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Micrococcus lysodeikticus Bacterial Walls as a Substrate Specific for the Autolytic Glycosidase of *Bacillus subtilis*

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The *Bacillus subtilis* 168 autolytic glycosidase degrades *Micrococcus lysodeikticus* cells or cell walls, whereas the *B. subtilis* autolytic amidase does not. The criteria used to establish this fact included: the determination of chemical bonds broken, heat-inactivated kinetics, pH dependence curves, and the physical separation of glycosidase from amidase. The physical separation involved LiCl elution from two different ion-exchange materials, walls from *B. subtilis* 168 strain βAO, and walls from mutant strain βA173 derived from strain βAO. No evidence was obtained for *B. subtilis* vegetative bacteria making any more autolysins than one autolytic amidase and one autolytic glycosidase.

Recent experiments (6) have shown that *Bacillus subtilis* makes two autolysins, an *N*-acylmuramyl-L-alanine amidase (alanine amidase) and a glycosidase. Either one or both of these wall degradative enzymes are involved in *B. subtilis* cell wall growth (5, 6) and cell separation (4, 6). It is easy to obtain mixtures of the two autolysins and much more tedious to separate the enzymes physically (6). Thus, to study the precise roles of each autolysin in growth and division, it is desirable to have simple assays specific for each autolysin so that the activity of either enzyme can be measured in the presence of the other.

Richmond (13) and Prasad and Litwack (12) have reported that *B. subtilis* makes at least one enzyme that can lyse *Micrococcus lysodeikticus*. The experiments presented in this paper show that lysis of *M. lysodeikticus* can be used to assay specifically for the *B. subtilis* glycosidase in the presence of the amidase.

MATERIALS AND METHODS

Unless otherwise stated, all materials and methods were described previously (3-6, 8).

Bacterial cells. *B. subtilis* cells were cultured and harvested as reported earlier (6). Heat-killed *M. lysodeikticus* cells were kindly provided by J. S. Anderson (University of Minnesota, St. Paul). They were a byproduct of the isolation of uridine diphospho-*N*-acetylhexosaminuronic acid. The detailed method of preparation has been described by Page and Anderson (11). *M. lysodeikticus* cells were also obtained from Sigma Chemical Corp. (St. Louis, Mo.). *M. lysodeikticus* cells from either source behaved similarly in the experiments described below. The principal difference was that Anderson's cells had walls which were lysed approximately twice as rapidly for the same concentration of *B. subtilis* autolysin added. In all experiments detailed in this paper, Anderson's cells or walls made from these cells were used.

Preparation of M. lysodeikticus SDS walls. *M. lysodeikticus* cells suspended in water were mixed with one-third volume, 25-μm glass beads and disrupted by sonic oscillation at 20,000 cycles/s for 15 min. The glass beads were allowed to settle, and sodium dodecyl sulfate (SDS)-treated cell walls were prepared from the remaining liquid by using the same differential centrifugation conditions and SDS treatment as were used to obtain *B. subtilis* SDS walls (6). For *M. lysodeikticus* walls, as for *B. subtilis* walls, a solution at 1 mg/ml had an approximate absorbancy at 540 nm (A_{540}) of 4.

Preparations of autolysin (3, 6). Exponential *B. subtilis* cultures with an A_{540} of 0.5 to 0.8 were concentrated by centrifugation. Walls were made from the concentrated cells and extracted with LiCl in either TK buffer (0.1 M KCl, 0.1 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.6) or TM buffer (0.05 M Tris, 0.05 M maleic anhydride-sodium hydroxide, pH 6.0). In both buffers, the *B. subtilis* glycosidase is removed optimally by 0.5 M LiCl and the amidase is removed optimally by 1.5 M LiCl (6). In 1 M LiCl extracts of *B. subtilis* walls (1 M LiCl autolysin), there is a majority of the glycosidase and small portion of the amidase which can be released by high salt concentrations. In 3 M LiCl extracts (3 M LiCl autolysin), both enzymes

are present in the maximal amounts obtainable by salt extraction. The ratio of autolysins released is very similar if either whole cells or walls from those cells are used in the LiCl extraction. Brown (1) also found that LiCl removes autolysins from whole cells.

Conditions for lysis of M. lysodeikticus substrate by B. subtilis autolysins. Unless otherwise stated, a LiCl-extracted autolysin preparation was added to *M. lysodeikticus* SDS walls or *M. lysodeikticus* cells, and the suspension was then diluted to reduce the LiCl concentration to 0.1 M or less. Then the A_{540} of the solution was measured at various times at 30 C (dilution assay). Alternatively, the walls in the solution were centrifuged (27,000 $\times$ *g* for 5 min) after incubation at 0 C for 5 min. Then the walls were resuspended in a smaller volume before incubation at 30 C to follow A_{540} loss (attached wall assay). Sometimes the walls were washed with 10 to 20 ml of water before the final suspension in the assay buffer. The latter technique permitted the assay of large volumes of LiCl extract with a small amount of walls since the final volume of the assay could be small relative to the LiCl extract added. Unless otherwise specified, the assay was performed in TM buffer. Enzyme activity was measured as the reciprocal of the half-life of the initial rate of A_{540} loss.

pH dependence curves. Autolysins in LiCl-containing solutions were attached to either *B. subtilis* or *M. lysodeikticus* substrate by dilution to reduce the LiCl concentration to 0.1 M or less. The substrate with enzyme attached was then centrifuged and suspended in TMB buffer at various pH values (0.1 M Tris, 0.1 M maleic, 0.1 M sodium borate, titrated to the desired pH with NaOH or HCl). The lytic rates were measured at 30 C.

Electron microscopy. *M. lysodeikticus* walls were examined by using techniques developed for *B. subtilis* walls (8).

RESULTS

B. subtilis autolysins are found attached to native cell walls, but they can be easily released in soluble form by exposing the walls to high salt concentrations (3, 6). When a preparation of autolysin made by extracting *B. subtilis* walls with LiCl was added to *M. lysodeikticus* cell walls, a lytic factor was bound to *M. lysodeikticus* walls and degraded them (Fig. 1). It can be concluded that the lytic factor bound to *M. lysodeikticus* walls because the walls were washed with water after exposure to the autolysin preparation and they still lysed when placed in the appropriate buffer. There was no lysis as measured by loss of A_{540} in experiments where the exposure to autolysin had been omitted. In the 1 M LiCl-extracted autolysin preparation used in the experiment of Fig. 1, there was mostly the autolytic glycosidase with traces of the amidase (6). From the changes in cell wall structure seen in the two samples of Fig. 1, it appears likely that at least one *B. subtilis* autolysin was capable of degrading *M. lysodeikticus* walls.

The maximal amount of salt-releasable autolysins, both amidase and glycosidase, was solubilized when 3 M LiCl was used to extract native *B. subtilis* walls (3). When such an extract was used to lyse *M. lysodeikticus* walls, it was found that reducing power was released simultaneously with loss of A_{540} (Fig. 2). It therefore seems probable that the *B. subtilis* glycosidase can degrade *M. lysodeikticus* walls. It can be seen (Fig. 3) that there was no trend toward an increase in the amount of N-terminal alanine residues released during the time it took to solubilize *M. lysodeikticus* walls completely. Thus the *B. subtilis* amidase, which cleaves the bond between the muramic acid and alanine residues in peptidoglycan (15), either does not act on *M. lysodeikticus* walls or does not bind to these walls.

In *B. subtilis* autolysin preparations made by extraction with high salt, there is always a high concentration of LiCl present. It was therefore interesting to find that LiCl, up to approximately 0.1 M, will stimulate the lysis of *M. lysodeikticus* by a *B. subtilis* autolysin mixture (Fig. 4). In all experiments the LiCl concentration was maintained at 0.1 M or below. In the experiment of Fig. 4, heat-killed cells of *M. lysodeikticus* were used instead of walls. Walls and cells always behaved similarly in experiments where lytic rates were measured. These lytic rates were calculated as the reciprocal half-life of loss of A_{540} from data such as those found in Fig. 1 and 2.

The rate of lysis of *M. lysodeikticus* walls by *B. subtilis* autolysin preparations was proportional to input enzyme if the extract was either added directly to the assay mixture or was bound to the walls which were then washed with water before the assay (Fig. 5). The maximal amount of enzyme used for the attached wall assay was 10 times that used in the dilution assay (Fig. 5). If all the added enzyme was adsorbed to walls in the attached wall experiment, the slope of plot A should be 10 times that of plot B (Fig. 5). The actual ratio of the slopes is slightly greater than 10, indicating that all of the added enzyme was indeed bound.

The suggestion that only the *B. subtilis* glycosidase acts on *M. lysodeikticus* walls was confirmed by physically separating the glycosidase from the amidase and then by testing the separated enzymes on the *M. lysodeikticus* substrate. This separation was accomplished by centrifuging native *B. subtilis* walls binding both amidase and glycosidase through a LiCl-sucrose gradient (Fig. 6A). As the walls passed

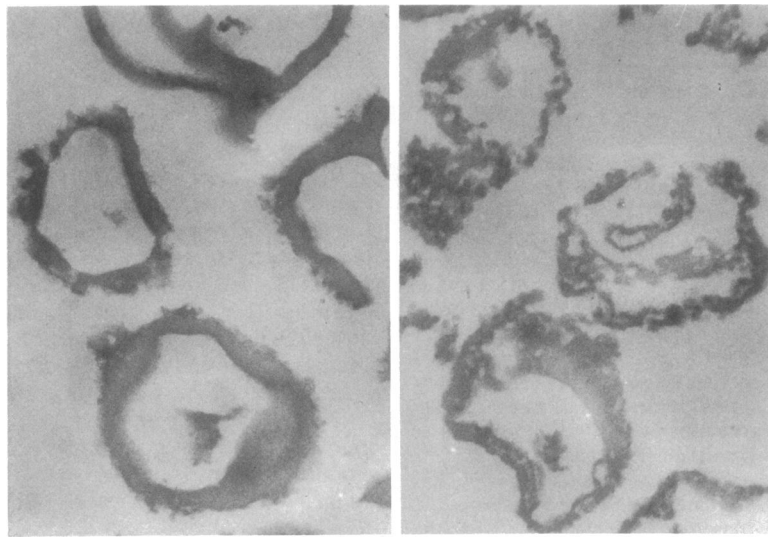

FIG. 1. *Ultrastructural changes resulting from B. subtilis autolysins acting on M. lysodeikticus walls. The autolysins in a 1 M LiCl autolysin preparation were bound to M. lysodeikticus SDS walls as described for the attached-wall assay. The walls were suspended in TM buffer and the A_{540} was followed with time. At two different times, samples were prepared and thin sections were made for electron microscope observation (×47,000). Left, sample at the beginning of the 30 C incubation; right, sample after the A_{540} of the suspension had dropped to 25% of the original.*

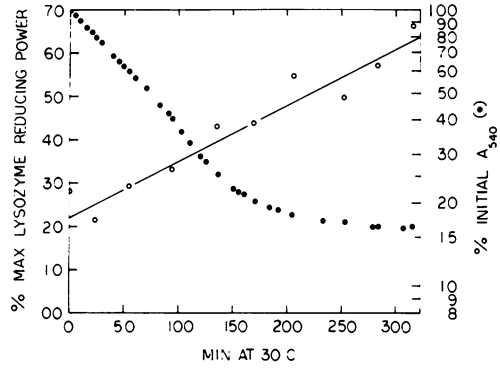

FIG. 2. *Release of reducing power during lysis of M. lysodeikticus walls by B. subtilis autolysins. LiCl autolysin (3M) from 1 liter of B. subtilis cells was mixed with 5.5 ml of M. lysodeikticus SDS walls at an A_{540} of 0.5. The mixture was assayed by using the attached-wall assay. Loss of A_{540} was followed with time at 30 C (●). Reducing power increase (Park-Johnson assay [6]) was measured on 0.5-ml samples boiled for 5 min to destroy residual enzyme (○). The reference value of 100% was obtained from an identical sample of walls digested to completion with egg white lysozyme. This value was equivalent to 100 nmol of N-acetyl-glucosamine per mg of walls.*

through the LiCl concentration at which an autolysin was removed, that autolysin was solubilized and, under the centrifugation conditions, effectively did not move in the gradient (6). Therefore, the glycosidase and amidase which were removed at different salt concentrations banded at different positions in the gradient. In earlier experiments (6) it had been established that, when a 0 to 2 M LiCl, 10 to 25% sucrose gradient was assayed by using *B. subtilis* walls as substrate at pH 8.6, the minor peak at low LiCl concentration belonged to the glycosidase, whereas the major peak at higher salt concentrations could be attributed to the amidase. Indeed, it can be seen (Fig. 6A) that the *M. lysodeikticus* lytic activity coincides with the glycosidase peak with no indication of any peak in the amidase region. The total enzyme activity recovered from the gradient was approximately 20% of the enzymes bound to the walls layered on the gradient. This figure is consistent with the 30% measured in previous experiments (6). When the assay was carried out with *M. lysodeikticus* as substrate at pH 8.6, the pH optimum for amidase activity, there was still no sign of any activity in the amidase region. The only difference between assays at pH 6.0 and 8.6 was that the glycosidase was inactivated during the assay, leading to incomplete digestion of the substrate. Also, at 45 C there was enzyme destruction during the assay at either pH 6.0 or 8.6. When freshly harvested, whole cells were centrifuged through the same LiCl-sucrose gradient as shown in Fig. 6A, amidase and glycosidase were also removed. In fact, in comparisons between gradients with whole cells and those with isolated walls, the

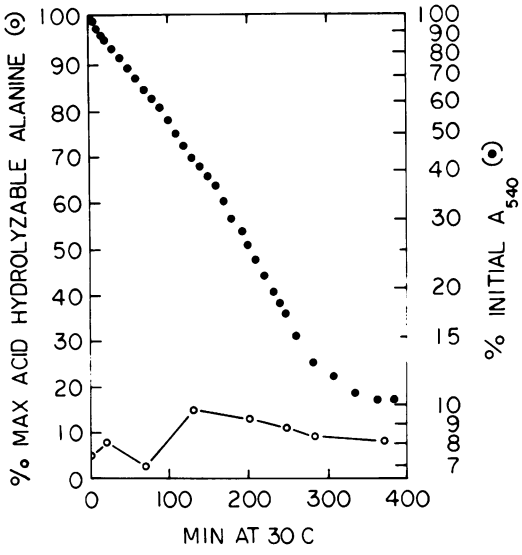

FIG. 3. *Lack of release of N-terminal alanine residues during lysis of M. lysodeikticus walls by B. subtilis autolysins. The autolysins in a 3 M LiCl autolysin preparation were bound to M. lysodeikticus SDS walls as in the experiment of Fig. 2, and then these walls were suspended in 0.1 M cacodylate-NaOH buffer, pH 6.0. The A_{540} was followed at 30 C (●). The TM buffer was not used because the amino groups in the buffer interfered with the chemical determinations. N-terminal alanine residues were determined at various times (○). Samples of 0.5 ml were boiled for 5 min, and then the pH was raised to 8.5 with $K_2B_4O_7$ before reaction with 1-fluoro-2,4-dinitrobenzene, acid hydrolysis, and thin-layer chromatography to isolate the dinitrophenyl derivative of alanine (6). As reference, a wall sample was acid-hydrolyzed before 1-fluoro-2,4-dinitrobenzene treatment to determine the total amount of alanine residues in the wall (1,000 nmol of alanine/mg of wall).*

two enzymes were found to be released at the same salt concentrations and in the same approximate ratios.

The evidence presented so far suggests that the amidase did not degrade *M. lysodeikticus* walls. If, in addition, the amidase did not bind those walls, then the glycosidase could be bound to *M. lysodeikticus* walls with no amidase attached to the same walls. Any glycosidase which could be released from these *M. lysodeikticus* walls by high salt would then be purified from contaminating amidase. To test whether there was any amidase binding to *M. lysodeikticus* walls, it was first necessary to demonstrate that amidase and glycosidase could be individually identified in a solution containing both enzymes. When a 3 M LiCl extract of *B. subtilis* cell walls was added to SDS-treated *B. subtilis* walls (Fig. 6B), the autolysins in the extract bound to autolysin-free walls. When *B. subtilis* walls were used in the assays of the gradient of Fig. 6B, the recovery of enzyme activity was 62% of that of the gradient of Fig. 6A. The comparable recovery when *M. lysodeikticus* walls were used was 84%. LiCl (3 M) releases about 30% of all autolysins bound to native walls (3), and 20 to 30% of native wall bound autolysins are released in a 0 to 2 M LiCl gradient (Fig. 6A). It therefore would be ex-

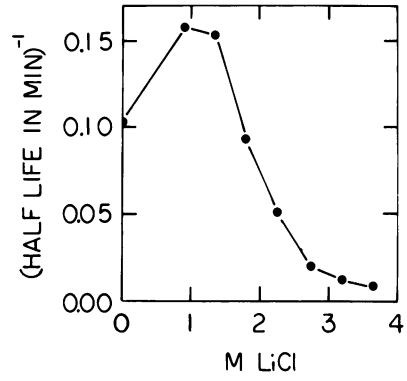

FIG. 4. *Effect of LiCl on the lysis of M. lysodeikticus cells by B. subtilis autolysins. The autolysins in a 1 M LiCl autolysin preparation were bound to M. lysodeikticus cells as described for the attached-wall assay. The cells were divided and assayed in TM buffer with LiCl at the final concentrations indicated. The lytic rates were measured at 30 C.*

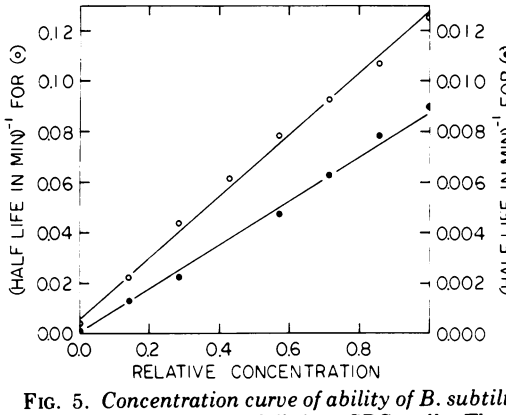

FIG. 5. *Concentration curve of ability of B. subtilis autolysins to lyse M. lysodeikticus SDS walls. Three M LiCl autolysin at various concentrations was assayed with a constant amount of M. lysodeikticus SDS walls using the attached-wall assay (○, plot A). In the most concentrated sample, autolysin from 400 ml of B. subtilis cells was assayed with 1 ml of M. lysodeikticus walls at an A_{540} of 0.1. The same 3 M LiCl autolysin preparation was assayed by using the dilution assay (●, plot B). The most concentrated 3 M LiCl autolysin used in the dilution assay was 10% of that used in the attached-wall assay.*

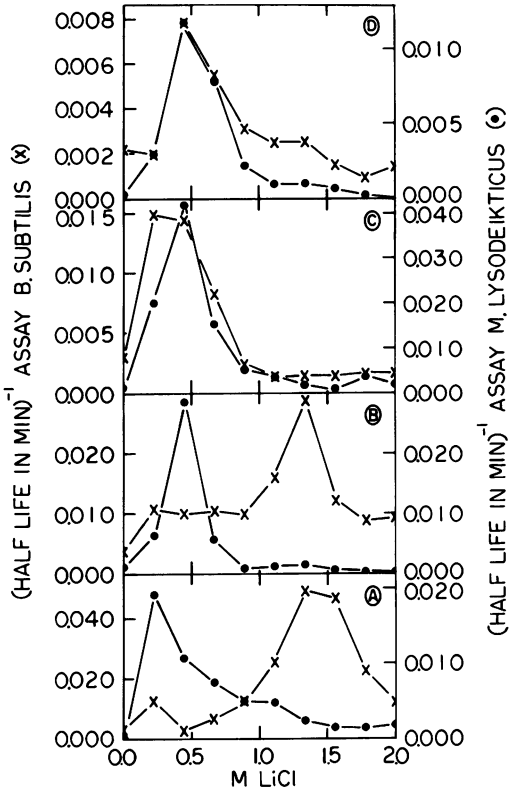

FIG. 6. *LiCl elution of B. subtilis autolysins from B. subtilis and M. lysodeikticus walls. In all experiments A to D, 1 ml of walls binding B. subtilis autolysins was centrifuged through an 11-ml 0 to 2 M LiCl, 10 to 25% sucrose gradient in TK buffer (6). Ten fractions were collected from each gradient, and half the enzyme from each fraction was assayed on 1 ml of B. subtilis SDS walls (×) at an A_{540} of 0.1 in by using the attached-wall assay. The assay was performed in TK buffer at 30 C. The remaining half of each fraction was assayed on 1 ml of M. lysodeikticus SDS walls (●) at an A_{540} of 0.1 by using the attached-wall assay. These assays were performed in TM buffer at 30 C. In all gradients, the enzyme was obtained initially from walls from 1 liter of B. subtilis cells. The same batches of native B. subtilis walls, B. subtilis SDS walls, and M. lysodeikticus SDS walls were used for all experiments so enzyme recoveries could be compared directly. Gradient A, Native B. subtilis walls were centrifuged through the LiCl-sucrose gradient. Gradient B, three M LiCl released autolysins were bound to 2 ml of B. subtilis SDS walls at an A_{540} of 0.5 as for the attached wall assay. Then the walls were centrifuged, suspended in water, and centrifuged through the gradient. Gradient C, same as gradient B except M. lysodeikticus SDS walls replaced the B. subtilis SDS walls. Gradient D, 3 M LiCl-released autolysins were attached to 2 ml of M. lysodeikticus walls at an A_{540} of 0.5 as for the attached-wall assay. Autolysins were extracted from these walls with 2 M LiCl in TK buffer in the same way that LiCl was used to extract autolysins from B. subtilis walls. The autolysins in this 2 M LiCl extract of M. lysodeikticus SDS walls were attached to B. subtilis SDS walls and run through the gradient as for gradient B.*

pected that the recoveries in the experiment of Fig. 6B would be about 20 to 30% that in the gradient of Fig. 6A if 3 M LiCl autolysins bound SDS-treated *B. subtilis* walls exactly as native walls bound native enzymes. Since the actual recoveries of 62 and 84% were much higher, the 3 M LiCl-removed autolysins seemed to bind *B. subtilis* walls principally in those sites from which autolysins can be removed by high salt. However, the fact that the elution patterns were the same in the experiments of Figs. 6A and 6B indicate that the autolysins removable by high salt were bound to the same kinds of sites in both native walls and SDS walls to which autolysins have been artificially reattached. It is also clear from Fig. 6B that the suspension of autolysin released by 3 M LiCl contained much more amidase than glycosidase activity when the substrate was *B. subtilis* walls assayed at pH 8.6.

When the same suspensions of autolysins released by 3 M LiCl were added to *M. lysodeikticus* walls and centrifuged through a LiCl-sucrose gradient (Fig. 6C), both the *M. lysodeikticus* and *B. subtilis* lytic activities banded in the same positions. The *M. lysodeikticus* lytic activity recovered from this gradient was about double that recovered from the gradient of Fig. 6B, suggesting that the glycosidase bound *M. lysodeikticus* walls better than *B. subtilis* walls. However, the recovery of *B. subtilis* lytic activity in the gradient of Fig. 6C was reduced to less than half of that in the gradient of Fig. 6B. This result shows that the amidase bound *M. lysodeikticus* walls less well than *B. subtilis* walls since the major *B. subtilis* lytic activity comes from the amidase and since the glycosidase seemed to bind better to *M. lysodeikticus* walls than *B. subtilis* walls. From the curve in Fig. 6C it is clear that *B. subtilis* autolysins were released from *M. lysodeikticus* walls by extraction with 2 M LiCl. Autolysins released in this procedure were attached to a fresh batch of SDS-treated *B. subtilis* walls and then analyzed in the experiment of Fig. 6D. In the gradient profile, there was a shoulder on the lytic activity peak when *B. subtilis* walls were used as substrate which was not present when *M. lysodeikticus* walls were used. This shoulder indicates that some amidase was released by 2 M LiCl from *M. lysodeikticus* walls which had been exposed to both amidase and glycosidase. In other experiments, the shoulder has some-

times been as high as the glycosidase peak. The recovery of activity as assayed on *B. subtilis* walls was 59% for the experiment of Fig. 6D as compared to that in the experiment of Fig. 6C. The corresponding figure for the *M. lysodeikticus* substrate was 32%. Therefore, it can be concluded that *M. lysodeikticus* walls bound the *B. subtilis* amidase in salt-releasable form but at lower efficiency than the glycosidase. When bound to *M. lysodeikticus* walls, both amidase and glycosidase were removed by the same salt concentration (Fig. 6C).

The finding that the amidase was much more heat stable than the glycosidase (6) provided yet another method of checking for amidase action on *M. lysodeikticus* walls. Heat-inactivation experiments were performed on a 3 M LiCl-extracted mixture of both *B. subtilis* autolysins. For assays on either *M. lysodeikticus* or *B. subtilis* walls, the lytic activity should drop, rapidly paralleling complete destruction of the glycosidase, and then the kinetics should show the slower exponential kinetics characteristic of the amidase. The rapid phase of the inactivation curves for assays on both substrates should be parallel on a semilogarithmic plot of inactivation with time. Also, the slow phases of the two inactivation curves should be parallel. The extrapolate of the slow exponential phase back to zero time should give the fraction of the total activity that was attributable to the amidase. This extrapolate for assays on *B. subtilis* walls at pH 8.6 was 95% (Fig. 7).

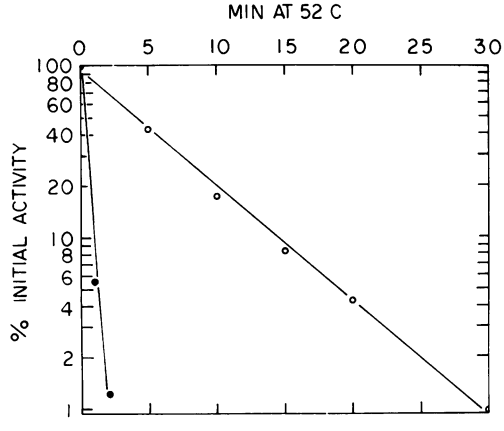

FIG. 7. *Heat inactivation of B. subtilis 3 M LiCl autolysin. LiCl autolysin (3 M) in TK buffer was heated at 52 C and sampled at various times for assays with B. subtilis SDS walls (O) and M. lysodeikticus SDS walls (●) as substrate. The maximal lytic activity with B. subtilis walls had a reciprocal half-life of 0.0534, and the corresponding number for M. lysodeikticus walls was 0.0163.*

This figure is not significantly different from the 80 to 90% obtained by using measurements from the gradients in experiments such as those in Fig. 6B. When *M. lysodeikticus* was used as substrate, there was no sign of a slow phase in the kinetics down to 98 to 99% inactivation. Thus the inactivity of the amidase on *M. lysodeikticus* walls was confirmed.

Since the amidase has a pH optimum at basic pH while the glycosidase is most active at lower pH (6), it would be predicted that a *B. subtilis* autolysin mixture acting on *M. lysodeikticus* cells would show a low pH optimum. Furthermore, if only the glycosidase can lyse *M. lysodeikticus* cells, preparations of isolated glycosidase and preparations of glycosidase mixed with amidase should give the same pH dependence curves. Both of these predictions were fulfilled in the experiment of Fig. 8. For comparison, a pH dependence curve for isolated glycosidase acting on *B. subtilis* walls is also presented in Fig. 8. All of the curves coincided in the region below pH 5. However, at higher pH there was apparently relatively more activity when *B. subtilis* walls were used as substrate than when *M. lysodeikticus* cells were used. The difference could arise from two sources. Either the glycosidase had different pH dependence for the two substrates, or the isolated glycosidase preparation was contaminated with small

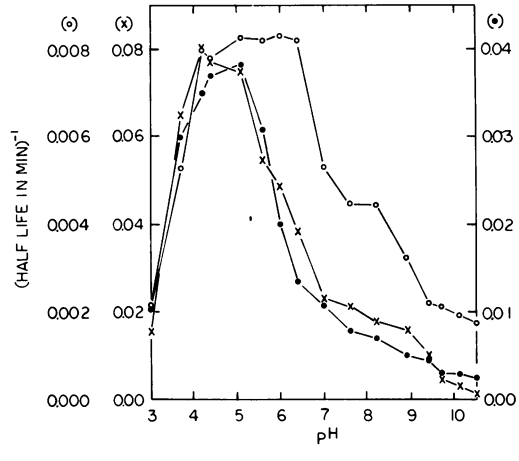

FIG. 8. *pH dependence curves of B. subtilis autolytic glycosidase (×). Crude 3 M LiCl autolysin was assayed at various pH with M. lysodeikticus cells as substrate. (●), 3 M LiCl autolysin was attached to B. subtilis βAO walls and centrifuged as in the experiment of Fig. 6B. The fractions with maximal M. lysodeikticus lytic enzyme were pooled and assayed with M. lysodeikticus cells. (O), Same as (●) except strain βAO walls were used instead of M. lysodeikticus cells for the assays.*

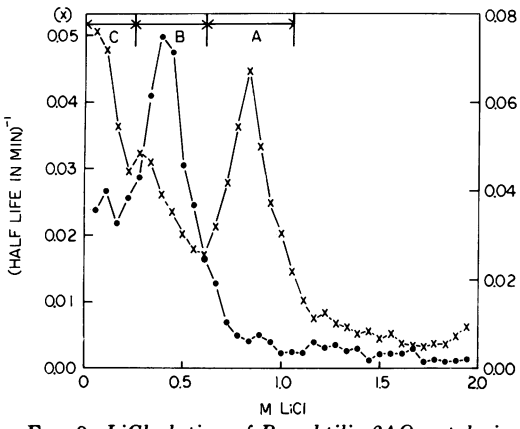

FIG. 9. *LiCl elution of B. subtilis βAO autolysins from B. subtilis βA173 SDS walls. LiCl autolysin (3 M) was obtained from 2 liters of strain βAO cells and was attached to 2 ml of strain βA173 SDS walls at an A_{540} of 0.5. The walls were suspended in 1 ml of water and centrifuged through a 30-ml 0 to 2 M LiCl, 10 to 25% sucrose gradient in TK buffer. The fractions were assayed by using strain βAO SDS walls in TK buffer (×) and by using M. lysodeikticus cells in TM buffer (●). All assays were performed at 45 C.*

amounts of amidase. The amidase is known to have a pH optimum at pH 8.6 and almost no activity below pH 5.0 (6). The explanation based on amidase contamination seemed more likely because the shape of the pH dependence curves with *B. subtilis* substrate varied from experiment to experiment at pH values above 5. Thus it appeared difficult to isolate, reproducibly, the glycosidase completely free from amidase by using differential elution from *B. subtilis* βAO walls.

In an effort to obtain cleaner separations of the two enzymes, salt elution from ion-exchange materials other than strain βAO walls were tried. Diethylaminoethyl (DEAE)-cellulose, DEAE-Sephadex, phosphocellulose, and carboxymethyl cellulose were found to be unsatisfactory (unpublished experiments). However, a mutant of strain βAO was found which had walls that could give, reproducibly, almost complete separation of amidase from glycosidase.

This mutant, strain βA173, was isolated from strain βAO after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (5) as a salt-sensitive mutant which could not grow at 51 C on antibiotic medium no. 2 (Difco) with LiCl added to 0.3 M. The exact explanation as to why strain βA173 did not grow on this medium while its parent strain βAO did is not clear. However, native strain βA173 walls autolyzed much more rapidly than native strain βAO walls, and strain βA173 SDS walls absorbed strain βAO autolysins with affinities different from those of strain βAO SDS walls (Fig. 9). There were three distinct salt concentrations at which lytic enzymes were released from strain βA173 walls (Fig. 9, regions A, B, and C). Peak A corresponded to the amidase since there was no *M. lysodeikticus* lytic activity in this region. The elution of this peak by less than 1 M LiCl clearly shows that strain βA173 walls are different from strain βAO walls from which amidase is eluted at 1.5 M LiCl (Fig. 6). Preliminary experiments suggested that the enzyme in region B was the glycosidase, whereas region C seemed to result from overloading the strain βA173 walls with enzyme. At low enzyme concentrations, the amidase apparently saturated the peak A sites and glycosidase appeared to fill the peak B sites. Any excess enzyme of both types seemed to bind loosely to the less-preferred peak C sites. Certainly, the fact that region C enzyme was released with the lowest salt concentration is consistent with the enzyme in this region being the least tightly bound.

If this hypothesis was correct, the amount of enzyme added in the experiment of Fig. 9 was already more than sufficient to fill all the sites of peaks A and B since the region C sites had at least begun to fill. Thus it was expected that, if even more enzyme were added to the same amount of walls, the only peak that could gain in size would be peak C, and then the height of peak C would be increased in relation to those of peaks A and B. In fact, in the experiment of Fig. 10 (top) it can be seen that when enzyme from 4 liters of strain βAO cells was added to 2 ml of strain βA173 walls at an A_{540} of 0.5, the relative size of peak C was much greater than in the experiment of Fig. 10 where enzyme from only 2 liters of strain βAO bacteria was used. In fact, the size of peak C was so large in the experiment of Fig. 9 that the resolution of peak B from peak C was not clean. However, in Fig. 10 (top) as in Fig. 9, the ratio of lysis of *B. subtilis* walls to *M. lysodeikticus* cells was greater in region C than in region B, showing that in both experiments the same type of enzyme separation was accomplished. One test of whether the enzyme in region C contained only peak A and peak B enzyme consisted of rebinding the enzyme isolated from peaks A, B, and C (Fig. 10, top) to strain βA173 walls and reanalyzing by using LiCl-sucrose gradients. From such a test (Fig. 10A, B, and C) it is clear that the region C enzyme did not band in its original position but moved to the positions of peak A and peak B enzyme. These latter enzymes were eluted with higher salt during the second centrifugation than in the original gradient. This phenomenon

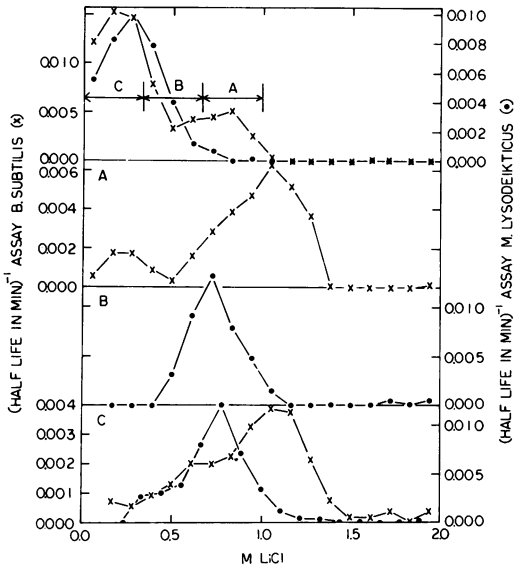

FIG. 10. *LiCl-sucrose gradient analysis of fractionated B. subtilis βAO autolysins attached to B. subtilis βA173 walls. In all gradients the sample was suspended in 0.5 ml of water, layered on top of a 4-ml 0 to 2 M LiCl, 10 to 25% sucrose gradient, and centrifuged at 35,000 × g (Spinco SW50L rotor at 21,000 rpm) for 45 min. Fractions were assayed with either strain βAO SDS walls (×) or M. lysodeikticus cells (●). Top, Sample was 3 M LiCl autolysin from 4 liters of strain βAO cells attached to 2 ml of strain βA173 SDS walls. Frames A, B, and C: Samples were fractions from the corresponding regions of the gradient of the top frame pooled and attached to 0.25 ml of strain βA173 SDS walls at an A_{540} of 0.5.*

is reproducible and might have resulted from some contaminating substance in the original extract which changed the ion exchange properties of the walls in the first gradient (Fig. 10, top). This contaminant might have been absent from the fractions collected from the first gradient for recentrifugation so the elution patterns in the second set of gradients (Fig. 10A, B, and C) were slightly different. The recoveries from this second set of gradients were all 30% or less so it might be argued that there was a third enzyme which eluted from strain βA173 walls in region C (Fig. 10 top) but which did not bind to or elute from strain βA173 walls in the experiment of Fig. 10C. This explanation appears unlikely since the strain βAO peak C enzyme could clearly be attached to, and extracted from, strain βA173 walls. The fact that region A amidase (Fig. 10 top) gave a small peak which eluted at very low salt concentrations (Fig. 10A) further supports the idea that amidase can band in both regions A and C.

Since all of the glycosidase from peaks B and C was eluted by less than 1 M LiCl, strain βAO autolysins attached to strain βA173 walls were often run on 0 to 1 M LiCl, 10 to 25% sucrose gradients to obtain better separations of peaks B and C in experiments where studies of the *M. lysodeikticus* lytic enzyme was the prime objective. From such gradients, enzyme fractions corresponding to peak B and peak C were pooled and used for pH dependence measurements (Fig. 11). These results confirmed the interpretation that enzyme corresponding to peak C was a mixture of enzyme corresponding to peaks A and B. Fractions corresponding to both peaks B and C, when tested on *M. lysodeikticus* cells, gave identical pH dependence curves. When assayed with *B. subtilis* walls, enzyme corresponding to peak B gave a curve almost coincident with those of glycosidase acting on *M. lysodeikticus*. The very small shoulder at high pH probably was due to slight amounts of remaining amidase in the fractions corresponding to peak B. However, enzyme corresponding to peak C gave basically a bimodal pH dependence curve consistent with this fraction containing two enzymes, the glycosidase with pH optimum at pH 5.0 and the amidase with pH optimum at pH 8.6 (6). The fractions corresponding to peak C clearly do not

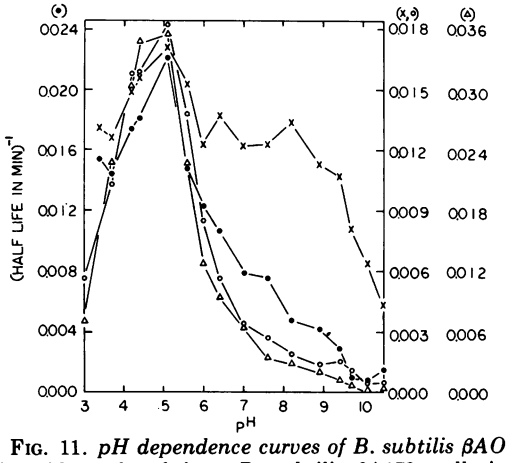

FIG. 11. *pH dependence curves of B. subtilis βAO glycosidase eluted from B. subtilis βA173 walls in LiCl-sucrose gradients. LiCl autolysin (3 M) from strain βAO cells was attached to strain βA173 SDS walls as in the experiment of Fig. 9. These walls were centrifuged through a 0 to 1 M LiCl, 10 to 25% sucrose gradient. Fractions corresponding to peaks B and C (Fig. 9) were pooled and used for measurements of pH dependence of lytic activity. (●), Peak B enzyme assayed by using strain βAO SDS walls. (×), Peak C enzyme assayed by using strain βAO walls; (○), peak B enzyme assayed by using M. lysodeikticus cells; (△), peak C enzyme assayed by using M. lysodeikticus cells.*

contain very large amounts of an additional autolysin with a new pH optimum. In three separate experiments, enzyme corresponding to peak B reproducibly gave almost identical pH curves acting on *M. lysodeikticus* cells and *B. subtilis* walls. These results show that the glycosidase can be separated efficiently from amidase by elution from strain βA173 walls and that, once separated, the glycosidase has essentially identical pH dependence for action on either *M. lysodeikticus* or *B. subtilis* substrates.

When native *B. subtilis* walls were allowed to autolyse at various pH, more reducing power was more rapidly liberated at pH 8.6 than at pH 5.0. This result might seem inconsistent with the conclusion that the glycosidase is more active at low pH than at high pH. However, due to the activity of large quantities of amidase attached to native walls, the loss of turbidity was also much greater at pH 8.6 than at pH 5.0 where essentially only the glycosidase was active. It therefore seems possible that the glycosidase attacks intact walls less efficiently than walls partially degraded by amidase.

DISCUSSION

Based on chemical analyses of wall breakdown products, pH dependence curves, heat-inactivation kinetics, and physical separations of *B. subtilis* autolytic amidase and glycosidase, it seems reasonable to conclude that *M. lysodeikticus* cells or cell walls can be degraded by the *B. subtilis* autolytic glycosidase but not the autolytic amidase. Furthermore, these studies fail to suggest the presence of a third autolysin. Mutational evidence presented in an accompanying paper (7) also indicates that *B. subtilis* has only one structural gene for the LiCl-releasable amidase.

From the reports about autolysates of *B. subtilis* walls (10, 14), it seems probable that the glycosidase is an *N*-acetyl-glucosaminidase. No activity was found when *p*-nitrophenyl-*N*-acetyl-glucosaminide was tested as substrate for the *B. subtilis* glycosidase at pH 7.0 in 0.1 M phosphate buffer in the presence of 0.1 M LiCl. Therefore, *M. lysodeikticus* remains the substrate of choice in assaying specifically for the *B. subtilis* glycosidase in the presence of the amidase.

The inactivity of the *B. subtilis* amidase on *M. lysodeikticus* walls could easily stem from differences in the peptide subunit of the glycan chains in the peptidoglycans of *B. subtilis* (10, 14) and *M. lysodeikticus* (9).

The experiments with strain βA173 walls show that overloading walls with either autolysin resulted in the saturation of one type of binding site and forced some of the enzyme to attach to less preferred binding sites. The same overloading pattern was found for strain βAO enzyme binding to strain βAO walls when the enzyme-to-wall ratio was too great. However, the separation of the glycosidase peak from the peak due to overloading was not so clean with strain βAO walls as with strain βA173 walls. In both cases, after the preferred sites were saturated, the excess enzyme bound loosely at sites from which elution occurred at low salt. The poor resolution of the overloaded enzyme from glycosidase might explain the difficulty of isolating pure glycosidase by using strain βAO walls as the ion exchange material. The fact that enzymes can bind at different sites in cell walls is not surprising since walls are complex ion-exchange materials with many different configurations of various charged groups.

The strain which was the original parent for all our mutants and which was used for all our biochemical experiments was *B. subtilis* βAO (4–8), a 168 strain from R. Epstein (Institute of Molecular Biology, University of Geneva, Switzerland). This strain was supposedly given to him by M. S. Fox (Massachusetts Institute of Technology, Cambridge). We have not been able to obtain evidence for *B. subtilis* βAO making more than two autolysins during vegetative growth, whereas Brown and Young (2) have reported three such enzymes from *B. subtilis* 168. One explanation for this discrepancy was the possibility that our strain βAO might not, in fact, be a 168 strain. Therefore, we studied another *B. subtilis* 168 strain, strain M, obtained from B. E. Reilly (School of Dentistry, University of Minnesota, Minneapolis, Minn). When walls from *B. subtilis* 168 strain M were centrifuged through a 0 to 2 M LiCl, 10 to 25% sucrose gradient, it was found that the autolysin peaks were eluted with the same salt concentrations and in the same ratios as was obtained with strain βAO walls by using either *M. lysodeikticus* cells or strain βAO SDS walls as substrate. Furthermore, 3 M LiCl autolysin from strain M attached to strain βAO walls and eluted in the same patterns as autolysins from strain βAO. Again no evidence was found for a third autolysin. We therefore suggest that our results can be generalized to other *B. subtilis* 168 strains with the difference in the number of autolysins found by ourselves and Brown and Young (2) being due to some cause other than the possibility that we were working with fundamentally different strains. Since LiCl extraction yields at most an estimated 40% of the total

autolysins, it is possible that a third autolysin which cannot be solubilized by LiCl has escaped our attention. However, as pH dependence curves of autolysis of *B. subtilis* βAO native walls (6) have shown, such a third autolysin cannot both be present in major amounts and have a pH optimum very different from those of the amidase and glycosidase we have studied.

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