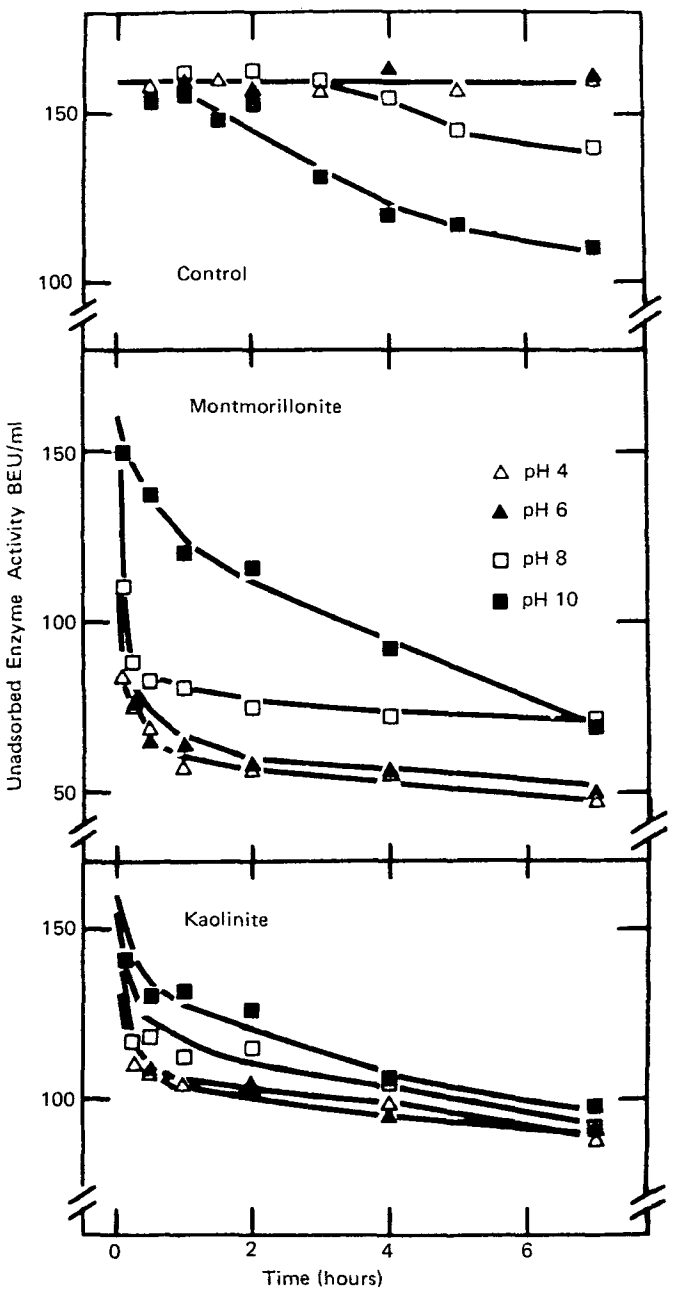

Fig. 1. The decrease of bacteriolytic activity at different pH in a cell-free culture solution from *M. virescens* in the presence or absence of montmorillonite (0.25 mg/ml) or kaolinite (20 mg/ml).

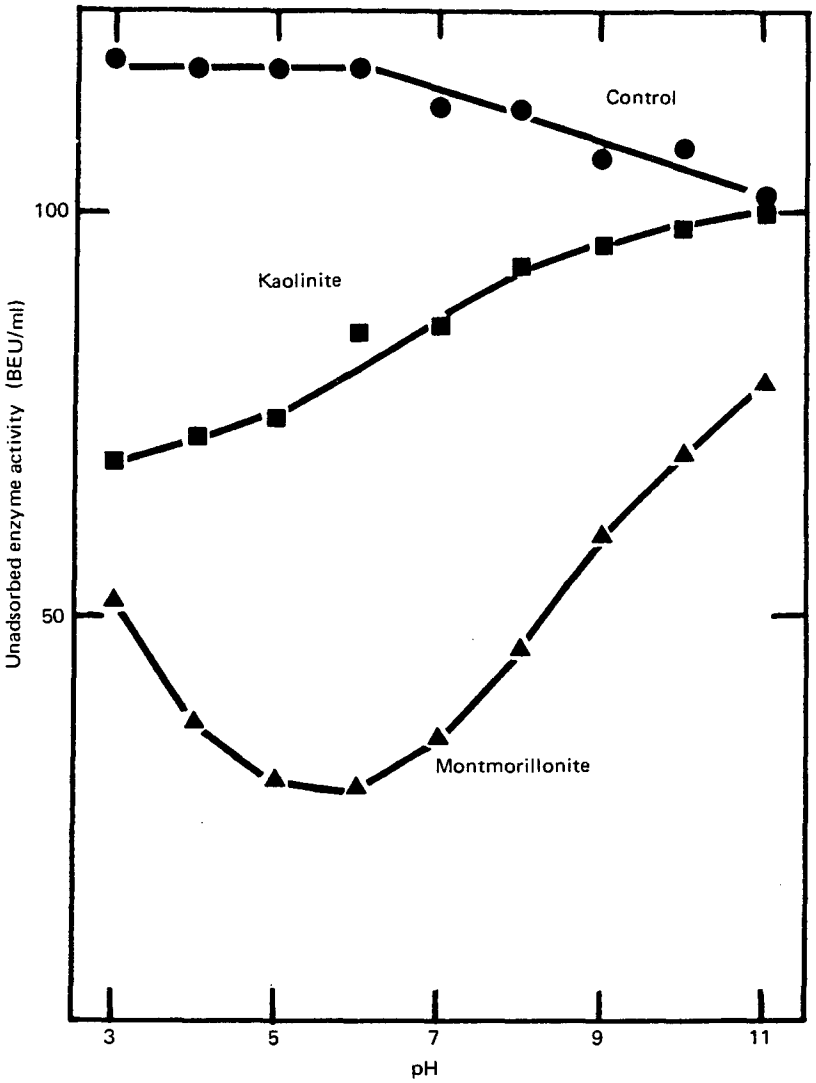

Fig. 2. Effect of pH on the adsorption of bacteriolytic enzymes onto montmorillonite (0.25 mg/ml) and kaolinite (20 mg/ml).

tion the activity remaining in the supernatant following centrifugation was assayed (Fig. 3). Controls showed that there was no inactivation of the enzymes (cf. Fig. 1 and 2). Thus, it could be assumed that the decrease in activity was really the result of the adsorption of the bacteriolytic enzymes onto clay minerals.

All points fall on a common isotherm and the maximal amount of enzyme which could be adsorbed was 300,000 and 3000 BEU/gm of montmorillonite

and kaolinite, respectively. These figures were the same as those obtained in the adsorption experiments concerning effect of time (see above).

Desorption. Some experiments with desorption of the enzymes from kaolinite were performed. Universal buffers of different pH and ionic strength had a

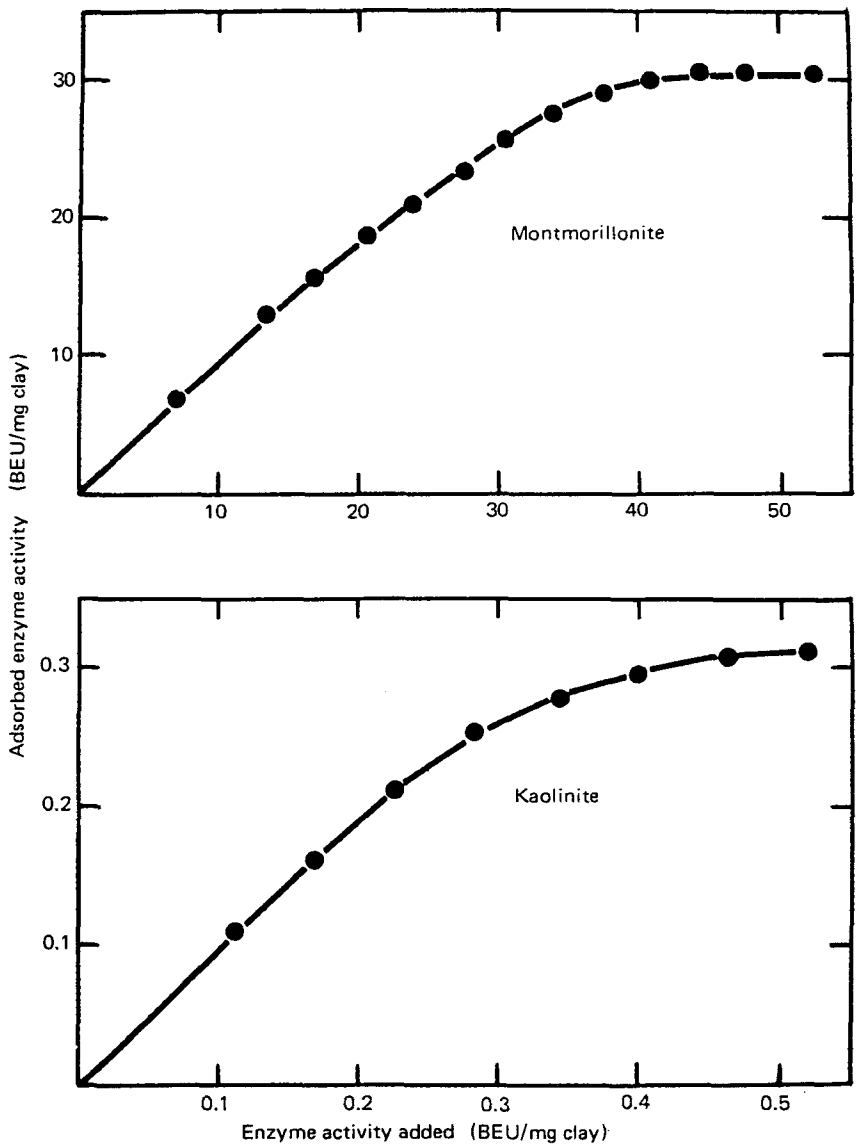

Fig. 3. Binding isotherms of bacteriolytic enzymes to montmorillonite and kaolinite at pH 6.0.

similar ability to desorb the enzymes as in the montmorillonite experiments (see below), but owing to large amounts of kaolinite the centrifugations were difficult. Because of these technical problems all subsequent desorption experiments were only performed with montmorillonite.

Figure 4 shows the result from an experiment where the bacteriolytic enzymes were eluted from montmorillonite with universal buffers of different pH and ionic strength. If the clay was suspended in a universal buffer with a pH of at least 9, bacteriolytic enzymes were eluted (Fig. 4A). The desorption was more efficient if the pH was increased to more than 10 but at this high pH the enzymes were inactivated rapidly. The ionic strength of the used universal buffers (pH 7.0 - 11.4) was nearly constant (0.09 - 0.10).

The first step in the desorption experiment with increasing ionic strength (Fig. 4B) was made with a universal buffer of pH 6 containing 0.1 *M* NaCl (μ = 0.18). Only small amounts of enzyme activity was desorbed. When the pellet was resuspended in a buffer of the same pH now containing 0.2 *M* NaCl (μ = 0.28), about 22% of the original activity was recovered. A second resuspension (0.5 *M* NaCl, μ = 0.58) gave another 22%. A further increase of the ionic strength desorbed only minor enzyme activity.

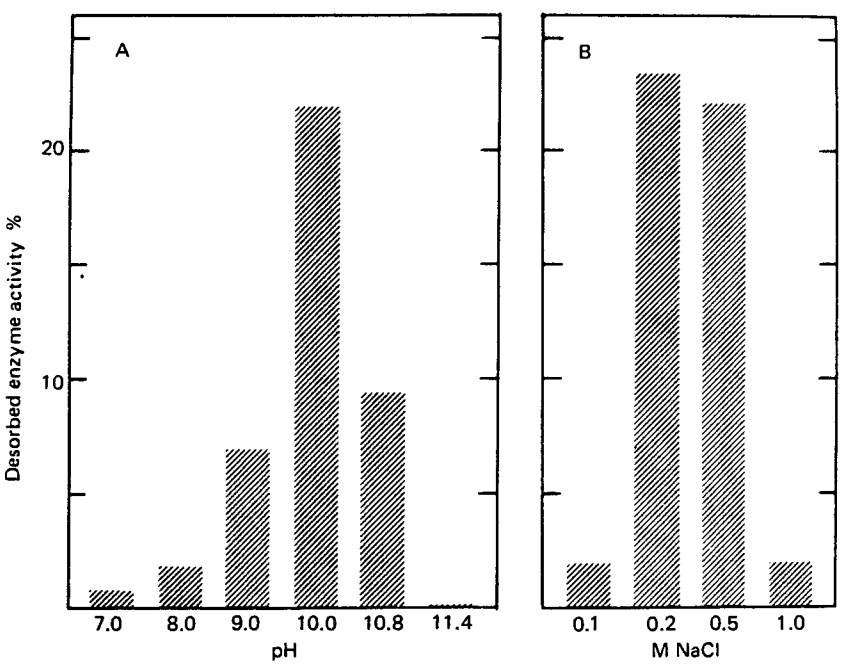

Fig. 4. Desorption of bacteriolytic enzymes from montmorillonite with universal buffer (A) increasing pH and (B) increasing sodium chloride concentrations at pH 6.0.

Other buffer systems could also be used for desorption of bacteriolytic enzymes with approximately the same yield: e.g., Tris-HCl (0.05 *M*, pH 7.5) + 0.4 *M* NaCl (μ = 0.44) and phosphate buffer (0.05 *M*, pH 6.0) + 0.4 *M* NaCl (μ = 0.50). Alderton *et al.* [2] used 5% aqueous pyridine, pH 5.0 (μ = 0.40), for the desorption of bentonite-adsorbed lysozyme. This buffer could also elute the bacteriolytic enzymes with high recovery (cf. below).

Characterization of the Desorbed Enzymes. Montmorillonite-adsorbed bacteriolytic enzymes were prepared and washed as described above. The enzymes were eluted from the clay with different solutions: (1) universal buffer,

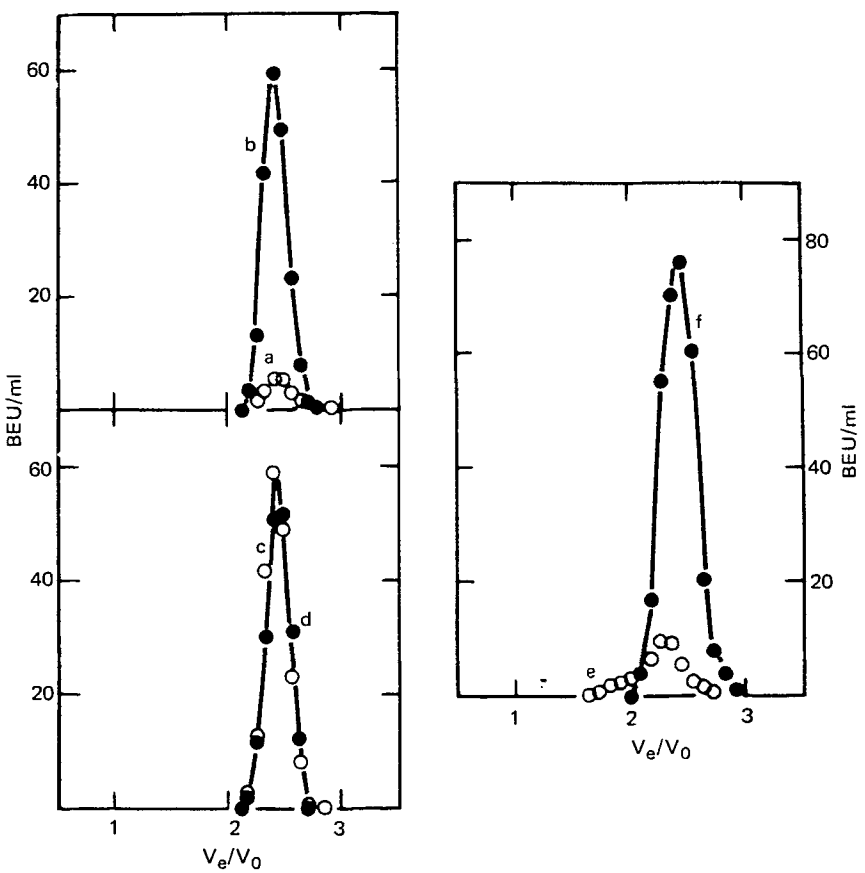

Fig. 5. Gel chromatography of desorbed bacteriolytic enzymes on Sephadex G-100. The enzymes were desorbed from montmorillonite by universal buffer pH 11 (a), phosphate buffer (0.05 *M*, pH 6.0) + 0.4 *M* NaCl (b), Tris-HCl buffer (0.05 *M*, pH 7.5) + 0.4 *M* NaCl (c), and 5% pyridine, pH 5.0 (d). As controls, an unpurified cell-free culture solution (e) and a purified alanyl-*N*-lysine endopeptidase solution (f) were used.

pH 11, (2) phosphate buffer (0.05 *M*, pH 6) + 0.4 *M* NaCl, (3) Tris-HCl (0.05 *M*, pH 7.5) + 0.4 *M* NaCl, and (4) 5% pyridine, pH 5.0. Two-hundred-and-fifty microliters of the desorbed enzyme solution (being equivalent to 3 ml, 870 BEU, of the initial culture solution) were chromatographed in Tris-HCl (0.05 *M*, pH 7.5) + 0.4 *M* NaCl on a Sephadex G-100 column (Fig. 5). Two controls concerning the chromatographic pattern were used: (1) 500 µl (145 BEU) of the unpurified culture solution and (2) 250 µl (400 BEU) of a purified enzyme III (alanyl-ε-*N*-lysine endopeptidase) solution [10, 11].

Washings of the montmorillonite-adsorbed enzymes with Tris-HCl buffer, phosphate buffer, or pyridine solution gave about the same yield of active enzymes. Universal buffer at pH 11.0 gave, however, only a low yield owing to the instability of the enzymes at too high a pH (cf. Fig. 1).

All the desorbed enzyme solutions formed only one peak when eluted from the Sephadex column. The V_e/V_0 values for these peaks were equal and they also agreed with that for a purified enzyme III solution (Fig. 5). Owing to a high content of viscous material it is difficult to perform gel chromatography experiments with untreated cell-free culture solutions. However, the curve in Fig. 5 shows that the bacteriolytic enzymes of an unpurified culture solution are distributed among several fractions. This in in agreement with earlier results [11] where it was found that three isolated enzymes were eluted at V_e/V_0 values of 1.7, 2.0, and 2.4, respectively. The gel chromatography experiments indicated that enzyme III was the only enzyme which was desorbed from montmorillonite. When enzyme III acts on *M. lysodeikticus* cell walls it degrades the walls and simultaneously liberates free amino groups but no reducing sugars or acetylamino sugars. The liberated amino groups originate exclusively from lysine ε-amino groups [12]. When the desorbed enzyme solution from montmorillonite digested *M. lysodeikticus* cell walls it liberated the same degradation products as enzyme III. Consequently the enzyme which was eluted from montmorillonite is the alanyl-ε-*N*-lysine endopeptidase previously characterized [12].

Discussion

Microorganisms living in soils are greatly influenced by the composition of their environment. For instance, it has been shown that clay minerals can stimulate the respiration of a wide range of bacteria by maintaining the pH of the environment suitable for sustained growth. It has also been supposed that surface interactions between soil particles and microbial cells or metabolites can profoundly influence the ecology of microbes in soil (see reviews of Stotzky [19] and Filip [6]).

The ability of different clay materials (especially montmorillonite) to adsorb proteins has been repeatedly pointed out. Most of the investigated pro-

teins have been pure. McLaren and co-workers have studied the adsorption of lysozyme and other proteins to kaolinite and montmorillonite [14-18]. They showed that lysozyme was rapidly adsorbed to the clays at a pH under the isoelectric point (about pH 11) and that active enzymes could be eluted with universal buffer at a high pH (11.1) or at a lower pH combined with increased ionic strength.

Myxococcus virescens produces at least three cell wall lytic enzymes when grown in casitone media (11). The alanyl-ε-*N*-lysine endopeptidase (enzyme III) probably is produced in largest amounts, but this is not certain because of the difficulties of assaying the activity of one special kind of bacteriolytic enzyme in a mixture of others.

Owing to the lack of information about the exact amounts of initial activity it is hard to know if the decrease in activity, observed when clay minerals are added to the cell-free culture solution (Fig. 1-3), depends on the adsorption of only one kind of the bacteriolytic enzymes or all. It seems probable, however, that all the enzymes can be adsorbed to the clay minerals if the clay concentration is high enough, as an addition of 0.12% of montmorillonite decreased the enzymatic activity of a culture solution by 97%.

It has been suggested that protein adsorption is a cation exchange reaction (e.g., 1, 8, 9, 14, and 18). The larger cation exchange capacity for montmorillonite than for kaolinite may thus explain the fact that the maximal adsorption capacity of the bacteriolytic enzymes was considerably larger for montmorillonite than for kaolinite. Also, Albert and Harter [1] found that smectite (montmorillonite) had a greater adsorption capacity than kaolinite when experiments were made with lysozyme. They also showed that the adsorption of lysozyme to smectite increased with increasing pH while the adsorption to kaolinite was almost independent of pH. From these results they concluded that lysozyme is bonded to kaolinite by mechanisms other than cation exchange.

The adsorption to clay minerals of the bacteriolytic enzymes from *M. virescens* was larger in more acidic conditions (Fig. 2), a result which does not agree with those of Albert and Harter [1]. This disagreement may be due to the fact that the myxobacterial culture solution contains a large amount of molecules of different sizes which can compete with the enzymes for the adsorption sites.

When the montmorillonite-enzyme complex was washed with solutions of high pH or high ionic strength, only enzyme III was eluted (Fig. 5). The lack of detection of other bacteriolytic enzymes may depend on inactivation or on the inability of the used solutions to desorb these enzymes. The fact that only enzyme III is eluted from the clay as well as the good recovery gives a basis for an easy and rapid purification method of enzyme III [12].

The myxobacteria presumably produce extracellular bacteriolytic enzymes when they are growing in soil. These enzymes have high isoelectric points and are rapidly adsorbed to the soil particles. If the bacteriolytic enzymes are eluted from the particles or if they can act in the adsorbed state, they can lyse other bacterial cells and the myxobacteria can then feed on the liberated nutrients. Yermoljeva and Bouinowskaja [20] have reported that kaolinite-adsorbed egg white lysozyme is inactive against *M. lysodeikticus* cells. However, in preliminary experiments I have shown that kaolinite- and montmorillonite-adsorbed bacteriolytic enzymes from *M. virescens* could lyse *M. lysodeikticus* cells. As to whether they are active on other bacteria as well and if only one of the enzymes is still active is not yet known.

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