Nutritional Factors Controlling Exocellular Protease Production by *Pseudomonas aeruginosa*

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A defined medium capable of supporting growth and exocellular protease production by clinical isolates of *Pseudomonas aeruginosa* has been developed. Control of protease production is effected by a mixture of three amino acids and glucose.

Elaboration of exocellular proteases is a variable but common characteristic of *Pseudomonas aeruginosa* isolates. Although several different types of proteases have been described, the protease with elastase activity has been the subject of most intensive investigation. In a previous study we have characterized the major (elastase) and minor exocellular proteases from our isolates and designated them proteases 1 and 2, respectively (6). Protease 1 has been given different designations by several other groups using different isolates of *P. aeruginosa* (7, 9, 13, 16). This elastase protease has been implicated as a virulence factor in experimental *Pseudomonas* infections (5, 12, 15). This requires that *P. aeruginosa* produce proteases in vivo. Preliminary evidence supports this theory in that antibodies to *P. aeruginosa* proteases are detectable in sera of patients with cystic fibrosis (8). However, production of exocellular proteases in other species has been shown to be sensitive to catabolite repression by utilizable carbohydrates and to end product inhibition by amino acids (4). If this sensitivity exists in *P. aeruginosa*, it might preclude protease production in vivo. This study was undertaken to examine the effects of nutritional factors on protease production and to develop a chemically defined medium supporting protease production.

P. aeruginosa strains 34362, 34369, and 34358 were isolated from cystic fibrosis patients suffering pseudomal pneumonia (C.H. Pai, Montreal Children's Hospital, Montreal, Canada), and strain PAKS-1 was isolated from a human urine specimen (B. Wretlind, Karolinska Hospital, Stockholm, Sweden). *P. aeruginosa* strains A, 24′, pos, and 11-50 are nonclinical isolates from our stock culture collection. Strain 34362 was used for developmental work on defined medium. Three media were used in this study: (i) complex medium (trypticase soy broth [TCS], BBL Microbiology Systems), (ii) glucose-ammonia-salts medium of Norris and Campbell (14), and (iii) defined medium (Table 1). All liquid cultures were incubated on a gyratory shaker (300 rpm) at 30°C for 24 h, maintaining a medium-to-flask volume ratio of 1 to 5. The protease activity of culture supernatants was measured by using the azocasein assay previously described (6). One unit of protease activity is defined as the amount of enzyme needed to produce an increase in absorbance at 370 nm of 1.0 in 1.0 min under standard assay conditions.

A recently described defined medium which supports exotoxin A production by *P. aeruginosa* (3) supported only poor levels of protease production (1.1 U/ml) by strain 34362 (in absence of nitrilotriacetate). The defined medium described in Table 1 was therefore developed independently by stepwise modification of the glucose-ammonia-salts medium of Norris and Campbell (14) to achieve optimum protease production. NaCl and K_2HPO_4 concentrations are those found in Trypticase soy broth. All other components of the medium were examined individually as to type and concentration required to yield maximum protease production.

No protease was produced in the glucose-ammonia-salts medium until ammonia was replaced by an organic nitrogen source. Glutamate and glutamine, as sole nitrogen sources, supported the best protease production of all the amino acids. Valine and phenylalanine, unsuitable as sole nitrogen sources, nonetheless enhanced protease production when added as supplements to glutamate-containing medium. The ability of a combination of amino acids to support protease production indicates that protein is not required to induce the production of protease, nor is production repressed by the presence of free amino acids.

Glucose was the best carbon source tested. Growth and protease production both increased with increasing glucose concentration up to 0.07 M and then declined at higher concentrations. In the absence of glucose, both, protease production and growth were poor despite the presence of glutamate, valine, and phenylalanine as

TABLE 1. *Composition of chemically defined medium for protease production by P. aeruginosa*

Component[a]	Concn (mM)
1. NaCl	85.6
2. K₂HPO₄	14.4
3. Sodium glutamate (mono)	92
4. Valine	24
5. Phenylalanine	8
6. Glucose	70
7. MgSO₄	1.33
8. CaCl₂	0.14
9. FeSO₄	0.0039
10. ZnSO₄	0.0085

[a] Components 1 through 5 were pooled, the pH was adjusted to 7.3, and the mixture was autoclaved. Components 6 through 10 were prepared and sterilized separately and added aseptically before use.

potential carbon sources. This stimulatory effect of glucose on protease production in the defined medium indicates that protease production by strain 34362 is not subject to control by catabolite repression. This is in contrast to the glucose-mediated repression of elastase production by *P. aeruginosa* reported by Miller and Bielefeldt (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B95, p. 33) and indicates that glucose repression is likely affected by other medium components. No magnesium requirement for growth or protease production could be demonstrated, but magnesium was provided as MgSO₄ because cells grown in its absence were clumped and osmotically fragile. Similarly, CaCl₂, although not stimulatory to growth or protease production, was included in the medium since Morihara reported it to be essential for production of alkaline protease (analogous to our protease 2) (10). Both iron and zinc were found to be required for optimum protease production. Pseudomonal elastase is a zinc metalloenzyme containing 0.9 mol of zinc per mol of enzyme (11). A culture supernatant of strain 34362 containing 8 U of protease 1 per ml would contain 9.8 nmol of protease 1 per ml, given a specific activity of 24 U/mg and a molecular weight of 34,850 (6). This level of protease 1 production would require 8.8 μM zinc in the growth medium which corresponds well with our observed requirement of 8.5 μM ZnSO₄.

Eight strains of *P. aeruginosa* were compared for the protease production in complex and defined medium (Table 2). Defined medium generally supported good growth with the exception of strain A. Protease production was variable in defined medium. For the limited number of strains tested, defined medium supported high levels of protease production for strains of clini-

cal origin, whereas nonclinical strains produced more protease in complex medium.

Electrophoretic analyses of culture supernatants from strains grown in complex and defined media were carried out by concentrating culture supernatants fivefold by lyophilization, dialyzing against 0.01 M Tris-hydrochloride buffer (pH 7.5) and electrophoresing on duplicate 7.5% polyacrylamide slab gels (2). Protein bands were located by staining one gel with 1% amido black in 7% acetic acid. Protease activity was located on the second gel by a modification of the procedure of Arvidson and Wadstrom (1). The gel was overlayered with a solution of azocasein in molten agarose prepared by mixing equal volumes of 0.5% azocasein in 0.02 M Tris-hydrochloride buffer (pH 7.5)–0.002 M CaCl₂ with 2% molten agarose (both at 45°C). The mixture was poured over the surface of the gel, incubated 1 h at 37°C, and then immersed in cold 5% trichloroacetic acid. All strains except 24′ had a major protein band coincident with purified protease 1 when grown either in complex or defined medium (Fig. 1). In contrast, the protein band coincident with purified protease 2 was clearly visible in complex medium-grown cultures but very faint or absent in defined medium-grown cultures. Strong bands of protease activity were seen to coincide with the major protein band (protease 1) for all strains except 24′. Protease 2 activity was apparent in all strains including 24′ when grown in complex medium, but not in defined medium. Purified protease 2 showed a secondary band of both protein and protease activity running just ahead of the main band. This material represents autodigestion products not present in freshly purified protease 2, but which develop upon storage. The minor bands of protease activity seen on our gels have not

TABLE 2. *Comparison of growth and protease production by P. aeruginosa strains grown in complex and defined media*

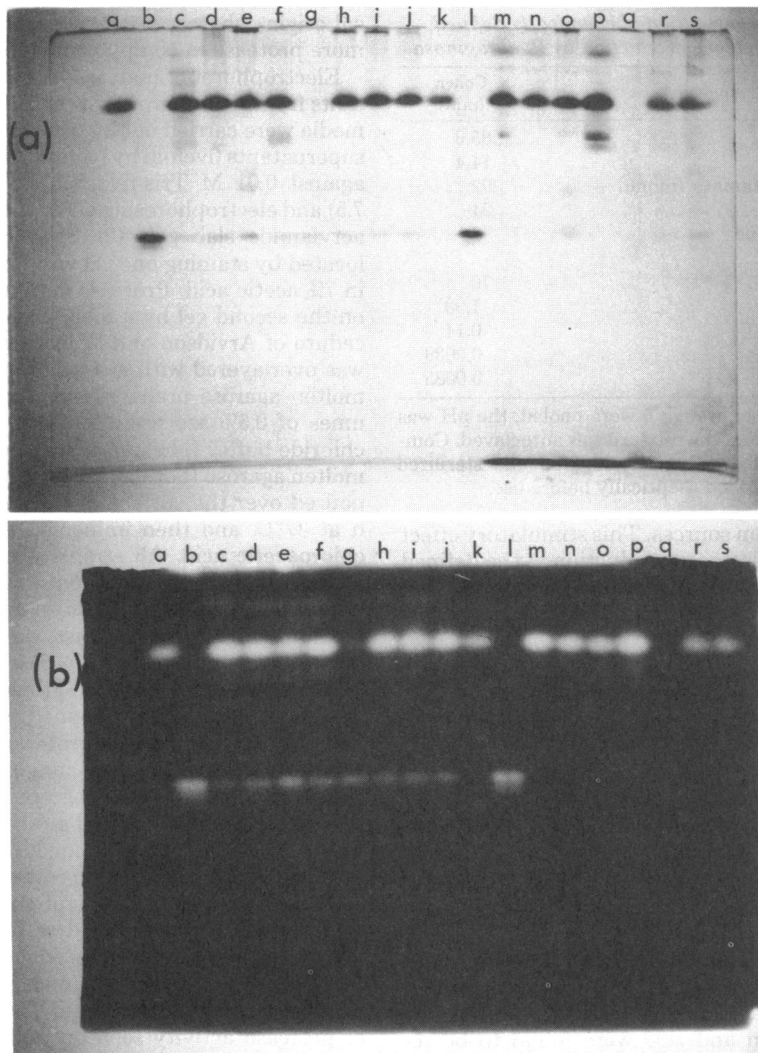| P. aeruginosa strain	Complex medium		Defined medium	
	Growth (OD₆₀₀)[a]	Protease (U/ml)	Growth (OD₆₀₀)[a]	Protease (U/ml)
Clinical isolates				
34362	8.3	6.4	11.7	6.7
34369	8.7	6.7	11.0	6.3
34358	7.4	6.0	14.6	6.7
PAKS-1	9.0	7.1	12.0	9.6
Nonclinical isolates				
24′	6.9	0.5	13.4	0.2
pos	7.8	6.0	12.9	2.5
11–50	7.8	6.1	13.8	3.5
A	8.8	6.6	0	0

[a] OD₆₀₀, Optical density at 600 nm.

FIG. 1. *Polyacrylamide gel electrophoresis of culture supernatants from eight strains of P. aeruginosa grown on complex and defined media. Culture supernatants were concentrated fivefold, and 12-µl volumes were applied to the sample wells of two identical slab gels. Amounts (6 µg) of purified proteases 1 and 2 were also applied. Each gel was then electrophoresed for 2.5 h at 15 mA. (a) One gel was stained for protein with amido black. (b) The other gel was treated to localize protease activity as described in the text. Wells a and k contained purified protease 1; wells b and l contained purified protease 2. Wells c to j contained complex medium culture supernatants of the following strains of P. aeruginosa: c, strain 34362; d, strain 34369; e, strain 34358; f, strain PAKS-1; g, strain 24'; n, strain pos; i, strain 11-50; and j, strain A. Wells m to s contained defined medium culture supernatants of the same strains as in wells c to i. Strain A would not grow in defined medium.*

previously been reported. Their detection here is presumably due to the sensitivity of this location technique.

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