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Autolytic Enzyme System of *Streptococcus faecalis*

V. Nature of the Autolysin-Cell Wall Complex and Its Relationship to Properties of the Autolytic Enzyme of *Streptococcus faecalis*

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Cell walls from exponential-phase cultures of *Streptococcus faecalis* ATCC 9790 contain an autolysin (a β-*N*-acetylmuramide glycanhydrolase, E.C. 3.2.1.17) which has been isolated from trypsin-speeded wall autolysates. The autolysin, which was excluded from Bio-Gel P-60, was further fractionated by diethylaminoethyl (DEAE)-cellulose chromatography or filtration on Bio-Gel P-200. After DEAE-cellulose chromatography, which removed most of the wall polysaccharide, autolysin activity was extremely labile and was rapidly lost at −20 C, even in the presence of albumin. The P-60-excluded enzyme was rapidly bound by walls at both 37 C (50% bound in about 1 min) and 0 C (50% bound in less than 4 min). Wall-bound autolysin could not be removed by 1.0 M ammonium acetate (*p*H 6.9). Autolysin was also bound by walls that had been extracted with 10% trichloroacetic acid or treated with 0.01 N periodate, suggesting that the nonpeptidoglycan wall polymers are not important for binding. Wall-bound autolysin was more stable than the soluble enzyme to proteinase digestion, acetone (40%), 8 M urea (at 0 C), or to inactivation at 56 C. Two bacterial neutral proteinases (which do not hydrolyze ester bonds) activated latent wall-bound autolysin, suggesting that activation results from the cleavage of one or more peptide bonds. The group A streptococcal proteinase activated latent autolysin but differed from the other proteinases in that it did not inactivate soluble autolysin. The results suggest that the autolysin is not covalently linked to the wall. The high affinity of the walls for the autolysin appears to be responsible for the firm, not easily reversed binding.

Exponential phase cultures of *Streptococcus faecalis* ATCC 9790 contain an autolytic enzyme. This autolysin, a β-*N*-acetylmuramide glycanhydrolase (E.C. 3.2.1.17), has been found to occur virtually exclusively in the cell-wall fraction (log walls) in both an active and a latent proteinase-activatable form (19). During autolysis of log walls, either in the presence or absence of trypsin, the autolysin was not released to the 25,000-g supernatant fraction until wall dissolution was virtually complete. In fact, trypsin-activated autolysin present in log walls failed to attack ^{14}C-labeled, sodium decylsulfate-inactivated log walls (SDS-walls), simultaneously present in the same incubation mixture, until dissolution of the unlabeled log walls was virtually complete (18). These results were interpreted to indicate that latent autolysin was activated by trypsin without being released from the wall substrate. A previously considered (18) possible explanation for these and other findings is that the autolysin is covalently linked to the wall so that it cannot easily be released or displaced to freshly added equivalent substrate. However, if the autolysin is covalently linked to the insoluble wall, it is difficult to visualize just how the enzyme can successfully cleave over 90% of the susceptible bonds in the same insoluble wall.

Consistent with a covalent linkage between autolysin and wall, we were previously unable to obtain an enzymatically active fraction that did not contain a considerable portion of the rhamnose and phosphorus-containing wall polysaccharides, even after complete dissolution of the wall substrate. We now report that, by ion-exchange chromatography or gel filtration, we can obtain enzymatically active fractions which contain only a tiny fraction of the wall polysaccharides initially present. This separation of autolysin from wall polysaccharides, as well as the facility with which the isolated soluble autolysin can, apparently irreversibly, bind to isolated walls even at 0 C, suggests that the autolysin is not covalently linked to the wall, but that it is bound to the wall by some other type of linkage. When wall-bound, the autolysin was more resist-

ant to heat, proteinase digestion, and denaturation by urea or acetone.

MATERIALS AND METHODS

Bacterial strains, growth conditions, cell disruption, and cell-wall isolation. *S. faecalis* ATCC 9790 was grown in highly buffered, synthetic medium and harvested during the exponential growth phase (log cells) (19). In most cases, cells were broken by shaking with plastic beads (19). In some cases, the cells were disrupted in a cell fractionator (U.S. Technical Development Co., New York, N.Y.) operated at 18,000 to 20,000 lb per in^2. Cell walls (log walls) were isolated, washed, and stored (19). For use as substrate, isolated log walls were inactivated by treatment with sodium decylsulfate (SDS or SDS-walls). The 10 carbon detergent rather than the 12 carbon (dodecyl or lauryl) compound was used because of its greater solubility, particularly at low temperature. In some cases, the inactivation was performed immediately after removing the crude walls from the broken cell preparation. Assays of lytic enzyme activity were carried out using SDS-walls as substrate in 0.01 M phosphate or ammonium acetate (*p*H 6.7) (19). One unit of enzyme was defined as the amount giving a decrease in optical density (OD) of 0.001 per hr.

Autolysin isolation. Log walls were allowed to autolyze in 0.01 M ammonium acetate (*p*H 6.7) in the presence of 0.05 μg of trypsin per ml. The lysates were concentrated by lyophilization, made up to a small volume in water, and centrifuged at 30,000 × *g* for 30 min to remove the small quantity of insoluble residue usually present. This supernatant fluid was usually applied to a Bio-Gel P-60 column to separate the autolysin-containing fraction (fraction II, excluded from the gel) from the disaccharide peptide oligomer (degraded peptidoglycan, fraction III) and trypsin (19). In one experiment (Fig. 2A), the crude wall lysate was directly applied to a Bio-Gel P-200 column. Fraction II contains large quantities of rhamnose and phosphorus and a small amount of free acetamido sugar in addition to autolysin activity. Fraction II preparations were further purified by diethylaminoethyl (DEAE)-cellulose chromatography by use of a slight modification of the method described by Young (21). A stepwise elution, by using 0.01, 0.1, 0.15, 0.2, and 0.5 M ammonium acetate (*p*H 6.9), was employed with type 20 DEAE-cellulose (Schleicher & Schuell Co., Keene, N.H.) having a capacity of 0.91 meq per g at 4 C. Intermediate elution steps were not found to result in increased resolution. Columns 12 by 1.5 cm at a flow rate of 30 ml per hr or 12 by 0.9 cm at a flow rate of 10 to 12 ml per hr were employed. Fractions of 1.3 ml were collected.

Binding of autolysin to cell walls. Initially binding experiments were carried out by exposing walls (2.1 mg) to soluble autolysin (50 to 350 units of fraction II) for a known time interval and sedimenting the walls at 30,000 × *g* for 30 min at 0 C; this was followed by one wash on the centrifuge. However, the rapidity with which binding occurred, even at 0 C, necessitated a more rapid means of separating enzyme from substrate. Filtration through cellulose acetate membrane filters (25 mm in diameter and 0.45 μm pore size) was found to separate walls from the autolysin in 1 to 2 min. All kinetic studies of binding, such as those shown in Fig. 4, were carried out by this filtration and followed by one wash in cold water. Even the short time required for filtration resulted in 20 to 45% of the autolysin being bound to SDS-walls (zero-time samples). Also, the filtration method gave somewhat inconsistent results. For example, in one case, after 40 min of binding at 37 C, 80 to 85% of the autolysin was found to be bound after filtration, whereas all of the enzyme was found to be bound after centrifugation. Therefore, maximal binding was determined after sedimenting the walls by centrifugation and, when possible, determining the nonbound activity remaining in the supernatant fluid as well as that bound to the walls.

Analytical methods. Rhamnose was determined by the method of Dische and Shettles (6). Phosphorus was determined either by the method of Boltz and Mellon (3) or by that of Lowry et al. (14). Acetamido sugars were determined by the Morgan-Elson reaction after 30 min at 100 C in 1% borate (9), with either *N*-acetylglucosamine or *N*-acetylmuramic acid as a standard.

Materials. The neutral proteinase of *Bacillus thermoproteolyticus* (crystalline) was a gift of K. Morihara, as were the group A streptococcal proteinase from T. -Y. Liu and the proteolytic enzyme of *S. faecalis* var. *liquefaciens* from L. Zimmerman. Leucine aminopeptidase (E.C. 3.4.1.1) was purchased from both Worthington Biochemical Corp. (Freehold, N.J.) and Mann Research Laboratories, New York, N.Y. Trypsin (E.C. 3.4.4.4., twice crystallized, salt-free) was purchased from General Biochemicals Corp., Chagrin Falls, Ohio. The neutral proteinase (crystalline) of *B. subtilis* was purchased from Miles Laboratories, Inc., Elkhart, Ind.

RESULTS

DEAE-cellulose separation. Fractionation of the Bio-Gel P-60-excluded fraction (fraction II) from a trypsin-speeded lysate of log walls, on a DEAE-cellulose column, consistently produced the elution pattern for rhamnose- and phosphorus-containing substances shown in Fig. 1. In the experiment shown in Fig. 1 (experiment 4B in Table 1), about 2 μg of bovine serum albumin was placed in each collecting tube, and 46% of the autolysin activity applied was recovered. Most of the activity (82% of that recovered) was found in the peak eluted with 0.1 M buffer. A smaller peak of activity (12% of that recovered) was eluted by 0.15 M buffer at the front of the rhamnose- and phosphorus-containing peak, and a trace of enzyme activity (6% of that recovered) was detected in the 0.01 M eluate. The addition of a larger amount (100 μg) of albumin to the collection tubes (experiment 5A, Table 1) increased the recovery of active autolysin to 78% from 0 to

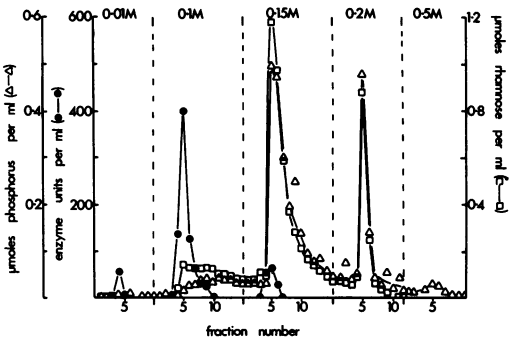

FIG. 1. *DEAE-cellulose chromatography of the Bio-Gel P-60-excluded fraction (fraction II) of a trypsin-speeded log-wall lysate. Fraction II from approximately 25 mg of log walls was applied to a column 12 by 0.9 cm and eluted in a stepwise fashion with the concentrations of ammonium acetate (pH 6.9) indicated.*

TABLE 1. *Purification of autolysin by DEAE-cellulose chromatography*[a]

Expt	Albumin (μg/tube)	0.01 M Eluate units	0.1 M Eluate units	0.15 M Eluate units	Total activity recovered[b]
1A	2	0	151	82	10
1B	0	7	158	57	11
2	2	690	630	270	
3	2	195	265	105	36
4A	2	40	775	130	42
4B	0	70	845	120	46
5A	100		876	158	78
5B	0		231		18

[a] Samples of fraction II (Bio-Gel P-60-excluded fraction) from 25 to 100 mg of log walls were placed on DEAE-cellulose columns (0.9 × 12 cm) which were eluted with ammonium acetate (*p*H 6.9) at 4 C. In all cases, elution patterns for rhamnose and phosphorus virtually identical with that shown in Fig. 1 for experiment 4B were obtained. In experiments 1, 4, and 5, parallel columns were run. Fractions (1.3 ml) from one column (A) were collected into tubes containing bovine serum albumin, whereas fractions from the second column (B) were not exposed to albumin. The sum of autolysin activity in all tubes in each peak is presented.

[b] Expressed as per cent of applied autolysin activity recovered in all fractions.

50%. Qualitatively, the elution pattern of autolysin activity was very consistent (Table 1), but in the absence of albumin (100 μg per tube) recovery was poor and erratic. In all fractionations, most of the activity (40 to 85% of that recovered) was found in 0.1 M eluate. A variable amount of autolysin (0 to 43%) was found in the 0.01 M eluate near the bed volume of the column. Although the columns were operated well below their theoretical capacity, this was probably due to failure of the autolysin to adsorb. The enzymatically active fractions eluted with 0.01 or 0.1 M buffer contained little rhamnose or phosphorus. Activity eluted with 0.15 M buffer (12 to 35% of that recovered) contained large amounts of both rhamnose and phosphorus, but the active fractions were consistently eluted at the front of this peak.

About 90% of the Morgan-Elson-positive acetamido sugars present in the wall lysates had been removed by Bio-Gel P-60 filtration before DEAE-cellulose chromatography. Therefore, it was impractical to determine the distribution of these reactive groups in the column fractions. At present, all that can be said is that a substantial portion was found in the pooled 0.15 M eluate after lyophilization.

Filtration on Bio-Gel P-200. Figure 2A shows the elution pattern on Bio-Gel P-200 of a trypsin-speeded wall lysate. Figure 2B shows the corresponding elution pattern on P-200 for fraction II (Bio-Gel P-60-excluded fraction) of a similar wall lysate. In both cases, about 80% of the activity applied was recovered, and a portion of the activity (about 25% in Fig. 2A and about 10% in Fig. 2B) eluted before the large peak containing the bulk of the rhamnose, phosphorus, and free acetamido sugar-containing material. The columns were eluted with water. Under these conditions the rhamnose- and phosphorus-containing polysaccharides and the Morgan-Elson-positive disaccharide peptide oligomer fraction eluted near the total volume of these columns. When the unfractionated wall lysate was filtered (Fig. 2A), autolysin activity was recovered in two peaks at about 1.8 and 2.3 times the void volume, respectively. When the Bio-Gel P-60-excluded fraction was filtered (Fig. 2B), activity was again recovered in two peaks, one very close to the void volume and the other at about three times the void volume. The presence of the disaccharide peptide oligomer fraction, removed by the Bio-Gel P-60 filtration, appeared to affect the filtration properties of the autolysin.

Changing the eluant from water to 0.02 M ammonium acetate (*p*H 6.0) resulted in another shift in the Bio-Gel P-200 elution pattern. Elution of an unfractionated, trypsin-speeded wall lysate with 0.02 M ammonium acetate resulted in the recovery of about 80% of the autolysin activity applied in a single peak at the void volume. About 15% of the Morgan-Elson-positive groups eluted in the void volume and the remainder eluted at about 3.3 times the void volume. It appears that adsorption phenomena, as well as

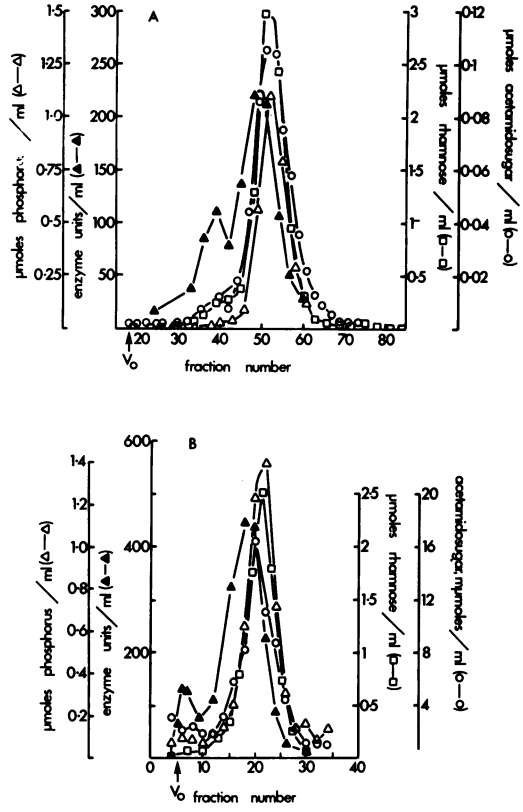

FIG. 2. *Filtration of a trypsin-speeded log-wall lysate (A) and of fraction II (B) from such a lysate on Bio-Gel P-200. Columns were eluted with water. In Fig. 2A, lysate of log walls (100 mg of walls) was applied to a column 31.5 by 2.5 cm, and 2.8-ml fractions were collected. In Fig. 2B, fraction II from about 65 mg of log walls was applied to a column 22 by 1.5 cm, and 1.0-ml fractions were collected. Please note difference in scales for acetamido sugars.*

interactions between the substances applied, are important factors in the elution patterns observed on these gels.

Stability of autolysin preparations. After DEAE-cellulose chromatography, the autolysin was extremely labile, even when stored frozen in the presence of albumin. For example, after storage at −20 C for 4 weeks, 65% of the activity present in the 0.1 M-eluted peak was lost. Thus, the DEAE-purified autolysin was far less stable than the enzymatically active fraction II preparations, which contain a large amount of rhamnose and phosphorus. Fraction II usually retained activity when stored at −20 C for extended periods; it has previously been shown to survive exposure to 37 C for 17 hr (19). The high degree of instability has thus far prevented further examination of the properties of the DEAE-purified autolysin. However, some striking differences were encountered when stability to various agents of soluble crude autolysin (fraction II) was compared with that of the same enzyme preparation after the activity was bound to SDS-inactivated walls. All of the experiments described below were performed with fraction II.

Heat stability. About 75% of the activity of the soluble autolysin was destroyed after 2 min at 56 C (Fig. 3). In contrast, when the isolated soluble enzyme was first bound to SDS-walls (*see* below) and the complex of autolysin bound to SDS-walls was then placed at 56 C, over 50% of the activity survived an exposure of 16 min. Thus, it seems that binding to the wall substrate protects the enzyme from heat. Resistance to inactivation at 56 C of both the active and latent (proteinase-activable) form of the autolysin, as found in preparations of log walls, was similarly tested. Log walls were exposed to 56 C for various intervals and then were tested for their ability to autolyze in the absence and in the presence of trypsin

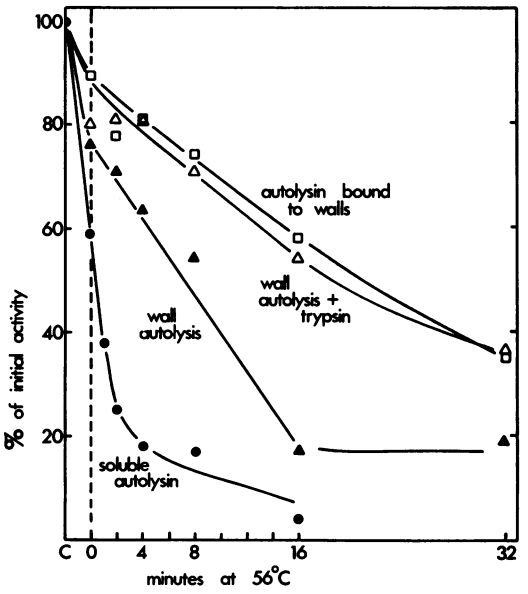

FIG. 3. *Comparison of heat sensitivity of wall-bound and soluble autolysin. Soluble autolysin (fraction II; ●), the same preparation of autolysin bound to SDS-inactivated walls (□), and untreated walls containing autolysin were heated to 56 C and held at that temperature for the times indicated and then were assayed. The untreated walls were assayed both in the presence (△) and in the absence (▲) of trypsin (50 μg/ml). Since the time taken to bring the isolated enzyme to 56 C caused considerable inactivation, the activities were compared with an unheated control (C).*

(50 μg per ml). Heat inactivation of log-wall autolysis (in the absence of trypsin) proceeded at a rate intermediate between that of soluble enzyme and isolated enzyme bound to SDS-walls. When the activity of the log walls was tested in the presence of trypsin, heat inactivation closely paralleled that of isolated autolysin bound to SDS-walls. These results suggest that the latent (proteinase-activable) form of the autolysin, which represents 75 to 80% of the activity present in log walls, is more stable to heat than is the active form.

Effect of proteolytic enzymes. Previously, we reported that a number of proteolytic enzymes can speed wall autolysis by activating a latent form of the autolysin in situ (18, 19). These proteolytic enzymes were also found to inactivate soluble autolysin (19). Exposure of soluble autolysin to pronase resulted in rapid inactivation, but after binding to SDS-walls the same enzyme preparation was resistant to pronase (Table 2). However, soluble autolysin was resistant to inactivation by the proteinase from a group A streptococcus (13). The streptococcal proteinase speeded the rate of lysis of log walls and resulted in the release of active autolysin to the supernatant fraction after wall dissolution was complete (Table 3). The resistance of unbound autolysin to this proteinase (Table 2) probably accounts for the relatively good yield (95 units) of autolysin obtainable even in the presence of 25 μg of this proteinase per ml. To obtain the maximal rate of wall lysis, a higher concentration of the streptococcal proteinase (about 3 μg per ml) than of trypsin (0.05 to 0.4 μg per ml) was required.

Wall autolysis was also found to be speeded by the neutral proteinases from *B. thermoproteolyticus* (Table 3) and *B. subtilis*. These enzymes have been shown to be capable of hydrolyzing peptide bonds but not ester linkages (15). Activation with the *B. thermoproteolyticus* proteinase resulted in the recovery of active autolysin (Table 3). The

TABLE 2. *Protection of autolysin by substrate binding*

Substrate	Concn	Time	Per cent inactivation[a]		
			Soluble autolysin	Wall-bound autolysin	Log walls
		hr			
Pronase	10 μg/ml	0[b]	86	3	
Pronase	10 μg/ml	1.0	86	3	
Streptococcal proteinase	50 μg/ml	1.0	0	—[d]	
Streptococcal proteinase	12.6 μg/ml	1.0	0	0	
Streptococcal proteinase	12.6 μg/ml	17	27	—[c]	
Acetone	40%	17 (0 C)	60	16[d]	0[d]
Urea	8 M	17 (0 C)	100	48[d]	0[d]
Urea	4 M	17 (0 C)	41	—	
Urea	8 M	1.0	94	91–100[d]	0–47[d]
Urea	6 M	1.0	100	61[d]	
Urea in 0.3 M Tris (*p*H 8.7)	6 M	1.0	100	95	95
Hydrochloric acid	0.01 N	1.0	100[d]	84[d]	

[a] Inactivation of soluble autolysin was determined as follows. Fraction II (*see* Materials and Methods) was incubated at 37 C (or 0 C where indicated) in the presence of the indicated concentration of the agent. After the specified incubation period, the tubes were brought to 0 C, and SDS-walls in phosphate buffer (0.01 M, *p*H 6.7) were added. With urea and acetone, autolysin activity was allowed to bind to the SDS-walls for 40 min at 37 C; the walls were centrifuged and washed once. The bound activity was assayed and compared to a suitable control. In all other cases, activity was determined directly. Inactivation of bound autolysin was determined after binding activity in fraction II to SDS-walls in 0.06 M phosphate (*p*H 6.7) for 40 min at 37 C. The walls containing bound enzyme were suspended in the agent in 0.36 M ammonium acetate (*p*H 6.7) to minimize dissolution of the wall substrate during this incubation. After incubation with the agent, the tubes were brought to 0 C; walls were removed by centrifugation, washed once with water, and suspended in 0.01 M phosphate (*p*H 6.7) for assay. Inactivation of autolysin present in log walls was determined by exposure of log walls to the agent, as described above for bound enzyme; assay was carried out in 0.01 M phosphate (*p*H 6.7) in the presence and in the absence of trypsin (0.05 μg/ml). A significant difference in the per cent inactivation of the active and latent activities was not observed.

[b] Zero-time sample, assayed immediately after addition of pronase.

[c] Not possible to test because of dissolution of substrate, even in 1.0 M buffer.

[d] Incubated in the absence of buffer.

TABLE 3. *Effect of proteolytic enzymes on wall lysis*[a]

Expt	Addition (μg/ml)	Lysis rate of log walls (units)		Activity of lysate on SDS walls (units)	
		Total	Minus control	Total	Minus control
1	None	120		25	
	Bacillus thermoproteolyticus proteinase				
	0.05	150	30	40	15
	0.1	250	110	35	10
	0.5	435	315	45	20
	1.0	530	410	70	45
	10.0	530	410	50	25
	Streptococcal proteinase				
	0.5	140	20	25	0
	1.0	245	125	30	15
	12.5	530	410	100	80
	25	530	410	120	95
2	None	105			
	Streptococcal proteinase				
	3.1	400	295		
	6.3	400	295		
	12.6	420	315		
	Trypsin				
	0.05	460	355		

[a] Log walls were allowed to autolyze (0.01 M ammonium acetate, *p*H 6.7) in the presence of the indicated concentrations of proteinase. The rate of lysis was determined and is expressed as units; one unit equals a decrease of 0.001 OD per hr. When wall lysis was complete, the small residue remaining was removed by centrifugation, and the activity of the supernatant fluid on SDS-walls was determined. The group A streptococcal proteinase was activated by incubation in 0.05 M β-mercaptoethanol for 25 min at room temperature before being added to the walls.

quantity of autolysin recovered was relatively small (maximum of 45 units), and this quantity decreased with a further increase of proteolytic enzyme concentration. This suggests that this proteinase was destructive to unbound autolysin, as were most of the proteolytic enzymes that have been tested (19). A proteolytic enzyme from an organism closely related to the *S. faecalis* strain used in this study, *S. faecalis* var. *liquefaciens* strain 31 (2), was also found to speed the lysis of log walls. Leucine aminopeptidase (37 μg per ml) failed to activate latent autolysin activity.

Inactivation with other reagents. Both soluble and wall-bound autolysin were inactivated by 0.01 N HCl (Table 2). Wall-bound autolysin was relatively resistant to inactivation by acetone (40%) and urea (8 M) at 0 C. At 37 C for 1 hr, 8 M urea (aqueous, *p*H 6.8) or 6 M urea in 0.3 M tris(hydroxymethyl)aminomethane (Tris), *p*H 8.7, inactivated both soluble and wall-bound autolysin. Wall-bound autolysin was somewhat more resistant than the unbound enzyme to 6 M urea (aqueous, *p*H 6.8). β-Mercaptoethanol (10 mM) had no effect on the inactivation in 6 M urea. Both the active and latent (proteinase-activatable) forms of the autolysin as they are found in log walls were less sensitive to inactivation by acetone or 8 M urea. Treatment of log walls with 6 M urea in 0.3 M Tris (pH 8.7) for 1 hr at 37 C did result in the inactivation of both forms of the autolysin. It appears that both the active and latent forms of the autolysin in log walls are even more resistant to inactivation by these reagents than is the isolated autolysin when bound to SDS-inactivated walls.

Exposure of soluble autolysin to iodoacetate (1.0 mM), *p*-hydroxymercuribenzoate (1.0 mM), or *N*-ethylmaleimide (10 mM) for 1 hr at 37 C failed to affect its activity. This suggests that free sulfhydryl groups are not essential to either the activity or binding of the enzyme.

Binding of autolysin to walls. Incubation of log walls in high salt concentrations (e.g., 1.0 M ammonium acetate, *p*H 6.9, for 1 hr at 37 C) resulted in no subsequent reduction of the autolysis rate of these walls either in the presence or in the absence of trypsin and in no detectable activity released to the supernatant fluid. Similarly, exposure to 8 M urea for 17 hr at 0 C (Table 2) failed to remove autolysin from log walls. As shown in Fig. 4, soluble autolysin was very rapidly bound to SDS-walls at 37 C (50% bound in 1 to 1.5 min) and with surprising speed even at 0 C (50% bound in 2 to 4 min). This binding occurred rapidly in 0.6 M phosphate as well as at the 0.06 M concentration shown in Fig. 4. After 40 min at 37 C, soluble autolysin was virtually completely bound in phosphate (*p*H 6.7) concentrations of 0.005 to 0.6 M and at *p*H values of from 5.8 to 8.0 (0.06 M phosphate). Binding to SDS-walls also occurred in 0.005 to 0.36 M ammonium acetate (*p*H 6.9). In distilled water (*p*H 6.0), only 21% of the free enzyme was bound in 40 min at 37 C (Fig. 4). Untreated log walls, as well as thickened walls from cells exposed to chloramphenicol (50 μg per ml) for 1 hr (17), were found to bind soluble autolysin (2 hr at 0 C). In all binding experiments, an excess of wall substrate was present. A significant difference in the percentage of autolysin bound to SDS-walls

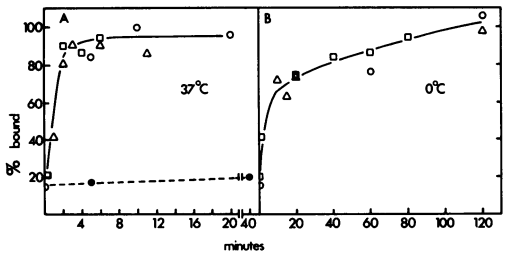

FIG. 4. *Binding of soluble autolysin to SDS-inactivated walls at 37 and 0 C. Soluble autolysin was mixed with SDS-walls in 0.06 M phosphate buffer (pH 6.7). Samples were removed at the indicated time intervals and filtered through 0.45-μM cellulose acetate filters. The walls on the filter were washed with cold water and resuspended in 0.01 M buffer for assay. Open symbols indicate values obtained in separate experiments. The values shown by the closed symbols were obtained when binding was done in distilled water (pH 6.0). The short time (about 1 min) required for filtration resulted in some binding of autolysin at time zero.*

in 2 hr at 0 C was not noted with concentrations of 70, 140, and 210 units of enzyme per 2.1 mg of walls. After the binding of 70 units of autolysin, a second 2-hr period of binding at 0 C resulted in the same percentage (87%) of autolysin bound.

SDS-walls (81 mg) were extracted with 20 ml of 10% trichloroacetic acid at 4 C for 5 days, re-extracted under the same conditions for 2 more days, and washed four times with water. Portions of the trichloroacetic acid-extracted walls were then allowed to bind soluble autolysin (145, 240, and 335 units) at 37 C, along with an SDS-wall control. At zero-time and after 10 min at 37 C, the trichloroacetic acid-extracted walls at all three enzyme concentrations bound the same percentage of autolysin as did the unextracted SDS-walls (40 to 45% at zero-time, 72 to 80% at 10 min). Since autolysin acts on trichloroacetic acid-extracted walls at only 20 to 30% of the rate at which it acts on SDS walls (19), after 40 min at 37 C the walls were removed by centrifugation in the cold, and the supernatant fluids were tested for activity on SDS-walls. The supernatant fluids contained from 2 to 5% of the total activity. No significant difference in the ability of trichloroacetic acid-extracted or SDS-walls to bind autolysin was observed. Similarly, binding of autolysin for 40 min at 37 C to SDS-walls that had been treated with 0.01 N sodium periodate at *p*H 6.1 for 17 hr at 4 C resulted in a supernatant fluid that was virtually without activity on SDS-walls.

DISCUSSION

The autolysin of *S. faecalis*, previously separated from the bulk of the disaccharide-peptide oligomer fraction by Bio-Gel P-60 (19), has now been separated from the bulk of the rhamnose- and phosphorus-containing polymers by either DEAE-cellulose chromatography (Fig. 1 and Table 1) or, less satisfactorily, by filtration on Bio-Gel P-200 (Fig. 2). Since the quantity of protein present in the wall of this organism is small (8, 19, 20), it is not possible to estimate the degree of purification in terms of specific activity. In this case, it seemed more meaningful to eliminate carbohydrate rather than protein from the enzyme preparation.

The traces of rhamnose and phosphorus still present in the most purified fractions can be attributed to incomplete fractionation or to a few sugar residues which remain covalently linked to the autolysin. The latter could be due either to the autolysin being a glycoprotein or to the remnants, left after hydrolysis of over 90% of the bonds between *N*-acetylmuramic acid and *N*-acetylglucosamine (19), of a covalent linkage to the wall. However, the sole activity detected was that of an *N*-acetylmuramidase and the release of low-molecular-weight products was not observed (19). The ability of soluble autolysin to rapidly bind, even at 0 C, to SDS-walls (Fig. 4) in a manner which renders it resistant to subsequent removal by high salt concentrations or to inactivation by 40% acetone or 8 M urea at 0 C strongly suggests that a similar type of interaction may be responsible for the difficulty in separating the autolysin from the wall polysaccharides. The observation that the elution pattern of autolysin from Bio-Gel P-200 was dependent on salt concentration and the presence or absence of the disaccharide peptide oligomer fraction supports the idea that the enzyme interacts with one or more of the wall polymers. Such an interaction also could explain the elution of activity in more than one peak on both DEAE cellulose and Bio-Gel P-200. Interactions which affect the gel filtration and ion-exchange properties of macromolecules have been observed in other systems (4).

The elution patterns for rhamnose and phosphorus, shown in Fig. 1, are very similar to those observed by Bleiweis et al. (1) for wall lysates of a different strain of a group D streptococcus. Even the asymmetry of the first peak containing both rhamnose and phosphorus is very much the same. In our experiments, this peak consistently eluted with 0.15 M buffer, and the second, narrower peak with 0.2 M buffer. This occurred even when there were several intermediate buffer concentration steps in our eluting system. In the experiments of Bleiweis et al. (1), peaks corresponding in both shape and relative size eluted at 0.1 and 0.15 M buffer, respectively. The elution systems differed in *p*H value. We used ammonium acetate (*p*H

6.9), whereas Bleiweis et al. used ammonium carbonate (*p*H 8.6). In our experiments, both of these peaks contained virtually twice as much rhamnose as phosphorus. The elution patterns reported by Bleiweis et al. also suggested a 2:1 ratio of rhamnose to phosphorus for the two main peaks, but their analyses of material isolated from these peaks and subjected to gel filtration showed ratios of 2:1 for the peak which they eluted at 0.15 M and 1:1 for the peak which they eluted at 0.2 M.

The elution of autolysin activity in two to three separate peaks by both ion-exchange and gel chromatography could result from the presence of more than one enzyme or more than one form of the same enzyme, or from the binding of the enzyme to different wall-derived polysaccharides. This is complicated by the activation of latent autolysin with trypsin. Walls contain a mixture of about one part active autolysin and four to five parts latent proteinase-activatable enzyme. The "naturally" active autolysin may well be the product of a proteinase with a different enzymatic specificity than trypsin, so that the resulting active products could differ in amino acid composition and chromatographic behavior.

Although it appears to be more difficult to separate the autolysin from the nonpeptidoglycan polymers than from the hydrolyzed peptidoglycan, it seems more likely that the enzyme binds to its substrate, the peptidoglycan polymer of the wall. Soluble autolysin in fraction II, which also contains a large fraction of the nonpeptidoglycan polymers, rapidly binds to walls at 0 C. The same enzyme preparation also efficiently binds to trichloroacetic acid-extracted or periodate-treated walls. Trichloroacetic acid removes the bulk of the nonpeptidoglycan polymers (teichoic acids?) from these walls, and sodium periodate destroys these polymers in walls of this species (A. R. Archibald and J. Baddiley, *personal communication*). Since (i) the nonpeptidoglycan polymers are probably covalently linked to the peptidoglycan [as has been demonstrated in walls from other bacterial species (7)], (ii) the treatments to remove or destroy nonpeptidoglycan polymers are probably not complete, (iii) the presence of these polysaccharides in the walls increases the reaction rate of the autolysin on the peptidoglycan, and (iv) the binding and catalytic sites of the autolysin may differ, binding of autolysin to the nonpeptidoglycan portion of the wall cannot be completely ruled out.

The sole action of conventional proteinases on walls of *S. faecalis* appears to be the activation of latent autolysin rather than hydrolysis of peptide bonds in the wall peptidoglycan (19). Activation with the *B. thermoproteolyticus*- and *B. subtilis*-neutral proteinases suggests that the process involves cleavage of peptide rather than ester bonds. Activation by any of a wide variety of proteinases appears to result from a relatively nonspecific cleavage of a polypeptide chain, which remains accessible to digestion when the autolysin is wall-bound. Binding to the wall seems to protect that portion of the protein required for enzymatic activity. For example, wall-bound autolysin is relatively resistant to pronase (Table 2), which rapidly degrades the soluble enzyme. At this time, we cannot discriminate between activation by cleavage of a single continuous polypeptide chain or of separate polypeptide chain(s). Of the total of 14 proteinases tested, both previously (19) and in these studies, only carboxypeptidase and leucine aminopeptidase failed to activate the latent autolysin in log walls.

Jollès (11) previously limited the classification of lysozymes to those bacteriolytic enzymes which possessed six important properties. However, Jollès and collaborators (5, 12) recently found that the properties of goose egg-white lysozyme differed from those of the other lysozymes in several important respects. As compared with hen egg-white lysozyme, the goose egg-white enzyme had a relatively low cystine and tryptophan content and was heat-labile; it had a higher specific activity, in its native state it could be digested by trypsin, and its activity was not inhibited by *N*-acetylglucosamine or tetra-*N*-acetylglucosamine. Jollès (10) now restricts lysozymes only to those enzymes which lyse bacteria, with *Micrococcus lysodeikticus* the classical example, and which hydrolyze the bond between *N*-acetylmuramic acid and *N*-acetylglucosamine. The autolysin of *S. faecalis* certainly fits this description, although its heat lability and sensitivity to proteolytic digestion may exceed those of the goose egg-white lysozyme.

The firmness of the binding, which results in the inability of fresh equivalent substrate to displace the substrate to which autolysin is bound (18), is certainly not typical of the conventional enzyme-substrate complex. Although perhaps useful in retaining the autolysin at the sites of new wall synthesis in the growing bacterial cell, this property makes it difficult to visualize the action of the enzyme in isolated cell-wall preparations. For example, after cleaving the accessible, susceptible bond(s), how does the wall-bound enzyme reach other bonds and continue towards virtual complete degradation and eventual dissolution of the insoluble substrate? (Obviously this would be an even more difficult problem for a covalently linked enzyme.) One possibility is that there are multiple (a minimum of 2) equivalent

binding sites. After hydrolysis of the accessible bond(s), the sites are no longer equivalent. The enzyme remains bound to site one but detaches from the second, no longer equivalent, site to immediately rebind to a neighboring site proximal to the next bond(s) to be cleaved. Multiple sites of binding would increase the firmness of the autolysin-wall interaction and would be consistent with the apparent irreversible nature of the binding.

The binding of soluble autolysin to SDS-walls seems to closely resemble the binding which may occur, in a much more organized and controlled manner, in vivo. Soluble enzyme is relatively labile to heat, urea, acetone, and proteinase action. Binding of soluble enzyme to SDS-walls decreases this lability to a level approaching that of autolysin "natively" bound to log walls. The lability of unbound enzyme, the increased stability after binding, the not easily reversible nature of the binding, as well as the requirement for proteinase activation of the latent autolysin, are all properties that are useful to the cell for the close control of a potentially dangerous process.

In reference to mitochondrial enzymes, Racker (16) has used the term "allotopy" to signify "an alteration in the properties of an enzyme when it is bound to the membrane." The differences in properties between soluble and wall-bound autolysin can be considered to be another example of allotopy merely by substituting "cell wall" for "membrane."

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