Autolytic Enzyme System from *Lactobacillus fermenti*†

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ABSTRACT: Cell walls of *Lactobacillus fermenti* (ATCC 9338) contain an autolytic enzyme system which is solubilized upon autolysis. The autolysis is accompanied by the release of reducing groups, hexosamines, and free amino groups in a manner indicating that the system is a mixture of several enzymes. These include an endo-*N*-acetylmuramidase, amidase(s) releasing N-terminal alanine and N-terminal glutamic acid, and possibly also an *N*-acetylhexosamine deacetylase. Intact cells, cell sap, isolated walls, and wall autolysates of *L. fermenti* induce lysis of *Micrococcus lysodeikticus*. This activity is destroyed by brief heating to 60–70° or by incubation in 2% sodium dodecyl sulfate. Low ionic strength ($\Gamma = 0.02$) seems requisite for functioning of the lytic system. Optimum conditions of pH and temperature have also been elucidated. Small amounts of trypsin (1–2 μg/ml) activate the lytic system only slightly or not at all. Higher levels of trypsin or subtilisin inhibit autolysis to a varying degree. Lysozyme inhibits autolysis completely. The autolytic enzyme system exhibits its greatest activity during the exponential phase of growth. It is hardly detectable during the stationary phase.

Although autolytic processes are very common in the microbial world, their biological significance is not well understood. Autolysis in growing cultures used to be considered a pathological process occurring whenever the metabolism of the cell was drastically disturbed. However recent work indicates that autolysis plays a vital physiological role. It seems indispensable for the insertion of newly synthesized β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl peptide fragments into the cell wall peptidoglycan (Shockman *et al.*, 1967, 1968; Shockman and Cheney, 1969; Coyette and Ghuysen, 1970).

We have previously studied the cell wall and peptidoglycan from *Lactobacillus fermenti* (Wallinder and Neujahr, 1971). We now show that this organism contains an autolytic enzyme system, with its greatest activity during the exponential growth phase. Most of the autolytic activity is associated with the cell wall, but some is found in the soluble cell fraction.

The autolytic system is solubilized upon autolysis of isolated cell walls. It appears to contain several enzymes including an endo-*N*-acetylmuramidase, amino acid amidase(s), and possibly also an *N*-acetylhexosamine deacetylase. The system is also active toward cells and isolated walls of *Micrococcus lysodeikticus*.

Materials and Methods

Reagents. Egg-white lysozyme, trypsin, subtilisin, deoxyribonuclease, muramic acid, and *N*-acetylglucosamine came from Sigma Co., St. Louis, Mo. All other chemicals came from KEBO AB, Sweden. All were reagent grade.

Organisms, Growth Media, and Conditions of Culture. L. fermenti 36 (ATCC 9338) were maintained, stored, and cultivated as earlier (Neujahr, 1966). The same semisynthetic medium was used to elucidate optimum autolysis conditions and for preliminary work on isolated cell walls. However, we have also used Rogosa medium (De Man *et al.*, 1960) for most cell-wall preparations and to find the relation between age of cultures and autolysis.

Cultures of 100 ml were usually grown overnight in 300-ml erlenmeyer flasks with side arms for direct reading in a Coleman spectrophotometer, Model 14. To elucidate growth curve parameters, 1.5-l. cultures were grown in 2.0-l. erlenmeyer flasks with side arms for sampling. Larger batches of cells came from 200-l. cultures in stainless steel fermentors. All cultures were grown statically at 37°. The cell suspension used as inoculum, 1% v/v, was diluted so that the logarithmic phase of growth began the following morning. Growth was followed by optical density measurements, by viable and total cell counts, and by dry solid determinations.

M. lysodeikticus was grown on a shaker in the PWYE medium of Salton (Salton and Freer, 1965).

Preparing Washed Cell Suspensions. Cells were harvested in a refrigerated Sorvall centrifuge (15 min at 4500*g*). They were washed at 2–4°, once in 0.9% sodium chloride, and then twice in 0.1 M Tris-HCl containing 0.01 M $MgCl_2$ (pH 7.5). The pellets, fresh or frozen, were resuspended in the buffer just before incubation. Initial density was adjusted to 1.0 at 420 nm. For other experimental conditions, see particular diagrams.

Preparation of Cell Walls. Thick suspensions of washed cells in deionized water were disrupted in the "X-press" (Edebo, 1960) and fractionated in Sorvall refrigerated centrifuge, essentially as before (Wallinder and Neujahr, 1971), but modified as follows. The sediment containing crude cell walls was suspended in 0.1 M Tris-HCl (pH 7.5) containing 0.01 M $MgCl_2$ and centrifuged at 10,000*g* for 30 min. Later we used 18,000*g*. This gave walls with essentially the same characteristics.

At this stage, crude wall suspensions in the above buffer were sometimes incubated with trypsin (0.5 mg/ml) at 37° for 30 min and then centrifuged at 10,000*g*. Control preparations without trypsin were kept in buffer alone at 2–4°. The supernatant from this last centrifugation was rejected. The sediment was washed twice with H_2O. The purified cell walls thus obtained were stored as frozen pellets until used.

Autolysis of cells or of isolated walls was measured as turbidity reduction at 420 nm using 10 ml of suspensions in

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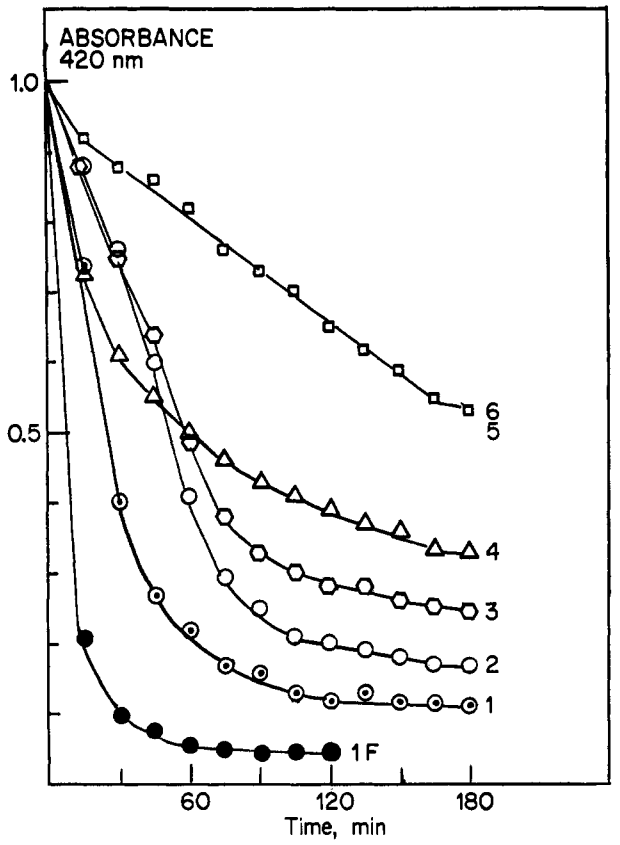

FIGURE 1: Autolysis of *L. fermenti* at low and high ionic strength. "F" denotes cells frozen before autolysis: (1) Tris-HCl, pH 7.2, $\Gamma = 0.02$; (2) as 1, but with $MgCl_2$ 0.001 M; (3) potassium phosphate, pH 7.2, $\Gamma = 0.02$; (4) Tris-HCl, pH 7.6, $\Gamma = 0.13$; (5) as 4, but with $MgCl_2$, 0.008 M; (6) deionized H_2O.

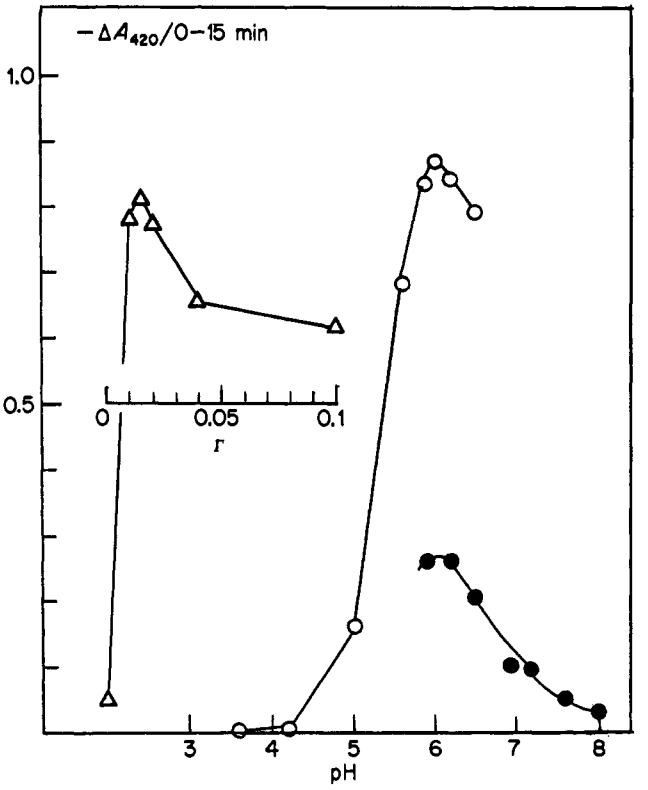

FIGURE 2: The effect of pH and ionic strength (Γ) on autolysis of *L. fermenti*. Rings indicate the initial autolysis velocities of frozen cells; dots indicate the highest autolysis velocities of fresh cells. All pH experiments were carried out at constant $\Gamma = 0.02$; all Γ experiments (triangles) at constant pH = 5.9. Buffers employed: pH 3.6–5.6 sodium acetate; pH 5.9–7.4 potassium phosphate; pH 7.2–8.0 Tris-HCl.

test tubes calibrated for direct reading in a Coleman spectrophotometer. Tubes were incubated in a water bath with gentle shaking.

Analytical Procedures. We followed the appearance during lysis of free amino groups, reducing groups, and amino sugars. Samples of lysing wall suspensions were withdrawn at intervals, inactivated at 70–75° for 5 min, and then centrifuged at 18,000*g*, 15 min, +2°. Amino groups, reducing power, total hexosamine, and *N*-acetylhexosamine were measured in the supernatants following Ghuysen *et al.* (1966), but with scaled-up procedures.

Procedure for the identification of reducing end groups was essentially that of Warth and Strominger (1971) using NaB^3H_4, but modified as follows. Each sample of lyophilized autolysate containing approximately 10 μmol of reducing end group equivalents was mixed with 2.5 ml of 0.2 M NaB^3H_4 in water (50 Ci/mol).

The ^3H-labeled samples were subjected to chromatography on Whatman No. 1 paper for 24 hr using 1-butanol–acetic acid–water (5:2:2, v/v) and to electrophoresis on paper at pH 2.0 (glycine-HCl), pH 3.9 (acetic acid–pyridine), and pH 8.0 (Veronal-acetate). Chromatograms and electrophoresis strips were evaluated in a Packard radiochromatogram scanner.

Results

Conditions Favoring Autolysis. Figure 1 shows how ionic strength and certain buffers influence the lysis. It goes faster at low ionic strength ($\Gamma = 0.02$)[1] than at higher ionic strength ($\Gamma = 0.13$) or in deionized water (*cf.* curve 1 with curve 4 or curve 2 with curves 5 and 6). Magnesium chloride at both low and high ionic strength clearly inhibits autolysis (curve 2 and curve 5). Figure 1 also shows that freezing cells before incubation gives them great initial activity.

Figure 2 summarizes the optimum conditions of pH and ionic strength. At $\Gamma = 0.02$, optimum pH lies close to 5.9. The same optimum pH applies to both frozen cells and fresh cells. Optimum ionic strength at pH 5.9 is $\Gamma = 0.015$. At optimum pH, variations in ionic strength over the range 0.04–0.10 scarcely affect the rate of autolysis.

Figure 3 shows how temperature influences autolysis. The initial rate of autolysis was highest at 45°. The insert in Figure 3, which compares autolysis at 30 and 60°, indicates heat inactivation of the autolytic system after 15 min at 60°.

Autolytic Activity of Isolated Cell Walls, Solubilization of the Autolytic System, and Its Activity toward M. lysodeikticus. Walls of *L. fermenti* were prepared from cells with high autolytic activity. Figure 4 demonstrates the autolysis of the isolated walls alone (curve 1) and when mixed with an equal proportion of *M. lysodeikticus* cells (curve 2). Both kinds of suspension were adjusted to the same initial turbidity. As seen in Figure 4 they both lysed to practically the same extent, whereas *M. lysodeikticus* alone did not undergo lysis (curve 4).

The autolytic activity of the walls was completely abolished

[1] Ionic strength is calculated as $\Gamma = (1/2)\Sigma(C_i Z_i^2)$, where C_i denotes concentration of the ion i, Z_i its valence.

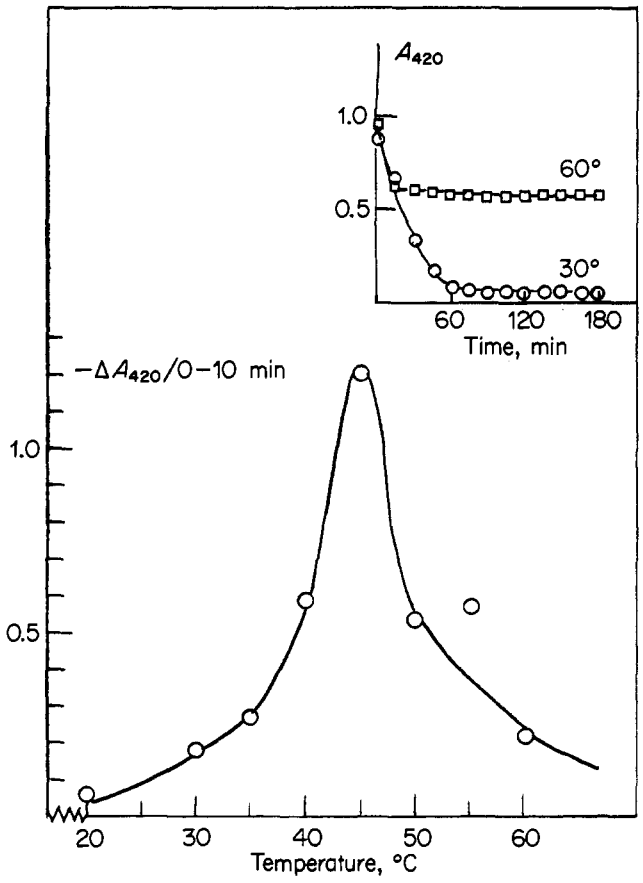

FIGURE 3: The influence of temperature on autolysis of *L. fermenti*. Potassium phosphate, pH 5.9, $\Gamma = 0.02$.

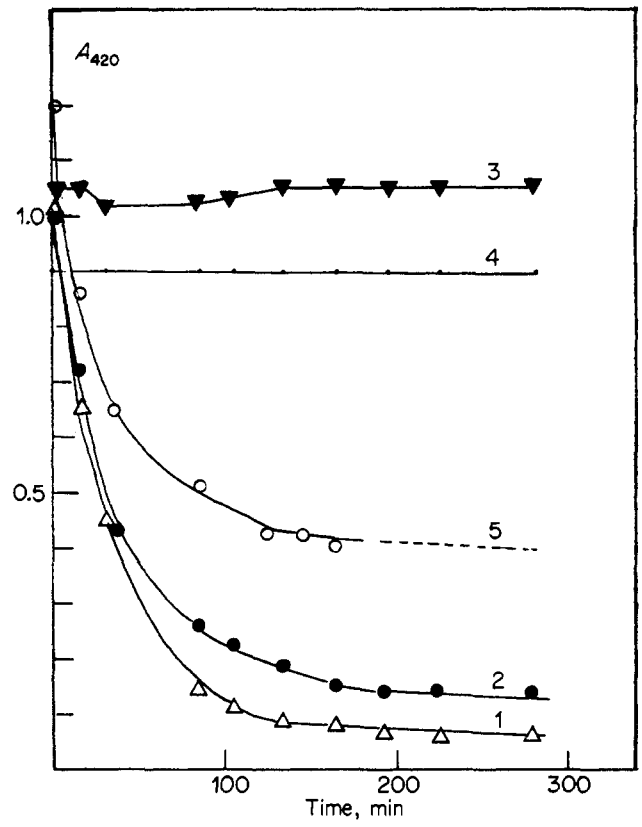

FIGURE 4: Lytic activity of isolated walls and solubilization of the system. Potassium phosphate, 0.02 M, pH 5.9, 45°: (1) walls from *L. fermenti*; (2) the same walls mixed with cells of *M. lysodeikticus* (1:1); (3) the same walls heated for 3 min at 70° and then mixed with cells of *M. lysodeikticus* (1:1); (4) cells of *M. lysodeikticus* alone; (5) cells of *M. lysodeikticus* suspended in 25,000*g* supernatant from autolyzed walls of *L. fermenti* (1 mg of walls/ml).

by brief heating at 70°, as it also was by incubation with 2% sodium dodecyl sulfate. When heat inactivated walls were mixed with cells of *M. lysodeikticus*, no clarification of the suspension could be observed (Figure 4, curve 3, *cf.* curve 2).

Walls of *L. fermenti* were allowed to autolyze for 75 min after which time the original turbidity of the suspension was reduced from 0.97 to 0.18 (420 nm). The solubilized material corresponded to 1 mg of walls/ml. The mixture was centrifuged at 25,000*g* and 10 ml of the supernatant was used to prepare a suspension of *M. lysodeikticus* cells (A_{420} 1.2). The extensive lysis of this suspension is shown in Figure 4 (curve 5). The amount of *M. lysodeikticus* solubilized in 165 min by the above wall autolysate corresponded to 0.37 mg of cells/ml.

The experiments summarized in Figure 4 thus demonstrate that the wall-associated autolytic enzyme system of *L. fermenti* is released into solution upon lysis. The system is also active toward *M. lysodeikticus*.

Mode of Action of the Autolytic System. Figure 5 describes certain chemical events concomitant with autolysis of isolated walls from exponentially growing *L. fermenti*. Curve R shows the release of reducing power, curve H the release of total hexosamine. These two curves closely follow each other and they are practically parallel to the turbidity reduction curve representing autolysis.

These results indicate that the autolytic enzyme system contains an endo-*N*-acetylmuramidase splitting the linkage between *N*-acetylmuramic acid and *N*-acetylglucosamine (*cf.* Ghuysen *et al.*, 1966). If all of the *N*-acetylmuramyl-*N*-acetylglucosamine linkages were opened during the autolysis of walls, one would expect at least twice the amount of reducing equivalents present as hexosamine. It can be seen in Figure 5 that after 2-hr incubation, when the turbidity of the wall suspension decreased to 20% of the original, the solution contained about 2.5 times more hexosamine equivalents than reducing groups. With prolonged time of autolysis this ratio remained essentially the same.

The reducing groups released during autolysis were identified as mainly derived from muramic acid. This is demonstrated in Figure 6 showing an electrophoretic separation at pH 3.9 of muramitol, glucosaminitol, and reduced autolysate. The major peak of the autolysate label coincided with the reduced muramic acid reference. Essentially similar results were obtained after electrophoresis at other pH values, whereas paper chromatograms were more difficult to interpret.

Determination of free *N*-acetylamino sugars during the course of lysis gave negative results. However, when the samples were subjected to acetylation by a procedure similar to that employed for the determination of total hexosamine, progressively increasing values were obtained (curve FH, Figure 5).

Figure 5 shows, in addition, that N-terminal amino groups are released during lysis (curve N). The initial rate of this release appears to be slower than in the case of reducing groups or hexosamine. There is a comparatively high initial content of free amino groups. Autolysate samples were taken after 2-hr incubation (80% turbidity reduction) and subjected to dinitrophenylation. Thin-layer chromatography of them revealed the presence of two major N-terminal amino acids, *viz.*, alanine and glutamic acid. Occasionally

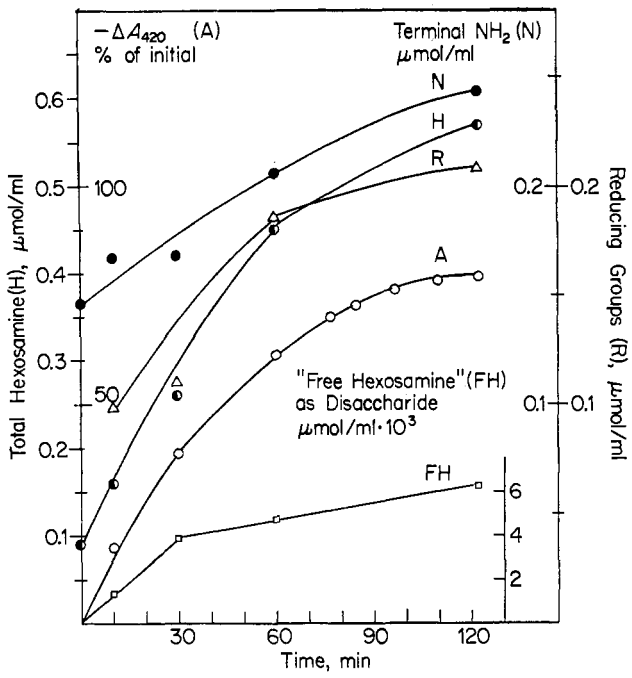

FIGURE 5: Release of reducing power (R), total hexosamines (H), "free hexosamine" (FH), and N-terminal amino groups (N) during autolysis of isolated walls from *L. fermenti*. Potassium phosphate, 0.02 M, pH 5.9, 45°. "Free hexosamine" was determined after acetylation of autolysate samples. There was no Elson–Morgan reaction without previous acetylation.

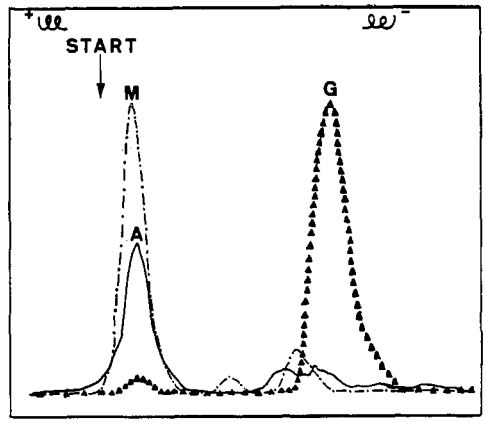

FIGURE 6: Proof that the reducing groups released during autolysis are mainly derived from muramic acid. Radioelectrophoregrams (pH 3.9) after reduction with NaB^3H_4: (A) wall autolysate; (M) muramic acid reference; (G) glucosamine reference.

minor amounts of aspartic acid could also be observed (see Table I).

Age of Cultures and Autolysis. Figure 7 shows the relation between age of the culture and autolytic activity of the corresponding washed cells. The culture was grown in Rogosa medium (De Man *et al.*, 1960). Growth was followed by measuring turbidity and pH, as well as by determining dry cell weight, viable count, and consumed glucose. The autolytic activity curve shows per cent reduction in A_{420} during the first 15 min. The growth curve exhibits three exponential phases. They correspond to successively decreasing generation times. This is because the pH drops below 5.4 after the first rapid growth phase. Autolytic activity peaks at the beginning of the second exponential phase. Then it decreases sharply as exponential growth ends and the stationary phase begins. There was no detectable autolysis in cells taken from the stationary phase of growth.

Autolytic activity of isolated walls and soluble cell fractions during various growth phases is illustrated in Figure 8. Curve WE shows the extensive autolysis of isolated walls obtained from cells growing exponentially. Curve CE indicates that the corresponding soluble cell fraction contains considerable amounts of the autolytic system. In contrast, both the isolated walls and the soluble cell fraction obtained from the stationary phase of growth show very little of the autolytic activity; *cf.* curve WS and curve CS with curves WE and CE.

Effect of Sucrose, Lysozyme, and Proteases. Sucrose (0.25–0.40 M) considerably decreased the rate of cell autolysis. The addition of lysozyme (400 μg/ml) completely inhibited the lysis of cells or of isolated walls. Walls prepared by the conventional method involving treatment with trypsin were practically devoid of autolytic activity. Walls were prepared

TABLE I: Thin-Layer Chromatography of Dnp Compounds from Wall Autolysate of *L. fermenti*.[a]

Autolysate	R_F	Authentic Compounds	R_F
Spot 1[b] (trace)	(0.20)	Aspartic acid	0.20
Spot 2	0.35	Glutamic acid	0.35
Spot 3	0.79	Alanine	0.79
Spot 4	0.90	3,4-Dinitrophenol	0.90
Spot 5	0.94	3,4-Dinitroaminobenzene	0.94
Spot 6	1.00	3,4-Dinitrofluorobenzene	1.00

[a] One-dimensional sequential development in solvents: (A) chloroform–methanol–acetic acid, v/v (85:14:1); (B) 1-butanol–1% ammonia, w/v (1:1, upper phase); (C) isobutyl alcohol–acetic acid–water, v/v (100:6:20). [b] Only in certain experiments.

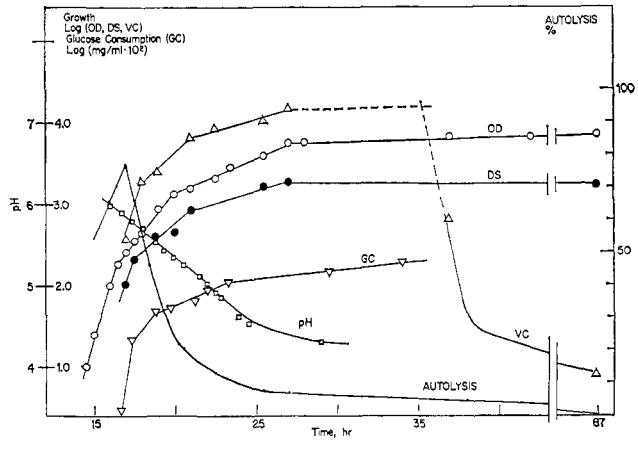

FIGURE 7: The relationship between the age of the culture and its autolytic activity. Growth given as increase in optical density, OD (log $A_{540} \times 10^3$), increase in dry solid content, DS (log mg/ml $\times 10^3$), and changes in viable count, VC (log cells/ml $\times 10^6$). Glucose consumption, GC (log mg/ml $\times 10^2$). Autolytic activity expressed as per cent decrease in absorbance at 420 nm of washed cell suspensions in potassium phosphate, 0.02 M, pH 5.9, during 0–15 min at 45°.

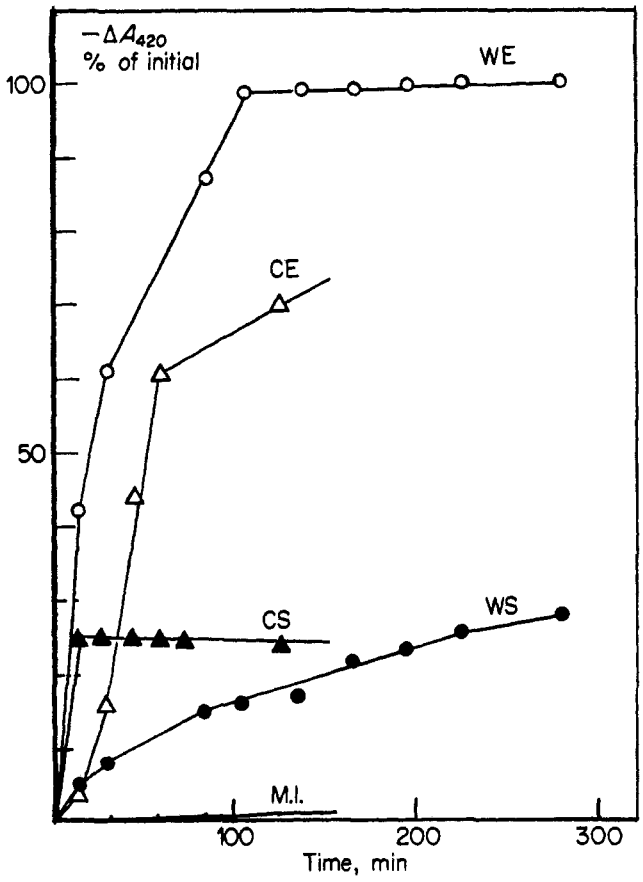

FIGURE 8: Lytic activities of walls (W) and soluble cell fractions (C) prepared from cells taken during exponential (E) and stationary (S) phases of growth. Potassium phosphate, 0.02 M, pH 5.9. Incubation of walls at 45°, incubation of soluble cell fractions mixed with cells of *M. lysodeikticus* at 35°. M.I. denotes control suspensions of *M. lysodeikticus* in above buffer, 35°.

without using trypsin and then subjected to autolysis in the presence of trypsin or subtilisin, both proteases at a level of 200 μg/ml, or without addition of a protease. Both proteases clearly inhibited autolysis.

The effect of trypsin was studied at 35 and 45° over a wide range of concentrations using suspensions of cells and walls, the latter prepared without trypsin. The results are summarized in Table II. There was no activation of autolysis of isolated walls at either temperature. Rather, increasing amounts of trypsin (0.5 μg/ml and higher) seemed to inhibit the autolysis to a correspondingly increasing degree. This inhibiting effect was more pronounced at 35° than it was at 45°, probably because trypsin is more active or less denatured at the lower temperature. Autolysis of intact cells was slightly activated (approximately 15%) by small amounts of trypsin (0.5–50 μg/ml), whereas higher concentrations were detrimental.

TABLE II: Effect of Trypsin on Autolysis of Cells and Isolated Walls of *L. fermenti* (0.02 M Potassium Phosphate, pH 5.9).

Trypsin (μg/ml)	Turbidity Reduction during 0–15 min			
	35°		45°	
	Walls (%)	Cells (%)	Walls (%)	Cells (%)
0	39	33	57	50
0.5	36	42	57	59
2.0	37	40	59	63
5.0		38		
10	25	37		
20	26	39		
30		37		
50	19	36	53	63
100	13	17	44	60

The effect of trypsin on the solubilized enzyme was studied in separate experiments. Walls were subjected to autolysis; any residue was removed by centrifugation and the clear supernatant tested for lytic activity toward *M. lysodeikticus* in the presence of varying amounts of trypsin (data not recorded). No activation by trypsin could be observed. With higher levels the rate of lysis drastically decreased. It can be concluded from the above results that the autolytic system of *L. fermenti* is not activable by trypsin.

Discussion

The results presented here demonstrate the occurrence in *L. fermenti* of an autolytic enzyme system which exhibits its greatest autolytic activity during the exponential growth phase. The activity is hardly detectable during the stationary phase. This type of autolytic activity indicates that the system may play a significant role during the synthesis of new cell walls and thus the cell division process. Only two other systems with a similar type of autolysis have been reported hitherto, *viz.*, the autolysin of *S. faecalis* (Shockman *et al.*, 1968) and that of *L. casei* (Coyette and Ghuysen, 1970). There are significant differences between these two systems and our system from *L. fermenti*. The latter contains not only an endo-*N*-acetylmuramidase, but also amidase (s), attacking the peptidoglycan peptide at two different sites. Bacterial amidases which split the linkage between acetylmuramyl and L-alanine have been observed in *Escherichia coli* and *Myxobacterium* (for review, see Ghuysen *et al.*, 1966), and recently also in *Clostridium botulinum* (Takumi *et al.*, 1971). An intrapeptide hydrolase releasing N-terminal-glutamic acid has, to our knowledge, not been reported earlier. Such an enzyme must split the peptide linkage between L-alanine and D-glutamic acid (Wallinder and Neujahr, 1971). Thus it appears to be a novel type of endopeptidase.

Another striking feature of *L. fermenti* autolysis is that the peptidoglycan fragments resulting from the action of the autolytic system are unacetylated. This could mean that the peptidoglycan in *L. fermenti* is to a large extent unacetylated—a phenomenon not reported with respect to other bacteria. An alternative and, in our opinion, a more plausible explanation would be that the the peptidoglycan fragments are deacetylated during wall lysis and/or solubilization. The latter interpretation would lead to the conclusion that our autolytic system includes an *N*-acetylhexosamine deacetylase in addition to the enzymes discussed above.

There are several differences in physiological aspects of the three autolysins (*S. faecalis*, *L. casei*, and *L. fermenti*) and their relation to proteolytic enzymes. In *L. fermenti* considerable lytic activity is found in the soluble cell fraction in addition to the activity found in walls. The wall associated and the soluble activity are most pronounced during the exponential phase and undetectable during the stationary phase of growth. In *S. faecalis*, only an inactive form of the enzyme (proenzyme) could be found in the cytoplasm (Shockman and Cheney, 1969; Pooley and Shockman, 1969). In *L.*

casei the autolytic activity of log and stationary walls was about the same, although there was a distinct difference between the intact cells from the corresponding growth phases.

The autolysin of *S. faecalis* is highly activable by trypsin and other proteinases (Shockman *et al.*, 1968), whereas our system from *L. fermenti* is not. Also the autolysin of *L. casei* has been reported as not being activable by trypsin (Coyette and Ghuysen, 1970). The autolysin recently described by Kawata *et al.* (1971) represents still another type as it contains an endo-*N*-acetylglucosaminidase instead of a muramidase. It is interesting to note the inhibition of *L. fermenti* autolysis in the presence of lysozyme. Also this contrasts with *S. faecalis* in which autolysis and lysis by lysozyme have been reported to partially overlap each other (Shockman *et al.*, 1968).

Finally, we would like to emphasize the advantages of using a tritium labelled reducing agent, NaB^3H_4 (Warth and Strominger, 1971). It facilitates the positive identification of the reducing groups released during lysis of bacterial cell walls. Ninhydrin is no longer needed for detection. Thus, there is no interference from amino acids and unreduced amino sugars. Because of the great sensitivity of the radioisotope method only minute amounts of samples are required for the identification of the reduced amino sugar(s). This eliminates the interference caused by sugars present in the nonpeptidoglycan wall portion. Employing the NaB^3H_4 method we have obtained best results with paper electrophoretic separation at various pH's, whereas paper chromatograms were more difficult to interpret.

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Complexes of Fructose Diphosphate Aldolase with Dihydroxyacetone Phosphate and Dihydroxyacetone Sulfate†

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ABSTRACT: Dihydroxyacetone sulfate has been tested as a substrate for fructose diphosphate aldolase from rabbit muscle. It has been found that it specifically forms a Schiff base with the enzyme but that it does not act as a substrate. The reactivity with CN^- and with BH_4^- of the dihydroxyacetone phosphate and sulfate complexes with aldolase has been studied and found to be differently affected by changes in the pH. The significance of these differences has been discussed and it has been proposed that the phosphate group of dihydroxyacetone phosphate, by interacting with the imine nitrogen of the protonated Schiff base, could modify the reactivity of the C-2 of the substrate.

The mechanism of action of fructose diphosphate aldolase from muscle involves the specific formation of a Schiff base between dihydroxyacetone phosphate and the lysyl residues of the active site of the enzyme (Grazi *et al.*, 1962; Kobashi *et al.*, 1966) (Scheme I). The protonated Schiff base has a preeminent role since it originates the carbanion intermediate (enamine), thus allowing the condensation to occur (Rose and Rose, 1969).

The factors which influence the specific recognition by dihydroxyacetone phosphate of the lysyl residues of the active site, as well as the formation and the deprotonation of the Schiff base, are not completely understood. They could also be strictly related and one of them could be represented by the phosphate group itself of dihydroxyacetone phosphate.

To test this possibility we have made a comparison of the behavior of dihydroxyacetone phosphate and of its analog dihydroxyacetone sulfate. The latter is capable of forming specifically a Schiff base with the enzyme but it does not act as a substrate. The different effects of pH on the reaction of aldolase with dihydroxyacetone phosphate and dihydroxyacetone sulfate have been discussed and it has been proposed that the phosphate group of dihydroxyacetone phosphate, by

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