Insect Pathogenic Properties of *Serratia marcescens*. Passive and Active Resistance to Insect Immunity Studied with Protease-deficient and Phage-resistant Mutants

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Many insects have a cell-free immune system in which small basic proteins called cecropins are the main defence against Gram-negative bacteria. We have earlier shown that an insect pathogenic strain of *Serratia marcescens* was resistant to insect immunity and that certain mutants resistant to phage ϕ J become sensitive to cecropins. We have found that protease-deficient mutants with and without resistance to ϕ J appear to be deficient in the mechanism of protease induction. Three different protease fractions exist and for two of the enzymes we describe a partial purification and characterization. The proteases show pronounced autodegradation which increases with the purity. Both enzymes are only partly affected by EDTA and they are highly toxic to *Drosophila melanogaster*. All three enzymes destroy cecropins in immune haemolymph from *Cecropia* pupae. However, *in vivo* experiments with different mutants indicate that in *Serratia*, passive resistance to insect immunity is more important for virulence in *Drosophila* than the production of proteases which can destroy cecropins.

INTRODUCTION

Serratia marcescens is a well known insect pathogen which can produce several hydrolytic enzymes (e.g. proteases and a chitinase), some of which have been shown to be toxins (Grimont & Grimont, 1978; Poinar *et al.*, 1979; Ourth & Smalley, 1980; Blazevic, 1980). *Serratia* strains isolated from insects often produce more proteolytic enzymes than do wild isolates from other sources (Miyata *et al.*, 1970; Decedue *et al.*, 1979). A partly purified, extracellular protease from *S. marcescens* was shown to be toxic to an insect (Kaška, 1976). However, besides toxin production, passive resistance to insect immunity is an important virulence factor of *S. marcescens* (Flyg *et al.*, 1980). Spontaneous mutants with decreased passive resistance can be isolated by selecting for resistance to phage ϕ J.

Using mutagens and phage ϕ J, we have now isolated a set of mutants with low levels of proteolytic enzyme activity with and without passive resistance to insect immunity. These strains were compared for their resistance to immune haemolymph from pupae of *Hyalophora cecropia* and also for their virulence to adult *Drosophila melanogaster*. A partial purification of two different proteolytic enzymes is reported, but autodigestion prevented homogeneous preparation.

METHODS

Parental strain, phage and growth media. All mutants described in this paper are descendants of strain Db11, a spontaneous streptomycin-resistant mutant of *Serratia marcescens* Db10 isolated from a culture of diseased *Drosophila melanogaster* (Flyg *et al.*, 1980). Strain Db11 is sensitive to bacteriophage ϕ J. The growth medium was normally the LB medium of Bertani (1951). LA agar plates (LB medium with 1.5% agar) were also used. For

Abbreviations: IH, Immune haemolymph; PMSF, phenylmethylsulphonyl fluoride; RAC, remaining active concentrate.

Table 1. *Strains of Serratia marcescens used and their properties*

Strain	Parental strain	Doubling time* (min)	Response to:†			Protease production		Comments	References
			Str	ϕ J	IH	Prot. 1	Prot. 2		
Db10	—	37	S	S	R	+	+	Isolated from diseased <i>Drosophila</i>	Flyg <i>et al.</i> (1980)
Db11	Db10	45	R	S	R	+	+		Flyg <i>et al.</i> (1980)
Db1109	Db11	50	R	R	S	+	+	Spontaneous ϕ J ^R mutant	Flyg <i>et al.</i> (1980)
Db1120	Db11	44	R	S	R	—	+	NG-treated	This paper
Db1121	Db1120	49	R	S	R	—	+	EMS-treated	This paper
Db1140	Db1121	51	R	R	HS	—	+	Spontaneous ϕ J ^R mutant	This paper

* Doubling time was recorded for liquid cultures grown in LB medium in a rotatory shaker at 37 °C.

† Sensitivity (S), high sensitivity (HS) or resistance (R) to streptomycin (Str), phage ϕ J and immune haemolymph from *Cecropia* (IH).

protease induction the C-A medium of Castañeda-Agulló (1956) was used, as modified by Bromke & Hämmel (1979) with gelatin as the protease-inducing protein. This medium was here supplemented with 10% sterile-filtered normal *Cecropia* haemolymph to resemble conditions in *in vivo* experiments. The growth rates for strains Db11, Db1109, Db1120 and Db1121 were approximately the same in this C-A haemolymph medium. All strains were stored frozen at -70 °C in LB medium containing 25% (v/v) glycerol.

Isolation of mutants. Washed cells of Db11 (about 3×10^8 ml⁻¹) were incubated at 37 °C for 30 min in 0.1 M-citrate buffer, pH 5.5, containing *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (250 µg ml⁻¹; Aldrich Chemical Co., Milwaukee, Wis., U.S.A.). After washing twice with 0.1 M-K₂HPO₄/KH₂PO₄ buffer pH 7.0, 0.1 ml bacterial suspension was added to 5 ml LB medium and allowed to grow overnight at 37 °C. Appropriate dilutions were spread on skim-milk plates which contained 10% (w/v) skim-milk powder and 2.8% (w/v) nutrient agar (Oxoid). After 15–40 h at 37 °C, colonies were selected which had no detectable, or only small, clearing zones. One such clone was, after purification, designated Db1120. To decrease the proteolytic activity further, Db1120 was subjected to a second mutagen treatment.

An overnight culture of Db1120 (about 2×10^9 ml⁻¹) was used as inoculum for approximately 100 min of growth in fresh LB medium, with 1% (w/w) ethylmethane sulphonate at 37 °C. After about two doublings, ethylmethane sulphonate was removed by four washings. The cells were then incubated for 120 min in 30 ml LB medium, spread on skim-milk plates and again screened for colonies without clearing zones. One mutant clone, Db1121, was retained for further studies. A spontaneous mutant resistant to ϕ J was isolated from Db1121. The procedure used for the isolation of this strain, Db1140, was the same as for Db1109 (Flyg *et al.*, 1980). Strains Db1109 and Db1140 are as resistant to Penicillin G as the parental strain Db11. A summary of all the *Serratia* strains used is given in Table 1.

Insects, immune haemolymph and toxicity tests. Immune haemolymph (IH), containing cecropins and other immune proteins, was obtained from diapausing pupae of *Hyalophora cecropia* immunized with live *Enterobacter cloacae* (Boman & Steiner, 1981). The assay for the antibacterial activity in the haemolymph followed the procedure of Boman *et al.* (1974) except that the haemolymph concentration was raised to 90%.

Drosophila melanogaster strain KAS 60 (wild-type), was grown and maintained on an autoclaved corn-yeast agar substrate at 25 °C, 65% relative humidity and 15.5 h light/8.5 h dark conditions. In all experiments male flies less than 5 d old were used. For immunization, the flies were injected with living *Enterobacter cloacae* attenuated by freeze-thawing. The concentration was 10^9 cells ml⁻¹ in 0.9% NaCl, according to Boman *et al.* (1972).

Injection experiments (i.e. toxicity tests including LD₅₀-determinations) were performed according to the methods of Flyg *et al.* (1980). The Ringer solution for injections of flies contained 25 mg KCl, 30 mg CaCl₂, 650 mg NaCl and 20 mg NaHCO₃ in 100 ml water. The solution was sterile-filtered after adjusting the pH to 6.3 by passage of CO₂.

Preparative growth and harvest of cultures. Cultures of 2.5 l each, were well aerated by using flasks of 4 l volume on a rotatory shaker (90 r.p.m., amplitude 2 cm). After 4 d growth at 37 °C, the cultures were centrifuged at 15000 g for 30 min. This step and all the following parts of the purification procedure were performed at 0–4 °C. The supernatants with the proteases were filtered through a pack of filters on vacuum. The pack consisted of (from the top) Whatman GF/D 2.2 µm, GF/A 1.6 µm, GF/B 1.0 µm (Whatman), Gelman 0.2 µm (Gelman Instrument Co., Mich., U.S.A.). This was necessary because 4 d old *Serratia* cultures are turbid even after centrifugation, presumably due to remaining cell wall fragments. This may also explain why filters sometimes become clogged. As

purity controls, cultures were streaked on LA plates with streptomycin ($100 \mu\text{g ml}^{-1}$) and tested for sensitivity to phage ϕJ (Flyg *et al.*, 1980).

Concentration, gel filtration and ion exchange chromatography. Filtrates were concentrated with an Amicon concentrator and Diaflo hollow fibres (Amicon Corp. Sci. Div. System, Mass., U.S.A.). When volumes had decreased to less than 200 ml, an Amicon cell model 202 with filter PM 10 (permeable to molecular sizes of less than 10000 Dal) was used until the final volume was 12 ml. These concentrated samples were analysed on a 320 ml gel filtration column ($65 \text{ cm} \times 2.5 \text{ cm}$) with Sephadex G-100 (Pharmacia). The column was in equilibrium with a 0.10 M-potassium phosphate buffer, pH 6.4, and the flow was maintained at 26 ml h^{-1} using a peristaltic pump Perplex 10 200 (LKB). Fraction volumes were 5.2 ml. To avoid possible contamination of proteases in columns, purification steps were always performed with Db1121 first, followed by Db1120 and last Db11. Active fractions were pooled and concentrated to 5 ml simultaneously as the buffer was changed to 0.02 M-Tris/HCl pH 7.8, using the Amicon cell 202. Ion exchange chromatography was performed on a 7.7 ml column ($5.0 \text{ cm} \times 1.4 \text{ cm}$) with DEAE-Sephacrose Cl-6B (Pharmacia) equilibrated with 0.02 M-Tris/HCl pH 7.8. After application of samples, the column was washed three times with the starting buffer and then eluted with a gradient 0–0.25 M-NaCl (150 ml), elution-time 3.5 h. Protease-active fractions were pooled and stored at -20°C . All filtrates from the Amicon cell were checked for proteolytic activity. No activity passed through the membrane.

Chromatofocusing. Pooled samples (1.0 ml and 1.5 ml, respectively) of the largest peaks of Proteases 1 and 2 from DEAE-Sephacrose chromatography were loaded on a 10 ml chromatofocusing column ($35 \text{ cm} \times 0.60 \text{ cm}$) with Polybuffer 74 (Pharmacia).

Assay for proteolytic activity. The procedure of Rinderknecht *et al.* (1968) was modified as follows. A sample was made up to 5 ml with 0.05 M-Tris/HCl pH 7.9 buffer solution. Hide Powder (20 mg; Sigma) was added to each tube and the reaction mixture incubated at 37°C for 2 h. The tubes were then centrifuged for 20 min at 3250 g. The absorbance of the clear supernatant was measured at 595 nm against a blank to which no enzyme was added. An increase in absorbance of 0.10 during 2 h incubation at 37°C was defined as one unit of proteolytic activity. Proteolytic activity in electrophoretograms was detected according to Westergaard *et al.* (1980) after separation of native proteins by PAGE.

Test with different inhibitors. Inhibitors, at a final concentration of 1 mM, were mixed with 50 μl enzyme and 4.85 ml Tris/HCl buffer. The mixture was then preincubated at 37°C for 1 h and finally assayed for proteolytic activity as above, except that the incubation time was 20 min.

Assay for protein. Protein was determined by the Lowry method except in the chromatofocusing experiment when we used the procedure of Bradford (1976).

Analytical PAGE. Electrophoresis of native proteins was performed essentially according to Maizel (1971), using 7.5% (w/v) gels and Pharmacia apparatus GE-4. SDS-PAGE of denatured proteins was carried out in 7.5% (w/v) gels, first according to Laemmli (1970), later as described by Neville (1971). Acidic electrophoresis was performed according to Hultmark *et al.* (1980) with an acrylamide/bisacrylamide ratio of 60:0.8. All gels were fixed in 45% ethanol/45% H_2O /10% acetic acid, stained with Coomassie brilliant blue R in 7.5% acetic acid, and destained in 7.5% acetic acid.

RESULTS

Comparisons of parental strain and mutants isolated

We first investigated our strains of *S. marcescens* for survival in IH from *Cecropia* pupae. About 4000 cells of each strain were incubated with 90% IH at 37°C for 2 h. At the intervals indicated, 5 μl samples were withdrawn for viable counts (Fig. 1). The survival values showed that our triple mutant Db1140 (lacking two proteases and resistant to the phage) was more sensitive to IH than our spontaneous phage-resistant mutant Db1109, but both were much more sensitive than our double protease-negative, phage-sensitive mutant Db1121. Thus, phage resistance decreased resistance to *Cecropia* immunity more than the absence of proteolytic enzymes.

When about 5 cells per fly were injected into the *Drosophila* haemocoel, the triple mutant Db1140 was found to be the least virulent strain because it did not differ from the control (Fig. 2). Strains Db11 and Db1121 (both sensitive to ϕJ) were highly virulent and killed all flies within 24 h. Thus, phage resistance decreased the virulence independently of the proteases. If the flies were immunized in advance with *E. cloacae*, the wild-type Db11 killed the flies a few hours sooner than did mutant Db1121, but both strains were also highly virulent for immunized flies.

In order to induce proteases, washed cells of the parental strain Db11, the phage-resistant mutant Db1109 and the two protease-deficient mutants Db1120 and Db1121 were inoculated

Fig. 1

Fig. 2

Fig. 1. Susceptibility of different strains of *S. marcescens* to immune haemolymph from *Cecropia* at 37 °C. ●, Db11, parental strain; ■, Db1121, protease-deficient mutant; □, Db1109, phage-resistant mutant; △, Db1140, protease-deficient and phage-resistant mutant. The relationship between the strains is given in Table 1. The incubation mixture (50 µl) contained 3.2×10^3 to 5.8×10^3 bacteria. The buffer used was 0.01 M-potassium phosphate, pH 6.4 and the haemolymph concentration was 90%. Normal haemolymph incubated with strain Db11 was the control (○).

Fig. 2. Survival of *Drosophila* flies after injection with about five viable cells of different *Serratia* strains. ●, Db11, parental strain; ■, Db1121, protease-deficient mutant; □, Db1109, phage-resistant mutant; △, Db1140, protease-deficient and phage-resistant mutant. Flies injected with Ringer's solution were the control (○). The relationship between the strains is given in Table 1.

Fig. 3. Induction of protease activity per living cell in a C-A medium without glycerol but supplemented with 5% gelatin and 10% normal *Cecropia* haemolymph. ○, Db11, parental strain; △, Db1109, resistant to phage ϕJ ; Db1120 (▲) and Db1121 (●), the protease-deficient mutants.

into a C-A medium (Bromke & Hammel, 1979) without glycerol, but supplemented with 5% gelatin and 10% normal *Cecropia* haemolymph. Plotting of proteolytic activity per living cell against time of growth, showed that the gelatin haemolymph medium induced protease synthesis in strains Db11 and Db1109, while the double protease-negative mutants Db1120 and

Fig. 4. Gel filtration of culture supernatants from the parental strain, Db11 (a) and two protease-deficient mutants, Db1120 (b) and Db1121 (c). A column (65 cm $\times$ 2.5 cm) with Sephadex G-100 was equilibrated with 0.1 M-potassium phosphate buffer, pH 6.4 and loaded with 12 ml concentrated supernatant. Flow rates were 26 ml h^{-1} and fraction volumes were 5.2 ml. Fractions were assayed for proteolytic activity (■) and A_{280} (Δ) was determined. The active fractions were pooled.

Db1121 gave only a very small response (Fig. 3). The absence of carbohydrates in this 'gelatin haemolymph medium' minimizes the catabolite repression of protease production, earlier investigated (Bromke & Hammel, 1979). The first points for mutants Db1120 and Db1121 in Fig. 3 (at $t = 30$) are just above the level of detection. When strain Db11 was grown in LB medium on a rotatory shaker for 7 d at 37 °C, proteolytic enzymes reached a plateau which was maintained for 6 d. Therefore cultures for protease purification were harvested after 4 d.

Purification of proteolytic factors

We have used two different purification schemes. Method 1 consisted of gel filtration on G-100, DEAE-chromatography and chromatofocusing. Method 2 used gel filtration on G-50 Superfine, DEAE-chromatography and gel filtration on S-200. In both cases, fractions had to be concentrated by ultrafiltration which was also used to change buffers when needed.

The areas below peaks of proteolytic activity in gel filtration of concentrated culture filtrates gave an estimate of the protease production of strains Db11, Db1120 and Db1121 (Fig. 4). Expressed as a percentage of the parental strain Db11, the amounts of proteolytic activities were 12% in mutant Db1120 and 7.9% in Db1121, respectively. This first gel filtration step effectively removes the brown material (fractions 40–70 in Fig. 4) originating from the growth medium.

Fig. 5. Anion exchange chromatography of pooled proteolytic peaks from gel filtration (Fig. 4) of material originating from the parental strain, Db11 (a), and two protease-deficient mutants, Db1120 (b) and Db1121 (c). A column (5.0 cm $\times$ 1.4 cm) with DEAE-Sephacrose CL-6B was washed with 0.02 M-Tris/HCl, pH 7.8 and then eluted with a linear gradient of NaCl increasing to 0.25 M. ■, Proteolytic activity; $\triangle$, A_{280} ; —, buffer concentration.

Chromatography on DEAE-Sephacrose showed that the protease activity in the parental strain Db11 consists of at least three components (Fig. 5). The largest peak is hereafter referred to as Protease 1 and the small one appearing early in the elution, Protease 2. The middle peak is probably an active cleavage product of Protease 2 but this proteolytic component has not been investigated further. Mutants Db1120 and Db1121 give only Protease 2 and the amount of enzyme is clearly lower in the double mutant Db1121. Peaks of Proteases 1 and 2 were always eluted at the same intervals of the gradient.

In an attempt to obtain further purification, Proteases 1 and 2 were subjected to chromatofocusing. This step gave an additional purification of about 8 and more than 25 times for Proteases 1 and 2, respectively, but autodigestion was very evident. Unfortunately, the degree of purification could not be judged by SDS-PAGE due to the small amount of the material obtained (70–80 ng ml⁻¹) and/or the buffer system used in chromatofocusing. Furthermore, LD₅₀ of the samples could not be determined because the Polybuffer itself was toxic to the flies. Thus, method 2 had to be used for further comparisons, especially for toxicity tests.

About 3% of the proteolytic activity of the sum of Proteases 1 and 2 did not adsorb to either

Fig. 6. Separation of native proteins by PAGE. (a) Lane 1, Protease 1; lane 2, Protease 2 and lane 3, concentrated supernatant of a Db11 culture. (b) Replica plating on zymogram of the same gel to indicate bands with proteolytic activity.

Table 2. *Properties of protease fractions obtained by method 2*

Protein concentration was determined by the Lowry method. Molecular weights were determined on 7.5% SDS-PAGE according to Laemmli (1970).

Properties	Protease 1	Protease 2	RAC fraction
A_{280}	0.702	1.105	11.8
Protein concn (mg ml ⁻¹)	1.0	0.40	ND
Proteolytic activity (U ml ⁻¹)	5675	3130	295
Specific activity (U mg ⁻¹)	5675	7825	ND
Mol. wt	55×10^3	78×10^3	ND

ND, Not determined.

CM-Sephadex or DEAE-Sephadex in equilibrium with 0.005 M-potassium phosphate buffer, pH 7.1. This material, 'remaining active concentrate' (RAC), is not highly purified but it was concentrated and investigated for action on IH.

Properties of purified proteases

Figure 6(a) shows a polyacrylamide gel of native Proteases 1 and 2 purified by method 2 (lanes 1 and 2) and a concentrated culture supernatant of strain Db11 (lane 3). A replica-plating of the same gel on a zymogram to indicate bands with proteolytic activity is shown in Fig. 6(b). Regions of Protease 1 and 2 are indicated. Toxicity tests, molecular weights, specific activities and other properties are summarized in Table 2.

Table 3. *Production of bacteriocin at different temperatures by protease-deficient mutants of S. marcescens*

The strains were spread on LA plates in 2.5 ml soft agar (0.7% agar) at a density of about 100 cells per plate. The plates were then overlaid with a second layer of soft agar and incubated at different temperatures overnight. After 24 h, the plates were exposed to CHCl_3 vapour for 1 min and then overlaid with 2.5 ml soft agar containing about 5×10^7 living cells of *E. coli* D21f2 (a heptose-less LPS mutant). Finally, the plates were incubated overnight once more in the same temperatures. Colonies of *Serratia* producing clear zones in the layer of *E. coli* are indicated by + and absence of a zone by -.

Temperature (°C)	Bacteriocin production by colonies of strain:		
	Db11	Db1120	Db1121
25	-	+	+
30	-	+	+
37	-	+	+
41	+	+	+

Table 4. *Effects of inhibitors on partly purified Proteases 1 and 2*

Inhibitor*	Proteolytic activity (% of control)	
	Protease 1	Protease 2
Control	100	100
Iodoacetamide	98	92
Soy bean trypsin inhibitor	96	96
EGTA	64	98
EDTA	57	90
PMSF	43	37
41 °C†	27	49

* Inhibitors were used at a final concentration of 1 mM in the assay, except for soy bean trypsin inhibitor (0.5 mg; Boehringer Mannheim). Iodoacetamide and PMSF were first dissolved in 0.10 ml of 95% ethanol.

† Before the assay, undiluted enzyme was preincubated for 30 min at 37 °C as a control (100% activity) and at 41 °C.

Foulds & Shemin (1969) described a temperature-sensitive factor which inactivated a bacteriocin produced by *Serratia*. To identify this factor, strains Db11, Db1120 and Db1121 were incubated at different temperatures, then overlaid with *Escherichia coli* D21f2 as indicator bacteria and finally incubated overnight at the same temperatures (Table 3). In the parental strain Db11, bacteriocin was detected only at 41 °C. Both of the protease mutants allowed production of bacteriocin at all tested temperatures.

Partly purified preparations of Proteases 1 and 2 were tested for effects of inhibitors (Table 4). EDTA and EGTA decreased the activity to about 60% of the control for Protease 1, but showed only a minor effect on Protease 2. Phenylmethylsulphonyl fluoride (PMSF) inhibited both proteases significantly, while there were no effects with soy bean trypsin inhibitor or iodoacetamide.

Protease digestion of Cecropia immune proteins

When examined by SDS-PAGE, Proteases 1 and 2 degraded immune protein P5 while most other bands were preserved (Fig. 7). The RAC fraction showed a clotting effect in agreement with an observation by Kučera & Lysenko (1977).

In another experiment, different dilutions of the proteases were incubated with IH for 30 min at 37 °C. Afterwards samples were spotted on gels and fractionated by acid-PAGE in combination with a bioassay for cecropins (Fig. 8). Proteases 1 and 2 both destroyed cecropins as well as P5 quite effectively. In contrast the RAC fraction preferentially acted on the cecropins.

Fig. 7. Effects of partially purified proteases on immune haemolymph (IH) of *Cecropia*. SDS-PAGE on a 7.5%–15% gradient gel with a 4.5% stacking gel, using a discontinuous buffer system. All samples contain IH. Lanes 1 and 2 are controls without and with 90 min incubation with 0.02 M-Tris/HCl buffer, pH 7.8 (proportions 1 part IH : 2 parts buffer); lane 3, IH with RAC fraction (1 : 2); lane 4, IH with Protease 2 (1 : 2) and lane 5, IH with Protease 1 (1 : 2). P4 and P5 are immune proteins.

DISCUSSION

Serratia marcescens is a common insect pathogen having at least four different proteases (Grimont & Grimont, 1978). We have found our strain of *Serratia* to produce three different protease fractions: Proteases 1 and 2 and the RAC fraction. From the chromatograms (Fig. 5) we conclude that mutant Db1120 is lacking Protease 1. In this strain, the peak for Protease 2 was significantly larger than in the parental strain Db11, a finding which indicates that during our growth and preparative work Protease 1 may degrade Protease 2. The difference between mutant Db1120 and the double mutant Db1121 seems only to be a reduced level of Protease 2. The (over-all) protease activity in the parental strain Db11 was inducible in a medium with *Cecropia* haemolymph (Fig. 3). Under the same conditions there was only a short period of increase in both Db1120 and Db1121. This indicates that strains Db1120 and Db1121 are mutants affected in the regulation of Protease 1.

One of our proteases could be identical to the bacteriocin-destroying factor described by Foulds & Shemin (1969). Table 3 shows that production of bacteriocin was detected by our parental strain Db11 only at 41 °C and at all temperatures by mutants Db1120 and Db1121. In addition, the proteolytic activity of the enzymes was decreased when they were incubated at 41 °C for 30 min. Since the two mutants lack Protease 1, we assume that this protease is the temperature-sensitive factor of Foulds & Shemin (1969).

Fig. 8. Effects of partially purified proteases on the cecropins. IH was incubated with different amounts of proteolytic enzymes for 30 min at 37 °C. Afterwards the samples were examined by electrophoresis in 15% polyacrylamide gels, pH 4. Gels contained samples with (a) Protease 1, (b) Protease 2 and (c) the RAC fraction. Lane 1 contained one part of IH mixed with one part of undiluted enzyme; lanes 2-5 contained twofold dilutions of the respective proteases. Lane 6 contained a control with one part IH added to one part buffer (0.02 M-Tris/HCl, pH 7.8). Pointers indicate different immune proteins.

Two groups have reported highly purified preparations of a protease from *S. marcescens* ATCC 25419 (Braun & Schmitz, 1980; Decedue *et al.*, 1979). Starting from *S. marcescens* strain BG, a similar protease was purified by Lyerly & Kregler (1979). These enzymes were reported to be metalloproteases inactivated by EDTA. Gorini (1950) found that for several bacterial proteases calcium was essential for function and stability. This was also confirmed by Castañeda-Agulló (1956). For Protease 2 we observed no effect with EDTA or EGTA (Table 4). With Protease 1, EDTA gave a reduction believed to reflect a stabilizing effect of a divalent metal ion. Taken together, we conclude that Proteases 1 and 2 differ from the metalloenzymes isolated from strains ATCC 25419 and BG. Autodigestion of highly purified proteolytic enzymes from *Serratia* was encountered in these studies as well as in our own work.

The biological role of the proteases in our strains was studied both by comparing two different protease-deficient mutants and by investigations of the toxicity of partly purified enzyme preparations. The LD₅₀ for both Protease 1 and 2 was around 0.5 ng per *Drosophila* fly. In addition, incubation of partly purified proteases with Cecropia IH (Figs 7 and 8) showed that the enzymes specifically destroyed cecropins, the components in IH which are acting against Gram-negative bacteria (Steiner *et al.*, 1981). Two factors that counteract the insect immune response have earlier been described; one from *Bacillus thuringiensis* (Siden *et al.*, 1979) and one from a nematode which needs a symbiotic bacterium (Götz *et al.*, 1981). In both cases these factors were shown to degrade cecropins in insects. Our proteases here show the same specificity *in vitro*.

Our *in vivo* experiments with *Drosophila* (Fig. 2) clearly show that the protease-negative double mutant Db1121 was almost as virulent as our parental strain Db11. Thus, we have to conclude that neither Protease 1 nor 2 contributes to the lethal effects of our *Serratia* in these injection experiments. We have used feeding experiments according to Flyg *et al.* (1980), but failed to obtain conclusive results concerning a role for the proteases.

We have used mutagens to obtain strains Db1120 and Db1121 and we must expect them to carry several undetected mutations. However, it is difficult to imagine how an undetected mutation could account for the fact that our two protease-negative mutants were always more resistant to Cecropia immunity than were the two spontaneous phage-resistant mutants Db1109 and Db1140 (Fig. 1). Thus, as an overall conclusion, our results demonstrate that, under our assay conditions, passive resistance to immunity is a more important virulence factor than the production of proteases even though they show a preference for cecropins.

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