

Biochemical Characterization of a Murein Hydrolase Induced by Bacteriophage Dp-1 in *Streptococcus pneumoniae*: Comparative Study Between Bacteriophage-Associated Lysin and the Host Amidase

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A phage-associated lysin recently isolated and purified from *Streptococcus pneumoniae* infected with bacteriophage Dp-1 has been biochemically characterized as an *endo-N*-acetyl-muramyl-L-alanine amidase. The purified peptides obtained after treatment of the cell wall with phage-associated lysin are composed of glutamic acid, alanine, lysine, glycine, serine, and aspartic acid in the molar ratios of 1.0:1.6:1.0:1.0:0.8:0.6. The N-terminal amino acid of this peptide has been characterized as alanine. This amidase and the inactive form of the amidase (E form) previously purified (J. V. Høltje and A. Tomasz, *J. Biol. Chem.* 251:4199-4207, 1976) from *S. pneumoniae* differ in their molecular weights, as well as in their capacity to be stimulated by reducing agents, and do not cross-react immunologically, although anti-phage-associated lysin serum was able to recognize and inhibit both phage-associated lysin and the active form (C form) of the host amidase.

The role of host murein-hydrolase activity in the release of progeny phage particles in *Streptococcus pneumoniae* infected with bacteriophage Dp-1 has been previously suggested (13). This suggestion has been recently reinforced with new experimental data (2, 4). In addition, we have found that a newly discovered phage-associated lysin (PAL) also plays a role in Dp-1 release and culture lysis (4). The PAL enzyme has been purified to electrophoretic homogeneity (2), and a number of properties of this enzyme resembled those of the host pneumococcal amidase, e.g., PAL will only lyse cell walls that contain choline in the teichoic acid (3). Høltje and Tomasz (8) have purified to electrophoretic homogeneity an inactive form (E form) of the amidase present in *S. pneumoniae* grown in ethanolamine-containing medium. The low-specific-activity E form of amidase was found to have a molecular weight of 35,000. Upon incubation in vitro with purified cell walls of pneumococci, the E form was converted to the active form (C form) that had a high molecular weight. In the present work we biochemically characterized for the first time a potent cell wall lysin associated with a virulent bacteriophage of *S. pneumoniae*. Since the PAL enzyme and the host amidase share important biological and chemical properties, we also carried out a comparative study between the PAL and the purified inactive form of the host pneumococcal amidase.

S. pneumoniae R6 is a derivative of the Rockefeller University Laboratory wild-type strain R36A. Strain *cwl* (9) is a mutant of R6 defective in the major autolysin (*N*-acetyl-muramyl-L-alanyl amidase) present in the wild-type strain. Bacteria containing teichoic acids with either choline or ethanolamine residues were produced as previously described (18). Isolation and purification of bacteriophage Dp-1 have been described elsewhere (10). Chemically defined growth medium (15), semisynthetic medium (16), and the preparation of the C form and E form of the autolysin (8) have been described in detail in previous communications. The isolation and purification of the Dp-1 PAL have been

recently described (2). Cell walls of choline-grown bacteria labeled with L-[4,5-³H]lysine monohydrochloride (40 Ci/mmol; Amersham Corp. and Searle Laboratories) or [³H]choline chloride (15 Ci/mmol; Amersham Corp. and Searle Laboratories) were prepared from mid-exponential-phase cultures (11).

To characterize the type of catalytic activity of the PAL, we analyzed the degradation products of [³H]lysine-labeled cell walls hydrolyzed with this lytic enzyme (Fig. 1). The lysine-labeled products were separated into two peaks (Fig. 1a). Peak II represents the lysine-labeled fragments from the

TABLE 1. Amino acid composition of the [³H]lysine-containing products

Amino acid ^b	No. of residues ^a in:		
	A1	A2	B1 ^c
Aspartic acid	0.9 (1)	0.9 (1)	0.6 (1)
Serine	0.9 (1)	0.9 (1)	0.8 (1)
Glutamic acid	1.4 (1 to 2)	1.2 (1)	1.0 (1)
Glycine	1.0 (1)	1.0 (1)	1.0 (1)
Alanine	1.8 (2)	1.7 (2)	1.6 (2)
Lysine	1.0 (1)	1.0 (1)	1.0 (1)
N-terminal residue ^d	Ala	Ala	Ala
Yield (mmol)	14.6	3.0	5.0

^a Calculated on the basis of 1.0 residue of lysine per mol of peptide. The numbers in parentheses are the assumed numbers of residues.

^b For the amino acids analysis, samples of [³H]lysine-labeled products were hydrolyzed with 200 μ l of 5.7 M HCl containing 0.05% (vol/vol) 2-mercaptoethanol in evacuated and sealed tubes at 110°C for 24 h. To determine the presence of muramic acid and glucosamine, samples of [³H]lysine-labeled products were hydrolyzed in 5.7 M HCl at 110°C for 2, 4, 6, and 24 h. The analyses were performed in a Beckman 121-MB analyzer, equipped with a Beckman integrator 126 data system. In control experiments, muramic acid and glucosamine were detected in hydrolysates of 1 to 2 nmol of *N*-acetylmuramic acid and *N*-acetylglucosamine carried out under the same acid hydrolysis conditions described above.

^c Further purified by high-performance liquid chromatography.

^d The N-terminal residues were allowed to react with dansyl chloride (5). The dansyl derivatives were separated by thin-layer chromatography as described by Woods and Wang (19).

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peptidoglycan of the cell walls (11). Peak II was rechromatographed on the same column, giving rise to two more clearly separated peaks (Fig. 1b, peaks A and B). These peaks were pooled, lyophilized, and rechromatographed separately. Two sharp peaks were obtained from peak A (Fig. 1c), whereas a single peak was obtained from peak B (Fig. 1d). These three peaks were collected, concentrated, lyophilized, and used for the chemical analysis. The amino acid compositions of peaks A1 and A2 are shown in Table 1. Peak B1 was further purified by high-performance liquid chromatography, and the amino acid composition is also shown in Table 1. All three lysine-containing products, A1, A2, and

B1, yielded alanine as the end-terminal residue. Furthermore, these peaks may represent low-molecular-weight cross-linked dimers and trimers of the peptide portions of the peptidoglycan as previously suggested (17). The composition of these fragments was glutamic acid, alanine, and lysine in the ratio of 1.0:1.6:1.0. In addition, aspartic acid, serine, and glycine were found in the ratio of 0.6:0.8:1.0 per lysine residue. It is interesting that such amino acids have been described as present in similar ratios in lysine-labeled products obtained by treatment of *S. pneumoniae* cell walls with the host amidase (11). Whether such amino acids are components of the peptide structure of the cell wall remains to be

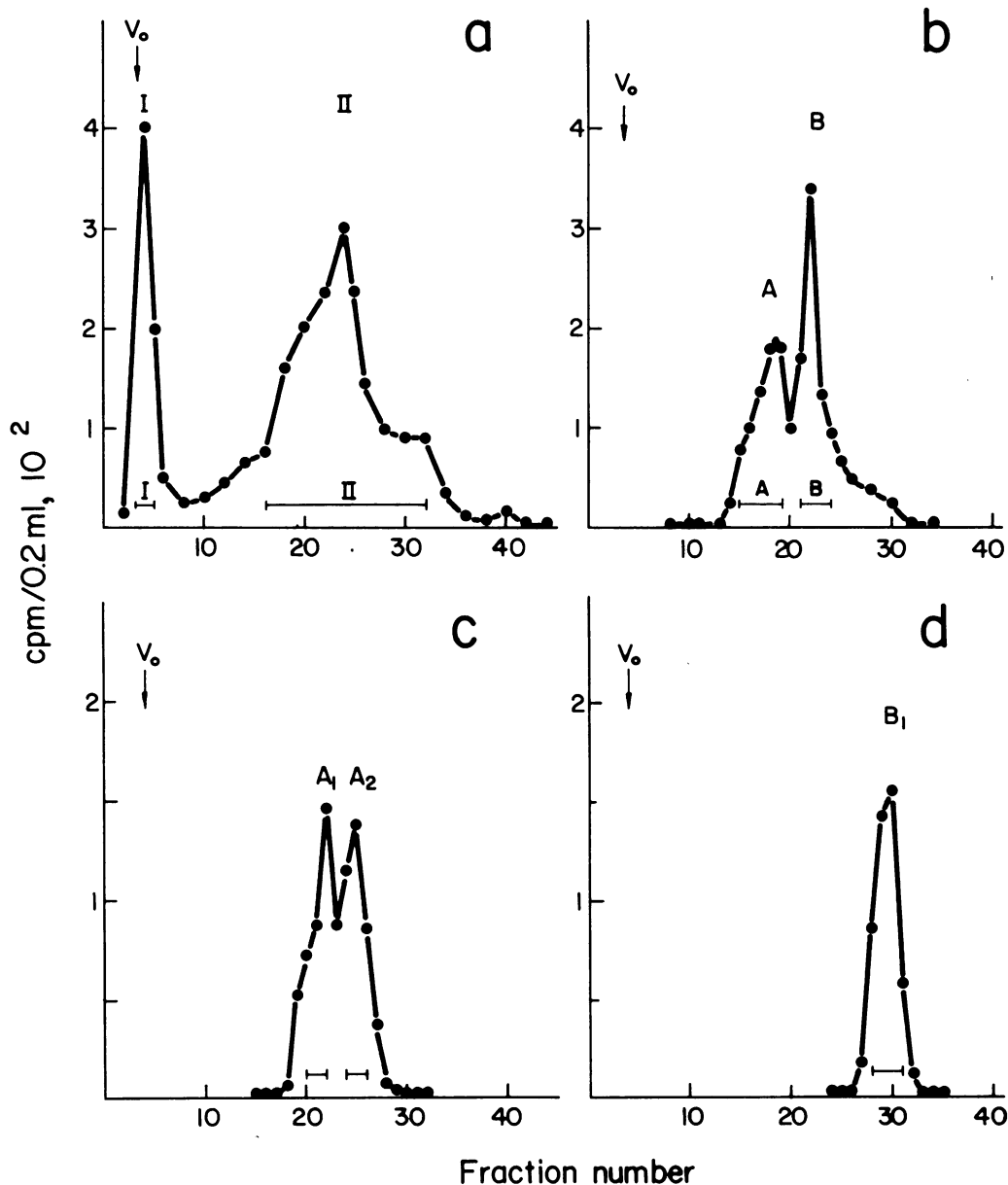


FIG. 1. Separation of [³H]lysine-labeled enzyme products of cell walls treated with PAL enzyme. Cell wall structures consisting of about 30 mg of carrier cell walls and tracer amounts of [³H]lysine-labeled cell walls were hydrolyzed with PAL (5,500 U) in a volume of 1.5 ml containing 10 mM dithiothreitol. After 20 h of incubation at 37°C the mixture was heated at 65°C for 10 min to inactivate the enzyme and centrifuged at 10,000 × g for 15 min, and the supernatant (ca. 1 ml) was removed and chromatographed on a Bio-Gel P-4 (Bio-Rad Laboratories) column (1.5 by 85 cm) with 0.1 M ammonium acetate (pH 6.9) as solvent. The fraction volume was ca. 1.7 ml in panels a and b and ca. 2 ml in panels c and d. Each fraction (100 μl) was tested for radioactivity. The horizontal brackets indicate fractions that were pooled to be rechromatographed. Arrows mark the void volume.

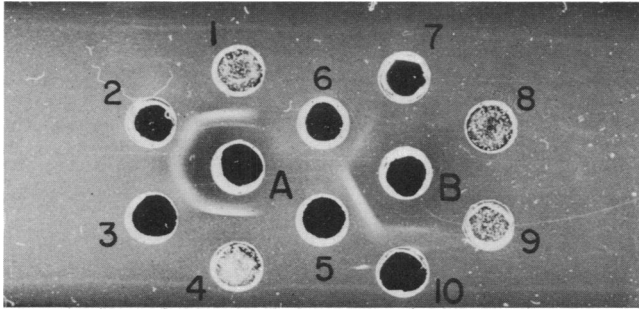


FIG. 2. Ouchterlony double-diffusion plate of amidases (E, C, and PAL) and antibodies. Double-diffusion experiments were performed on 1% agarose-coated microscope slides by using the Ouchterlony technique (12). Wells: A, anti-PAL serum; B, anti-E serum; 1, 4, 8, and 9, PAL (40 U); 5 and 6, E form amidase (40 U); 2, 3, and 10, C form amidase (40 U); 7, charalopsis muramidase choline walls products (7) (0.5 mg). The preparation of rabbit antisera against the purified pneumococcal amidase and against the purified PAL has been described in previous communications (1, 3).

investigated. The analysis of *N*-acetylmuramic acid and *N*-acetylglucosamine in the products A1, A2, and B1 indicated that neither of these two cell wall components was present. These results taken together demonstrate that PAL is an *endo-N*-acetylmuramyl-L-alanine amidase.

We have previously shown that this PAL shares many properties with the host amidase (2), e.g., the inability to hydrolyze ethanolamine-containing pneumococcal cell walls and sensitivity to inhibition by the pneumococcal membrane teichoic acid (Forssman antigen) (2, 7), although both amidases differ in some other properties; e.g., their M_r and capacity to be stimulated by reducing agents. The PAL enzyme purified to electrophoretic homogeneity from Dp-1-infected cultures of the autolysin-defective strain of *S.*

pneumoniae cwl showed in sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis an apparent molecular weight of about 31,000 (2), whereas Höltje and Tomasz (8) reported a molecular weight of 35,000 for the low-specific-enzyme activity E form of the pneumococcal amidase. Using the purified forms of both enzymes, we carried out an analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to test the differences between the reported molecular sizes, and we found that PAL can indeed be resolved from the E form, confirming that PAL is truly smaller than the E form (data not shown). In addition, when we carried out a comparative experiment between these enzymes in the presence and in the absence of dithiothreitol, neither the E form nor the C form of the host amidase was stimulated by reducing agents, in sharp contrast to the situation found with the purified PAL enzyme (data not shown). In a previous communication we demonstrated that this property was also present in the nonpurified form of PAL (4).

The functional interaction between autolytic enzymes and the cell wall substrate containing the appropriate teichoic acid is required for the activity of the amidases isolated and purified in *Bacillus subtilis* (6) and *S. pneumoniae* (8). The PAL induced in *S. pneumoniae* by phage Dp-1 also shows this functional requirement (3), suggesting again a general role for wall teichoic acids as autolysin-binding ligands. Furthermore, preliminary analyses of the amino acid compositions and the tryptic peptide maps of the PAL and E enzymes indicate a remarkable similarity, but not identity, between the primary structures of both enzymes (unpublished data). Taking into account all these data and the fact that these enzymes were obtained from two closely related strains (i.e., an autolysin-defective mutant infected with Dp-1 and the wild type) and that they cleave the same bond, it seemed possible that the two enzymes might also be immunologically related.

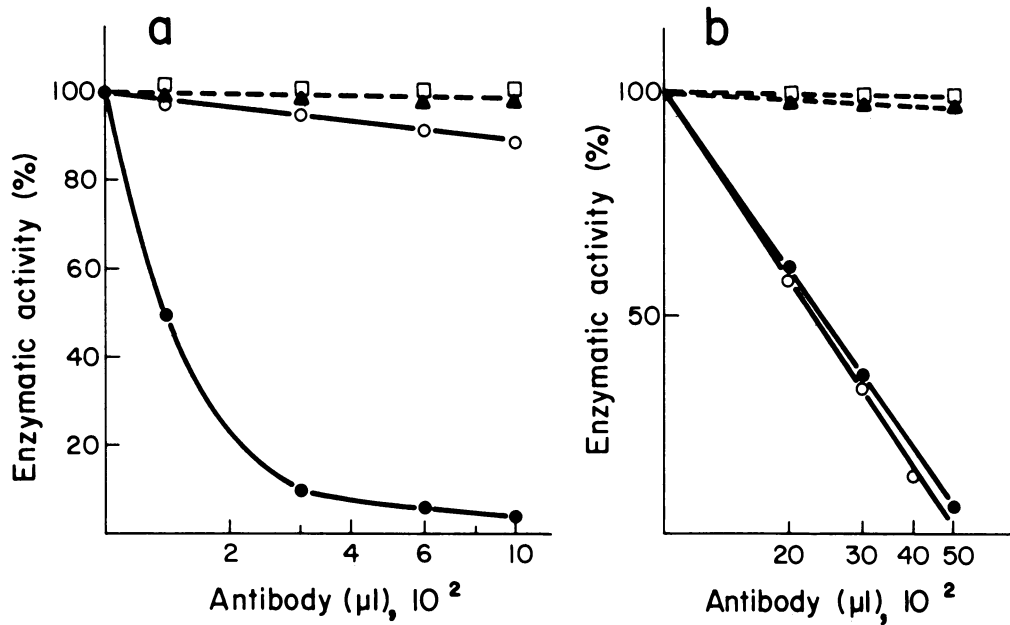


FIG. 3. Inhibition of PAL or host amidase activities by antibodies. (a) Effect of anti-E antibody against purified pneumococcal amidase (C form, 200 U) (●) or purified PAL (225 U) (○). (b) Effect of anti-PAL antibody against purified pneumococcal amidase (C form, 200 U) (●) or purified PAL (225 U) (○). In both panels normal rabbit serum (▲) and anti-Dp-1 serum (□) were used as controls. The samples with antibodies were incubated for 5 min at 37°C; then the incubation was continued for 30 min at 37°C, and the samples were tested for PAL or for pneumococcal amidase activities following the standard assays previously described (2, 8).

Figure 2 presents an Ouchterlony plate showing that antibodies against the PAL precipitated the homologous enzyme, as well as the active form of the host amidase (C form), and the precipitin lines indicated antigenic identity. Nevertheless, the anti-PAL serum did not form any band when tested against the inactive form (E form) of the host amidase. Figure 2 indicates that antibody against the host amidase (E form) could recognize both the E and the C forms of the host enzyme, but this antibody did not form any precipitin band against PAL. In addition, charalopsis muramidase choline walls products did not form any band against anti-E serum (Fig. 2) or against anti-PAL serum (data not shown) when used as controls. These degraded walls were used instead of intact ones since the latter would not diffuse into the agarose. Several experiments were performed to test the effect of the antisera on the activity of the enzymes. In vitro cell wall degradation by PAL was completely inhibited in the presence of anti-PAL serum; antibodies against the E form of host amidase, anti-Dp-1 serum (antiserum prepared against highly purified bacteriophage Dp-1), or normal rabbit serum had no inhibitory effect. In contrast, in vitro cell wall degradation by the C form of the pneumococcal amidase was inhibited both by the anti-PAL and the anti-E sera; anti-Dp-1 or normal rabbit serum had no effect (Fig. 3). These results fully complement those found on Ouchterlony double-diffusion plates and are in agreement with the previous observations that anti-PAL serum inhibited the lysis of an amidase-deficient strain of pneumococcus (*lyt* 4-4) infected with Dp-1, whereas anti-E serum did not (3). It has been pointed out that conformation can play a central role in immunodominance (14), i.e., the helical form of a polymer can be more important to determine its immunodominant feature than a particular amino acid residue of the determinant. In our case, it may happen that the antiserum obtained against the PAL, the active form of the phage-induced amidase, can recognize the conformation of the active C form of the host amidase, which differs remarkably in its conformation from the inactive E form, as was pointed out before (8). This, in turn, would mean that the E form and PAL are not closely related in conformation.

We can conclude that the PAL enzyme is an amidase similar, but not identical, to the *S. pneumoniae* host amidase. The present work is a necessary step in the investigation of whether Dp-1-induced amidase is derived from modified preexisting host amidase or is completely phage encoded. However, further experiments will be necessary before any conclusion can be drawn about the relationship between these two amidases.

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