AUTOLYTIC ENZYME OF STREPTOCOCCUS FAECALIS: RELEASE OF SOLUBLE ENZYME FROM CELL WALLS

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Cultures of Streptococcus faecalis 9790, in the exponential phase of growth, will autolyze under a variety of conditions that prevent continued cell wall synthesis (Shockman et al, 1961). Stationary phase (valine- or threonine-deprived) cultures resist autolysis. Proneness to lysis of exponential phase bacterial cultures has been reported for many other bacteria, such as Bacillus subtilis (Young and Spizizen, 1963), Bacillus cereus (Mohan et al, 1965), Escherichia coli (Mohan et al, 1965; Weidel et al, 1963), other strains of S. faecalis (Bleiweis and Krause, 1965; Montague, 1964) and Staphylococcus aureus (Mitchell and Moyle, 1951; Rogers and Garrett, 1965). The autolytic enzyme systems of such cultures have been thought to be related to an ordered cleavage of the more or less continuous cell wall peptidoglycan (mucopeptide) shell surrounding the bacterial cell, so that growth of the wall can occur by means of the insertion of new polymer units (Weidel et al, 1963; Shockman, Kolb and Toennies, 1958; Shockman, 1965). Autolytic enzymes have also been related to competence of bacteria for the incorporation of exogenous DNA macromolecules (Young and Spizizen, 1963). From autolysates of isolated and purified walls of exponential phase cells (log walls) we have now obtained a soluble enzyme that is capable of dissolving log walls. Low concentrations of trypsin speed autolysis apparently by activating or releasing the lytic enzyme in the wall preparations.

Previous studies with S. faecalis have shown that the potential for cell

autolysis can be measured by placing washed cells in a suitable buffer (Shockman et al, 1961). Proneness to autolysis of cells reaches a maximum toward the end of the exponential growth phase and rapidly falls off thereafter (Shockman, 1965). Attempts to study the autolytic mechanism with whole cells proved to be unfruitful and complex. The initial reaction is obviously rapidly followed by a variety of events. Since it seemed likely that the initial reaction of cell autolysis was an enzymatic action on the cell wall peptidoglycan, this reaction was investigated. Isolated, purified walls (Shockman et al, 1958) were used as assay substrate. Log walls autolyzed, while those from stationary phase cells did not. The rate and extent of autolysis of log walls was variable and differed with different preparations of walls from the same lots of cells. Wall autolysis could be partially inactivated by 10 to 15 mins at 56° C or, more completely, by extraction of walls with sodium decylsulfate (SDS). Relatively large concentrations of the 25,000 or 105,000 g supernatant fraction (2 to 8 mg protein/ml) of disrupted exponential phase cells lysed log walls but were inactive on walls of older cells. Attempts at fractionation and purification of lytic activity from the cytoplasmic fraction were inconsistent. Supernates of autolyzed walls were, at that time, also inactive when tested on either untreated walls or SDS or heat inactivated walls.

Montague (1964) found that trypsin caused a solubilization of rhamnose and phosphate from walls pretreated with lysozyme and interpreted this to indicate that trypsin had a direct action on walls of his strain of S. faecalis. Examination of the effect of trypsin on walls of our strain of S. faecalis (Table I) made it clear that while trypsin greatly increased the rate of autolysis of untreated log walls, the enzyme did not affect the final O.D. reached, nor did it result in significant lysis of either SDS inactivated log walls or of walls from stationary phase cells. Trypsin increased the rate of log wall autolysis at very low concentrations. In other experiments, 2 μg/ml lysed 700 μg of log walls at their maximal rate (decrease of about 0.25 O.D./hour), with walls reaching their minimum density (15% of the initial turbidity) in

about 90 minutes. Even 0.02 μg/ml increased the rate of lysis to about

TABLE I. EFFECT OF TRYPSIN ON AUTOLYSIS OF CELL WALLS

Cell walls from	Trypsin mg/ml	Lysis Rate[a] units	Lysis Extent[b] %
Log phase cells	-	34	15
	0.1	200	14
SDS treated[c]	-	6	87
	0.1	7	87
Stationary phase			
Valine deprived cells	-	6	78
	0.1	9	75
Threonine deprived cells	-	3	92
	0.1	4	90

[a]Lysis rate - one unit equals a decrease of 0.001 O.D./hr at 37° of 0.7 mg/ml walls in 0.02 M phosphate buffer, pH 7.0, read at 450 mμ in 12 mm diameter tubes in a Spectronic 20 equipped with an A. H. Thomas Co. absorbancy digital readout.

[b]Extent of lysis - percent of initial turbidity remaining after 22 hours at 37°

[c]Walls extracted twice with 2% sodium decylsulfate in the cold and washed four times to remove the SDS. The SDS treated walls used in this experiment were not completely inactivated.

Conditions of culture growth and of cell wall isolation were essentially as previously described (Shockman et al, 1958).

0.12 O.D./hour and minimum turbidity was reached in about 3 hours. Except for carboxypeptidase, treatment with any of a number of proteolytic enzymes such as papain, ficin, pronase, subtilisin BPN' (nagarse) or pepsin, increased the rate of lysis of log walls. Thus, it seems that trypsin increased the rate of wall autolysis either by activating or releasing an endogenous wall autolytic enzyme.

Log walls (700 μg/ml) were allowed to autolyze in the presence of trypsin (0.02 to 0.05 μg/ml). The lysate was chilled upon reaching its minimum turbidity (1½ to 2 hr) and a small residue was removed by centrifugation at 25,000 g. The supernatant fraction contained an enzyme which increased the rate of autolysis of log walls or dissolved SDS treated log walls at rates proportional to the amount of enzyme added (Figure 1).

The activity of trypsin prepared lysates was variable from preparation to preparation, probably because some trypsin was present. Lytic enzyme was not only released from walls by trypsin but was also rapidly destroyed when lysates

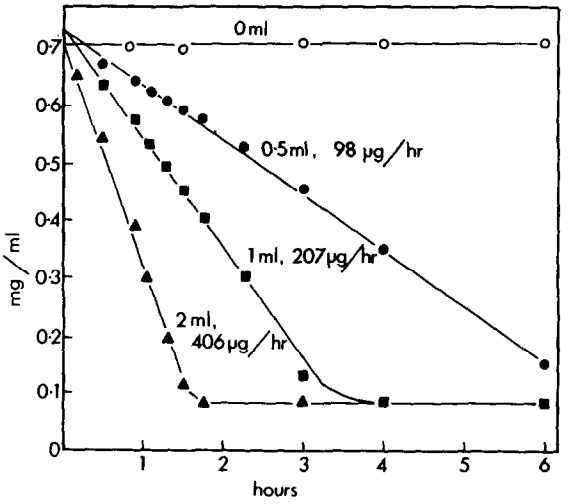

Figure 1. Lysis of SDS treated log walls by lytic enzymes obtained from the supernatant fraction of trypsin activated wall autolysis. The lysis rates are indicated as decrease in wall weight per hour (converted from optical density readings), using the conditions described in Table I.

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were incubated with added trypsin (30 µg/ml) or any of several proteolytic enzymes, such as pronase, papain, ficin or pepsin. The lytic enzyme was separated from trypsin by passing twenty-fold concentrated enzyme through a column of polyacrylamide gel (Biogel P-60). Lytic enzyme was recovered in the excluded volume, while a trypsin marker was eluted at about twice the void volume. About 90% of the lytic activity put on the column could be recovered in the void volume. All other fractions were inactive. All of the P-60 excluded lytic activity was recovered in the supernatant fraction after centrifugation at 105,000 g for 2 hours. Lytic activity was also recovered in a sharp peak at abou 1.2 times the void volume on a Biogel P-200 column. These results indicate a molecular weight of the lytic enzyme of between 60,000 and 150,000. However, results of experiments to be presented elsewhere indicate that amino sugars, rhamnose and organic phosphate may also be in the P-60 excluded fraction. We do not know if the presence of amino sugars in this fraction is coincidental or if the lytic enzyme is still attached to a wall fragment. The amounts of protein even in 20 fold concentrates of our most active preparations are below the level detectable by conventional methods.

Removal of trypsin from the P-60 excluded fraction was indicated by the increased stability of these preparations. The P-60 excluded fraction decreased in activity by less than 3%/hour in the absence of trypsin and by about 9%/hour in the presence of 30 μg trypsin/ml at 37° C. Bringing the trypsin free enzyme to 56° C caused a complete loss of activity.

Liberation of lytic enzyme with time is shown in Table II. In Experiment 1, a decrease in lytic activity of lysates occurred after wall lysis was complete. In Experiment 3, without added trypsin, lysis was slower and enzyme yields were smaller. The lability of the enzyme probably accounts for some of the previous difficulties encountered in isolating active enzyme from wall lysates. All 3 experiments indicate that little or no enzyme is released unless wall dissolution takes place. Most of the enzyme released was found toward the end

TABLE II. TIME COURSE OF LIBERATION OF LYTIC ENZYME FROM LOG WALLS

Experiment	1		2			3		
Trypsin Conc.:	0.04 μg/ml		0.05 μg/ml			0.0 μg/ml		
Time min.	% initial O.D.	Enzyme units[a] /ml	Time min.	% initial O.D.	Enzyme units[a] /ml	Time min.	% initial O.D.	Enzyme units[a] /ml
0	100	0[b]	0	100	-	0	100	-
30	93	0[c]	35	82	-	240	42	0[b]
60	55	4	60	50	8	300	28	0[c]
90	15	95	70	37	14	360	14	17
120	15	74	80	20	41	420	11	25
180	15	54	90	15	76	480	11	21

0.7 mg/ml of log walls were incubated in 0.02 M sodium phosphate buffer, pH 7.0, containing 0.04 μg trypsin/ml. Samples were removed, chilled and centrifuged at 25,000 g for 30 min. Supernates were tested for lytic activity on SDS treated log walls (0.7 mg/ml) in 0.02 M phosphate, pH 7.0.

[a] One enzyme unit = a decrease of 0.001 O.D./hr. Conditions as in Table I.

[b] No activity detected.

[c] A 15% decrease in turbidity was observed after 23 hours.

of wall lysis when only a small fraction of the initial wall turbidity was still present. These and similar experiments indicate that the lytic enzyme may be integrally associated with the cell wall despite the very small amounts of "non wall" amino acids present in these wall preparations. Autolysis of log

wall, wall lysis in the presence of trypsin and the action of the trypsin-free enzyme on SDS inactivated log walls all have similar pH optima between 6.2 and 7.0. The optimum phosphate buffer concentration for lysis by trypsin-free enzyme was found to be between 0.005 M and 0.02 M, the same as that for wall autolysis. These optima are also essentially the same as those previously found for autolysis of exponential phase S. faecalis cells (Shockman et al, 1961). However, exponential phase S. faecalis exhibits a second optimum for autolysis of cells at buffer concentrations over 0.2 M (Shockman et al, 1961), suggesting that autolysis of cells in high buffer concentrations may involve other factors.

For all three types of wall lysis, the decrease in turbidity was accompanied by a corresponding increase of rhamnose, reducing power and amino sugar in the supernatant fraction. No detectable release of amino terminal groups was observed, even in the presence of trypsin.

Evidence to be presented elsewhere indicates that the enzyme cleaves the linkage between N-acetylmuramic acid and N-acetylglucosamine and in this way is similar to egg white lysozyme. However, the autolytic enzyme seems to be larger than lysozyme and exhibits a different specificity. For example, walls of Micrococcus lysodeikticus (the classical substrate of lysozyme) and of lysozyme sensitive valine or threonine deprived stationary phase S. faecalis were less sensitive to dissolution by the enzyme than were SDS inactivated log walls. Lytic enzyme was not isolated from walls of valine or threonine deprived cells with or without trypsin. The biochemical reasons for the apparent absence of lytic enzyme from these walls are not yet known, but could be related to the role of such an enzyme during wall growth (Shockman, 1965).

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