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Activity of an Autolytic *N*-Acetylmuramidase During Sphere-Rod Morphogenesis in *Arthrobacter crystallopoietes*

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Cells of *Arthrobacter crystallopoietes* grow as spheres in glucose-mineral salts (GS) medium and can be induced to form rods by addition of peptone, succinate, and certain other compounds (1). Changes in cell wall polysaccharides and peptides during morphogenesis have been reported (2, 3). The polysaccharide "backbones" of the sphere cell wall peptidoglycan were heterogeneous in size and averaged less than 40 hexosamines per chain. Those of the rod cell walls were more homogeneous in size and averaged 114 to 135 hexosamines per chain. We have investigated the relationship between autolytic enzymes and morphogenetic events. The results reveal the presence of an *N*-acetylmuramidase; the activity of this enzyme during morphogenesis correlates positively with the observed changes in the polysaccharide backbones. The preparative and analytical procedures employed have been described previously (2, 3).

Purified cell walls from spherical and rod-shaped cells were examined for autolytic activity. Wall preparations from spherical cells possessed greater activity of an autolysin than did those from rods (Table 1). Turbidity of the sphere cell wall suspensions decreased 10 times as rapidly as the turbidity of rod wall suspensions. The relative rate of reducing power release was less dramatic; by this criterion, the sphere walls were lysed 30% faster. This disparity in autolytic rate between reducing sugar release and turbidity measurement may be a reflection of the comparative chain lengths of the polysaccharide backbones in the two walls. Cleavage of a glycosidic bond in the sphere walls, which are constructed of short polysaccharides, may be a more drastic event in terms of solubilization than would be cleavage of the glycosidic bond of the rod walls, which have a long polysaccharide backbone. When interlinking long carbohydrate ribbons, the peptide cross-bridges would more effectively maintain structural integrity during random attack by a glycosidase.

The autolysin appeared to be a glycosidase, since reducing power and not amino groups were freed during autolysis. Cell walls from both spherical and rod-shaped cells autolyzed optimally at *p*H 5.5. Potassium phthalate-NaOH buffer (0.01 M, *p*H 5.5) was used for subsequent assays. Boiling the walls or addition of 10^{-3} M fluorodinitrobenzene caused complete inhibition of autolysis. Addition of 10^{-4} M phosphate, several cations, and sodium thioglycolate resulted in inhibitions ranging from 11 to 38%.

TABLE 1. *Autolytic activity of purified cell walls of A. crystallopoietes at pH 7.5*[a]

Cell wall prepn from	Absorbancy (650 nm) decrease per hr	Amt (moles/mole) of glutamic acid released in 10 hr	
		Reducing power	Amino groups
Spheres			
1	0.17	0.33	0
2	0.12		
Rods			
1	0.01	0.20	0.01
2	0.03	0.23	0.01

[a] Approximately 1.0 mg of cell walls per 1.0 ml of 0.01 M tris(hydroxmethyl)aminomethane (Tris)-chloride buffer (*p*H 7.5) was incubated at 37 C. Decrease in absorbancy was measured. Reducing power and amino groups were measured at zero-time and after 10 hr of incubation.

The mechanism of action of the glycosidase was determined. Rod and spherical-stage cell walls were allowed to autolyze for 10 hr. Samples of the autolysates were reduced with $NaBH_4$. Acetic acid was added to destroy excess $NaBH_4$, and the samples were dried. Reduced and unreduced samples from the autolysates were acid-hydrolyzed, diluted, and analyzed with the amino acid analyzer. The reduced autolysate samples contained less than half of the *N*-acetyl-

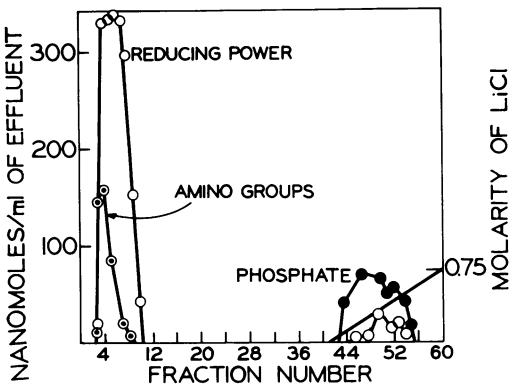

FIG. 1. *Fractionation of a cell wall autolysate of A. crystallopoietes on ECTEOLA-cellulose. A 37-mg amount of cell walls from spherical cells of A. crystallopoietes was permitted to autolyze at 37 C in 0.01 M potassium phthalate-NaOH buffer, pH 5.5. The soluble fraction (equivalent to 15.5 mg) was recovered, lyophilized, and resuspended in 2.5 ml of water. This material was applied to an ECTEOLA-cellulose column (21.5 by 1.5 cm) with a void volume of about 15 ml. The column was eluted with water, and then with a linear gradient produced from 100 ml of water and 100 ml of 1.0 M LiCl. Fractions (5 ml) were collected and assayed for phosphate, reducing power, and total amino groups.*

TABLE 2. *Autolysis of A. crystallopoietes sphere cell walls in the presence of proteolytic enzymes*[a]

Enzyme added	Concn of protease	Absorbancy decrease per hr
	μg/ml	
None	0	.20
Nagarse	4	.20
	10	.34
	20	.38
	50	.25
	100	.17
	200	.03
	1,000	.08
Trypsin	4	.35
	20	.06
	100	.09
	1,000	.04
Pepsin	4	.22
	20	.07
	100	.05

[a] Reaction mixtures containing 0.5 mg of cell walls per ml of potassium phosphate-NaOH buffer (0.01 M, *p*H 5.5) were incubated at 37 C with the enzymes indicated. Changes in absorbancy at 650 nm were corrected for changes resulting from digestion of boiled cell wall preparations with similar concentrations of the enzymes. In no case was there a large turbidity drop in these controls.

muramic acid present in the unreduced samples, whereas the amounts of *N*-acetylglucosamine were equivalent. This indicated that the enzyme is an *N*-acetylmuramidase.

An autolysate consisting of 37 mg of sphere cell walls was centrifuged to sediment the insoluble residue. The soluble material was fractionated on ECTEOLA-cellulose (Fig. 1). The fractionation pattern was that expected for an *N*-acetylmuramidase digest. The amount of reducing power released was approximately equal to that expected from complete digestion of 15.5 mg of cell walls. The total amount of phosphate was equivalent to only about 15% of the phosphate present in 15.5 mg of undigested cell walls. Analyses of the insoluble residue from the autolysate showed that there was an increase, in this material, of the components of the phosphate-containing polymer of the cell walls. The increase in these components was consistent with solubilization of only 6.4% of the phosphate

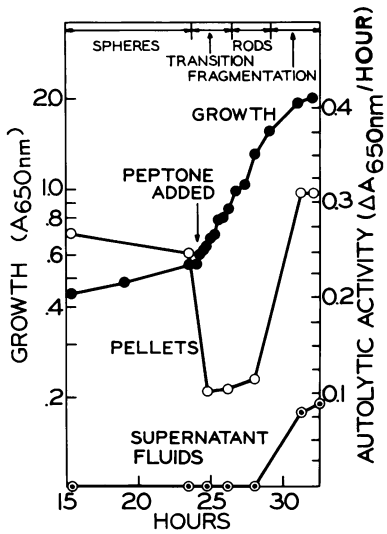

FIG. 2. *Sphere-rod morphogenesis and N-acetylmuramidase activity in A. crystallopoietes. Cells were grown in glucose-mineral salts medium for 24 hr, and then 0.5% peptone was added. The turbidity of the cultures was monitored. The stages of morphogenesis are indicated at the top of the figure. At intervals, samples of the cultures were diluted with assay buffer to a final volume of 50 ml, with a turbidity of 36 Klett units, centrifuged, and suspended in 5 ml of assay buffer. The cells were broken by sonic disruption and were centrifuged at 13,000 × g. Supernatant fluids were assayed for lytic activity on boiled cell walls. The cell wall fractions of the pellets were resuspended in 2 ml of buffer and were assayed for autolytic activity in the presence of 20 μg of nagarse per ml. The curve labeled "pellets" refers to the autolytic activity of the cell walls; A = absorbancy.*

polymer relative to solubilization of 42% of the cell wall mass. This polymer is strongly bonded to the peptidoglycan (2). The significance of the enrichment of the phosphate polymer in the undigested residue is not clear. The peptidoglycan bonded to the polymer may be protected from the autolysin.

When added at high concentrations, nagarse, trypsin, and pepsin inactivated the autolysin (Table 2). Low concentrations of nagarse and trypsin stimulated autolytic activity. A similar effect of proteases has been observed with the *N*-acetylmuramidase from *Streptococcus faecalis* (4).

The activity of the autolytic *N*-acetylmuramidase was determined at different stages of sphere-rod morphogenesis (Fig. 2). Autolytic activity of sphere wall fractions was relatively high. Very shortly after induction of morphogenesis by the addition of peptone to the spherical cells, autolytic activity decreased. The level of activity remained low when the cells were growing as rods. During subsequent fragmentation of rod-shaped cells to produce spherical cells, the wall fractions again showed high activity of the autolysin. Only during fragmentation was any activity detected in the supernatant fluids from broken cells. The activity in these fluids was the same regardless of whether rod or sphere cell walls were used as substrate. The pattern of change in activity of the autolytic *N*-acetylmuramidase during morphogenesis correlates positively with the changes in the backbone of the walls and may be causally related to these alterations.

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