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Characterization of a Small Proteolytic Enzyme Which Lyses Bacterial Cell Walls

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ABSTRACT

ENSIGN, J. C. (University of Wisconsin, Madison), AND R. S. WOLFE. Characterization of a small proteolytic enzyme which lyses bacterial cell walls. J. Bacteriol. 91:524–534. 1966.—An enzyme isolated from a myxobacter possesses both cell-wall lytic and proteolytic activity. The enzyme has been purified over 600-fold and is electrophoretically homogeneous upon cellulose acetate at several *p*H values and upon polyacrylamide gel columns. A single peak was obtained upon ultracentrifugation and density gradient centrifugation. Based upon Sephadex gel filtration, a molecular weight of 8,700 was determined for the enzyme. Albumin and casein were extensively degraded by the enzyme, with approximately one-third of the peptide bonds present in these proteins being hydrolyzed. The enzyme lyses cell walls by hydrolyzing peptide bonds in the glycosaminopeptide.

We have described previously (5) the isolation of a myxobacter, strain AL-1, which produces an exocellular enzyme capable of lysing intact cells and cell walls of a wide variety of bacteria. The enzyme preparation also exhibited proteolytic activity. Since cell-wall lytic and proteolytic specific activities increased proportionally at each stage of purification, it was suggested that one enzyme catalyzed both processes. The present communication describes further purification of the enzyme, as well as some of its properties and the mechanism by which it hydrolyzes cell walls.

MATERIALS AND METHODS

Procedures for maintaining cultures, for obtaining large batches of growth liquor, for isolating cell walls from *Arthrobacter crystallopoietes*, and for assaying cell-wall lysis and proteolysis, including the assay with dinitrofluorobenzene (DNFB), were those described previously (5).

In certain experiments, proteolysis was measured by the enzymatic solubilization of dye complexes from Azocoll, degraded collagen coupled to an azo dye (Calbiochem). To a 25-ml Erlenmeyer flask were added a 0.1- to 0.5-ml amount of enzyme, 1.0 ml of 0.05 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer containing 10^{-4} M ethylenediaminetetraacetate (EDTA) at *p*H 9.0, and water to a total volume of 2.5 ml. The reaction was started by the addition of 10.0 mg of Azocoll. After exactly 10 min of shaking in a water bath at 38 C, the reaction mixture was filtered quickly through a sintered-glass filter. The absorbancy of the filtrate was determined at 580 mμ with a Beckman DU spectrophotometer. A reaction mixture containing Azocoll, but no enzyme, was similarly incubated and filtered; the absorbancy of this mixture was used as a blank. Eight flasks could be assayed easily in each experiment by making the Azocoll additions at 1-min intervals. A unit of proteolytic activity with Azocoll as substrate is defined as an increase in absorbancy at 580 mμ of 0.001 in 10 min at 38 C. Specific activity is defined as units per milligram of protein. The Azocoll assay was used for most experiments, but is not as sensitive as the DNFB assay. In all enzymatic assays, the reaction time and the amount of enzyme used were chosen so as to be within the linear range of enzyme action and to be valid for the amount of enzyme used.

To determine the mechanism by which the enzyme hydrolyzes cell walls of *A. crystallopoietes*, 0.1 ml of purified enzyme was incubated at 38 C with 50 mg of cell walls in 5.0 ml of 0.025 M Veronal buffer (*p*H 9.0). Duplicate 0.1-ml samples were removed periodically to determine reducing sugars by the method of Park and Johnson (19) and free amino groups by the procedure of Ghuysen and Strominger (7).

Density gradient centrifugation of the enzyme was done by the technique of Martin and Ames (14). A 0.1-ml sample of enzyme containing 0.3 mg of protein with a specific activity of 75,000 was layered on top of a linear 5 to 20% (w/v) sucrose gradient in each of two Lusteroid centrifuge tubes. As a control, a third tube was layered with 0.1 ml of a mixture containing 0.5 mg of lysozyme and 0.5 mg of bovine serum albumin. The tubes were centrifuged at 102,000 × *g* for 22 hr at 5 C in a SW-39 rotor in a model L Spinco centrifuge. After centrifugation, the bottom of each

tube was punctured with a no. 21 hypodermic needle, and 7-drop fractions were collected; 1 ml of distilled water was added to each fraction. Fractions showing absorbancy at 280 mμ were assayed for cell-wall lytic and proteolytic activities.

Ultracentrifugal analysis was conducted in a Spinco model E ultracentrifuge with a 0.3% solution of purified enzyme in distilled water. The AN-D rotor was employed at a speed of 59,780 rev/min. A temperature of 20 C was maintained, and exposures were taken at 32-min intervals with the Schlieren optics.

The molecular weight of the enzyme was determined by the gel filtration technique described by Andrews (1). A column (1.5 by 61 cm) of Sephadex G-100 was prepared and washed with 0.1 M KCl in 0.05 M Tris buffer at *p*H 9.0. A 1.0-ml sample of enzyme containing 3.0 mg of protein with a specific activity of 74,000 was added to the top of the column and was washed through with the KCl-Tris solution at a flow rate of 8 ml/hr. Samples of 1.0 ml were collected; absorption of each sample at 280 mμ was determined, and those fractions containing absorbing material were assayed for cell-wall lytic and proteolytic activity. The molecular weight of the enzyme was determined by comparing its elution volume with those of several proteins of known molecular weight.

The procedure of Reisfeld, Lewis, and Williams (21) was employed, with minor modifications, for polyacrylamide-gel disc electrophoresis studies. A 1-ml amount of a small-pore gel solution was placed in glass tubes (0.5 by 9.0 cm) and was allowed to polymerize. Over this was layered 0.15 ml of large-pore solution which was then photo-polymerized. On top of this layer, 0.15 ml of large-pore solution containing 100 μg of purified enzyme was added and photo-polymerized. The tubes, sample uppermost, were attached to the anode buffer tray, which was then filled with β-alanine-acetate buffer (*p*H 4.5). The cathode tray containing the same buffer was placed directly below, and the gel tubes were immersed in the buffer. The cathode tray was placed in an ice bath and was agitated with a magnetic stirrer to minimize heating of the gel tubes. A current of 5 ma per tube was applied for 90 min. The gel columns were then removed from the tubes. One column was stained with Amido Black, and excess stain was removed electrophoretically. Another gel column was sliced into 1.5-mm segments; each section was placed in a separate test tube, and a 0.5-ml amount of 0.01 M Tris buffer (*p*H 9.0) was added to each. Each disc was macerated with a glass rod, and the tube was shaken for 20 min at room temperature. A 0.25-ml sample was removed from each tube and assayed for cell-wall lytic activity; the remaining material was assayed for proteolytic activity.

Electrophoresis on cellulose acetate was conducted by use of a Gelman electrophoresis chamber and Sepraphore III support strips (Gelman Instrument Co., Ann Arbor, Mich.). The strips, 2.5 by 17 cm, were soaked overnight in buffer before use, and, after they were lightly blotted a 10-μliter sample was applied to each. The strips were placed in the chamber with the origin located approximately 1 cm from the anode compartment, and a current of 1.5 to 2.0 ma per strip was applied for 1 hr. The strips were stained with Amido Black, and excess stain was washed out with 7% acetic acid. Experiments were conducted with the following buffers at a concentration of 0.025 M: phosphate (*p*H 7.0), Veronal (*p*H 8.0 and 9.0), and glycine (*p*H 10.0 and 11.0).

Lysozyme was purchased from Calbiochem; Sephadex G-50 and G-100, from Pharmacia Fine Chemicals, Inc., New York, N.Y.; diisopropyl-fluorophosphate (DFP), *p*-chloromercuribenzoate (PCMB), and cytochrome *c* (horse heart-type III), from Sigma Chemical Co., St. Louis, Mo.; ovalbumin, bovine serum albumin, soy bean trypsin inhibitor, ribonuclease (bovine pancreas), trypsin, casein, gelatin, and diethylaminoethyl (DEAE) cellulose and carboxymethyl (CM) cellulose, from Mann Research Laboratories, Inc., New York, N.Y.; and hydroxylapatite, from Clarkson Chemical Co., Inc., Williamsport, Pa.

RESULTS

We established previously (5) that both cell-wall lytic and proteolytic enzymatic activities were exocellular and could be precipitated by acetone or ammonium sulfate. A viscous material also was precipitated by these procedures, which complicated and limited subsequent purification procedures. Upon further investigation, a procedure has been developed for the selective precipitation of the enzyme by zinc chloride, with the viscous material remaining in the supernatant fluid. The results of a typical purification of cell-wall lytic and proteolytic activities are summarized in Table 1.

A 1-liter amount of growth liquor containing 440 units per ml of cell-wall lytic activity and 85 units per ml of proteolytic activity was cooled to 4 C, and the *p*H was adjusted to 6.7 with 3 N HCl. All subsequent purification steps were carried out at 4 C in a cold room. As this solution was vigorously stirred, 20 ml of a 10% solution of $ZnCl_2$ which had been filtered through filter paper was added dropwise to the growth liquor. A flocculent gray precipitate formed and, after standing for 20 min, was collected by centrifugation at 10,000 × *g* for 15 min. The clear amber supernatant fluid was decanted, and a 10-ml sample was dialyzed for 36 hr against four changes of 10^{-4} M sodium EDTA in 0.01 M Tris buffer (*p*H 9.0). The dialyzed supernatant fluid contained no cell-wall lytic or proteolytic activity. The remaining supernatant solution which contained over half of the total starting protein was discarded. The precipitate was suspended in 200 ml of 0.05 M Tris buffer (*p*H 9.0) which contained 10^{-3} M sodium EDTA, and was blended for 2 min in a chilled Waring Blendor. The suspension was centrifuged at 10,000 × *g* for 10 min, resulting in the separation of an amber supernatant solution and a gray precipitate. The supernatant

TABLE 1. *Summary of purification data for the myxobacter AL-1 enzyme*

Step	Assay	Units/ml	Total units $\times 10^{-3}$	Purification (fold)	Specific activity*	Per cent recovery (activity)
Growth liquor	C†	440	440.0	1	120	100.0
	P‡	85	85.0	1	23	100.0
Supernatant of Zn ppt	C	0	0.0	—	—	—
	P	0	0.0	—	—	—
Tris-EDTA wash of Zn ppt	C	85	17.0	—	35	3.9
	P	19	3.8	—	8	4.5
Citrate wash of Zn ppt	C	1,840	276.0	192	23,000	63.0
	P	344	51.6	187	4,300	60.6
Effluent from DEAE cellulose	C	2,978	268.0	412	49,500	61.0
	P	572	51.5	414	9,500	60.6
Effluent from G-100 Sephadex column	C	10,600	265.0	632	76,000	60.3
	P	2,000	50.0	620	14,300	59.0

* Units of activity per milligram of protein.

† Cell-wall lytic assay: one unit is defined as a decrease in optical density of 0.001 at 660 mμ of a standard suspension of cell walls of *Arthrobacter crystallopoietes* in 15 min at 38 C.

‡ Proteolytic assay: solubilization of dye from Azocoll. One unit is defined as an increase in optical density of 0.001 at 580 mμ in 10 min at 38 C.

fluid contained 13% of the original protein and exhibited 3.9% of the total cell-wall lytic and 4.5% of the total proteolytic activities. Extensive dialysis of the supernatant solution did not increase the level of cell-wall lytic or proteolytic activity. The precipitate was homogenized in 100 ml of 0.1 M citrate buffer (*p*H 5.0) in a Waring Blendor for 2 min. The suspension was shaken gently for 15 min and was then centrifuged at 10,000 × *g* for 15 min. The clear, light-amber supernatant solution was decanted. The precipitate was homogenized for 2 min with 50 ml of the citrate buffer and was centrifuged. The two supernatant fluids were combined and dialyzed against 20 liters of 0.01 M Tris buffer (*p*H 9.0) containing 10^{-3} M EDTA for 24 hr (two changes). A gray pellet remained after the second extraction. No enzyme activity was solubilized by a third citrate buffer wash of this precipitate.

The dialyzed, citrate-buffer supernatant solution contained 63% of the original cell-wall lytic activity with a 192-fold increase in specific activity. The values for proteolytic activity were approximately the same, 187-fold purification with 60.6% recovery.

The dialyzed material was concentrated under vacuum to 30 ml, and 1.2 ml of 0.5 M glycine-NaOH buffer (*p*H 10.5) was added to give a final concentration of 0.02 M. This solution was applied to a column (2 by 23 cm) of DEAE cellulose which had been equilibrated with the 0.02 M glycine buffer. The column was washed with 300 ml of 0.02 glycine buffer at *p*H 10.5, followed by a linear NaCl gradient to 1.0 M. Fractions of 5.0 ml were collected and assayed for absorbance at 280 mμ and for cell-wall lytic and proteolytic activity. The results are presented in Fig. 1. All of the cell-wall lytic and proteolytic activity was washed through the column without being adsorbed. A dark-brown band which adsorbed at the top of the column was eluted partially by the NaCl gradient. This material contained no enzymatic

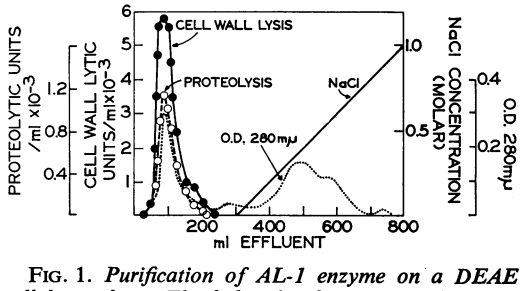

FIG. 1. *Purification of AL-1 enzyme on a DEAE cellulose column. The dialyzed and concentrated enzyme obtained from 1 liter of growth liquor by zinc precipitation was applied to a column (2 × 23 cm) at pH 10.5. The column was washed with 300 ml of 0.02 M glycine buffer (pH 10.5), followed by a linear NaCl gradient to 1.0 M. Fractions were assayed for cell-wall lytic and proteolytic activities and for absorbance at 280 mμ. In this and subsequent plots, numbers on the ordinate have been multiplied by 10^{-3} or 10^{-2} where indicated.*

TABLE 2. *Adsorption of the myxobacter AL-1 enzyme upon various materials*

Expt	Adsorbent	*p*H	Protein	Protein adsorbed	Units of activity after treatment		Specific activity‡
					C*	P†	
			mg/ml	%			
1	None (control)	—	0.11	—	2,120	380	19,300
2	$Ca_3(PO_4)_2$ gel	6.5	0.03	78	190	35	6,330
3	Hydroxylapatite	6.5	0.01	90	105	20	10,500
4	CM cellulose	6.5	0.05	55	42	79	8,400
5	DEAE cellulose	7.2	0.09	18	1,840	110	20,500
6	DEAE cellulose	8.0	0.09	18	1,880	108	20,900
7	DEAE cellulose	9.0	0.06	45	2,070	118	34,000
8	DEAE cellulose	10.0	0.05	66	2,030	125	40,600

* Cell-wall lytic assay as in Table 1.
† Proteolytic assay as in Table 1.
‡ Units of cell-wall lytic activity per milligram of protein.

activity. Essentially all of both cell-wall lytic and proteolytic activity was recovered from the column with an increase in specific activity for each of over twofold.

The fractions containing enzyme activity were pooled, concentrated under vacuum to 5 ml, and applied to a column (2.2 by 47 cm) of Sephadex G-100. The enzyme was washed through the column with 0.025 M Tris buffer (*p*H 9.0), and 2.0-ml fractions were collected. Each fraction which exhibited absorbance at 280 mμ was assayed for cell-wall lytic and proteolytic activities. Both activities emerged from the column in a single sharp peak (Fig. 2). The specific activity of the cell-wall lytic and proteolytic activities increased by 65 and 66.5%, respectively, and high recoveries of both were obtained (Table 1). With the use of India ink, a void volume for the column of 26 ml was found. The enzyme peak emerged from the column at 61 ml. The ratio of the effluent to void volume is 2.35 which, according to the data of Whitaker (23), indicates that the enzyme has a molecular weight of less than 10,000.

The completed purification resulted in a product with an increase in specific activity for cell-wall lysis of 632-fold and for proteolysis of 620-fold. The overall recoveries were essentially the same, 60.3% for cell-wall lysis and 59% for proteolysis.

A variety of ion-exchange materials were tested batchwise to see whether a differential adsorption of the two enzymatic activities could be obtained. A 1-g amount of the resin, from which excess moisture had been removed with a Büchner funnel, was added to 2.5 ml of the enzyme, which had been partially purified by zinc precipitation but not treated with DEAE cellulose or Sephadex. In each instance, the adsorbent and enzyme previously were equilibrated with the same buffer. The mixture was shaken for 30 min in an ice bath, and the adsorbent was removed by filtration. Assays of protein content, cell-wall lytic activity, and proteolytic activity were performed before and after treatment. The results are summarized in Table 2. In no instance was one activity adsorbed to the exclusion of the other. The enzyme was adsorbed by calcium phosphate gel and by the cation exchangers, hydroxylapatite and CM cellulose. DEAE cellulose in excess adsorbed less than 20% of the total protein at *p*H 7.2 or 8.0; nearly half was adsorbed at *p*H 9.0, and at *p*H 10.0 two-thirds of the protein was adsorbed. The number of units of activity remained nearly constant before and after DEAE cellulose treatment at all *p*H values, indicating that protein other than the enzyme was being adsorbed.

An estimate of the homogeneity and molecular weight of the enzyme was obtained by employing the density gradient technique of Martin and Ames (14). The results (Fig. 3) show that both cell-wall lytic and proteolytic activities are found in a single band remaining at the top of the gradient. Lysozyme with a molecular weight of 14,400 to 14,700 (11) was located slightly further from the meniscus, indicating a similar and probably lower molecular weight for the myxobacter AL-1 enzyme. That cell-wall lytic and proteolytic activities remain together in the centrifugal field is further evidence that a single enzyme catalyzes both reactions. The possibility of two enzymes each with a low molecular weight cannot be ruled out by this experiment.

The ultracentrifugation pattern of the purified enzyme is shown in Fig. 4. The results, which show a slow migration of the enzyme in the centrifugal field, indicate that it is of low molecular weight. It was not possible to calculate an accurate molecular weight from the sedimentation rate, for

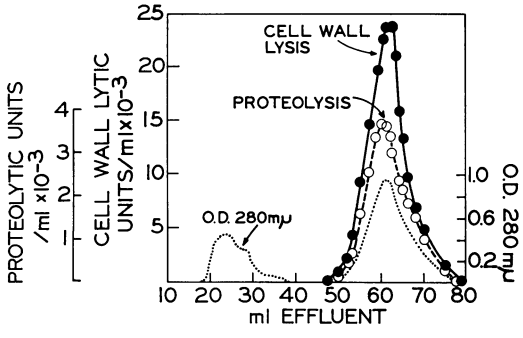

FIG. 2. *Purification of AL-1 enzyme on a Sephadex G-100 column. A 5-ml amount of the purified enzyme from DEAE cellulose was applied to a column (2.2 × 47 cm), and the enzyme was washed through with 0.025 M Tris buffer (pH 9.0) at a rate of 12 ml/hr. Fractions containing 280 mμ absorbing material were assayed for cell-wall lytic and proteolytic activities.*

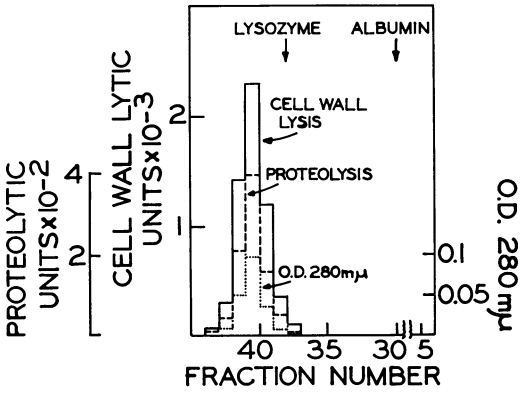

FIG. 3. *Sedimentation of the AL-1 enzyme in a sucrose gradient. The purified enzyme (0.3 mg of protein) was layered on a 5 to 20% linear gradient and was centrifuged at 102,000 × g for 22 hr. Fractions collected from the bottom of the centrifuge tube were assayed for cell-wall lytic activity and proteolytic activity (DNFB procedure) and for absorbance at 280 mμ. The positions obtained for lysozyme and for albumin in an identical experiment also are shown.*

diffusion of the enzyme caused a gradual reduction of the boundary peak. At 96 min, a sedimentation value of 1.04 Svedberg units was calculated, which indicates a molecular weight in the region of 10,000. The single sharp peak provides evidence that the preparation is homogeneous.

The results of the gel filtration (Fig. 2), sucrose gradient centrifugation (Fig. 3), and ultracentrifugation (Fig. 4) indicate that the enzyme is of low molecular weight. The molecular weight was determined by comparing the effluent volume of the enzyme from a Sephadex G-100 column with those of several proteins with established molecular weights. The molecular weights of the proteins used in standardizing the column were taken from the literature as follows: cytochrome *c* (13), ribonuclease (10), lysozyme (11), soybean trypsin inhibitor (24), trypsin (3), and ovalbumin (22). As shown in Fig. 5, a linear relationship exists between the effluent volumes and the logarithm of the molecular weights of the proteins tested. The curve can be extrapolated to include the experimental point for the effluent volume of the myxobacter AL-1 enzyme. From the position of this point, a molecular weight for the enzyme of 8,700 was calculated. The same result was obtained with a similarly calibrated Sephadex G-75 column.

The results of electrophoresis of the enzyme on cellulose acetate strips reveal that it migrates toward the cathode as a single band at *p*H values ranging from 7.0 to 11.0 (Fig. 6). Migration toward the cathode at *p*H 11.0 indicates that the enzyme is a basic protein.

The technique of disc electrophoresis on polyacrylamide gel has proved to be extremely sensitive for the determination of protein homogeneity (4, 18). The results of applying this procedure to the purified AL-1 enzyme are shown in Fig. 7. The enzyme preparation was resolved into one major and two very faint bands. The gel was cut into segments, each of which was assayed for lytic activity. Cell-wall lytic and proteolytic activities were found only in two adjacent segments,

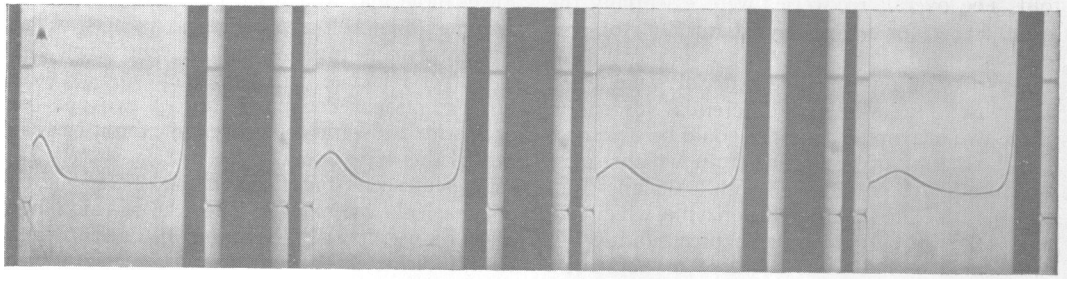

FIG. 4. *Sedimentation pattern of purified AL-1 enzyme, and 0.8% solution in distilled water. AN-D rotor, 59,780 rev/min. Exposures at 32, 64, 96, and 128 min (left to right).*

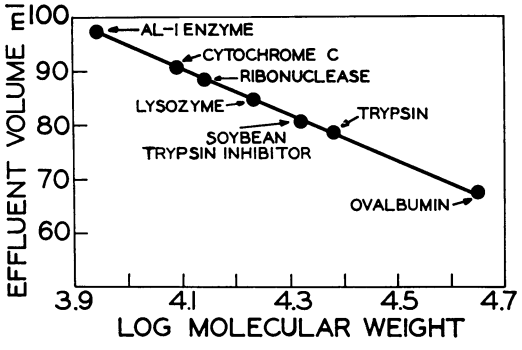

FIG. 5. *Determination of the molecular weight of the AL-1 enzyme by gel filtration on a Sephadex G-100 column (1.5 × 61 cm) calibrated with proteins of known molecular weights.*

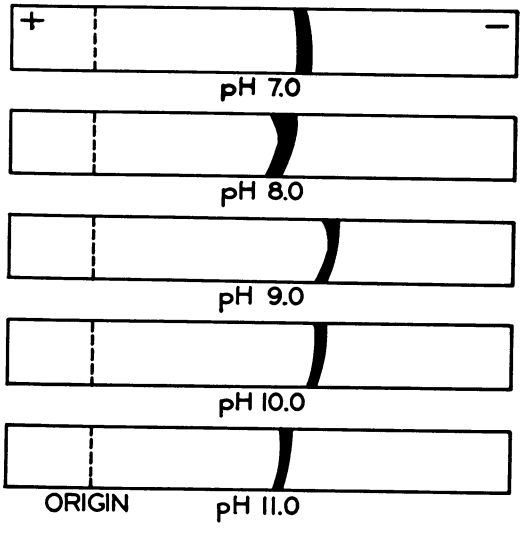

FIG. 6. *Drawing of the appearance of the electrophoresis of purified AL-1 enzyme on cellulose acetate strips at different pH values. A 10-μliter amount of enzyme solution was applied in a streak at the origin. A current of 1.5 to 2.0 ma was applied to each strip for 1 hr. Stained with Amido Black.*

the position of which corresponds to that of the major protein band (Fig. 7). No lytic activity was detected in the segments containing the faint bands; these may represent denaturation of the enzyme during electrophoresis.

The evidence thus far presented is compatible with the concept that a single enzyme catalyzes both cell-wall lysis and protein hydrolysis. A series of experiments were performed to define and compare some of the properties of the two activities. A sharp *p*H optimum at 9.0 was observed for both cell-wall lysis and proteolysis (Fig. 8). Each of the four buffers listed was used at a concentration of 0.02 M, the breaks in the

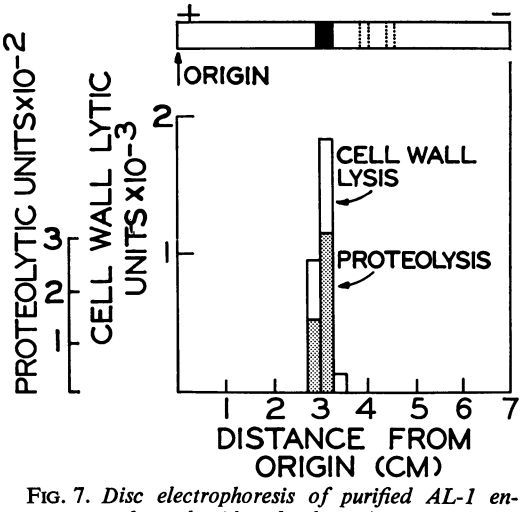

FIG. 7. *Disc electrophoresis of purified AL-1 enzyme on a polyacrylamide gel column (0.5 × 7.0 cm). A current of 5 ma per tube was applied for 90 min. One column was stained with Amido Black; the other was sliced into 1.5-mm segments, and each was tested for cell-wall lytic activity and proteolytic activity (DNFB procedure). At the top is shown a reproduction of the stained gel and at the bottom the position of enzyme activity in the corresponding sections.*

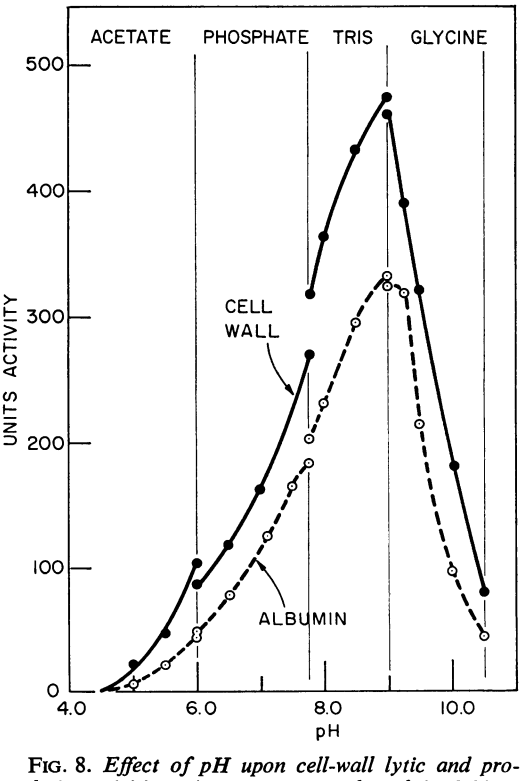

FIG. 8. *Effect of pH upon cell-wall lytic and proteolytic activities. Assays were conducted in 0.02 M buffers at the indicated pH values. Proteolysis was assayed by the Azocoll procedure.*

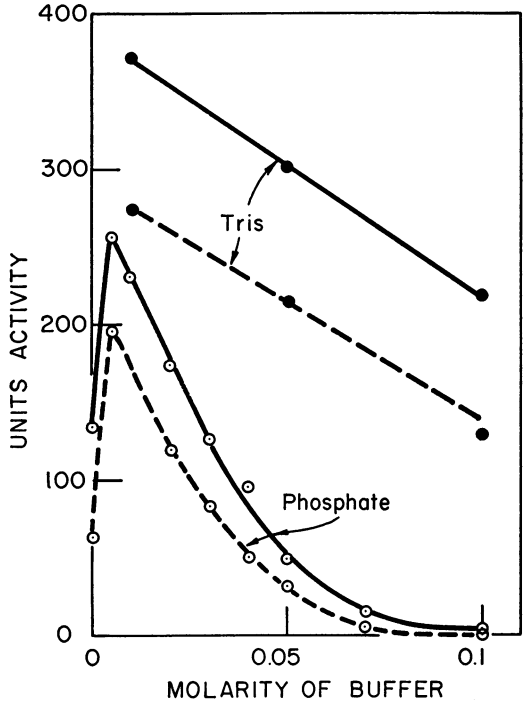

FIG. 9. *Effect of Tris and phosphate buffer concentration upon cell-wall lytic and proteolytic activities. Solid lines, cell-wall lysis. Dashed lines, proteolysis (Azocoll procedure).*

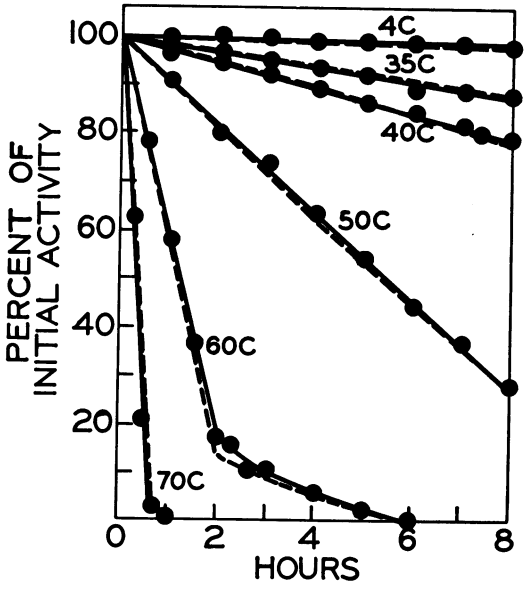

FIG. 10. *Stability of AL-1 enzyme at different temperatures. Purified enzyme was incubated at the temperature shown, and at the times represented by the points on the graph 0.1-ml samples were removed and assayed for cell-wall lytic activity (solid lines) and proteolytic activity (dashed lines) by the Azocoll technique.*

curves being a result of variations in activity of the enzyme in the presence of the different buffers. The effect upon the enzyme of varying the concentration of Tris buffer at *p*H 9.0 and phosphate buffer at *p*H 7.8 is shown in Fig. 9. Cell-wall lytic and proteolytic activities are equally susceptible to increased ionic strength of the two buffers. Phosphate buffer was particularly inhibitory, the enzyme being completely inactive at 0.1 M and nearly so at 0.05 M. Tris buffer was less inhibitory, the level of activity at 0.1 M being 58% of that at 0.005 M. In the absence of added buffer, the enzyme was weakly active.

The stability of the enzyme in 0.02 M Tris buffer (*p*H 9.0) at different temperatures was determined. As shown in Fig. 10, at each temperature the decrease in activity with time of cell-wall lysis and proteolysis was essentially identical. The enzyme was stable at 4 C for 8 hr, and after 48 hr 93% of the activity remained; at 35 C the enzyme retained 87% of its activity after 8 hr; at higher temperatures, the stability of the enzyme decreased rapidly.

The effect of *p*H upon the stability of the enzyme was determined. The enzyme was incubated at 45 C with 0.02 M buffers ranging from *p*H 3.0

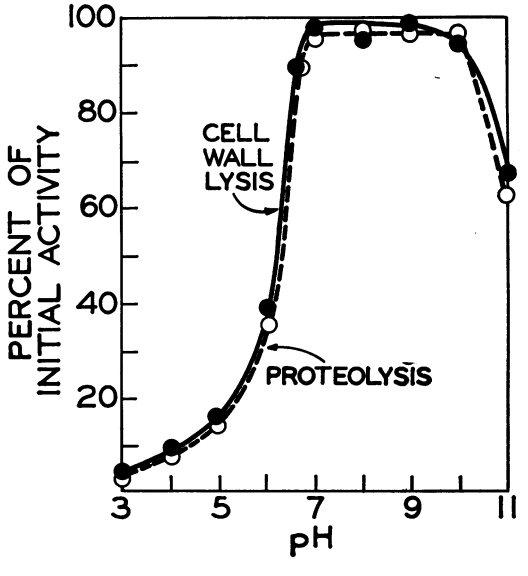

FIG. 11. *Stability of AL-1 enzyme at different pH values. Purified enzyme incubated with 0.02 M buffers ranging from pH 3.0 to 11.0 at 45 C. Cell-wall lytic activity and proteolytic activity (Azocoll assay) were determined at zero-time and after 1 hr of incubation.*

to 11.0. At zero-time and after 1 hr of incubation, 0.1-ml samples were removed, and the cell-wall lytic and proteolytic activities were determined. As shown in Fig. 11, the two activities responded identically. The enzyme exhibited maximal stability from *p*H 6.5 to 9.5.

TABLE 3. *Effect of metal salts, chelating agents, and inhibitors upon proteolytic and cell-wall lytic activities of the myxobacter AL-1 enzyme*

Agent tested	Molarity	Units of activity	
		Cell-wall lysis*	Proteolysis†
None (control)	—	450	340
$HgCl_2$	10^{-4}	0	0
	10^{-5}	280	210
	10^{-6}	376	285
$ZnCl_2$	10^{-4}	52	30
	10^{-5}	320	235
	10^{-6}	402	310
$Ca(C_2H_3O_2)_2$	10^{-3}	356	270
	10^{-4}	374	290
	10^{-5}	395	305
	10^{-6}	430	345
$CuSO_4$	10^{-3}	44	18
	10^{-4}	156	120
	10^{-5}	320	245
	10^{-6}	390	295
$MgCl_2$	10^{-3}	430	325
	10^{-4}	455	340
	10^{-5}	450	350
$MnCl_2$	10^{-3}	130	92
	10^{-4}	210	165
	10^{-5}	342	250
	10^{-6}	380	290
$AgNO_3$	10^{-3}	0	0
	10^{-4}	160	105
	10^{-5}	440	345
NaCl	10^{-1}	20	14
	10^{-2}	77	45
	10^{-3}	450	340
PCMB	1.4×10^{-4}	439	355
	4.2×10^{-4}	444	345
DFP	10^{-3}	428	350
	10^{-4}	410	335
	10^{-5}	452	340
Soybean trypsin inhibitor	100 μg	458	352
DNFB	10^{-4}	432	345
Sodium EDTA	5×10^{-2}	0	0
	10^{-2}	530	965
	10^{-3}	780	700
	10^{-4}	565	480
	10^{-5}	530	420
	10^{-6}	520	440
Sodium citrate	10^{-1}	0	0
	10^{-2}	640	780
	10^{-3}	590	370
	10^{-4}	465	355

* Cell-wall lytic assay as in Table 1.
† Proteolysis assay as in Table 1.

In Table 3 are presented the results of an investigation of the effect of some metal salts, inhibitors, and chelating agents upon cell-wall lytic and proteolytic activity. In no instance was there a significant difference in the effect of a particular agent upon the two activities. No stimulation of activity resulted from the addition of metal salts. The heavy metal salts of mercury, zinc, copper, and silver were inhibitory at 10^{-3} to 10^{-4} M but not significantly so at 10^{-6} M. Magnesium had no effect at the highest concentration tested, 10^{-3} M. Calcium and manganese produced partial inhibition. Sodium chloride was inhibitory at 10^{-2} M but not at lower concentrations. The chelating agents EDTA and citrate were inhibitory at concentrations greater than 10^{-2} M but stimulated activity at lower concentrations. With EDTA, maximal stimulation of cell-wall lysis occurred at 10^{-3} M and proteolysis at 10^{-2} M. Definite stimulation occurred with EDTA at 10^{-5} and 10^{-6} M, whereas at 10^{-4} M citrate no longer stimulated. The marked stimulation of proteolytic as compared with cell-wall lytic activity by EDTA and by citrate may be a result of the presence of inhibitory metals in the Azocoll substrate, which were removed by the chelating agents. Both activities were stimulated proportionally by 10^{-3} M EDTA when proteolysis was assayed by the DNFB procedure with albumin as substrate. The enzyme proved insensitive to PCMB, DFP, DNFB, and soybean trypsin inhibitor.

An estimate of the extent of proteolysis of three proteins (albumin, casein, and gelatin) was made by comparing quantitatively for each protein the amino groups released enzymatically with those released upon acid hydrolysis. To 1.0 ml of a

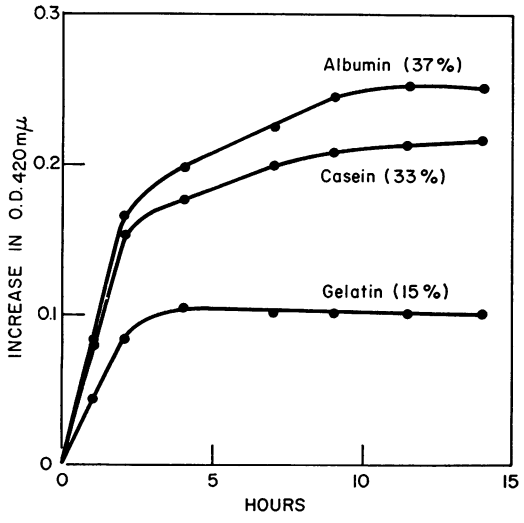

FIG. 12. *Release of DNFB-reactive amino groups from proteins by purified AL-1 enzyme. The figures in parentheses refer to the percentage of amino groups released by the enzyme compared with those released by acid hydrolysis.*

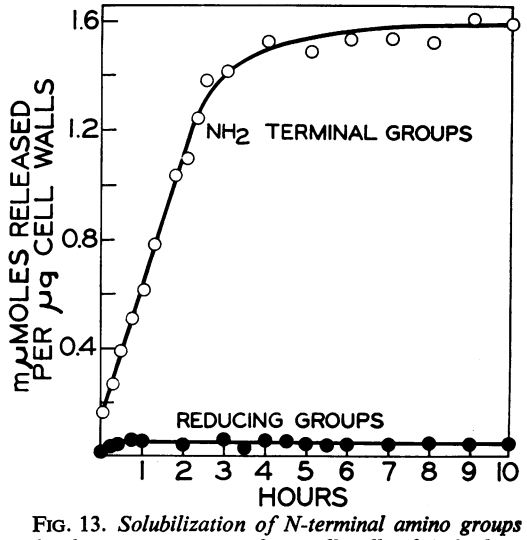

FIG. 13. *Solubilization of N-terminal amino groups and reducing sugar groups from cell walls of Arthrobacter crystallopoietes by purified AL-1 enzyme.*

0.2% solution of each protein were added 1.0 ml of 0.05 M veronal buffer (*p*H 9.0) and 0.1 ml of enzyme. Each reaction mixture was incubated for 14 hr at 38 C. At various time intervals, duplicate 0.1-ml samples were removed and assayed for release of amino groups by the DNFB procedure. For acid hydrolysis, 1.0 ml of each of the 0.2% protein solutions was pipetted into a separate Pyrex test tube (1.3 by 10 cm). A 1-ml amount of concentrated HCl was added to each, and each tube was sealed under vacuum and heated at 110 C for 24 hr. The top of each tube was removed; the sample was evaporated to dryness, and 2.1 ml of water was added to each. Triplicate 0.1-ml samples of each hydrolysate were then assayed by the DNFB procedure (Fig. 12). Amino groups were freed enzymatically at a rapid linear rate for 2 hr, followed by a more gradual release from albumin and casein and an abrupt leveling off for gelatin. The numbers in parentheses in Fig. 12 refer to percentage of amino groups released by the enzyme compared with those released by acid hydrolysis. Thus, for albumin, 37%, for casein, 33%, and for gelatin, 15% of the total amino groups were released by the enzyme.

The release of amino groups and of reducing sugars during digestion of cell walls by the enzyme was followed. The results (Fig. 13) are presented as millimicromoles of reducing sugars or amino groups released per microgram of cell walls. These values were obtained with D-glucosamine and L-alanine as standards in the respective assays. Free amino groups were released from the cell walls at a rapid linear rate for 2.5 hr, followed by a decrease in the rate. A maximal number of 1.6 mμmoles of free amino groups per μg of cell-wall material was reached after 8 hr. There was essentially no release of reducing sugars from the cell walls. It is evident that the enzyme lyses cell walls of *A. crystallopoietes* by hydrolyzing peptide bonds in the mucopeptide complex.

DISCUSSION

The isolation of an enzyme capable of degrading both proteins and cell walls is without precedent, and it is logical to suspect that more than one enzyme is involved. With this in mind, we have employed many techniques in an attempt to separate or to detect differences between the cell-wall lytic and proteolytic activities; in no instance was there obtained a separation of or differentiation between the two. Some of the techniques employed, such as gel filtration, density gradient centrifugation, ultracentrifugation, and electrophoresis on polyacrylamide gel or cellulose acetate, are extremely sensitive, and most certainly would have yielded a separation if more than one enzyme were present. In addition, the two activities exhibit: an identical *p*H optimum, a similar response to increased ionic strength of buffers as well as to heat inactivation, and an equal reaction to metal salts, inhibitors, and chelating agents.

The enzyme solubilizes cell walls by hydrolyzing peptide bonds. This fact is of utmost significance in supporting the claim of one enzyme. Had the lytic mechanism involved a cleavage of

the carbohydrate "backbone," the single enzyme concept would be untenable. Since the structurally rigid components of bacterial cell walls contain peptides with some of the amino acids in the L configuration, it is perhaps not surprising to find a proteolytic enzyme capable of hydrolyzing these. This especially would be feasible if the enzyme were of low molecular weight, and were capable of penetrating to the substrate. The AL-1 enzyme with a molecular weight of less than 9,000 fulfills this requirement. The relatively low specificity of the enzyme as a protease is probably of significance in relation to its cell-wall lytic capacity. The enzyme hydrolyzes 33% of the peptide bonds of casein. In comparison, Nomoto, Narahashi, and Murakami (17) reported that a protease from *Streptomyces griseus* (pronase) hydrolyzed 75%, a protease from *Bacillus subtilis* 40%, trypsin 23%, pepsin 31%, and papain 30% of the peptide bonds in casein. The figure for pronase of 75% proteolysis is deceiving, because Hiramatsu and Ouchi (9) have succeeded in fractionating the commercial preparation into at least three different proteases. Few proteolytic enzymes have been specifically tested for cell-wall lytic activity. Kato et al. (12) mentioned that the protease, pronase, did not lyse cell walls of *Staphylococcus aureus*. A recent paper by Montague (16) verifies that under some conditions a proteolytic enzyme may attack cell walls. He showed that trypsin had almost no effect on intact cell walls of *Streptococcus faecalis;* after partial lysis by lysozyme, the cell walls were attacked rapidly.

Most of the cell-wall lytic enzymes that have been characterized adequately hydrolyze bonds between the amino sugars of the glycosaminopeptide. Some enzymes, however, have been described which attack the peptide moiety. The enzyme F_2B of Ghyusen, Leyh-Bouille, and Dierickx (6) from *Streptomyces* splits the amide bond between muramic acid and the peptide in cell-wall fragments of *Micrococcus lysodeikticus*. The L_{11} enzyme isolated from a species of *Flavobacterium* by Kato et al. (12) hydrolyzes the muramic acid-alanine bond and at least one other bond in cell walls of *Staphylococcus aureus*. The lytic principle in an enzyme preparation from a species of *Staphylococcus* was shown by Browder et al. (2) to be a peptidase which liberates glycine and alanine from cell walls of *S. aureus*. Young, Tipper, and Strominger (25) reported that an autolytic enzyme associated with cell walls of a strain of *B. subtilis* hydrolyzes the amide bond between muramic acid and L-alanine in the cell walls of this organism. Peptidase activity was detected by Pelzer (20) in a mixture of autolytic enzymes from *Escherichia coli* B. Proteolytic activity was not reported for these enzymes.

On the basis of gel-filtration data, the AL-1 enzyme has a molecular weight of 8,700. The technique of employing gel filtration for molecular-weight determinations was reported by Whitaker (23) and Andrews (1) to be highly accurate for most proteins. Basic proteins of low molecular weight, such as lysozyme and ribonuclease, have been shown by Miranda, Rochat, and Lissitzky (15) and by Glazer and Wellner (8) to be adsorbed onto Sephadex gels when placed on the columns in the absence of salts or buffer. To avoid this complication, we used 0.1 M KCl + 0.02 M Tris buffer to equilibrate and elute the columns employed for molecular-weight determination. According to Andrews (1), this should be sufficient to overcome the adsorption problem. Using a column of Sephadex G-100, Whitaker (23) obtained a molecular weight for lysozyme of 7,700, which is approximately one-half the true value. The elution volume was independent of ionic strength, thus ruling out ion-exchange adsorption as a factor. The author theorized that lysozyme might have formed a complex through its active site with the dextran gel. The possibility exists that the AL-1 enzyme also behaves anomalously upon gel filtration, giving a false low molecular weight. However, the results obtained with density gradient centrifugation and ultracentrifugation verify that the enzyme is indeed very small.

Any new bacteriolytic enzyme has potential value as a tool in studies of cell-wall chemistry. The results of an investigation conducted in collaboration with D. J. Tipper and J. L. Strominger indicate that the AL-1 enzyme will prove very valuable in this respect. Undegraded polysaccharide "backbone" fragments, free from all peptide residues, have been isolated from enzyme digests of cell walls of several bacteria. A manuscript describing this work in detail is in preparation. Studies of the structure and properties of the AL-1 enzyme are being continued.

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