JOURNAL OF CLINICAL MICROBIOLOGY, July 1980, p. 131-133
0095-1137/80/07-0131/03$02.00/0

Vol. 12, No. 1

Production of Alkaline Protease by *Pseudomonas aeruginosa*

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A highly sensitive and specific radioimmunoassay has been developed for *Pseudomonas aeruginosa* alkaline protease. Production of alkaline protease was found to be strain variable and medium dependent.

Recently, a number of investigations have indicated that proteases produced by *Pseudomonas aeruginosa* may play a role in the establishment and maintenance of various types of infections (3, 5, 12, 13). Three distinct *P. aeruginosa* proteases have been isolated and characterized (9, 10, 13). Studies on the role of the individual proteases have been greatly hindered by the lack of specific and sensitive assays for each of these enzymes. In an attempt to aid future studies involving the alkaline protease, we have sought to develop a highly sensitive and specific assay for this enzyme. In this communication we describe a liquid-phase radioimmunoassay (RIA) specific for alkaline protease antigen and report on the production of this protease by various strains of *P. aeruginosa* under different growth conditions.

Highly purified *P. aeruginosa* alkaline protease and elastase were the kind gift of K. Morihara (9). The purified alkaline protease was homogeneous on electrophoresis and ultracentrifugation (9). Alkaline protease antisera were prepared by injecting 50 μg of alkaline protease, emulsified in an equal volume of Freund complete adjuvant, intraperitoneally, subcutaneously, intramuscularly, and into the rear foot pads of rabbits. Three and 4 weeks later the injection course was repeated using a total of 50 μg and 25 μg, respectively, suspended in Freund incomplete adjuvant. Ouchterlony double-diffusion analysis utilizing the alkaline protease antiserum showed only one precipitin band with culture filtrate, which showed a line of identity with the purified alkaline protease (data not shown). The bacterial strains PA103 (11), PA01 (6), WR5 (1), PAKS-1 (12), and EZ-3 (15) have been previously described. Cultures were grown at 32°C for 22 h with maximum aeration in a reciprocating shaker (Lab Line Instruments, Melrose Park, Ill.). The following media were utilized: (i) a dialysate of Trypticase soy broth (TSBD) treated with chelating agent, Chelex 100 (Bio-Rad Laboratories, Richmond, Calif.), as follows—Chelex 100 (10 g) was added to 30 g of Trypticase soy broth dissolved in 90 ml of distilled water, and the mixture was stirred at 22°C for 8 h before overnight dialysis at 4°C; (ii) a defined medium, T8S, similar in composition to T8A medium (4), to which was added Zn^{2+} (1 mM as $ZnSO_4 \cdot 4H_2O$), Fe^{2+} (0.05 μg/ml as $FeSO_4 \cdot 7H_2O$), succinate (21 mM), and 0.6 ml of a trace salts solution (R. DeBell, personal communication); and (iii) A Casamino Acids-yeast extract based medium, MTYG, previously utilized for *Pseudomonas* protease production (14). Culture supernatants were sterilized using membrane filtration (0.45 μm; Millipore Corp., Bedford, Mass.) before being tested for alkaline protease content by RIA. Total protein content of each supernatant was measured by the method of Bradford (2), modified by using a commercial reagent (Bio-Rad Protein Dye Reagent Concentrate, Bio-Rad Laboratories). Ovalbumin (Sigma Chemical Co., St. Louis, Mo.) was used as a protein reference standard. Purified alkaline protease and elastase were tested for proteolytic activity in a casein digestion assay as previously described (14). Radiolabeled alkaline protease was prepared in the following manner: 1 mCi of carrier-free $Na^{125}I$ (Amersham Corp., Arlington Heights, Ill.) was added to 50 μg of alkaline protease and 10 μg of lactoperoxidase in 1 ml of phosphate-buffered saline (pH 7.2). The iodination reaction was initiated by adding 25 μl of 0.03% hydrogen peroxide (H_2O_2). After a 5-min incubation, an additional 25 μl of 0.03% H_2O_2 was added, and the reaction was continued for 5 min. The labeled preparation was dialyzed for 18 h at 4°C against 1 liter of phosphate-buffered saline before chromatography on a Sephadex G-100 column. Labeled preparations routinely possessed a specific activity of greater than 2 μCi/μg of alkaline protease protein. Radioimmunoassay was carried out in polystyrene test tubes (10 by 75 mm) with a final volume of 0.5 ml. Reagents were added in the following sequence; 375 to 465 μl of reaction buffer [150 mM NaCl, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride, 5 mM ethylenediaminetetraacetic acid, pH 7.2, 1 mg of bovine serum albumin per ml, and 0.05% Nonidet P-40 (Particle Data Lab-

oratories, Elmhurst, Ill.)], 10,000 cpm of labeled alkaline protease in 10 μl, culture supernatant (10 to 100 μl per assay) or nonradioactive antigen (10 to 100 μl per assay), and 15 μl of a 1:100 dilution of anti-alkaline protease rabbit serum (sufficient to precipitate between 50 and 60% of the total counts in the absence of competing antigen). The reaction tubes were incubated for 10 min at 22°C, and then 50 μl of a 10% (vol/vol) preparation of IgG Sorb (Enzyme Center, Boston, Mass.) was added to each sample. Assays were incubated for an additional 5 min and centrifuged for 5 min at 3,000 × *g* (4°C). Pellets were washed twice with 1 ml of cold reaction buffer and counted in a Beckman Biogamma counter.

The standard curve for the alkaline protease RIA was linear when the concentration of homologous antigen was between 5 and 200 ng per assay (Fig. 1). Purified *P. aeruginosa* elastase (protease II) exhibited no cross-reactivity with alkaline protease. The addition of growth medium (100 μl per assay) resulted in no displacement of labeled antigen from precipitated immune complexes (data not shown). As little as 5 ng of alkaline protease per ml was detected by RIA assay. In contrast, we found the lower limit of detectability of alkaline protease in the casein digestion assay to be 10 μg (data not shown).

Table 1 illustrates the ability of various strains of *P. aeruginosa* to produce alkaline protease in Chelex-treated TSBD medium. All strains tested were found to produce alkaline protease antigen, although the amount varied from strain to strain by as much as sixfold (3.22 μg/ml for EZ-3 as compared to 0.54 μg/ml for WR5).

To determine whether media composition affected the production of alkaline protease, we compared the ability of three different media to support alkaline protease production. Table 1 shows the quantity of alkaline protease produced by five strains of *P. aeruginosa* grown in these different media. Alkaline protease production was found to be medium and strain dependent. Maximal production of alkaline protease by strains PA103 and EZ-3 was found to occur in Chelex-treated TSBD medium, whereas with strains WR5, PA01, and PAKS-1, growth in MTYG medium was optimal for alkaline protease production. The quantity of alkaline protease produced in different media by a given strain varied from a 2-fold difference (PA103) to greater than a 30-fold difference (PAKS-1). Although not all strains produced maximal yields of alkaline protease in MTYG medium, the ratio of alkaline protease to total extracellular protein in all strains tested was highest in MTYG medium. These findings extend those of previous studies which demonstrated that total protease production was dependent on media composition (8, 10).

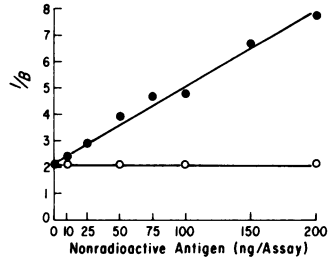

FIG. 1. *RIA for alkaline protease. 1/B, where B represents the percent of total radiolabeled antigen bound.* (●) *Purified alkaline protease;* (○) *purified elastase.*

TABLE 1. *Effect of growth medium on the production of alkaline protease by P. aeruginosa*

Strain	Medium	Protease concn (μg)	
		Per ml of culture supernatant[a]	Per mg of extracellular protein[a]
PA103	TSBD	0.77	0.7
	T8S	0.48	0.8
	MTYG	0.40	4.0
PA01	TSBD	2.27	3.1
	T8S	0.37	8.2
	MTYG	7.69	92.7
EZ-3	TSBD	3.22	3.4
	T8S	0.49	2.2
	MTYG	0.49	6.1
WR5	TSBD	0.54	0.3
	T8S	0.35	0.6
	MTYG	2.41	4.0
PAKS-1	TSBD	0.94	0.6
	T8S	0.52	0.8
	MTYG	16.25	3.2

[a] Represents average values from two independent experiments. Protease values were determined by RIA.

The sensitivity and specificity of the RIA described in this report should greatly facilitate studies on the role alkaline protease plays in the pathogenesis of *P. aeruginosa* infections, and the physiology and genetics of alkaline protease production.

We thank John Bradley for outstanding technical assistance.

This investigation was supported by Public Health Service grant AI-14671 from the National Institute of Allergy and Infectious Disease.

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