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In Vitro Production of an Extracellular Protease by *Legionella pneumophila*

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Extracellular protease activity was measured in liquid cultures of representative strains from six serotypes of *Legionella pneumophila*. A variety of substrates were degraded, including denatured casein, skim milk, gelatin, and hide powder azure, but not elastin.

Legionella pneumophila is the etiological agent of Legionnaires disease (8), which is commonly recognized as a form of pneumonia (3, 16). Currently, the mechanism(s) involved in the pathogenesis of *L. pneumophila* infections is not understood.

The pulmonary and extrapulmonary manifestations which reportedly accompany infection with *L. pneumophila* suggest the possible involvement of extracellular bacterial products (5, 15). Several extracellular products have recently been described, including a cytotoxin (1, 6), a hemolysin (1), and a gelatinase (15). A previous report has indicated that *L. pneumophila* organisms, when incubated with human serum, degraded 5 of 23 serum proteins (10). This study describes a protease produced in vitro in several media by representative strains of serogroups one through six. This protease digests casein, hide powder, and gelatin.

Eight strains of *L. pneumophila* representative of serotypes 1 through 6 were obtained from the Centers for Disease Control, Atlanta, Ga. Strains tested included Knoxville 1, Bellingham 1, and Philadelphia 2 (serogroup 1); Togus 1 (serogroup 2); Bloomington 2 (serogroup 3); Los Angeles 1 (serogroup 4); Dallas 1-E (serogroup 5); and Chicago 2 (serogroup 6). Purity of all cultures was monitored by microscopic morphology, characteristic growth, pigment production, and by absence of growth on blood agar or brain heart infusion plates. Media used in this study included a complex liquid medium with and without ferric pyrophosphate (GC-FC) (14), a chemically defined medium (14), and yeast extract broth (12). Innocula were obtained from cultures grown on GC-FC agar slants for 3 to 4 days. Bacterial cells were washed from slants with the medium chosen for growth. Acid-washed nephelometer flasks or disposable plastic flasks were inoculated with sufficient cells to obtain an initial turbidity of approximately 35 Klett units. Cultures were incubated at 35°C on a gyratory shaking water bath at 200 rpm.

Bacterial cells were removed from cultures by centrifugation at 12,000 × g for 30 min. Culture supernatant fluids were then filter sterilized (0.22-μm membrane filters, Millipore Corp., Bedford, Mass.), aliquoted, and frozen at −70°C. Proteolytic activity was tested for by a modified skim milk plate (13) and gelatin plate assay (4), by a denatured casein assay (17), by an elastin Congo red assay for elastase (2), and by a modified colorimetric assay utilizing hide powder covalently labeled with Remzol brilliant blue (11). A commercial preparation of hide powder azure was utilized for the latter assay (Calbiochem, La Jolla, Calif.).

Proteolytic activity was measured in culture filtrates from all *Legionella* organisms tested grown in GC-FC, yeast extract broth, and defined media. No elastase activity was detected with Knoxville 1 culture filtrate utilizing the elastase assay. Proteolytic activity was observed by the production of a well defined zone of clearing on skim milk plates (Fig. 1) as well as on gelatin plates, by release of perchloric acid-soluble products from heat-denatured casein, and by release of dye from insoluble hide powder. Activity was first observed in early to mid-log cultures in all three media; however, maximum proteolytic activity was found in early stationary cultures of *L. pneumophila*. Maximal levels of activity were found to be approximately equal in GC-FC and yeast extract broth media, but somewhat lower in defined medium.

All of the characterization studies in this report were performed on early stationary (42-h) culture filtrates of Knoxville 1 grown in GC-FC liquid medium. Added ferric iron, although

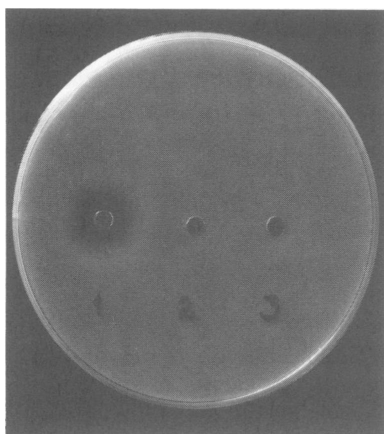

FIG. 1. *Comparison of proteolytic activity on skim milk plate assay of culture filtrate. Plate incubation 37°C, 24 h, 4-mm wells. (1) Knoxville 1 culture filtrate; (2) GC-FC media; (3) culture filtrate heated 60°C for 30 min.*

needed for maximal growth (14), did not increase proteolytic activity in GC-FC liquid medium. The proteolytic activity observed in the skim milk plate assay was visible as a zone of clearing of approximately 1 mm by 2 h at 37°C. All strains tested gave approximately comparable zones. An incubation period of 24 h at 37°C was chosen for the skim milk assay to give an easily measured zone of clearing. Incubation for longer times gave larger zones, but boundaries were diffuse, making accurate measurement difficult. Gelatin plate assays were conducted for 2 h at 37°C. Zones of clearing were well defined and easily measured using these conditions.

The conditions for the hide powder protease reaction were optimized with respect to buffer, temperature, substrate concentration, and agitation. The optimized hide powder assay was performed in polypropylene culture tubes charged with 50 mg of substrate and 2.5 ml of buffer. Culture filtrates or media were added to the tubes at room temperature, and the tubes were immediately capped and shaken at 37°C for 1 h. The reaction was terminated by chilling the reaction tubes on ice and then filtering the contents through Whatman 4 filter paper. Filtrates were analyzed for dye release by measuring absorbance at 595 nm. Reaction buffers investigated covered the pH range of 4.5 to 9.0. Buffers tested included tris(hydroxymethyl)-aminomethane-maleate, tris(hydroxymethyl)-aminomethane-hydrochloride, citrate, acetate, phosphate, ADA, and imidazole. The pH optimum of protease activity against hide powder in all reaction buffers tested, except citrate, appeared to have a broad peak between pH 6.0 and 7.0. No activity was observed in citrate buffer. Maximal proteolytic activity was obtained with 100 mM sodium phosphate buffer (pH 6.0). Proteolytic activity was inhibited in this buffer by the addition of sodium chloride (80% inhibition with 150 mM added NaCl) or by increasing the concentration of phosphate buffer. The addition of $CaCl_2$ to the reaction was also inhibitory (50% at 10 mM). Maximal activity was found at 54°C; approximately a threefold increase in dye release was observed in the assay at 54°C over that observed at 37°C in a 30-min assay. Background dye release, however, was likewise proportionally increased; therefore, 37°C was chosen as the standard assay temperature.

Temperature and pH stability studies on proteolytic activity in Knoxville 1 culture filtrate and culture filtrate dialyzed against 25 mM sodium phosphate (pH 7.0) were performed in the skim milk, the gelatin plate, and the optimized hide powder assay. Protease activity in both dialyzed and untreated culture supernatants showed virtually identical sensitivity to heating (Table 1). Proteolytic activity was present at or above control activity (samples kept on ice) with a preincubation temperature ranging up to 45°C (30-min preincubation time). Approximately half of the proteolytic activity as measured in the hide powder assay remained in these preparations after preincubation at 54°C for 30 min. Protease activity from strains tested demonstrated similar heat sensitivities in all three assays. No gelatinase or hide powder activity was observed after incubating culture filtrates from all strains at 80°C for 30 min.

The sensitivity of the proteolytic activity to temporary alterations in pH was determined by

TABLE 1. *Effect of preincubation at elevated temperature on proteolytic activity of L. pneumophila culture filtrate*[a]

Preincubation temp (°C)	Remaining activity (%)	
	Culture filtrate	Dialyzed culture filtrate
On ice	100	100
25	100	103
30		92
35	110	92
40	104	96
45	89	89
50	75	75
55	31	27
60	02	0
65	02	0
80	0	0

[a] Hide powder azure assay, standard conditions. Thirty minutes of preincubation of samples at each temperature.

titrating samples with 0.1 N HCl or NaOH to a measured pH and then incubating the sample at 37°C for 4 h. Samples were then neutralized, diluted to equal volume, and assayed for remaining activity. The results of this experiment are presented in Table 2. A broad plateau in proteolytic stability was observed from pH 5.5 to 8; at pH extremes, activity dropped rapidly in the hide powder, skim milk, and gelatin assays (Table 2).

The similarities in pH and temperature stability profiles observed in the skim milk, gelatin, and hide powder assays may indicate that these activities are due to a single enzyme with multiple substrate specificities, not an uncommon feature of bacterial proteases.

The effects of various protease inhibitors were studied using the hide powder assay. The serine protease inhibitor phenylmethyl-sulfonyl fluoride and Trasylol had no effect on proteolytic activity when preincubated with culture filtrates at 0.5 to 50 μg/ml and 2 to 10 U/ml, respectively. Pretreatment of culture filtrates with the anionic detergent sodium dodecyl sulfate (SDS) had an activating effect at low concentrations (0.05 to 0.5%). At 1% and higher concentrations, SDS inactivated the proteolytic activity. No dye was released by comparable concentrations of SDS in the hide powder assay. Urea substantially inhibited proteolytic activity when preincubated with culture filtrate at 4 to 6 M concentrations. Reducing agents such as 2-mercaptoethanol and dithiothreitol had no effect on proteolytic activity when preincubated with culture filtrates at concentrations up to 10 mM.

Proteolytic activity measured by the hide powder assay was reduced by preincubation of culture filtrate with a variety of chelating agents (Table 3). Chelating agents used in these studies were neutral solutions of ethylenediamine tetraacetate, disodium salt (EDTA), nitrilotriacetic acid, ethyleneglycol-bis(β-aminoethyl ether)-N,N′-tetraacetic acid, and o-phenanthroline. EDTA appeared the most efficient chelating agent tested in inhibiting the proteolytic activity. Activity was not significantly recovered by adding back Mg^{2+}, Mn^{2+}, or Ca^{2+} salts to the culture filtrate after EDTA pretreatment. Proteolytic activity was almost entirely regained however by adding back Zn^{2+} salts before assay. Proteolytic activity was also regained by dialyzing EDTA-treated culture filtrate overnight against 50 mM Na_2HPO_4 (pH 7.0). These results imply the formation of a reversible complex of EDTA and protease. Whether the enzyme is in fact a metalloprotease remains to be demonstrated.

Knoxville 1 culture filtrates were incubated with 0.5 or 1.0 mg of trypsin per ml at 37°C for 30 min, trypsin inhibitor (7) was added, and incubation was continued for an additional 30 min. Under these conditions, the proteolytic activity in culture filtrate was not altered by trypsin treatment.

Protease activity was precipitable from culture filtrate with 40 to 65% ammonium sulfate. Preliminary studies using dialyzed, ammonium

TABLE 2. *Effect of acid and alkali pretreatment of proteolytic activity of L. pneumophila Knoxville 1 culture filtrate*

pH	Hide powder azure[a] (% of control)	Skim milk[b] (zone of clearing, mm)	Gelatin[c] (zone of clearing, mm)
2	0		
3	0	0	0.5
4	10	0	1.0
5	42	6.0	1.3
6	82	8.0	1.5
7	82	8.0	2.0
7.4[d]	100	8.0	2.0
8	63	8.0	2.0
9	36	0	1.3
10	28	0	0
11	0		

[a] One hour of incubation at 37°C, neutralized culture filtrate.
[b] Twenty-four hours of incubation at 37°C, measured zone of hydrolysis diameter − well diameter (4 mm).
[c] Two hours of incubation at 37°C, measured zone of hydrolysis diameter − 3 mm.
[d] Untreated culture filtrate.

TABLE 3. *Effect of chelating agents on proteolytic activity in L. pneumophila Knoxville 1 culture filtrates*[a]

Treatment	Concn (mM)	Remaining activity (%)
No pretreatment		100
EDTA	0.1	53
	5.0	5
EGTA[b]	0.1	58
	5.0	27
NTA[c]	0.1	95
	5.0	67
O-phenanthroline	0.1	95
	5.0	23
EDTA, 5 mM, postdialysis		100
Citrate	1.0	80

[a] Hide powder azure assay. Concentrations given for 30 min preincubation period. Concentration for the actual protease assay is 0.0075 that shown.
[b] EGTA, Ethylene glycol-bis(β-aminoethyl ether)-N,N-tetraacetic acid.
[c] NTA, Nitrilotriacetic acid.

sulfate-concentrated protease have demonstrated an approximate molecular weight of 40,000 by gel filtration on Sephadex G-100. Enzymatic activity measured with the hide powder and gelatin plate assays also eluted as a single peak with an approximate molecular weight of 33,000 from SDS-polyacrylamide gels after electrophoresis. Activity eluted at the same R_f when the sample was heated at 37°C for 30 min with SDS or SDS and 2-mercaptoethanol. Analytical scale isoelectric focusing demonstrated an isoelectric point of approximately 4.1 to 4.3 for the protease in the same assays.

In conclusion, we have found that eight strains of *L. pneumophila*, representative of serogroups one through six, produce measurable proteolytic activity when grown in complex and defined media. This protease digested denatured casein, gelatin, and hide powder, but not elastin. The activity was produced at approximately the same levels in all strains tested. Protease activities produced by all strains were similarly inactivated by heating, were stable to dialysis but sensitive to chelating agents, urea, and treatment with pH extremes. *L. pneumophila* protease appears to be a neutral (possibly metallo-) protease, with a molecular weight of approximately 40,000 (by gel filtration) and an isoelectric point of approximately 4.3.

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