Purification and characterization of an antibacterial protein from haemolymph of *Sarcophaga peregrina* (flesh-fly) larvae

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Three antibacterial proteins were induced when the body wall of *Sarcophaga peregrina* (flesh-fly) larvae was injured with a hypodermic needle. These proteins were separated and one was purified to homogeneity. The molecular weight of the purified protein was 5000 and its amino acid composition was similar to that of cecropins, which are antibacterial proteins in *Hyalophora cecropia* (cecropia moth) pupae. This protein was found to have bactericidal activity and to be effective at a concentration of 0.1 µg/ml against certain Gram-negative and Gram-positive bacteria.

It is known that insects have both humoral and cellular defence systems to protect themselves from the invasion of various pathogens and to scavenge unnecessary own-tissue fragments that are produced during metamorphosis (Briggs, 1958; Stephens, 1962; Hink & Briggs, 1968; Boman *et al.*, 1974; Pye & Boman, 1977; Scott, 1971; Gagen & Ratcliffe, 1976; Schmit & Ratcliffe, 1977). Of these systems, humoral immunity is particularly interesting because it can be induced by injection of certain dead or live bacteria and so is analogous to antibody production in vertebrates, although its specificity is not always stringent (Chadwick, 1975; Boman, 1981). The molecular basis of the lethal effect on bacteria of substances induced in the haemolymph is unknown. Moreover, it is not certain whether substances other than protein are involved in this humoral immunity (Stephens & Marshall, 1962; Gingrich, 1964).

Recently, a family of inducible antibacterial proteins called 'cecropins' was purified from the haemolymph of immunized pupae of *Hyalophora cecropia* (cecropia moth) and their complete amino acid sequences were determined (Hultmark *et al.*, 1980; Steiner *et al.*, 1981). Cecropins are not lysozymes but small basic proteins and they show high antibacterial activity against several Gram-negative bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa* (Boman & Hultmark, 1981). Similar proteins were also found in immune haemolymph of various species of Lepidoptera by a convenient technique developed by Hultmark *et al.* (1980) to locate antibacterial activity on polyacrylamide gels (Hoffmann *et al.*, 1981).

Previously we reported the induction of bactericidal protein in the haemolymph of third-instar larvae of *Sarcophaga peregrina* (flesh-fly) by injection of a light suspension of *E. coli* into the abdominal cavity (Natori, 1977). Subsequently we found that the injection of bacteria was not necessary, but that wounding of the body wall with a hypodermic needle was essential for induction of bactericidal protein. Not only bactericidal protein but also a lectin with ability to activate mouse macrophages was found to be induced in the haemolymph under these conditions (Komano *et al.*, 1980, 1981, 1983; Nakajima *et al.*, 1982).

The present paper describes the identification of three antibacterial proteins in the haemolymph of injured *Sarcophaga* larvae and purification and characterization of one of the three proteins. The molecular weight and amino acid composition of the purified protein were found to be similar to those of cecropins, suggesting that parts of the inducible antibacterial proteins are derived from common ancestors.

Materials and methods

Animals and collection of haemolymph

Third-instar larvae of *S. peregrina* were used throughout. When kept in contact with water at room temperature (20°C), the larvae could be used for isolation of haemolymph for up to 5 days after leaving their food. For induction of antibacterial proteins, larvae were anaesthetized by keeping them on a glass board in ice for a few minutes and then the posterior half of their body wall was injured with a stainless-steel hypodermic needle. One prick per

Abbreviation used: SDS, sodium dodecyl sulphate.

animal was sufficient for induction of antibacterial proteins.

Haemolymph was collected by cutting off the anterior tip of each larva with fine scissors and collecting the drop of haemolymph that exuded in a Petri dish on ice. Gut contents did not contaminate haemolymph when this method was used. Usually, 1 ml of haemolymph was collected from about 150 larvae. The resulting haemolymph was centrifuged for 5 min at 1000 rev./min (100g) to remove haemocytes and the clear supernatant was stored at -80°C .

Assay of antibacterial activity

Two assay methods were used. For tests on bactericidal activity, samples were incubated with $(1-3) \times 10^7$ cells of *E. coli* K12 594 (streptomycin-resistant) in 0.2 ml of insect saline (130 mM-NaCl/5 mM-KCl/1 mM- CaCl_2) for 60 min at 37°C . Then the mixtures were diluted 20000-fold with insect saline and samples of 50 μl of the diluted mixture were spread on Difco nutrient-agar plates containing 100 μg of streptomycin/ml to inhibit growth of bacteria contaminating the mixture during the experiment. The plates were incubated for 18 h at 37°C and the numbers of colonies in test and control plates were compared, as described previously (Natori, 1977).

Since this assay takes a long time, the following simple method was routinely used to evaluate antibacterial activity throughout the purification of antibacterial protein. *E. coli* K12 594 was grown in antibiotic medium (Difco). Cells in the exponential phase were collected and suspended in insect saline at an $A_{650}^{1\text{cm}}$ of 0.3 (2.5×10^8 cells/ml) determined in a Shimadzu 150-02 spectrophotometer. The sample (200 μl), antibiotic medium (190 μl) and *E. coli* suspension (10 μl) were mixed in a test tube and incubated at 37°C for 140 min with shaking. Then the mixture was rapidly chilled and the A_{650} was measured. For quantification of antibacterial activity, samples were serially diluted with 10 mM-phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.0) containing 130 mM-NaCl and 0.2% bovine serum albumin, and antibacterial activity was assayed at each dilution. One unit of antibacterial activity was defined as the amount that caused 50% inhibition of bacterial growth relative to the control.

Polyacrylamide-gel electrophoresis

Electrophoresis under non-denaturing conditions was carried out in 15% (w/v)-polyacrylamide slab gels (80 mm $\times$ 80 mm $\times$ 1 mm) by the method of Gabriel (1971). Electrophoresis was carried out at 200 V for 6 h at 4°C , under acidic conditions (pH 4.0). Protein bands with antibacterial activity were located on the gel essentially by the method of Hultmark *et al.* (1980). After electrophoresis, the

gels were incubated in antibiotic medium containing 0.2 M-phosphate buffer, pH 7.4, and 100 μg of streptomycin/ml. The gels were then overlaid with 5 ml of the same medium containing 0.6% agar and 2×10^5 viable *E. coli* K12 594 cells kept at 47°C . The soft-agar medium containing *E. coli* cells was usually kept at 47°C for less than 1 min before pouring. Another layer of agar without bacteria was overlaid on this, and the gel was incubated at 37°C for 18 h.

Electrophoresis on SDS/polyacrylamide slab gels was carried out by the method of Laemmli (1970). The protein sample (1–3 μg) was put into 4 mm-wide cells in 1 mm-thick gels with a small amount of Bromophenol Blue. The stacking gel (3% acrylamide) was about 2 cm long and the separating gel (15% acrylamide) was about 7 cm long. Samples were subjected to electrophoresis at 50 V and when the tracking dye entered the separating gel the voltage was increased to 100 V. After electrophoresis, the gels were stained by the method of Fairbanks *et al.* (1971).

Antibacterial specificity

The effect of purified protein on the growth of *E. coli*, *Shigella sonnei*, *Proteus vulgaris*, *Corynebacterium bovis*, *Bacillus megaterium*, *Micrococcus flavus*, *Ps. aeruginosa* and *Sarcina lutea* was tested by mixing it at various concentrations with 400 μl of Difco antibiotic medium or 0.5% polypeptone solution and inoculating the test bacteria. After incubation at the optimum temperature for each bacterium for 6–24 h, the minimum concentration of the protein that inhibited the growth of each bacterium (minimum inhibitory concentration) was determined by measuring the turbidity of the medium.

Results

Antibacterial proteins in the haemolymph of injured larvae

The antibacterial activity in the haemolymph was assayed in samples obtained at various times after injury of the body wall. As Fig. 1 shows, antibacterial activity increased with time, reaching a maximum after 2 days, and then decreased rapidly, almost disappearing in about 5 days. This pattern of change depended on the temperature at which the injured larvae were kept; for instance, the antibacterial activity in the haemolymph was maximal within 24 h when larvae were kept at 28°C . To see how many antibacterial components were induced, we collected haemolymph 2 days after injury of the body wall, subjected it to electrophoresis, and located antibacterial activity by the method of Hultmark *et al.* (1980). Three spots of material inhibiting bacterial growth were found in the

Fig. 1. Change in antibacterial activity in the haemolymph

Larvae were kept at room temperature (18–20°C) after injury to their body wall. At the times indicated, haemolymph was collected and bactericidal activity was determined. ●, haemolymph from injured larvae; ○, haemolymph from untreated larvae.

haemolymph of injured larvae, but none in the haemolymph of normal larvae, as Fig. 2 shows. Thus at least three antibacterial proteins seemed to be induced on injury of the body wall. The material that migrated fastest under these electrophoretic conditions had the most activity when *E. coli* cells were used as a target.

Hoffmann *et al.* (1981) observed multiple antibacterial proteins in the immune haemolymph from different Lepidoptera species and the presence of cecropin-like protein in all of them. Therefore, from the viewpoint of comparative immunology, it is important to see whether Diptera species also contain cecropin-like protein. To characterize the antibacterial proteins in the haemolymph of *Sarcophaga peregrina* larvae, we attempted to purify them.

Separation of three antibacterial proteins

Since haemolymph from injured larvae contains three antibacterial activities, we first tried to isolate each component. For this, about 30 ml of haemolymph, prepared from about 4500 larvae, was diluted with 4 vol. of buffer A (10 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.0) and loaded on a column of CM-cellulose (Whatman CM52, 3 cm × 4 cm) that had been equilibrated with the same buffer. After extensive washing with 25 mM- NaCl in buffer A,

Fig. 2. Antibacterial protein in haemolymph

Haemolymph samples (4 µl) prepared from injured larvae (a) and untreated larvae (b) were subjected to electrophoresis under non-denaturing conditions. Then soft agar containing *E. coli* was overlaid and incubated for 18 h at 37°C to locate spots of antibacterial material.

adsorbed material was eluted with 250 mM- NaCl in buffer A. Antibacterial activity was exclusively recovered in this fraction, not in the flow-through or washout fractions. The active fraction from CM-cellulose was heated for 10 min at 100°C to denature most of the protein, and a clear supernatant was recovered after centrifugation for 10 min at 40000 g. This fraction was found to contain three antibacterial activities like the original haemolymph, with no appreciable loss of activity, indicating that antibacterial proteins are heat-stable. Previously we reported loss of bactericidal activity when haemolymph from injured larvae was heated. At that time we heated the haemolymph without dilution, and so it was probable that the bactericidal proteins were aggregated with denatured proteins and co-precipitated with them, resulting in apparent loss of activity on heating. The resulting supernatant was concentrated by ultrafiltration through a membrane

Fig. 3. *Chromatography of antibacterial activity on a column of Sephadex G-50*

A heat-treated sample (2 ml) was fractionated on a column (1.5 cm $\times$ 60 cm) of Sephadex G-50. Fractions (2 ml) were collected and antibacterial activity was assayed with 10 μ l of each fraction. Antibacterial activity was measured as inhibition of bacterial growth. O, A_{280} ; ●, bacterial growth.

Fig. 4. *Chromatogram of antibacterial activity on a column of CM-cellulose*

The G-II fraction from Sephadex G-50 was fractionated on a column (2.0 cm $\times$ 4.0 cm) of CM-cellulose. Antibacterial activity was eluted from the column at NaCl concentrations of 130 mM and 260 mM. The arrows indicate the positions where the salt concentration in the elution buffer was changed. Fractions (3 ml) were collected and antibacterial activity was assayed with 10 μ l of each fraction. O, A_{280} ; ●, bacterial growth.

filter (UF disc type A, Spectrum) and the concentrated solution was stored at -20°C .

About 2 ml of concentrated solution obtained after heat treatment was applied to a column of Sephadex G-50 (1.5 cm $\times$ 60 cm) that had been

equilibrated with buffer A containing 130 mM-NaCl. At this step, antibacterial activity was separated into two peaks with absorbance at 280 nm, as shown in Fig. 3. Material in the first peak, named 'fraction G-I', was found to contain only one activity,

whereas that in the second, named 'fraction G-II', contained two activities as shown by polyacrylamide-gel electrophoresis. Fraction G-II was therefore further fractionated on a column of CM-cellulose. For this, the G-II fraction from Sephadex G-50 was diluted with 4 vol. of buffer A and applied to a column of CM-cellulose (2.0 cm $\times$ 4 cm) that had been equilibrated with the same buffer. The column was washed well, and then adsorbed material was eluted stepwise with increasing salt concentrations in buffer A. As Fig. 4 shows, two distinct antibacterial activities were eluted from the column at NaCl concentrations of 130 mM and 260 mM respectively. These fractions were named 'C-I' and 'C-II' in order of their elution. The electrophoretic profiles of antibacterial activity in the G-I, C-I and C-II fractions are shown in Fig. 5. Each fraction contained only one activity, and the spots of antibacterial material located on poly-

Fig. 5. Antibacterial activities of the G-I, C-I and C-II fractions

The electrophoretic profile of antibacterial activity in the G-I, C-I and C-II fractions on polyacrylamide gel was determined and compared with that of the original haemolymph. (a) Haemolymph from injured larvae; (b), G-I fraction (0.4 unit); (c), C-I fraction (1 unit); (d), C-II fraction (0.2 unit).

acrylamide gel coincided with those in the original haemolymph, indicating that the three antibacterial proteins were separated from each other by these procedures.

Purification of an antibacterial protein from fraction C-II

Approx. 45% of the antibacterial activity was recovered in fraction C-II, therefore the antibacterial protein in this fraction was purified further on a column of hydroxyapatite. About 18 ml of fraction C-II was diluted 10-fold with buffer A and applied to a column (2 cm $\times$ 2 cm) of hydroxyapatite that had been equilibrated with buffer A. The column was washed with 50 mM-phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, pH 6.0) and adsorbed material was then eluted with 100 mM-phosphate buffer. Since the amount of protein was very small, the elution of protein from the column was monitored by measuring $A_{215}-A_{225}$, which is an indicator of the absorption due to peptide bonds. Two protein peaks were eluted from the column and the position of antibacterial activity coincided with that of one of these peaks, as shown in Fig. 6. This peak fraction contained almost homogenous protein, as judged by polyacrylamide-gel electrophoresis both under denaturing and non-denaturing conditions, as shown in Fig. 7. The position of antibacterial activity coincided with the single band on polyacrylamide gel under non-denaturing conditions.

The purification of the antibacterial protein is summarized in Table 1. About 70 μg of pure protein was obtained from 120 ml of haemolymph. The

Fig. 6. Chromatogram of fraction C-II on a hydroxyapatite column

The C-II fraction was applied to a column (2 cm $\times$ 2 cm) of hydroxyapatite. The column was washed with 50 mM-phosphate buffer, pH 6.0, and then at the point indicated by an arrow the elution buffer was changed to 100 mM-phosphate buffer. Fractions (6 ml) were collected and antibacterial activity was assayed with 10 μl of each fraction. $\circ$, $A_{215}-A_{225}$; $\bullet$, bacterial growth.

Fig. 7. Polyacrylamide-gel electrophoresis of purified bactericidal protein

The active fraction from hydroxyapatite was subjected to electrophoresis under denaturing (a) and non-denaturing (b) conditions. The amounts of protein applied were 2.4 μ g for (a) and 5.3 μ g for (b). The arrow indicates the band with antibacterial activity located with overlaying soft agar. α -Chymotrypsinogen (mol.wt. 25000), myoglobin (17800) and aprotinin (6500) were used as molecular-weight markers.

specific activity was increased about 30000-fold compared with the original haemolymph and did not change appreciably when this fraction was fractionated further on a column of CM-cellulose, indicating that this fraction was almost pure.

Characteristics of purified antibacterial protein.

Purified protein was sensitive to tryptic digestion, the activity being lost on brief incubation with trypsin. The molecular weight determined by gel-filtration through Sephadex G-50 was 5000, which was consistent with the value obtained by SDS/polyacrylamide-gel electrophoresis. The amino acid composition of this protein is shown in Table 2. The protein seems to contain 40–42 amino acid residues. The contents of alanine, glycine, glutamic acid/

Table 1. Summary of purification of antibacterial protein

A 130 ml sample of haemolymph was used as starting material. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard, except where marked *, which was determined from A_{215} – A_{225} as described by Murphy & Kies (1960).

Purification step	Total protein (mg)	Specific activity (unit/mg)	Recovery (%)
Haemolymph	18 100	0.75	100
First CM-cellulose	156	82	97
Heat-treated supernatant	31	410	93
Concentrated supernatant	—	—	62
Sephadex G-50			
G-I	19	85	12
G-II	8.2	850	51
Second CM-cellulose			
C-I	3.4	33	1
C-II	1.1	5500	45
Hydroxyapatite	0.07*	27 000	14

Table 2. Amino acid composition of antibacterial protein

The sample was hydrolysed for 22 h at 100°C in 6M-HCl and the resulting amino acids were analysed in a Hitachi 835 amino acid analyser. Hydrolysis was performed in the presence of β -indolepropionic acid to prevent the decomposition of tryptophan.

Amino acid	Composition	
	Mol/100 mol	No. of residues
Asx	7.84	3
Thr	6.04	1–2
Ser	2.73	1
Glx	10.06	4
Gly	11.49	5
Ala	12.25	5
Val	5.87	2
Cys	0.38	0
Met	1.48	1
Ile	6.74	3
Leu	4.10	2
Tyr	2.56	1
Phe	1.27	0–1
Lys	10.37	4
His	3.91	1–2
Arg	6.78	3
Pro	4.54	2
Trp	1.52	1
Total		40–42

glutamine and lysine are higher than those of other amino acids. These findings are similar to those for cecropin A and B purified from *Hyalophora cecropia*, which consist of 37 amino acids (Steiner *et al.*, 1981).

Table 3. *Bacterial specificity of purified antibacterial protein*

Purified protein at various final concentrations of below 1 µg/ml was mixed with Difco antibiotic medium (A) or polypeptone solution (P), and the minimum inhibitory concentration was determined. *Pseudomonas aeruginosa* A3 and *Sarcina lutea* PCI 1001 were resistant to this protein at a concentration of 1 µg/ml.

Bacteria	Strain	Culture conditions			Minimum inhibitory concentration (µg/ml)
		Medium	Temperature (°C)	Time (h)	
<i>Escherichia coli</i>	K12	P/A	37	6	0.15
<i>Shigella sonnei</i>	JS11746	P	37	12	0.08
<i>Proteus vulgaris</i>	OX19	P	37	12	0.3
<i>Corynebacterium bovis</i>	1810	A	37	24	0.3
<i>Bacillus megaterium</i>	APF	P	37	6	0.08
<i>Micrococcus flavus</i>	FDA16	A	27	12	0.3

The minimum inhibitory concentration of this protein on *E. coli* in liquid culture was 0.1 µg/ml, and it was effective even in the presence of 10% (w/v) calf serum. The antibacterial specificity of this protein was tested with various bacteria in liquid culture. As summarized in Table 3, this protein was effective towards certain Gram-negative and Gram-positive bacteria. So far as tested, *E. coli*, *S. sonnei* and *B. megaterium* were extremely sensitive to this protein and the minimum inhibitory concentration on these bacteria was below 0.2 µg/ml. However, *P. aeruginosa* and *S. lutea* were resistant to this protein under these culture conditions.

The mode of action of this protein seems to be bactericidal, because *E. coli* treated with this protein lost colony-forming activity.

Discussion

The present paper reports the purification of one of three antibacterial proteins of *Sarcophaga* larvae induced on injury of the body wall. It is known that antibacterial activity is induced in some insects on administration of bacterial vaccine. However, the activity has not been well-characterized, except in the case of cecropins, which are antibacterial proteins of *Hyalophora cecropia* pupae (Hultmark *et al.*, 1980; Steiner *et al.*, 1981). More recently, many cecropins with minor differences in amino acid composition have been found in the haemolymph of *Hyalophora cecropia* and *Antheraea pernyi* (Chinese oak silk moth) pupae (Hultmark *et al.*, 1982; Qu *et al.*, 1982). Thus an interesting problem is to investigate the organization of cecropin genes. In Diptera, however, no such protein has been identified. The antibacterial protein purified from *Sarcophaga* had a similar molecular weight and amino acid composition to cecropins, suggesting that it may be a cecropin.

We demonstrated three antibacterial activities in the haemolymph of injured larvae. Hoffmann *et al.*

(1981) reported the presence of multiple antibacterial activities in various Lepidoptera. It is likely that these proteins have different lethal effects or different antibacterial specificities and together construct a potent defence system to prevent bacterial infection. In addition to antibacterial proteins, a lectin with activity to enhance phagocytic activity of mouse macrophages was also shown to be induced in the same haemolymph (Nakajima *et al.*, 1982; Komano *et al.*, 1983). Thus it is likely that many proteins participating in the defence mechanism of this insect are activated by the same stimulus of injury of the body wall.

The purified protein repressed the growth of bacteria at a concentration of 0.1 µg/ml *in vitro*, which is comparable with the effective concentrations of various antibiotics, and it was effective even in the presence of 10% (w/v) foetal-calf serum. This is important, because the activity of most peptide antibiotics is markedly decreased in medium containing serum, owing to their adsorption to serum protein. The antibacterial protein of *Sarcophaga* may bind preferentially to bacteria and has lower affinity to serum protein. These results suggest that the protein could be used as an antibacterial substance. But for that purpose, many more experiments must be done: first, it must be determined if the antibacterial protein is antigenic when injected into animals. Once the sequence of this peptide is determined, it may be synthesized chemically. For determination of its active site, the protein must be obtained in large quantity. Genetic engineering might be useful for large-scale production of this protein, although a eukaryotic system may have to be used, since the protein is toxic to *E. coli*.

At the moment nothing is known about the mechanism of the lethal effect of this protein on bacteria. We only assume that *E. coli* is killed by this protein, because once it is treated with this protein it could not form colonies on agar plates, even in the absence of this protein.

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