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Wall Autolysin of *Lactobacillus acidophilus* Strain 63 AM Gasser*

Jacques Coyette and Jean-Marie Ghuysen

ABSTRACT: The autolysin of *Lactobacillus acidophilus* strain 63 AM Gasser has the specificity of an endo-*N*-acetylmuramidase. It hydrolyzes both *N*-acetylmuramic acid and *N*, *O*-diacetylmuramic acid linkages. It does not exhibit any amidase or endopeptidase action. It is present in both log-phase and stationary-phase cells.

In stationary-phase cells its action upon the wall peptidoglycan is inhibited.

Experiments carried out with several Gram-positive bacteria (Cole, 1965) and with *Escherichia coli* (Schwarz *et al.*, 1969) strongly suggest a zonal growth of the wall peptidoglycan at least during cell division. Biochemical study of the biosynthesis of the peptidoglycan also strongly suggests that the insertion of newly synthesized β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl peptide fragments requires the creation of nonreducing *N*-acetylglucosamine receptor sites in the glycan strands (for a review, see Ghuysen, 1968). Wall lytic endo-*N*-acetylmuramidases could thus play the role of providing those receptor sites a nd it may be that the safe enlargement of the peptidoglycan sacculus is due, in fact, to a strict coordination between the biosynthetic and the hydrolytic processes. So far is known, *Streptococcus faecalis* appears to be one of the simplest model with regard to the study of wall growth at the cellular level. Indeed, the active wall-bound autolytic system consists of a single enzyme that has the specificity of an endo-*N*-acetylmuramidase (Shockman *et al.*, 1967b). Moreover, it has been shown that the cell equator is the site where the active autolysin is located and the region where new wall material is inserted (Shockman *et al.*, 1967a; Shockman and Martin, 1968; Shockman and Cheney, 1969; Pooley and Shockman, 1969). In contrast to *S. faecalis* which exhibits a single plane of division, other spherical microorganisms that are characteristically arranged in clusters such as *Staphylococcus aureus* (Tipper, 1969), and rod-shaped bacteria such as *E. coli* (Weidel and Pelzer, 1964) and *Bacillus subtilis* (Young, 1966a,b) have complex autolytic systems. These systems contain enzymes such as amidases, endopeptidases, and endo-*N*-acetylglucosaminidases that do not appear to be consistent with a role in wall biosynthesis but that might be involved in other phenomena such as competence, excretion, and permeation of large molecules.

The experiments hereby presented show that *L. acidophilus* 63 AM Gasser is another simple model that may be useful for the study of wall expansion and cell division in a rod-shaped microorganism.

Materials and Methods

Growth conditions, analytic techniques (measurement of reducing groups, acetamido sugars, and N-terminal groups), and *walls preparation and structure* have been described (Coyette and Ghuysen, 1970).

Enzymes. Streptomyces F_1 endo-*N*-acetylmuramidase was used (Ghuysen, 1968).

Experimental Section

Autolysis and Bacterial Growth. Strains of *Lactobacilli* are known to autolyze (Knox and Brandsen, 1962). Preliminary experiments carried out with *L. acidophilus* 63 AM Gasser showed that the rate of autolysis of log-phase cells suspension was maximal in a 0.05 M citrate buffer, pH 5. The specific autolytic activity during growth of *L. acidophilus* was determined as follows: cells were harvested at various times and washed by centrifugations with cold distilled water. The pellets were resuspended in 0.05 M citrate buffer, pH 5, and the turbidity of each cell suspension was adjusted to an optical

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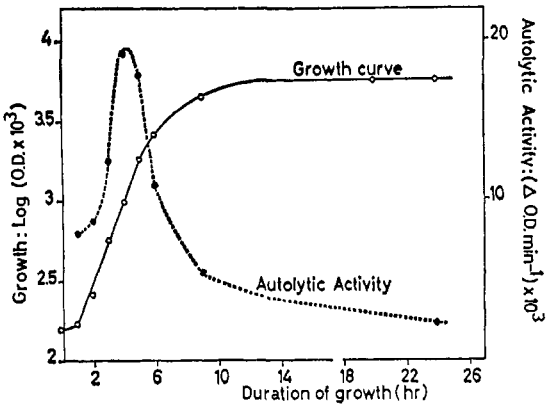

FIGURE 1: Specific activity of autolytic enzyme during growth. For experimental conditions, see text.

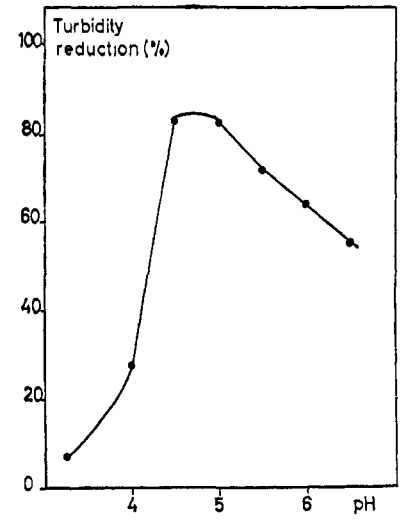

FIGURE 2: Effect of pH on the rate of autolysis of log-phase walls. Wall suspensions (1 mg/ml) were made in 0.05 M citrate buffers and incubated for 3 hr at 37°.

density of 0.5 at 550 mμ. The cell suspensions were then incubated at 37°. The autolytic activity was expressed in terms of rate of clarification, *i.e.*, the decrease, per minute, of the optical density of the cell suspension. It was calculated on the basis of the incubation time required for turbidity reduction of 40% of the original value. As shown in Figure 1, the specific autolytic activity of the cell population increased with time during the exponential phase of growth, until it reached a maximal value which coincided with the transition from the log phase to the stationary phase. This transition was accompanied by a marked loss of the autolytic activity of the cell population.

Specificity of the Autolysin. Walls prepared from log-phase cells (*i.e.*, log-phase walls) readily autolyzed. The rate of autolysis was maximal in 0.05 M citrate buffers, pH 4.5 to 5 (Figures 2 and 3), and the wall solubilization was paralleled by the liberation of reducing groups. Heat-treated (10 min at 100°) log walls did not autolyze, but underwent solubilization when incubated, at 37°, in the presence of *Streptomyces* F_1 endo-*N*-acetylmuramidase (in 0.05 M citrate buffer, pH 5; ratio enzyme to substrate 1 to 100). Both log wall autolysates and F_1 digests of heat-treated log walls (at completion of the reactions) did not contain any terminal amino groups of L-alanine. Both preparation contained equal amounts of terminal amino groups of aspartic acid (160 mμmoles/mg), which therefore represent the native terminal amino groups of the walls, in this preparation, (Coyette and Ghuysen, 1970), and equal amounts of reducing groups. These reducing groups were equivalent to 300 mμmoles, per milligram of walls, of a mixture of β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid and β-1,4-*N*-acetylglucosaminyl-*N*,*O*-diacetylmuramic acid disaccharides. The disaccharide units were actually isolated and characterized (Coyette and Ghuysen, 1970). The *L. acidophilus* enzyme, like the *S. faecalis* endomuramidase autolysin (Shockman *et al.*, 1967b), exhibited a narrow lytic spectrum. All the walls tested (Table I) were found partially or completely resistant to the *L. acidophilus* autolysin with the exception of those of *Bacillus megaterium* KM of which the sensitivity was similar to that of the *L. acidophilus* heat-treated log walls.

Cellular Localization of the Autolysin. The autolytic activities of log-phase (4.5 hr) cells, log-phase walls, stationary-phase (24 hr) cells, and stationary-phase walls were measured (Figure 4). Though the stationary-phase cells were very poorly autolytic, the corresponding walls underwent rapid solubilization. It thus follows that, during the stationary phase, the cell population maintains a high potential for lysis but that the cells have lost the capability of using the autolysin which, somehow, is prevented from reaching the wall peptidoglycan substrate. By mechanical disruption of the cells, the autolysin is liberated and adsorbed by the walls which, consequently, become susceptible to lysis. As shown in Figure 5, the lysis of a purified log-phase wall suspension was accompanied by the progressive release in the supernatant of free lytic enzyme in an active form. There is thus no necessity to obtain a complete dissolution of walls to obtain a soluble autolysin. In contrast to this, it had been observed that a complete wall dissolution was a prerequisite for obtaining the *S. faecalis* autolysin in so-

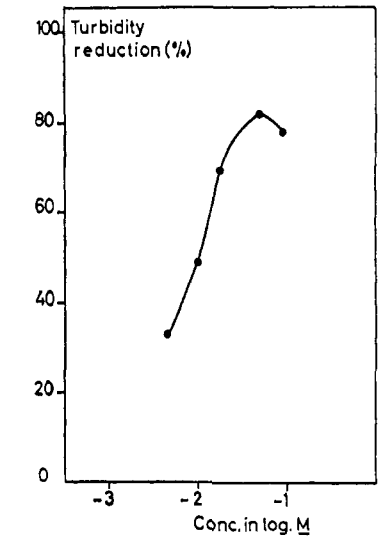

FIGURE 3: Effect of salt concentration on the rate of autolysis of log-phase walls. Walls suspensions (1 mg/ml) were made in citrate buffers, pH 5, and incubated for 3 hr at 37°.

TABLE I: Lytic Spectrum of *L. acidophilus* Autolysin.[a]

Walls	Clarification, in Per Cent, of the Wall Suspensions after Various Times of Incubation	
	3 hr	42 hr
L. acidophilus heat-treated log walls	95	100
B. megaterium KM	95	100
Corynebacterium anaerobium Prévot 3471	0	19
Micrococcus lysodeikticus	0	39
Streptomyces albus G	0	24
Corynebacterium fermentans 3211 (IP)	0	0
Clostridium perfringens BP 6K	0	0
Staphylococcus aureus Copenhagen	0	0
Micrococcus citreus	0	0
Streptococcus pyogenes group A type 14	0	0

[a] *L. acidophilus* log wall autolysates (equivalent to 1 mg of original walls) were incubated at 37° with 500 μg of bacterial walls, in 500 μl (final volumes) of 0.05 M citrate buffer, pH 5.

lution (Shockman *et al.*, 1967b). This observation may indicate that the *L. acidophilus* walls, as they are prepared, contained saturating amounts of autolysin. The observation that after disruption of the cells the soluble fraction always contained some autolytic activity is in agreement with this hypothesis. Finally, another property of the *L. acidophilus* wall autolysin which stands in contrast with that of *S. faecalis* (Shockman *et al.*, 1967b), is that trypsin did not enhance the rate of wall autolysis and did not induce the release of more enzyme.

Discussion

The autolytic system of the rod-shaped *L. acidophilus* 63 AM Gasser is composed of one or more enzymes that have the specificity of an endo-*N*-acetylmuramidase. No *N*-acetylmuramyl-L-alanine amidase or endopeptidase activities were detected. This endomuramidase hydrolyzes all the *N*-acetylmuramic acid and *N,O*-diacetylmuramic acid linkages in the walls. Thus, it is similar to the autolytic system of *S. faecalis* ATCC 9790 (Shockman *et al.*, 1967b) though it is not known whether or not the *S. faecalis* endomuramidase can hydrolyze *N,O*-diacetylmuramic acid linkages. The *L. acidophilus* endomuramidase appears to be an integral part of the walls only during the logarithmic phase of growth. Simultaneously with the transition to the stationary phase, *i.e.*, with the loss of the cell capability of growing and dividing, the endomuramidase seems to be prevented from reaching the wall peptidoglycan receptors and would remain bound to intracellular or cytoplasmic sites. It might be possible that during log phase the enzyme, or at least all of it, is not in the wall, but can be transported to it and that in the transition to stationary phase this ability is rapidly lost. Evidently, this observation is in agreement with the prevailing hypothesis that autolytic endomuramidases are involved in peptidoglycan biosynthesis by providing new acceptor sites for the transfer of disaccharide peptide units (see earlier). This observation also provides support to the hypothesis (Schwarz *et al.*, 1969) that the signal which releases the peptidoglycan hydrolases during cell division would be transmitted *via* the membrane to which these enzymes and the replicating DNA would be structurally bound. It has been shown that in log-phase cells of *S. faecalis* an inactive latent autolysin (proenzyme) is mainly located in the cytoplasm and that it is transported to equatorial wall sites where it becomes activated through proteolytic process (Pooley and Shockman, 1969). The facts that, in contrast to the *S. faecalis* autolysin, the *L. acidophilus* autolysin is not activated by proteinases, indicate that the regulatory mech-

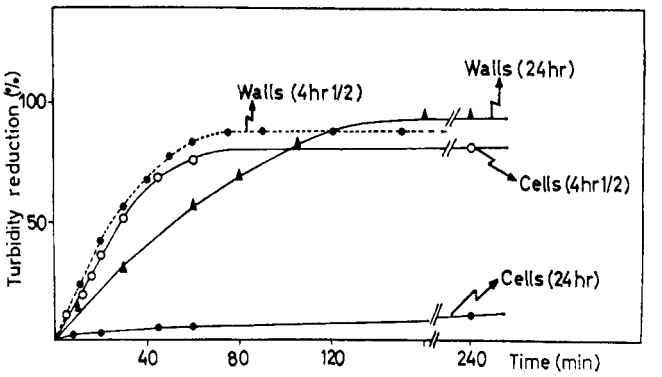

FIGURE 4: Kinetics of autolysis of log-phase cells, log-phase walls, stationary-phase cells, and stationary-phase walls, in 0.05 M citrate buffer, pH 5: wall suspensions, 1 mg/ml; cell suspensions, initial optical density: 1.0.

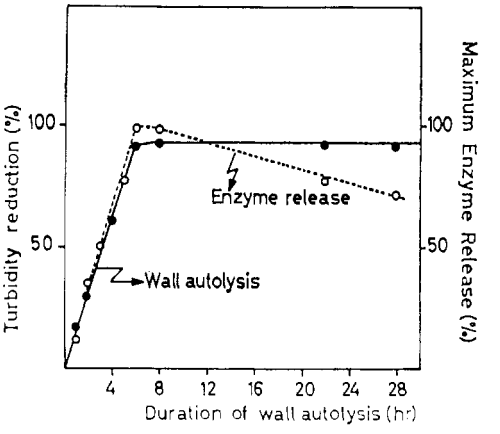

FIGURE 5: Release of soluble autolysin during log walls autolysis. A log-phase wall suspension (2 mg/ml) in 0.05 M citrate buffer, pH 5, was incubated at 37°. The clarification of the suspension was followed (solid line) by measuring optical density at 550 mμ (left ordinate). Samples were removed at various time intervals and centrifuged at 0° at 17,000*g*. Each of the supernatants was added to equal volume of a heat-treated log-phase wall suspension (2 mg/ml) in 0.05 M citrate buffer, pH 5. The mixtures were incubated at 37°. Enzyme activity is expressed as per cent of maximum lytic activity released (right ordinate), *i.e.*, as per cent of the lytic activity of those samples removed just after completion of wall autolysis (6 to 8 hr after beginning of autolysis). Under the above conditions, these samples reduced the turbidity of the heat-treated wall suspension to 50% of its original value after 6-hr incubation.

anisms may not be identical in these two microorganisms.

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Isolation of DD Carboxypeptidase from *Streptomyces albus* G Culture Filtrates*

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ABSTRACT: *Streptomyces albus* G secretes a soluble DD carboxypeptidase whose catalytic activities are similar to those of the particulate DD carboxypeptidase from *Escherichia coli*. Both enzymes hydrolyze the C-terminal D-alanyl-D-alanine linkage of UDP-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine and the enzyme–peptide interactions have identical Michaelis constants. Like the *E. coli* enzyme, the *Streptomyces* DD carboxypeptidase exhibits endopeptidase activities. The *Streptomyces* enzyme is lytic for those walls in which the peptidoglycan interpeptide bonds are mediated through C-terminal D-alanyl-D linkages. There is no strict requirement for a specific structure of the C-terminal D-amino acid residue. The tripeptide N^{α},N^{ϵ}-bisacetyl-L-lysyl-D-alanyl-D-alanine is an excellent substrate for the *Streptomyces* DD carboxypeptidase.

The recent recognition of the presence in *Escherichia coli* of a particulate DD carboxypeptidase has excited considerable interest. This enzyme exhibits two important properties. It hydrolyzes the C-terminal D-alanyl-D-alanine sequence of the wall nucleotide precursor uridine-5′-pyrophosphoryl-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanyl-D-alanine (Araki *et al.*, 1966; Izaki and Strominger, 1968). It also exerts an endopeptidase action upon the peptide dimer of the *E. coli* wall peptidoglycan (Bogdanovsky *et al.*, 1969) by hydrolyzing the C-terminal D-alanyl-(D)-*meso*-diaminopimelic acid interpeptide bond (van Heijenoort *et al.*, 1969). Such an enzyme might thus be involved in the regulation of the size of the peptide moiety of the *E. coli* peptidoglycan either by limiting the number of wall peptide precursors or by hydrolyzing interpeptide bonds in the completed wall. More recently, particulate DD carboxypeptidase activities were also shown to occur in *Bacillus subtilis* (Strominger *et al.*, 1969; Matsuhashi *et al.*, 1969) and in the blue-green alga *Anabaena variabilis* (Matsuhashi *et al.*, 1969).

The wall peptidoglycan of *Streptomyces* sp. (Leyh-Bouille *et al.*, 1970a) belongs to a chemotype entirely different from that of *E. coli* (van Heijenoort *et al.*, 1969), but it presents structural features that indicate the active presence of a DD carboxypeptidase. The extent of peptide cross-linking is low and the C termini of the peptides are never D-alanyl-D-alanine. A program was therefore initiated whose aim was the study of the DD carboxypeptidase in strains of *Streptomyces*. The purpose of the present paper is to report the isolation of a soluble bacteriolytic DD carboxypeptidase which is secreted by *Streptomyces albus* G.

Materials and Methods

Analytical Techniques. Amino acids (fluorodinitrobenzene technique), D-alanine (enzymatic procedure), and N- and C-terminal groups (fluorodinitrobenzene and hydrazinolysis

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