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New Centrifugation Technique for Isolating Enzymes from Large Cell Structures: Isolation and Characterization of Two *Bacillus subtilis* Autolysins

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Bacillus subtilis cell walls can be centrifuged through a linear gradient of 0 to 2 M LiCl and 10 to 25% sucrose so that different autolysins are removed by different salt concentrations and banded in separate positions as the walls pass through the gradient. Using this technique we have found that *B. subtilis* cell walls are isolated with two autolytic enzymes attached. One autolysin, a glycosidase, can be eluted from walls with 0.5 M LiCl, has a *p*H optimum between 5 and 8, is relatively heat-sensitive, and has a molecular weight of 60,000. The other autolysin, an alanine amidase, can be eluted from walls with 1.5 M LiCl, has a *p*H optimum around 8, is relatively heat-stable, has a molecular weight of 35,000, and is present in quantities ten times greater than the glycosidase.

The bacterium *Bacillus subtilis* makes autolytic enzymes which can degrade the walls of the cells making the enzymes. Recent work (7) has shown that such autolysins are used during the growth process to open gaps in the cell wall to permit surface expansion and are also used to separate daughter cells which remain linked after septum formation (6, 8). These enzymes might also be involved in making competent cells capable of being transformed by deoxyribonucleic acid (DNA) (1, 11), although there is still disagreement on this subject (12). The present communication describes two methods for isolating two different autolysins from the cell walls of *B. subtilis*.

MATERIALS AND METHODS

Unless otherwise stated, all materials and methods were as described previously (5-7).

Buffers. TM buffer was 0.05 M tris(hydroxymethyl)aminomethane (Tris), 0.05 M maleic anhydride, *p*H adjusted to 6.0 with NaOH. TK buffer was 0.1 M Tris, 0.1 M KCl, *p*H adjusted to 8.6 with HCl. HK buffer was 0.1 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.1 M KCl, *p*H adjusted to 8.0 with NaOH. For *p*H optima experiments, the TMB buffer was 0.2 M maleic anhydride, 0.2 M Tris, 0.2 M boric acid, *p*H adjusted as desired with NaOH; this last buffer does not store well without undergoing a change in *p*H, so the *p*H was always checked before the beginning of each experiment.

Cell wall preparation. Exponential cultures [absorbancy at 540 nm (A_{540}) = 0.5 to 0.8] of *B. subtilis* βAO growing in Penassay Broth (Difco) at 37 C were harvested by centrifugation. Then the cells were resuspended in distilled water and disrupted by sonic oscillation. All subsequent centrifugations for wall isolation were performed in water for experiments involving a LiCl-sucrose gradient. All preparations of enzyme released by 3 M LiCl in TK buffer were made as previously reported (5).

Autolysin assays. The substrate was exponential cell walls treated with sodium dodecyl sulfate (SDS) to remove residual autolysins (7). Two assay methods were used. (i) Dilution assay: Enzyme samples in LiCl solutions were assayed by dilution into a suspension of SDS-treated walls to reduce the LiCl concentration below 0.03 M. Then the loss of A_{540} due to digestion of the walls was followed spectrophotometrically as a function of time at 45 C. (ii) Attached wall assay: When the enzyme concentration was very low, it was often desirable to concentrate the enzyme before the assay. In these cases the enzyme was first attached to SDS-treated walls in dilute suspension, and then the walls were concentrated for the assay. In such experiments, the samples were added to SDS-treated walls and then diluted with water to reduce the LiCl concentration to below 0.03 M. After 5 min at 0 C, all the free enzyme was attached to the SDS-treated walls since in low LiCl concentrations free enzyme will bind walls (5). The walls were collected by centrifugation at 27,000 × *g* (15,000 rev/min in a Sorvall RC2-B centrifuge with an SS-34 rotor) for 5 min. Sometimes the walls

were washed with the same volume water as originally added. The SDS-treated walls with enzyme attached were finally resuspended in 0.8 ml of buffer, and A_{540} was followed with time at 45 C. The A_{540} of the walls at the beginning of all assays was usually approximately 0.1 in about 1 ml of buffer. The enzyme activity in both assay procedures was measured as the reciprocal of the half-life of loss of A_{540} (5). In the enzyme concentration ranges used, this measurement has been shown previously to be linear with enzyme concentration (5). Autolytic rate was measured in the same way at 45 C.

Teichoic acid extraction. Cell walls were suspended in 10% trichloroacetic acid and incubated at room temperature over one or two nights (13). Then remaining trichloroacetic acid was removed by exhaustive washing with H_2O.

Phosphate content. Phosphate determinations were made using a modification of the procedure of Chen, Toribara, and Warner (4).

Reducing group release. Measurements using the Park-Johnson assay were made according to the procedure of Ghuysen, Tipper and Strominger (9). The turbidity of some samples due to unlysed walls was considerable. Therefore, each sample was read against a blank in which all components were identical except that just before reading of the A_{690} all parts of the reaction mixture were mixed together at the same time and not boiled.

N-terminal alanine release. The procedure was as described by Ghuysen, Tipper and Strominger (9). After wall lysis, the samples were treated with fluorodinitrobenzene, hydrolyzed, and chromatographed. After chromatography, the spot corresponding to the dinitrophenol derivative of alanine was suspended in buffer, and the A_{360} was read. No other spots on the chromatograms change intensity with wall hydrolysis.

LiCl-sucrose gradient centrifugation. A linear gradient from 0 to 2 M LiCl was constructed in a centrifuge tube using equal volumes of two solutions: 10% sucrose in TK buffer, and 25% sucrose and 2 M LiCl in TK buffer. The sucrose was present for gradient stability. A sample of walls which had been frozen and thawed in water was layered on top of the gradient. The maximum sample volume was 10% of that of the gradient. The gradient was centrifuged in a swinging bucket rotor at 10,000 $\times$ g (8,000 rev/min in a Sorvall RC2-B centrifuge with an HB-4 rotor) for 70 min. After this time all of the walls were pelleted. To empty the gradient, a 35% sucrose solution was pumped into the centrifuge tube at the bottom displacing the lighter gradient volume which was collected in a fraction collecter. When the wall sample to be passed through the gradient was frozen and thawed there was formation of clumps which sedimented under the conditions given. However, if the sample had not been frozen or if a frozen and thawed sample were agitated vigorously to obtain dispersal of the clumps, not all walls pelleted. In these cases the gradient was centrifuged at 51,000 $\times$ g (17,000 rev/min in a Spinco ultracentrifuge using an SW27 rotor with large buckets) for 1 hr to sediment all of the walls in the sample. The recovery of enzyme was approximately the same with either technique.

Sephadex G-75 chromatography. Sephadex G-75 was equilibrated with 3 M LiCl in TK buffer and then poured to give a column bed 2.5 by 90 cm. The column and elution buffers were both 3 M LiCl in TK buffer because the sample was stable in this solution. The elution buffer was forced from the bottom of the column to the top by a peristaltic pump to give a flow rate of 0.5 ml/min. To obtain better separation, the sample was sometimes recycled through the column. For this procedure, an enzyme mixture, including some blue dextran 2000 (Pharmacia) to mark the void volume, was loaded onto the column. Then inflow and outflow tubes were joined, and liquid was passed through the column by using a peristaltic pump with the same setting as above. When the blue dextran was about to exit from the column a second time, the outflow tube was connected to a fraction collector and the inflow tube to a 3 M LiCl in TK buffer reservoir. The pumping was then continued to obtain eluted fractions.

RESULTS

The starting material for the enzyme isolation was native exponential *B. subtilis* βAO cell walls. Such walls have autolysins already attached (5, 14). As previously reported (5), autolysin can be released from walls by soaking the walls with 3 M LiCl. We decided to study at what LiCl concentration the actual extraction took place by testing for autolysin release with various LiCl molarities. The method used for exposing walls to different LiCl concentrations was to centrifuge a layer of native cells through a sucrose gradient which had also incorporated a LiCl gradient extending from 0 to 2 M. As the walls passed through the LiCl concentration at which enzyme was released, the enzyme would dissociate and move with the velocity of free protein, while the walls, being much larger, would continue their way to the bottom of the gradient at a much faster rate. Since it will be shown later that the released autolysins have molecular weights of approximately 35,000 and 60,000, it can be computed that the liberated enzymes have moved less than 1% of the total distance down the gradient during the entire period of centrifugation. Therefore, for all practical purposes the enzymes are found in the gradient at the positions at which they were released by high salt. Experimental confirmation of this conclusion comes from the finding that when centrifugation times are increased threefold there is no change in the profiles of the released enzyme. Thus, fractions collected from the gradient would represent elution of the cell wall with different LiCl con-

centrations.

The results of the assays from such a gradient are presented in Fig. 1. The gradient itself was calibrated by reading samples from the fractions in an Abbé refractometer (Fig. 1, center frame). Since our calibration measurements showed that the increase in refractive index is proportional to the LiCl and sucrose concentrations, the data in Fig. 1 show that the gradient was linear with respect to salt and sucrose except at the two ends. The low reading at the low LiCl concentration end is from the water left from the sample of walls layered on the gradient. The high reading at the other end is from the high sucrose concentration used to displace the gradient during the collection process. Since the refractive index readings from the gradient reflect both the sucrose and LiCl present in the gradient, these readings do not directly give LiCl concentrations. To obtain values for the LiCl molarity at various positions in the gradient, the buffers used to make the gradient were mixed in different ratios to give known LiCl concentrations. The refractive indexes were then read. Fractions from the gradient with corresponding refractive indexes were assumed to have corresponding LiCl concentrations. The samples were read for A_{260} and A_{280} (Fig. 1, center frame). It can be seen that most of the ultraviolet-absorbing material stays with the sample which was layered on. In the region of the gradient where the autolysins are released, there is very little ultraviolet-absorbing material, indicating that any enzymes found in the gradient are quite pure. Since all fractions contained different LiCl and sucrose concentrations it was decided to mix each fraction with a corresponding fraction from an identical gradient to which no sample was added and from which collection was in reverse order. In this way the appropriate amount of sucrose and LiCl was added to all fractions except the end ones to give a final sucrose concentration of 17.5% and a final LiCl concentration of 1 M (Fig. 1, center frame). Thus the results of any biological or biochemical test using these diluted samples could not be attributed to differences in sucrose and salt levels.

These diluted samples were assayed for ability to lyse SDS-treated walls under two

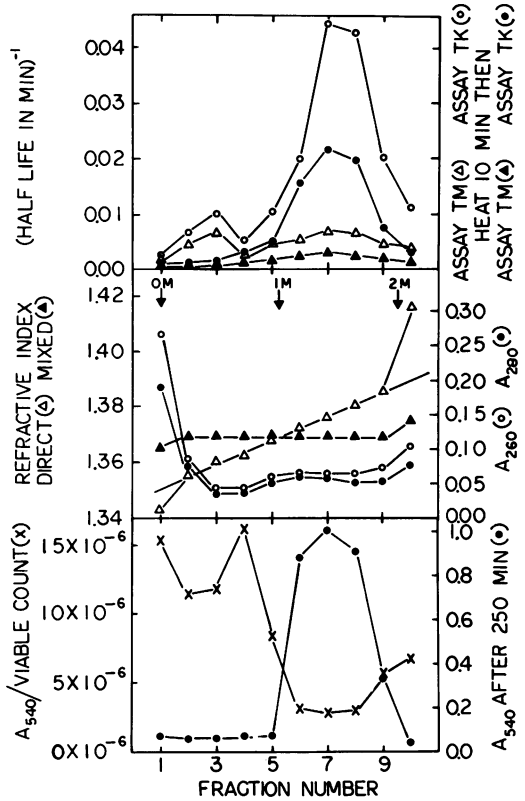

FIG. 1. *Elution of two autolysins from walls of Bacillus subtilis βAO in a LiCl-sucrose gradient. Cell walls were made from an exponential culture of 3 liters of bacteria, layered on a 20-ml 0 to 2 M LiCl, 10 to 25% sucrose gradient, and centrifuged as described in the text. Fractions of 2 ml were collected, and the refractive indexes were read (△, center frame). The correspondence between refractive index and LiCl molarities is indicated by the vertical arrows. Absorbancy at 260 nm (A_{260}) (○) and A_{280} (●) readings are also given in the center frame. After these readings were made, a reverse gradient was mixed as described in the text. Then the refractive indexes were read again (▲, center frame), and all other measurements were made on these mixed fractions. From each fraction, 0.4 ml was assayed for wall lytic activity (top frame) using the attached wall assay technique in either TK buffer (○) or TM buffer (△). From each fraction, 0.8 ml was heat-inactivated for 10 min at 52 C and then assayed as above (top frame) in either TK buffer (●) or TM buffer (▲). Long chains of βAO were produced by growth at 48 C, as previously described (6). The average chain length was determined after a 20-min incubation of 0.2 ml of each fraction with a 2-ml chain-containing culture at A_{540} = 0.35 (×, bottom frame). As previously reported (6), the chain length can be obtained by dividing the A_{540} of culture by the viable count. The ability of fractions to stimulate the growth of mutant βA177 was tested (●, bottom frame) by adding 0.2 ml of each fraction of 2 ml of growth-inhibited cells and then reading the A_{540} of the culture after 250 min of aeration at 51 C (7). The more the growth enhancement, the higher is the A_{540} after the incubation.*

different sets of conditions. One set was that standardly used in our laboratory for assaying autolysins (TK buffer at *p*H 8.6). From Fig. 1 it can be seen that two peaks of enzyme activity are found, one of which is much smaller than the other. In different experiments the ratio varies with the activity of the minor component, being usually about 10% of that of the major peak. One enzyme is optimally released at 0.5 M LiCl and, as later experiments will show, is a glycosidase. The other peak, eluted maximally by 1.5 M LiCl, will be shown to be an alanine amidase. Since it had been reported that *B. subtilis* made an autolysin with *p*H optimum around 5.5 (3), it was decided to assay the gradient at *p*H 6.0. Again two peaks were found (Fig. 1); however, the ratio of activity was quite different, with the major peak at *p*H 8.6 now being the minor peak. The different efficiencies of the assay at different *p*H values suggested that the two peaks were composed of separate enzymes each with a different *p*H optimum.

Further evidence was obtained when the fractions from the gradient were heat-inactivated at 51 C for 10 min. In earlier experiments (6, 7) it had been shown that after such treatment of 3 M LiCl-released enzyme, considerable autolysin activity still remains. The results given in Fig. 1 show that the amidase is much more heat-stable than the glycosidase. Furthermore, the fact that after heat inactivation there is practically no activity left in the glycosidase peak indicates that the enzyme separation is quite clean with almost no overlap.

Enzyme recovery calculations could be made since it had been found that, in the concentration range studied, the rate of lysis was directly proportional to amount of enzyme present on walls (5). Therefore the concentration of autolysin on walls is given by the lytic rate. Hence, amount of enzyme can be measured as the rate of the lysis times the quantity, or A_{540} value, of walls studied. For enzyme recovery studies after gradient separation, the SDS-treated walls used for the assay came from the same batch of walls as was used for the gradient. It was assumed that, for a given lytic rate, the same quantity of enzyme per amount of wall would be required for either autolysis where native enzyme is attached to native wall or lysis of SDS-treated walls where enzyme is added from gradient fractions. In the experiment of Fig. 1 it was calculated from assays in TK buffer at *p*H 8.6 that 29% of all autolysins were released into the gradient and 19% of the autolysins pelleted with the walls which passed through the gradient. Thus roughly half of the enzyme layered onto the gradient could not be accounted for. This loss of enzyme could be due to release by LiCl and subsequent inactivation so that only half of the activity could be recovered. The amount of enzyme recovered is comparable to earlier experiments (5) in which it was found that, over a LiCl concentration range extending up to 6 M, the maximum amount of activity released was about one-third of the total. These enzymes recovery computations are only approximations and may be underestimates if the endogenous autolysins acting on native walls are more efficient than LiCl-extracted enzymes acting on SDS-treated walls. When the gradient range was increased to 0 to 6 M LiCl, there was no new peak of activity released. As shown previously (5), above 6 M there is an inactivation of released enzyme.

Earlier experiments indicated that wall lytic enzymes can be added to growing cultures to dechain cells held together by septum material after septum completion (6). The experiment of Fig. 1 shows that both enzyme peaks can separate *B. subtilis* growing as long chains at 48 C since the chain lengths become shorter if enzyme from either peak is added. The shortening is less pronounced when the glycosidase is added, suggesting that the conditions for enzyme action on living cells in the culture was equivalent to in vitro conditions at high *p*H so that amidase activity was greater than glycosidase activity.

A mutant strain of *B. subtilis*, βA177, was isolated (7) in which growth could be limited under conditions where not enough autolysins were being produced to open enough gaps in the cell wall to allow for an optimum rate of surface expansion. Growth could be enhanced by addition to the culture of either lysozyme or *B. subtilis* autolysins removed from walls by 3 M LiCl. The results of the test of whether both the glycosidase and amidase could stimulate growth of this mutant are presented in Fig. 1. There is a clear positive effect due to the amidase as reflected in greater A_{540} readings 250 min after addition of fractions from the gradient. There seemed to be no effect of the added glycosidase. This lack of stimulation could easily be due to this enzyme being added below the threshold needed to produce an effect. It is known that the response to autolysins extracted with 3 M LiCl is not linear and a threshold level is often required before any enhancement is seen (7). Lysozyme which is known to be a glycosidase will also enhance the growth of this mutant (7). Therefore it can

be concluded that growth of mutant βA177 can be stimulated by disruption of either the polysaccharide backbone or the peptide cross bridges in the peptidoglycan. The suggestion from these results is that degradation of the peptidoglycan in any way can probably lead to growth enhancement.

The suggestion that the glycosidase and amidase had different *p*H optima (Fig. 1) was strengthened by pooling peak fractions from a gradient similar to the one described in Fig. 1, attaching the released autolysins to SDS-treated walls, and determining lytic rate as a function of *p*H. It is clear from the experiment of Fig. 2 that the amidase has a *p*H optimum of about 8 whereas the glycosidase assay shows a much broader *p*H dependence with a peak of activity between *p*H 5 and 8. The dip in activity in the middle of the glycosidase peak is not reproducible and is due to fluctuation in the experimental measurements. When TK buffer was used, the assay of the glycosidase at *p*H 8.6 was more efficient than under any other condition tried. This result was to be expected from the curves of Fig. 1, in which the glycosidase lytic rate is slightly higher in TK buffer than in TM buffer at *p*H 6.0. Therefore, routine assays of both enzymes were performed in TK buffer. For comparison the *p*H dependence of autolysis of native walls is also plotted (Fig. 2). This curve is essentially identical to the curve for the amidase alone, with a small shoulder in the *p*H region where the glycosidase activity is most prominent, consistent with the idea that native walls have attached amidase and glycosidase in about the ratio found for enzymes released in a LiCl-sucrose gradient. If *B. subtilis* walls have another autolysin, as reported by Brown and Young (3), with *p*H optimum at 9.5, it seems to be largely absent from walls of strain βAO since even native walls do not show a *p*H optimum at 9.5 (Fig. 2). In all cases, at *p*H 9.5 and above there is inactivation of autolysin during the assay since the rate of autolysis rapidly diminshes toward zero.

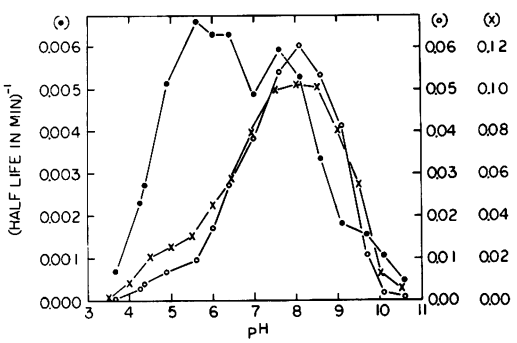

FIG. 2. *The pH dependence of autolysis of native Bacillus subtilis βAO walls and of isolated amidase and glycosidase acting on SDS-treated walls. Native βAO walls harvested from exponential cells were suspended in TMB buffer adjusted to various pH values, and the autolytic rate was measured* (×). *From a gradient similar to the one given in Fig. 1, fractions corresponding to the peak of glycosidase activity were pooled and the enzyme was attached to SDS-treated walls as described in the text. Then the walls were suspended in TMB buffer at various pH values, and the lytic rate measured* (●). *The same experiment was performed on pooled fractions from the peak of amidase activity* (○).

Brown and Young (3) have reported that one *B. subtilis* autolysin is a glycosidase, and Hughes (10) has suggested that such an enzyme would be an endo-β-*N*-acetylglucosaminidase. The results of the experiment identifying the enzyme eluted with 0.5 M LiCl as a glycosidase are given Fig. 3. Autolysins were fractionated in a gradient as described in Fig. 1. Then all fractions were added to SDS-treated walls. A portion of the mixture was used to follow lytic activity, while another por-

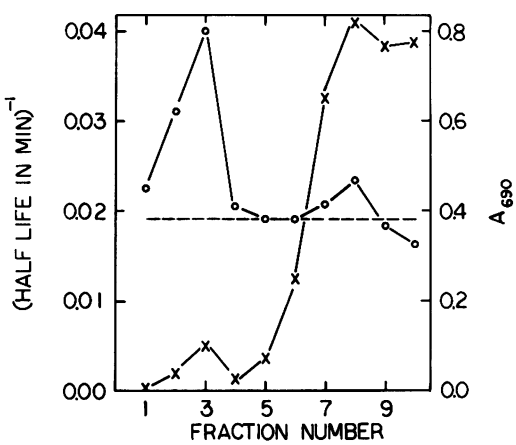

FIG. 3. *Release of reducing groups by autolysins fractionated in a LiCl-sucrose gradient. A gradient was run as described in Fig. 1, and 2-ml fractions were collected. SDS-treated walls (1 ml at* A_{540} = *0.5) were added to 1 ml of each fraction. After dilution to attach enzyme to the walls, the walls were collected by centrifugation and resuspended in 0.6 ml of TK buffer. A small portion (0.08 ml) was diluted into TK buffer for assay of lytic rate* (×). *The rest was incubated for 1,200 min at 45 C and heated at 95 C for 20 min to inactivate the remaining enzyme. Then each sample was divided into two equal portions, one-half of which was used for the reducing group assay and one-half of which was used as the blank for the assay as described in the text* (○). *Dashed line gives the reducing group assay performed on a sample of walls treated as above but without added enzyme.*

tion was left to lyse for 1,200 min at 45 C and then was used in the Park-Johnson assay for reducing groups. The dashed line (Fig. 3) gives the background amount of reducing found in cell walls with no added enzyme. Therefore any groups generated by an autolysin would be given an A_{690} reading significantly higher than the dashed line. It can be seen that reducing groups were released only by enzyme corresponding to the minor peak. Based on four experiments, the reducing group release by the enzyme of the smaller peak is reproducible, whereas the slight amount of additional reducing power in the region of the major peak is not and represents fluctuation in the assay. Thus the enzyme eluted by 0.5 M LiCl is a glycosidase capable of generating free reducing groups.

Young (14) and Hughes (10) have reported that the principal autolysin produced by *B. subtilis* is an alanine amidase cleaving the bond between *N*-acetylmuramic acid residues and L-alanine residues in peptidoglycan. Therefore, alanine amidase activity was checked for the two autolysins separated in the gradient. After cleavage by the enzyme extracted by 1.5 M LiCl, N-terminal alanine was released (Fig. 4). HK buffer was used for the lytic digestion instead of TK buffer because the latter buffer itself has a large number of N-terminal groups. The *p*H of HK buffer was 8.0, not 8.6, in order to maintain good buffering capacity. The efficiency of the assay of either amidase or glycosidase in this buffer was about 75% of that in TK buffer. In this particular gradient very little glycosidase was present. However, in another gradient where more glycosidase was present, the incubation before the assay of N-terminal alanine was prolonged so that the peak fraction of glycosidase could completely digest the substrate. Even under these conditions there was no significant increase in the N-terminal alanine above the background amounts found in tubes 1 through 4 in the gradient shown in Fig. 4. Thus, all of the alanine amidase activity coincided with the 1.5 M LiCl-released enzyme.

To explain the report that a *B. subtilis* autolysin is tightly bound to teichoic acid (2), it might be proposed that autolysins bind teichoic acid before acting to degrade the peptidoglycan. If this proposal were true, then walls stripped of teichoic acid might act less well as substrate for autolytic enzymes. Therefore, SDS-treated walls were extracted with trichloroacetic acid to remove teichoic acid and then tested as substrate for both the amidase and the glycosidase. In two separate experiments teichoic acid was removed to the extent of decreasing phosphate content 80 and 90%, respectively. In both cases, remaining walls acted as substrate for both enzymes with essentially no loss in efficiency. Therefore autolysin activity is not dependent on walls having the majority of their teichoic acid still attached. Measurements of teichoic acid attached to glycosidase or amidase after a gradient isolation were abandoned when it was found that the gradient buffer interfered with the phosphate analysis.

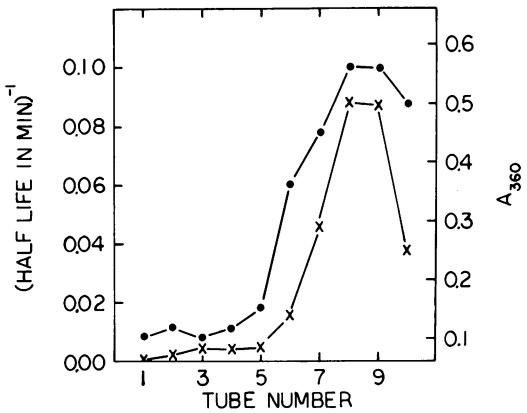

FIG. 4. *Release of N-terminal alanine by autolysins fractionated in a LiCl-sucrose gradient. A gradient was run and treated identically to the one described in Fig. 3 up to the point of resuspension in TK buffer. Instead of TK buffer, HK buffer was used. Then 0.08 ml of the resuspended walls was diluted and assayed in HK buffer* (×) *as in the experiment of Fig. 3. The rest of the sample was incubated for 200 min at 45 C and heated at 95 C for 20 min. Then each sample was hydrolyzed and assayed for N-terminal alanine as described in the text* (●).

In earlier experiments (6) it was proposed on the basis of exponential heat inactivation kinetics that there was principally one enzyme found in 3 M LiCl extracts of native walls. It was of interest to determine whether both the glycosidase and amidase were present in such extracts. Therefore, enzyme released in 3 M LiCl in TK buffer was mixed with SDS-treated walls and then diluted with water to permit the autolysin to bind the walls. These walls with 3 M LiCl-released autolysins attached were then concentrated and centrifuged through a LiCl-sucrose gradient to separate the amidase and glycosidase. Upon assay of the fractions collected, it was found that there was the same approximate ratio of glycosidase to amidase as was found with native walls. Using amidase and glycosidase isolated in a gradient, heat inactivation experiments were performed in 3 M LiCl in TK buffer, the same conditions as used when exponential heat inactivation

kinetics were found earlier (6). The results were the same as those given in the experiment of Fig. 1, namely that the glycosidase was much more heat-labile than the amidase. Therefore, in the heat inactivation curve of a 3 M LiCl extract of native walls, the enzyme activity would be expected to show a steep drop to about 90% of the initial value, representing complete destruction of the glycosidase, followed by a more gradual decay characteristic of the amidase. This small perturbation in the measured kinetics would have looked like experimental error in the previously published data (6). The small amount of glycosidase present in 3 M LiCl autolysins and the high background in the glycosidase assay could also account for the fact that in earlier experiments (7) no glycosidase activity was found associated with 3 M LiCl extracted autolysins.

Since 3 M LiCl will release both enzymes in relatively stable form, approximate molecular weights could be obtained by fractionation on a gel filtration column. Exponential cell walls were treated with 3 M LiCl, and the extract was passed through a Sephadex G-75 column (Fig. 5). A single peak of enzyme activity was found which had a shoulder on the left. There was no enzyme activity from fraction 40 through fraction 110. Since the recovery of enzyme activity from such Sephadex columns is 100% within 10%, the enzyme activity of the major peak could be attributed to the amidase while that of the shoulder could be assigned to the glycosidase. The K_D of the amidase could be obtained unambiguously from the elution profile, and the K_D of the glycosidase could be estimated by the position of the shoulder. In another experiment (Fig. 5, inset) the enzyme was passed twice through the same Sephadex G-75 column just described. In this case the two enzyme activities could be completely separated. The K_D of the glycosidase could now be obtained more cleanly by noting the position of the peak between that of the amidase and the void volume. As expected, the range of ratios of glycosidase to amidase as separated by Sephadex G-75 filtration was within the range obtained by passing native walls through a LiCl-sucrose gradient. When the column was calibrated with bovine serum albumin, ovalbumin, chymotrypsinogen, and cytochrome c, it was found that the amidase banded between ovalbumin and chymotrypsinogen, with an estimated molecular weight of 35,000. The glycosidase peaked between bovine serum albumin and ovalbumin, and a molecular weight of 60,000 was obtained using K_D estimated either by the position of the shoulder or by the position of the isolated peak after passage twice through the column. From the A_{260} and A_{280} readings, it can be seen that very little protein coincided with either isolated enzyme. Most of the ultraviolet-absorbing material in the 3 M LiCl extract of the walls moves in either the void or total volume.

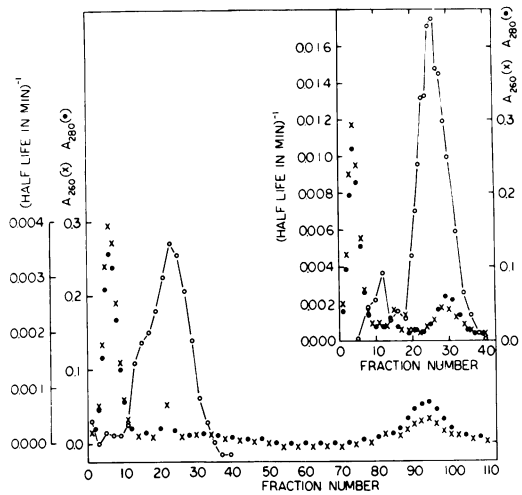

FIG. 5. *Sephadex G-75 column separation of amidase and glycosidase. Main graph: Cell walls from 4 liters of exponential βAO cells were extracted with 3 M LiCl in TK buffer as previously described (5). This autolysin-containing solution (3 ml) was mixed with 0.1 ml of 5% blue dextran 2000 and loaded onto the column. Fractions of 3 ml were collected and analyzed for* A_{260} (×), A_{280} (●), *and lytic activity* (○) *by the dilution assay mixing 10 μliters of fraction and 1 ml of SDS-treated walls at* $A_{540} = 0.1$. *Void volume was at fraction number 6, and total volume was at fraction number 93. Inset: The same column was run as above except twice as much sample at the same enzyme concentration was loaded onto the column. The material was passed twice through the column as described in the text. Fractions of 6 ml were collected and assayed for lytic activity by the attached wall method using 0.1 ml of each fraction and 1 ml of SDS-treated walls at* $A_{540} = 0.1$ (○). *The* A_{260} (×) *and* A_{280} (○) *were also measured. Only the portion of the column with enzyme activity is pictured. Void volume was at fraction number 4.*

DISCUSSION

The experiments reported above indicate that isolated *B. subtilis* βAO walls have attached an alanine amidase, presumably an *N*-acetylmuramyl-L-alanine amidase (10, 14), and a glycosidase, presumably an endo-β-*N*-acetylglucosaminidase (10). The amidase is the major enzyme when the assay is performed in TK buffer at *p*H 8.6 where both enzymes exhibit maximal activity. The enzymes can be separated either by elution of walls with different LiCl concentrations or by chromatog-

raphy of 3 M LiCl-extracted enzyme mixture through Sephadex G-75 columns. In both cases about one-third of the autolytic activity of native walls can be recovered. The autolysins isolated by either technique are largely free from contamination by other proteins since the ultraviolet abosrption in the regions of enzyme peaks indicate that there is almost no protein present. Phosphate analyses on gradient fractions were impractical so that contamination of isolated enzyme by teichoic acid was not measured directly. However, the finding of small molecular weights for enzymes obtained from Sephadex G-75 columns suggests that any contamination by teichoic acid must be minimal since autolysin with teichoic acid bound is much larger and elutes with the void volume even when Sephadex G-200 columns are used (2).

Our result that the amidase has a higher *p*H optimum than that of the glycosidase is in agreement with analyses of crude autolysates (3), which show that glycosidase activity is present principally at *p*H values below 9.5, whereas substantial amidase activity is found at that high *p*H.

Our LiCl-sucrose gradient technique should have general applicability for eluting proteins from any large cell structure including cell membranes. For example, for proteins removable from membranes by a detergent, a detergent gradient can be used in place of the LiCl gradient, so that at different detergent concentrations different proteins will be eluted. In fact, for structures less dense than the gradient solution, passage through the gradient can take place by flotation, with the sample initially placed at the bottom of the centrifuge tube and the gradient layered on top.

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