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PURIFICATION AND CHARACTERIZATION OF THE AUTOLYTIC GLYCOSIDASE OF *STREPTOCOCCUS PNEUMONIAE*

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A new lytic enzyme isolated from *Streptococcus pneumoniae* has been purified to electrophoretical homogeneity. The enzyme, showing a M_r of 64000, has been characterized as an *endo*-β-1,4-*N*-acetylglucosaminidase that requires choline in the teichoic acid of the cell wall substrate for catalytic activity. In vivo experiments demonstrate that the glucosaminidase behaves as an autolytic enzyme.

Peptidoglycan hydrolases that cause dissolution of cell walls and/or cells have been called autolysins. An *N*-acetylmuramoyl-L-alanine amidase was until recently the only autolysin found in *Streptococcus pneumoniae* (1). This enzyme does not seem to play a role in wall growth or septum formation, although we have recently demonstrated, in a direct experimental way, the participation of the amidase in daughter-cell separation at the end of cell division (2). Since the bacterial sacculus is considered as a close bag, a fundamental role of the lytic enzymes in murein biosynthesis has been proposed (3). Glycosidases and certain peptidases are the only murein hydrolases that are compatible with a role in currently accepted schemes of cell wall biosynthesis (3). The isolation of a mutant of pneumococcus (M31) deleted in the *lytA* gene coding for the pneumococcal amidase (4) has allowed the detection of a new lytic activity in this species, preliminarily characterized as a glycosidase (5). M31 multiplies at an apparent normal growth rate (4) suggesting that this mutant posseses the complete machinery for wall growth and septum formation. The suppression of the strong amidase activity present in the wild type strain of pneumococcus makes M31 the ideal strain to purify the new lytic activity detected , a fundamental step to assign a precise biological role to this enzyme. We describe here the purification, characterization and properties of a new autolytic enzyme of pneumococcus.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* strain M31, a mutant deleted in the *lytA* gene (4) was used as source of the new lytic activity reported in this work. This strain was grown in C medium (6)

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supplemented with yeast extract (0.08%, Difco Laboratories). Growth was followed with a Coleman nephelometer.

Preparation of walls containing autolysin: autolytic walls. Cultures of M31 in the exponential phase of growth were rapidly cooled in ice-water and harvested by centrifugation (10000 x g, 10 min). All subsequent operations were carried out at 4°C unless otherwise stated. The bacteria were washed with phosphate/magnesium buffer (50 mM potassium phosphate buffer, pH 6.5, containing 10 mM $MgCl_2$), resuspended in the same buffer and disrupted in a French Pressure Cell Press (American Inst. Comp., MD, USA) at 140 MPa. After removal of unbroken cells by low-speed centrifugation (1000 x g, 10 min), cell walls were recovered from the supernatant by centrifugation (27000 x g, 15 min). These walls were suspended in 0.1% Brij-58 in phosphate/magnesium buffer and, after 30 min incubation at 4 °C to remove membranes, cell walls were pelleted and treated again with Brij. After centrifugation, cell walls were resuspended in 0.1% Brij in phosphate/magnesium buffer containing 0.1% choline.

Enzyme purification. Autolytic walls of M31 strain prepared as described above were allowed to autolyse by overnight incubation at 30°C. In order to solubilize the lytic activity that was still in a cell wall-bound form, 2% choline (final concentration) was added to the autolysate and, after standing for 15 min at 0°C, the mixtures were centrifuged at 40000 rpm for 3 h at 4°C in a SW50.1 rotor. Following this procedure most of the enzyme was found in the supernatant. Afterwards, the supernatant was diluted with 50 mM Tris-maleate buffer pH 6.5 and applied on a choline-Sepharose 6B column and eluted according to a procedure previously described (7).

Preparation of cell walls and assay for enzymatic activity. Cells walls labeled with [methyl-^{3}H]choline (specific activity, 60 Ci/mmol, Amersham Searle) or [2-^{14}C]ethanolamine (specific activity, 44 Ci/mmol, Amersham Searle) were prepared by biosynthetic labeling of the bacteria and the walls were purified as previously described (8). Pneumococcal lipoteichoic acid (LTA) was prepared following published procedures (9). One unit of pneumococcal glucosaminidase activity was defined as the amount of enzyme that catalyzes the hydrolysis of 1 μg of cell wall material in 1 h. Under standard assay conditions, enzyme activity was determined by measurement of the amount of radioactivity that was solubilized from [^{3}H]choline- or [^{14}C]ethanolamine-labeled cell walls after incubation at 37°C for 15 min, following a procedure previously described (8).

Characterization of free reducing groups. The characterization of the free reducing groups liberated by the lytic enzyme studied in this work was carried out by the procedure described by Ward (10). Samples of pneumococcal walls were treated with the purified enzyme, reduced with NaB.H. (0.5 Ci/mmol in 10 mM NaOH; Amersham) overnight at 4 °C, hydrolyzed with 4 M HCl at 100 °C for 4 h, and chromatographed on a Dowex 50WX4 (200/400 mesh) column. Muramitol and galactosaminitol for use as markers were prepared in our laboratory from muramic acid and galactosamine obtained from Sigma.

RESULTS AND DISCUSSION

Few bacterial autolytic enzymes have been purified due, in most cases, to their presence in small amounts in the cell and to their high affinity for binding to the cell wall. We have previously observed that when the mutant M31 was mechanically disrupted most of the lytic activity appeared bound to the cell wall (5) and initial attempts to release the lytic enzyme(s) present in this mutant by using high salt concentration or alkali were unsuccessful. We have recently observed that all the lytic enzymes of S. pneumoniae and its bacteriophages have a high affinity for choline, a property that has been successfully used for purification of one of these

Table 1

Purification of the pneumococcal glycosidase

Steps	Volume	Total protein (a)	Total activity (b)	Specific activity	Yield (b)	Purification factor
	ml	mg	units ($\times 10^{-3}$)	units/μg	%	-fold
1.Crude extract	40	248	133	0.5	100	1
2.Cell wall fraction	10	7.6	38	5	29	9
3.Autolysed cell walls	10	7.0	98	14	74	26
4.Supernatant ultrac.	9	4.5	63	14	47	26
5.Choline-Sepharose (c)	3	0.3	30	100	22	186

(a) Protein concentration was determined by the method of Bradford (12).
(b) The enzymatic activities determined in steps 1 and 2 are underestimated since the protein is in an insoluble, cell wall-bound form (see Results).
(c) Choline-Sepharose was prepared as described before (7).

lysins by affinity chromatography on choline-Sepharose (7). We have also taken advantage of this property to purify the lytic activity present in M31 from walls containing autolysin (autolytic walls) following a procedure similar to that suggested by Brown (11) to isolate lytic enzymes strongly attached to their substrate (see Materials and Methods). Table 1 summarizes the steps followed for the purification with the yields achieved. It should be noted that the values reported in step 1 and 2 for total enzyme activity are most probably an underestimation of the real ones since it is conceivable that a competition to bind the enzyme present in M31 was established between the autolytic walls and the [^{3}H]choline-labeled walls used in the enzymatic assay, due to the high affinity of the lytic activity by the cell walls. The active fractions obtained from the column of choline-Sepharose 6B were collected, concentrated with polyethylene glycol 20000 and examined for homogeneity by SDS-PAGE (Fig. 1). A single band with an apparent M_r of 64000 was visualized. This purified enzyme was capable of degrading choline-containing walls (3200 cpm were solubilized out of a total of 6000 cpm) but not ethanolamine-containing ones (60 cpm were solubilized out of a total of 4500 cpm) as previously demonstrated using crude extracts obtained from M31 (5).

We have previously suggested that the lytic activity found in M31 corresponds to a glycosidase since hydrolysis of purified pneumococcal cell walls was accompanied by an increase in the reducing power of the suspension without a concomitant increment in the number of free amino groups (5). To determine the nature of this glycosidase activity, samples of untreated or glycosidase-treated walls were reduced with NaB^3H_4 to label the reducing groups present. The reduced samples were hydrolyzed and, after chromatography on a Dowex 50WX4 column, the radioactivity was found mainly as [^{3}H]glucosaminitol (Fig. 2) demonstrating that the lytic enzyme isolated from M31 was an endo-β-1,4-N-acetylglucosaminidase. The small peak of radioactive material in the position where muramitol eluted might be

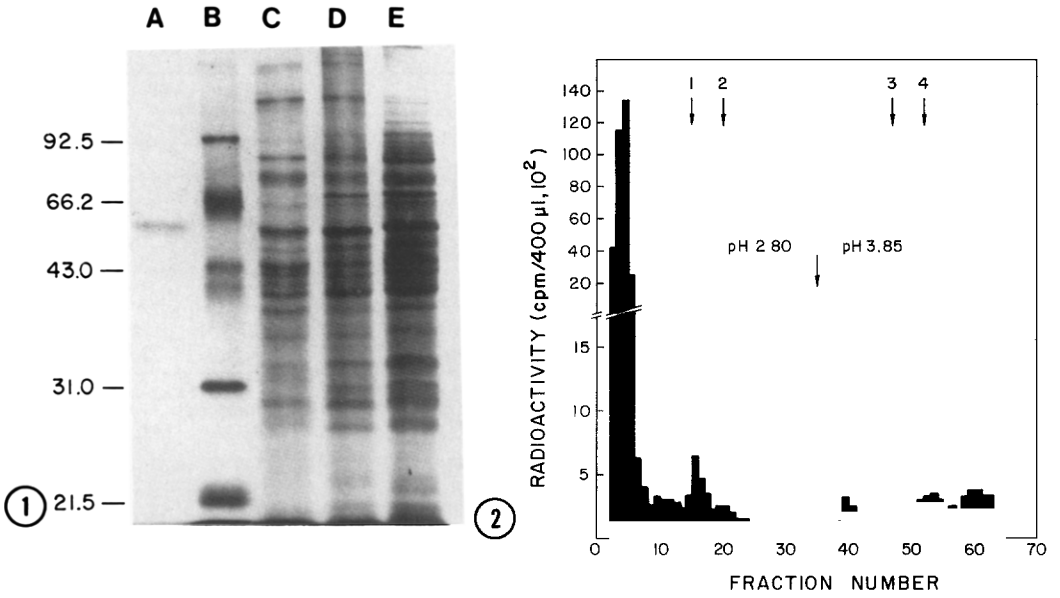

Fig. 1. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified pneumococcal lysin. Lane A, Purified M31 lysin; lane B, protein standards (phosphorylase B, bovine serum albumin, ovalbumin, carbonic anhydrase, and soybean trypsin inhibitor. The M_rs are indicated on the left in kilodaltons. Lane C, supernatant obtained after ultracentrifugation; lane D, extract obtained from autolytic walls, and lane E, crude extracts obtained from strain M31. SDS-PAGE was carried out on 10% slab gels as described (13) and stained with Coomassie Brilliant Blue R-250.

Fig. 2. Analysis of the free reducing groups in hydrolyzed cell walls of S. pneumoniae. Samples of the pneumococcal cell walls (6 mg/ml) were treated with the purified M31 lysin (26000 U/ml) at 30 °C for 18 h, reduced with NaB^3H_4 (0.5 Ci/mmol) as indicated under MATERIALS AND METHODS and applied to a Dowex 50WX4 (200/400 mesh) column (0.9 by 20 cm) equilibrated in 0.1 M pyridine-acetate buffer (pH 2.8). Fractions (2 ml) were eluted with the same buffer (70 ml) and then with 0.133 M pyridine-acetate buffer (pH 3.85) (arrow). Samples of the fractions were analysed for radioactivity. Numbered arrows: 1, muramitol; 2, muramic acid; 3, glucosaminitol; 4, glucosamine.

attributed to a lack of specificity of the glucosaminidase as previously suggested in other system (14).

Some properties of the purified pneumococcal glucosaminidase have been determined. The enzymatic activity was maximal at pH 6.5 in 20 mM phosphate buffer when the reaction was carried out at 30°C. The reaction rate was proportional to the enzyme concentration up to 2 ug of enzyme per ml. Under standard assay conditions, no stimulation of the activity of the enzyme was found in the presence of several ions tested (i.e., Li^+, Na^+, K^+, Ca^{2+}). The dependence of the reaction rate on substrate concentration followed Michaelis-Menten kinetics and from the Lineweaver-Burk plot of the data, an apparent K_m of 0.045 g/liter was found. An activation of the enzyme was achieved at concentration of choline around 0.1 % and above this concentration the activity dropped and was completely abolished at 2%. LTA acts as a strong inhibitor of the glucosaminidase activity as previously reported for other pneumococcal lytic enzymes (1,7) (data not shown).

We have reported that the pneumococcal glucosaminidase behaves as an autolysin causing the lysis of the cultures upon incubation at 30°C (5).

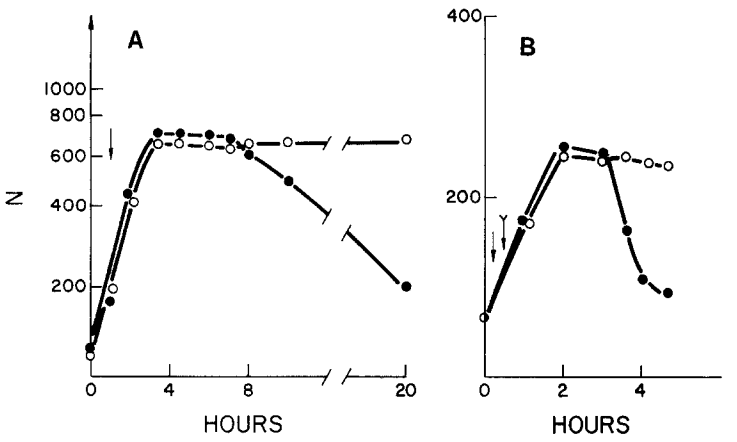

Fig. 3. Sensitization of S. pneumoniae strain M31, to exogenous pneumococcal glucosaminidase. A. Stationary phase lysis in a "cured" mutant culture. Exponentially growing cultures of M31 (7 ml) received purified glucosaminidase (400 units) at the time indicated by the arrow (●) and incubated at 37°C. A control culture did not receive enzyme (○). B. Sequential treatment of M31 with penicillin and glucosaminidase. An exponentially growing culture was treated with penicillin (0.1 U/ml), added at the time indicated by the arrow and 30 min later the culture received purified glucosaminidase (winged arrow) and incubated at 37°C (●). A control culture received no additions (○).

Nevertheless, we did not exclude a more subtle activity of this enzyme at 37°C, the optimal temperature of growth for S. pneumoniae. In fact, when the purified glucosaminidase was added to a culture of M31, incubated at 37 °C, at a concentration much higher than that normally present in this strain, the lytic activity was kept under the regulatory control of the 'cured' cells (cells that have adsorbed the lytic enzyme added), possibly through the LTA (1,7), until the culture reached the stationary phase of growth and then the culture started to lyse, in contrast with the results found for control, 'non cured' M31 cells (Fig. 3A). In addition, the well established fact that penicillin induces the liberation of LTA to the culture medium in S. pneumoniae (15) should explain the lysis induced by penicillin in the case of glucosaminidase-'cured' cells of S. pneumoniae (Fig. 3B). These experiments demonstrate that the glucosaminidase behaves as an autolytic enzyme since the protein adsorbed onto M31 mutant reverts this strain to the wild type phenotype in several properties such as lytic response to penicillin and stationary phase lysis as previously shown for the pneumococcal amidase (15).

The results reported here demonstrate that choline-containing teichoic acids also conditioned the activity of the pneumococcal glucosaminidase, as already described in the case of the pneumococcal amidase (16) and of the muramidase encoded by the pneumococcal phage Cp-1 (17) which could suggest that the domains responsible for choline recognition have evolved from a common ancestor as previously postulated (17). On the other hand, it has been suggested that some autolysins present in minor amounts might be important in 'remodeling' the cell wall during cell enlargement (18) and also in the development of the competent state for genetic transformation

(19). The precise assignment of the biological role of the glucosaminidase described in this paper must wait until mutants (deleted or otherwise) in the gene coding for the pneumococcal glucosaminidase can be obtained. The purification and characterization of the enzyme reported here as well as our ongoing studies to clone and sequence the gene coding for this enzyme are fundamental steps to achieve this goal.

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