Effect of Substrate on the Regulation of Exoprotease Production by
Pseudomonas aeruginosa ATCC 10145

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(Received 7 May 1982; revised 11 October 1982)

Exoprotease production by Pseudomonas aeruginosa ATCC 10145 was growth-associated when cultures were grown on complex substrates such as proteins but it occurred during the decelerating growth phase when the organism was grown on amino acids, mixtures of amino acids or simple carbon sources. NH₄Cl and simple carbon sources caused repression. Exoprotease was produced in chemostat cultures in response to growth under any of the nutrient limitations studied (carbon, nitrogen or phosphate). Furthermore, by growing at rates less than approximately 0.1 h⁻¹, the repression of enzyme production could be overcome to a large degree. At low growth rates there was an inverse relationship between growth rate and exoprotease production. Thus, exoprotease production was depressed by available energy sources and was increased in response to any nutrient limitation.

INTRODUCTION

Pseudomonas aeruginosa is involved in spoilage of meat (Lawrie, 1979) and milk (Juffs & Doelle, 1968) and is also of medical importance due to its invasion of burn wounds as an opportunist pathogen (Lowbury, 1975). The proteolytic action of the organism has been shown to be important in many of these effects.

The exoprotease produced by P. aeruginosa ATCC 10145 has been purified and characterized in this laboratory (O’Callaghan, 1981) and it is a Ca²⁺-containing enzyme of molecular weight 40 to 44 × 10³, with an optimum pH range for activity of 7 to 8.5 and a temperature optimum of 60 °C. The specific activity of the purified enzyme was 10⁻³ × 10³ units per mg protein. This single protease fraction is similar to that named ‘Fraction II’ by Morihara (1964). The enzyme was found to be an extracellular enzyme and not a periplasmic or intracellular enzyme released by lysis.

In the present study the regulation of production of this exoprotease was studied and was found not to comply with the definitions of inducible, catabolite repressible or end-product repressible enzymes as outlined by Demain (1971). Instead, production of this enzyme appeared to be controlled by a metabolic type of repression which seemed to be mediated by the energy status of the cells.

METHODS

Media. Pseudomonas aeruginosa ATCC 10145 was maintained on nutrient agar slopes at 4 °C and stock cultures were transferred at monthly intervals. The medium consisted of a basal salts solution containing (g l⁻¹): NaCl, 0.1; KCl, 0.2; K₂HPO₄, 0.2; CaCl₂, 0.5; MgSO₄.7H₂O, 0.5; Tris, 6.5. A trace element solution (5 ml per l) of the following composition (g l⁻¹) was also added: FeSO₄(NH₄)₂SO₄, 0.16; H₂BO₃, 0.232; CoCl₂.6H₂O, 0.410; CuSO₄.5H₂O, 0.008; MnSO₄.4H₂O, 0.008; (NH₄)₆MoO₃.24H₂O, 0.022; ZnSO₄.7H₂O, 0.174. Carbon and nitrogen sources were added in the form of (a) protein (casein or BSA), (b) Casamino acids as the sole source of carbon and nitrogen, or (c) either glucose, glycerol or citrate as the carbon source and NH₄Cl as the nitrogen source. The pH of the medium was adjusted to 7.2 with 0.2 M-HCl before autoclaving.

Preparation of inoculum. The organism was subcultured from a nutrient agar stock culture on to Pseudomonas F
Agar (Difco) and incubated for 24 h at 27 °C and then subcultured into 100 ml of the basal salts medium, containing the appropriate carbon and nitrogen sources, in 250 ml conical flasks and incubated on a reciprocal shaker at 27 °C and 150 r.p.m. until the culture reached the mid-exponential phase of growth. Cells were then harvested by centrifuging for 10 min at 14000 g at 4 °C and resuspended in 0.9% (w/v) NaCl to approximately 1.0 mg dry wt ml⁻¹. One ml of this suspension served as an inoculum for 100 ml medium.

Growth conditions. For studies on biomass and exoprotease production on the Casamino acids and protein media, 250 ml flasks (with sidearms) containing 100 ml medium, were used. The flasks were incubated on a reciprocal shaker at 27 °C and 150 r.p.m. Studies using media containing glucose, glycerol or sodium citrate as carbon sources necessitated pH control because growth resulted in a rapid drop in pH. These incubations were carried out in 1 l glass fermentation vessels containing 500 ml medium. pH was controlled at 7.5 ± 0.2 by the automatic addition of 0.5 M-NaOH. Mixing was achieved by means of a magnetic stirrer assisted by baffles on the vessel walls. Aeration rate was maintained at 1 l min⁻¹ and the temperature of the culture vessel was maintained at 27 °C. Foaming was prevented by adding 1 ml of a silicone-based antifoam solution (Antifoam Emulsion A, Sigma) as required.

Continuous culture. The system consisted of a glass vessel to which medium was added aseptically by means of a Watson Marlow MHRE flow-inducer fitted with an interval timer. The effluent was continuously discharged through an overflow tube designed to allow the retention of 470 ml culture fluid in the fermenter. pH was controlled at a preset value by the automatic addition of 0.5 M-HCl or 0.5 M-NaOH. If required, a few drops of antifoam were added to the culture. Aeration rate was maintained at 1 l min⁻¹. The temperature of the culture vessel was maintained at 27 °C. Mixing was achieved by means of a magnetic stirrer assisted by baffles on the vessel walls. Steady state conditions were established when biomass and exoprotease levels were constant in three consecutive determinations at the same dilution rate, each taken after three volume changes. To minimize the chance of selecting mutants adapted for a particular dilution rate, fresh chemostat runs were started from stock cultures.

Determination of bacterial mass. Growth was monitored turbidimetrically at 650 nm during all the experiments and converted into dry weights using a standard curve.

Exoprotease assay. Exoprotease activity was determined by a modified Lowry method. The substrate used was a 1% (w/v) buffered casein solution at pH 7.5. Freshly mixed 0.1 M-Na₂HPO₄/NaH₂PO₄ was added to maintain this pH. To 1 ml casein solution 1 ml of an appropriate dilution of culture cell-free supernatant was added. The tubes were incubated at 30 °C for 20 min. Three ml of 5% (w/v) TCA was then added and the tubes were left to stand for 30 min. The precipitated protein was separated by filtering through Whatman no. 1 filter paper. To 1 ml filtrate, 3 ml 7.5% (w/v) Na₂CO₃ was added, and after 10 min, 1 ml of a 1:3 dilution of Folin–Ciocalteau reagent was added to each tube. The tubes were allowed to stand for 30 min at 30 °C and then the absorbances were measured at 660 nm. Units of enzyme activity (U) were calculated from a standard curve and are expressed as μg tryosine released from casein per hour at 30 °C.

Chemicals. BSA was obtained from Sigma. Casein (vitamin-free) was obtained from Difco. All other chemicals were BDH AnalaR grade.

RESULTS

Initial studies concentrated on the production of exoprotease in media where protein (casein or BSA at 0.5%, w/v) was the sole source of carbon and nitrogen. After a lag period of approximately 10 h, the organism grew exponentially for approximately another 20 h in these media before entering the decelerating growth phase (Fig. 1). Exoprotease production commenced during the early-exponential growth phase and continued throughout the growth cycle.

Casamino acids (a mixture of amino acids from the breakdown of casein) were also examined as substrates for exoprotease production. It was found that exoprotease production was repressed during the exponential phase on Casamino acids media and production only commenced in the decelerating phase. Thus the availability of substrate appeared to exert a repressive effect. To examine further the nature of this repression, the effects of various other substrates were examined in defined media.

Carbon sources

The carbon sources examined were citrate (1%, w/v), glucose (1%, w/v) and glycerol (2%, w/v) in the basal salts solution with NH₄Cl (0.1%, w/v) as the nitrogen source. Cultures were incubated at 27 °C for 40 h and the pH was maintained at 7.5. The efficiency of exoprotease production per unit biomass on the different media followed the pattern: glycerol > glucose >
Exoprotease production by *P. aeruginosa*

Fig. 1. Biomass and exoprotease production by *P. aeruginosa* growing in protein media (0.5%, w/v) at 27°C. Biomass on casein (○), exoprotease on casein (●), biomass on BSA (□), exoprotease on BSA (■).

Fig. 2. Biomass and exoprotease production by *P. aeruginosa* growing in simple carbon/nitrogen media at 27°C. Biomass on glucose (○), biomass on glycerol (●), exoprotease per unit biomass on glucose (□), exoprotease per unit biomass on glycerol (■).

citrate. Further examination of the pattern of exoprotease on the glucose and glycerol media revealed that higher biomasses and low exoprotease activities were produced on the glucose medium compared with the glycerol medium (Fig. 2).

It was apparent that a correlation existed between growth rate and substrate complexity, with highest growth rates on the simplest substrates. This fact was reflected in the stage of growth at which exoprotease was produced. In general, it appeared that on simple substrates production began in the decelerating phase of growth (Fig. 2), whereas on complex media production began in the early-exponential phase of growth and continued throughout growth (Fig. 1).

Supplementation of a protein medium with various readily utilizable carbon sources resulted in increased growth rates and biomass production and in decreasing exoprotease production. Again, it was apparent that simpler substrates such as glucose and citrate had a stronger effect than more complex substrates such as glycerol and Casamino acids. Furthermore, the effect was more pronounced with increasing concentration of the supplemented carbon source (data not shown).
Table 1. *Effect of carbon sources on repression of exoprotease production by* *P. aeruginosa*

Carbon sources (0.5%, w/v) were added to 1% Casamino acids cultures producing exoprotease (i.e. cultures entering the decelerating growth phase) and biomass and exoprotease activity determined 4 h later. Values shown are means of triplicate analyses on each of three batches of culture ± standard deviation.

<table>
<thead>
<tr>
<th>Carbon source added</th>
<th>Biomass (g dry wt l⁻¹)</th>
<th>Exoprotease activity (U ml⁻¹)</th>
<th>Exoprotease units per unit biomass [U (mg dry wt⁻¹)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.44 ± 0.03</td>
<td>930 ± 87</td>
<td>646</td>
</tr>
<tr>
<td>Citrate</td>
<td>1.58 ± 0.02</td>
<td>323 ± 71</td>
<td>204</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.57 ± 0.06</td>
<td>549 ± 44</td>
<td>350</td>
</tr>
<tr>
<td>Glycerol</td>
<td>1.58 ± 0.02</td>
<td>774 ± 150</td>
<td>490</td>
</tr>
<tr>
<td>Casamino acids</td>
<td>1.70 ± 0.02</td>
<td>885 ± 11</td>
<td>521</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of supplementation of a BSA medium (0.5%, w/v) of a Casamino acids medium (CAA, 0.5%, w/v) with NH₄Cl on exoprotease and biomass production by *P. aeruginosa* growing at 27 °C; 70 h after inoculation in the case of BSA medium and 12 h after inoculation in the case of Casamino acids medium. □, Biomass; ■, exoprotease.

Various simple and complex carbon substrates when added to cultures of *P. aeruginosa* already producing exoprotease, caused an increase in biomass production and repressed exoprotease production, with the greatest repression attributable to the simplest substrates (Table 1).

*Nitrogen sources*

Supplementation of complex media with simple inorganic nitrogen sources was also examined. NH₄Cl inhibited exoprotease production on BSA medium and this inhibition was concentration-dependent. However, NH₄Cl had a similar inhibitory effect on biomass production which was expected as exoprotease production is essential for utilization of the protein and, consequently, growth (Fig. 3). To examine further the effect of simple nitrogen supplementation on exoprotease production, a complex medium, where exoprotease was not required for growth, was examined. Supplementation of a 0.5% Casamino acids medium with NH₄Cl resulted in a decrease in exoprotease production with increasing concentrations, but biomass levels were unaffected (Fig. 3).
Table 2. Effect of different nutrient limitations on biomass and exoprotease production by *P. aeruginosa* in continuous culture at a growth rate of 0.1 h⁻¹

Values shown are means of triplicate analyses on each of three runs ± standard deviation.

<table>
<thead>
<tr>
<th>Limitation</th>
<th>Biomass (g dry wt l⁻¹)</th>
<th>Exoprotease units (U ml⁻¹)</th>
<th>Exoprotease units per unit biomass [U (mg dry wt)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate</td>
<td>0.7 ± 0.01</td>
<td>60 ± 18</td>
<td>85.5</td>
</tr>
<tr>
<td>Carbon (glucose)</td>
<td>1.22 ± 0.04</td>
<td>110 ± 30</td>
<td>90</td>
</tr>
<tr>
<td>Nitrogen (NH₄Cl)</td>
<td>1.01 ± 0.05</td>
<td>192 ± 51</td>
<td>189</td>
</tr>
<tr>
<td>Nitrogen (Casamino acids)</td>
<td>1.62 ± 0.02</td>
<td>558 ± 84</td>
<td>345</td>
</tr>
</tbody>
</table>

Fig. 4. Effect of growth rate on biomass (○) and exoprotease production (●) by *P. aeruginosa* under conditions of glucose limitation.

NH₄Cl was also shown to repress exoprotease production when added to a Casamino acids culture producing exoprotease and this repression was also concentration-dependent. The effects of simple carbon sources and ammonia on exoprotease production suggest that there are two different patterns of repression controlling production.

**Growth limitation**

The effects of carbon, nitrogen and phosphate limitation on biomass production were studied in continuous culture. Under all of these limitations, exoprotease was produced (Table 2). However, the efficiency of exoprotease per unit biomass depended on the limitation, with greatest exoprotease activities produced under conditions of nitrogen limitation.

**Effect of growth rate**

Under all of the limitation conditions studied, it was found that as the growth rate decreased, exoprotease production increased, whereas at high growth rates, no exoprotease production occurred. The general trend found under all limitation conditions is shown in Fig. 4 where carbon limitation was studied. It was also found that biomass decreased at very low dilution rates. Decreasing the growth rate of a culture by limiting aeration also resulted in increased exoprotease production as was evident from comparing growth and exoprotease production on
aerated and aeration-limited cultures of 0.5% Casamino acids/salts medium (Fig. 5). Final biomass levels were also lower on the aeration-limited cultures.

DISCUSSION

Pseudomonas aeruginosa when incubated in a medium with protein as the sole source of carbon and nitrogen, produced exoprotease throughout the growth cycle (Fig. 1). However, exoprotease was also found to be produced in non-protein media, either with Casamino acids as the sole source of carbon and nitrogen or with glycerol, glucose or citrate as the carbon source and NH4Cl as the nitrogen source. This suggests that protein is not essential to induce exoprotease and that the enzyme is not wholly inducible, but is partially constitutive. Indeed, the ability of free amino acids to support exoprotease production indicates that the enzyme is not wholly susceptible to specific end-product repression. Similar phenomena were observed for exoprotease production by clinical isolates of this organism (Jensen et al., 1980).

A general trend emerged from studies with other carbon sources, indicating that the simpler the substrate (i.e. the more readily metabolizable) the less efficient it was at supporting exoprotease production. Similar trends have been observed for exoprotease production by Micrococcus (McDonald & Chambers, 1966), Aeromonas proteolytica (Litchfield & Prescott, 1970a, b) and Pseudomonas lachrymans (Keen & Williams, 1967); and for a-amylase production by Bacillus subtilis (Sekiguchi & Okada, 1972).

Supplementation of protein media with simple carbon substrates also resulted in reduced activities of exoprotease and addition of these substrates to cultures already producing exoprotease resulted in repression (Table 1). Again, it was found that in general the simpler the substrate added, the greater the repression observed.

Exoprotease production on substrates other than protein only commenced in the decelerating growth phase and proceeded into the stationary phase (Fig. 2, Fig. 5). This pattern of production...
Exoprotease production by P. aeruginosa

is a common feature of many, though not all, bacteria synthesizing extracellular enzymes (Glenn, 1976).

It was also found that the simpler the carbon substrate, the higher the growth rate and the smaller the amount of exoprotease produced. This effect can be explained in terms of availability of substrate. The more complex the substrate, the less readily metabolizable it is; therefore, the growth rate of the culture is limited by the ease with which the cell can utilize the substrate as an energy source. Continuous culture studies also confirmed this inverse relationship between growth rate and exoprotease production (Fig. 4).

This repression of enzyme synthesis by readily metabolizable carbon substrates is referred to as catabolite repression (Paigen & Williams, 1970). Indeed, it may be suggested that the derepression of extracellular enzyme synthesis in the decelerating phase in batch cultures (and at low growth rates in continuous culture) when a growth substrate becomes exhausted, is caused by a decrease in catabolite repression by the substrate. This has been postulated by several authors (Fayyaz Ud Din & Chaloupka, 1970; Stinson & Merrick, 1974; Saito & Yamomota, 1975).

A similar type of catabolite repression to that found in this organism has been found to be involved in the regulation of extracellular protease synthesis in other organisms. For example, various organic acids have been shown to repress exoprotease synthesis in Pseudomonas maltophilia (Boethling, 1975) and several carbon sources, including amino acids were found to repress exoprotease synthesis in Vibrio parahaemolyticus (Tanaka & Iuchi, 1971) and Vibrio SA1 (Wiersma et al., 1978).

That exoprotease production was regulated by repression rather than induction was evident from the following facts: (a) no specific inducer was required for production to occur, which was confirmed by the ability of the organism to produce exoprotease on a wide variety of substrates; (b) the pattern of exprotease production in continuous culture (Fig. 4) was the same as that for enzymes whose production was regulated by repression (Wiersma & Harder, 1978).

The type of repression observed was not classical catabolite repression since the imposition of some limitation (phosphate, nitrogen or oxygen) even in the presence of excess carbon, permitted exoprotease production; nor was it end-product repression, as evidenced by the ability of the organism to produce exoprotease while growing on the end-products of its action, a mixture of amino acids.

The ability of non-carbon substrates, for example, inorganic nitrogen, to repress exoprotease production without affecting growth rate could be attributable to a different type of control mechanism (Liu & Hsieh, 1969). We believe that the type of repression exerted here was some kind of metabolic repression dependent on the energy status of the cell since availability of substrate can be equated directly with availability of energy. Wiersma & Harder (1978) came to a similar conclusion concerning regulation of exoprotease production by Vibrio SA1.

From the above results, it is apparent that exoprotease production on a protein medium would follow the pattern: production commences after a lag period, triggered by nutrient limitation, and subsequent production is kept in balance by the energy status of the cells. For example, if too much exoprotease is produced then the level of free amino acids in the medium increases, and the presence of this more readily available substrate results in an increased growth rate and a subsequent decrease in enzyme production. This decrease in enzyme production eventually results in a decrease in the level of available substrate (amino acids) and a decrease in growth rate. This, in turn, results in derepression of exoprotease. Therefore, a balance exists between the level of substrate, growth rate and the amount of enzyme produced when growth is dependent on protein substrates.

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