Age-dependent changes in protease and elastase production in cultures of *Pseudomonas aeruginosa*

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Summary. The intracellular and extracellular protease and elastase contents of *Pseudomonas aeruginosa* were studied in relation to the age of the culture. The intracellular protease and elastase content of the organisms decreased as the culture grew older, whereas extracellular protease and elastase greatly increased 24 h after incubation commenced, suggesting that host tissue injury by *P. aeruginosa* infection may increase in proportion to the duration of tissue colonisation.

Introduction

The extracellular proteases of *Pseudomonas aeruginosa* (alkaline protease and elastase) are considered to be important virulence factors in several human infections caused by this opportunist pathogen (Sabath, 1980). Woods *et al.* (1981) noted an important role of protease activity in the adherence of *P. aeruginosa* to mammalian buccal epithelial cells. Furthermore, *P. aeruginosa* elastase is of interest on account of its ability to produce corneal ulcers (Kreger and Grey, 1978), necrotic skin lesions (Meinke *et al.*, 1970) and pulmonary haemorrhages (Kreger and Gray, 1978) and to inactivate the human plasma α_1 -proteinase inhibitor (Moriyama *et al.*, 1979).

In the present study, age-dependent changes in the intracellular and extracellular excretion of protease and elastase by cultures of *P. aeruginosa* were investigated.

Materials and methods

Pseudomonas cultures

P. aeruginosa strain TN-1 was isolated from a bronchiectatic patient suffering from pseudomonal pneumonia (Kawasaki Medical School Hospital, Kurashiki, Japan). It was lyophilised and stored at -70°C until use. Before each experiment lyophilised bacteria were inoculated into 25 ml of Mueller-Hinton broth—(L) beef extract 19.75 g, casamino acids 1.15 g, bacto-soluble starch 0.10 g—and incubated for 18 h at 37°C . *P. aeruginosa* TN-1, initially adjusted to $(1-2) \times 10^5$ cfu/ml, was grown in 25 ml of Mueller-Hinton broth at 37°C with

continuous shaking (60 cycles/min) in a temperature gradient bio-photorecorder Model TN-112D (Toyo Kagaku Sangyo Co. Ltd, Tokyo). Organisms were harvested at the early exponential (1.1×10^6 cfu/ml, after incubation for 2 h), mid-exponential (1.1×10^7 cfu/ml after 4 h) and late exponential (8.6×10^8 cfu/ml after 8 h) phases, and at the early stationary (1.0×10^{10} cfu/ml after 18 h) and late stationary (1.2×10^{10} cfu/ml after 24 h) phases. At the specified time, a sample of the culture was centrifuged at 4000 *g* for 30 min and the protease and elastase contents of the supernate were measured. The pellet was washed twice with sodium phosphate-buffered saline (PBS, pH 7.4), resuspended in PBS and disintegrated in 2-ml portions by ultrasonic treatment for 1 min with the microtip of a Branson sonifier (Model W-200P) set at no. 2. The pellet solution was used for the measurement of intracellular protease and elastase.

Protein concentration was determined according to the method of Lowry *et al.* (1951) with bovine serum albumin as the standard.

Protease assay

Generally, supernate and pellet protease contents were assayed by a hide powder azure dye-release method (Rinderknecht *et al.*, 1968); 0.1 ml of the culture supernates (or pellets) and 4.9 ml of 0.01 M Tris buffer (pH 8.0) were incubated with 20 mg of remazol brilliant blue hide powder for 45 min at 37°C in a shaking waterbath. The reactions were terminated by cooling with iced water and the reaction mixture was centrifuged for 5 min at 2000 *g*. In the resultant supernates, the absorbance of dye released by protease was measured at 595 nm.

Elastase assay

Elastase content was assayed in the same manner except that 0.25 ml of each culture supernate (or pellet

solution) was incubated with 20 mg of orcein-elastin in 2.5 ml of Tris buffer (pH 8.0) with shaking for 90 min. The reactions were stopped by cooling with iced water and the reaction mixtures were centrifuged for 5 min at 2000 *g*. In the resultant supernates, released orcein was measured as absorbance at 590 nm (Johnson *et al.*, 1967).

The following chemicals were obtained from commercial sources: Trizma base, bovine serum albumin, trypsin, and elastase (Sigma Chemical Co., St Louis, MO.); Mueller-Hinton broth (Difco Laboratories, Detroit, MI.); remazol brilliant blue hide powder/azure, elastin-orcein (Nakarai Chemicals Ltd., Kyoto, Japan). Other chemicals were of the highest purity available from commercial sources.

Results

Fig. 1 shows changes in the supernate and pellet protein contents with the age of the culture. Although there were no significant changes in the supernate protein content with age, the pellet protein content/ 10^6 cfu gradually decreased. Fig. 2 shows age-dependent changes in the supernate and pellet protease contents. Whereas the supernate protease content greatly increased with the age of the culture, by the stationary phase, the pellet protease content had decreased to 20% of its content in the early exponential phase. In addition, the supernate elastase content greatly increased at the stationary phase (fig. 3). In the stationary phase, the pellet elastase content decreased to 65% of its content at the mid-exponential phase. Pellet elastase decreased less than pellet protease with the age of the culture (figs. 2 and 3).

Discussion

As reported previously (Kanungo, 1980; Umeki *et al.*, 1983), older cultures are generally characterised by a reduction in physiological activity and a reduced ability to respond to environmental changes. The availability of nutritional factors modifies the physiological state of many micro-organisms (Umeki and Wozawa, 1983). Banno *et al.* (1982) reported that *Tetrahymena*, a ciliate protozoan, secreted large amounts of intracellular protease into its culture medium as the culture aged. The extracellular proteases of *P. aeruginosa*, an opportunist pathogen which causes fatal infections in the immunocompromised host (Feigin and Shearer, 1975), have been purified by various investigators Morihara and Tsuzuki, 1977; Wretling and Wadström, 1977). However, little has been reported about changes in the contents of intracellular and extracellular proteases in relation to the age of the culture. This micro-organism secretes several extracellular enzymes that contribute to its pathogenesis, including at least two proteases, elastase and alkaline protease. In the present study, changes in the intracellular contents of *P. aeruginosa* protease and elastase and their secretion into the culture medium in relation to the age of the culture were investigated.

Our results showed age-related increases in extracellular and decreases in intracellular protease and elastase content. *P. aeruginosa* rarely causes infection in immunocompetent hosts, even though the micro-organism may be present in the gastrointestinal and upper respiratory tracts or on the skin. *P. aeruginosa* lung infections, however, fre-

Fig. 1. Age of culture and age-dependent changes in supernate (s) and pellet (p) protein concentrations of *P. aeruginosa*. Each point is the mean (bar = SD) of 3 or 4 separate experiments. ●---●, organism density; ●—●, s protein; ○—○, p protein.

Fig. 2. Changes in contents of supernate (s) and pellet (p) intracellular proteases of *P. aeruginosa* in relation to the age of the culture. Each point is the mean (bar = SD) of 3 or 4 separate experiments, ●----●, organism density; ●—●, s protease content; ○—○, p protease content.

Fig. 3. Changes in contents of supernate (s) and pellet (p) intracellular elastases of *P. aeruginosa* in relation to the age of the culture. Each point is the mean (bar = SD) of 3 or 4 separate experiments. ●----●, organism density; ●—●, s elastase content; ○—○, p elastase content.

quently occur in patients with specific underlying diseases (Aduan and Reynolds, 1979). Once *P. aeruginosa* invades the lower respiratory tract, it multiplies at a rate influenced by the particular conditions of the pulmonary micro-environment, which include underlying diseases such as cystic fibrosis and bronchiectasis. *P. aeruginosa* is generally considered to adhere more frequently to the mucosal epithelial cells of hosts which have been colonised already than to those of non-colonised hosts (Christensen *et al.*, 1985; Umeki, 1987). Long-term colonisation with *P. aeruginosa* runs parallel to the severity of the underlying disease and the

difficulty of eradication of this micro-organism with antimicrobials. The present results suggest that a large volume of protease and elastase secreted extracellularly by *P. aeruginosa* during long-term colonisation may produce great damage to its hosts. The protease content in sputum from 14 patients with bronchiectasis and *P. aeruginosa* lung infection (Umeki, 1987) was also determined. The mean content of sputum protease was 85.7 mg/mg of protein, which is much greater (about 300-fold) than the in-vitro extracellular protease in this study.

Specific colonisation mechanisms can be advantageous to the participants in a host- (or tissue-)

pathogen relationship. Colonisation by a particular bacterial species may vary considerably according to the host species and tissue (Christensen *et al.*, 1985) These conditions are known as host tropism and tissue tropism respectively. Extracellular pro-

tease and elastase may play an important role in establishing these host and tissue tropisms. Partial purification and characterisation of these proteases are currently under investigation in our division.

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