INSECT IMMUNITY:
INDUCIBLE ANTIBACTERIAL PROTEINS FROM
HYALOPHORA CECROPIA

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ABSTRACT - A powerful bactericidal activity can be induced in the hemolymph of many insects as a response to an injection of bacteria. The nature of the effector molecules of this immune response was investigated, using pupae of the Cecropia moth, Hyalophora cecropia. Three major types of antibacterial proteins were found: the cecropins, the P5 proteins, and lysozyme. They appear in the hemolymph as a result of de novo synthesis.

Six different cecropins were purified and characterized. The full amino acid sequences of the three major cecropins A, B and D were determined, as well as partial sequences of the three minor cecropins C, E and F. The cecropins are all very small ($M_r = 4,000$) and basic ($pI > 9.5$) proteins, and they show extensive homology in their sequences. The three major cecropins are products of different genes. Their C-terminals are blocked by uncharged groups, which can be removed by mild acid hydrolysis. The minor cecropins are closely related to the major forms, and may be unblocked precursors or, in one case (cecropin F), a minor allelic form. The cecropins were shown to be lytic, and to be efficient against several Gram positive and Gram negative bacterial strains, but not against mammalian cells.

The P5 proteins are bactericidal proteins, larger than the cecropins ($M_r = 20,000 - 23,000$). Six forms, differing in isoelectric point, were isolated. They form two closely related groups, the basic (P5 A-D) and the acidic forms (P5 E-F). Within each group, the different forms have almost identical amino acid compositions.

The Cecropia lysozyme is similar to lysozymes isolated from other insects, as well as to that from hen egg white. It is lytic to a restricted number of Gram positive bacteria.

The presence of cecropins and other antibacterial factors was demonstrated also in other lepidopterans, notably Galleria mellonella, and may explain earlier observations of antibacterial factors in the latter species.
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PAPERS INCLUDED IN THIS THESIS, References (1-5):
(1) Insect immunity. Purification and properties of three inducible bactericidal proteins from hemolymph of immunized pupae of Hyalophora cecropia.
  Eur. J. Biochem. 106, 7-16. 23

(2) Insect immunity: Galleria mellonella and other lepidoptera have cecropia-P9-like factors active against Gram negative bacteria.
  Insect Biochem. 11, 537-548. 35

(3) Sequence and specificity of two antibacterial proteins involved in insect immunity.
  Nature 292, 246-248. 49

(4) Insect immunity: Isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae.

(5) Immune protein P5 from the moth Hyalophora cecropia, a heterogenous family of antibacterial proteins.
  (Manuscript, to be completed before publication). 69
INTRODUCTION

Insects are strikingly different from ourselves. Their entire body plan and virtually every organ system show examples of functional solutions that are different from those found in vertebrates. These solutions have furthermore proved to be highly successful. Insects have held a dominating position in the terrestrial fauna, at least since the middle carboniferous, when they first appear in large numbers in the geological record. The differences between insects and vertebrates go back to a long history of independent evolution of the two groups since the very early division of higher metazoa in two major branches, the protostomia and the deuterostomia.

However, on the more fundamental level of biochemistry and genetics, there is a greater unity of life. For problems in these areas insects have often provided good experimental models, the results being applicable to animals in general. Thus, when we study insect function, we can hope to gain insight both in the basal attributes of life, and in the various forms in which it can be expressed.

The aim of this investigation was to throw some further light on a less well-known aspect of insect function – the humoral mediators of insect resistance to bacterial infection.

BACKGROUND

The study of insect immunity is an old subject, the foundations of which were laid in the first decades of this century. In fact, the existence of immunity in insects was noted already by Pasteur, who was able to raise a strain of silkworms that was resistant to pébrine, a disease caused by a microsporidian (6). An extensive review of the older literature was given by Whitcomb et al. (7) and more recent work was covered by Boman (8). It is now well documented that insects can cope with heavy inocula of various microorganisms, including many that are lethal pathogens to man. Invading microbes are attacked by a cellular defense, as well as by humoral factors. The cellular defense reactions include such mechanisms as phagocytosis, nodule formation and encapsulation, and are often collectively referred to as the "hemocytic reaction".
Leaving melanisation and other humoral aspects of the encapsulation reaction out of the discussion, the earlier work on the humoral factors of insect immunity can briefly be summarized as follows:

1) A constitutive level of lectins and other agglutinins is often present. The role of these agglutinins is not well understood, and they will not be further discussed in this essay. Invertebrate lectins were recently reviewed (9, 10). Constitutive bactericidal factors have also sometimes been demonstrated.

2) A powerful bactericidal and bacteriolytic activity is found in the cell-free hemolymph of many insects after a previous injection of live or dead bacteria.

3) This response is in most cases rather unspecific; a broad spectrum of bacteria are attacked, irrespective of what organism is used as vaccine. In fact, even the injection of sterile saline will induce a weak response, the "wound effect".

4) There is general agreement that no immunoglobulins are present.

In terms of molecular mechanisms, however, these early investigations give little information. Partially purified antibacterial fractions were obtained in several instances as summarized in Table 1, but the results reported are difficult to integrate to a coherent picture. Lysozyme-like activity was often demonstrated, and it has been claimed that lysozyme is the major effector of antibacterial activity in insect hemolymph (19). Later, lysozyme has been purified and characterized from several insect sources (20-23).

Adopting pupae of the Cecropia moth, Hyalophora cecropia, and other Saturniid moths as an experimental system, Boman and co-workers demonstrated that a specific set of proteins was synthesized simultaneously with the appearance of antibacterial activity in the hemolymph. These "immune proteins" were designated P1-P8, according to increasing mobility in SDS polyacrylamide gel electrophoresis (24). Using diapausing pupae, it was possible to incorporate radioactive amino acids selectively into these proteins, since the synthesis of other proteins during diapause is reduced to a very low level. This selective incorporation was used as a convenient assay in the purification of the two major immune proteins, P4 (25) and P5 (26). However, none of these proteins were found to have any antibacterial activity of their own.

In a new attempt to isolate and characterize the humoral factors of insect immunity, it was decided to resort to biological assays for antibacterial activity. Due to their large size and their diapause, pupae of H. cecropia proved to be very suitable for this biochemical work. This approach has led to the discovery of a new family of bacteriolytic peptides, the cecropins. In addition, it was also established that P5 represents a heterogeneous family of proteins with antibacterial activity.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Source</th>
<th>Technique</th>
<th>Properties</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stephens &amp; Marshall, 1962</td>
<td>Galleria mellonella</td>
<td>Dialysis</td>
<td>Relatively small $M_r$, Weakly acidic, Heat stable (100°C, 5 min), Unaffected by trypsin, Probably non-protein</td>
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<tr>
<td>(11)</td>
<td></td>
<td>Ion exchanger</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethanol precipitation</td>
<td></td>
</tr>
<tr>
<td>Gingrich, 1964</td>
<td>Oncopeltus fasciatus</td>
<td>Electrophoresis</td>
<td>Associated with acidic proteins, Heat stable (75°C, 60 min), Not precipitated by TCA, Resists 1 M HCl and NaOH, Non-protein</td>
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<tr>
<td>(12)</td>
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<td></td>
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<td>Hink &amp; Briggs, 1968</td>
<td>Galleria mellonella</td>
<td>Gel filtration</td>
<td>$M_r = 7,000$, Heat stable (100°C, 5 min)</td>
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<td>(13)</td>
<td></td>
<td>Factor A:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Factor B:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Both:</td>
<td></td>
</tr>
<tr>
<td>Kawarabata, 1970</td>
<td>Bombyx mori</td>
<td>Gel filtration</td>
<td>Heat stable (70°C, 15 min), Destroyed by trypsin and periodic acid, Eluting with lysozyme-like activity</td>
</tr>
<tr>
<td>(14)</td>
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<td>Bakula, 1970</td>
<td>Drosophila melanogaster</td>
<td>Acid extraction</td>
<td>Basic, Destroyed by trypsin</td>
</tr>
<tr>
<td>(15)</td>
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<td>Natori, 1977</td>
<td>Sarcophaga peregrina</td>
<td>Gel filtration</td>
<td>$M_r &lt; 10,000$, Heat sensitive (at temperatures &gt; 60°C, 20 min), Destroyed by trypsin, Activity lost by dialysis, Protein</td>
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<td>Kinoshita &amp; Inoue, 1977</td>
<td>Bombyx mori</td>
<td>Chitin column Gel filtration</td>
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<td>Croizier, 1979</td>
<td>Spodoptera littoralis</td>
<td>Heat treatment Ion exchangers</td>
<td>$M_r = 20,000$, Acidic, Heat stable (85°C, 15 min), Protein</td>
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<td>(18)</td>
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<td></td>
<td></td>
</tr>
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</table>
TECHNICAL CONSIDERATIONS.

Analytical methods

Earlier investigations in this laboratory were aided by the development of a biological assay for antibacterial activity, the "killing assay" (27). Antibacterial preparations were incubated with live E. coli, strain D31, in phosphate buffer, and after different time intervals samples were diluted and the remaining number of living bacteria was counted by spreading on agar plates. In addition, two rapid, qualitative assays were used (28): In an assay for lysis the decrease in turbidity in a bacterial suspension was measured after the addition of the antibacterial sample, and in the "spot test" the material to be assayed was applied on top of plates seeded with bacteria. Zones of growth inhibition indicated antibacterial activity.

Though sensitive and conceptually straightforward, the killing assay was too time-consuming when large numbers of fractions were to be assayed, especially when quantitative results were needed. For this reason I investigated the quantitative aspects, first of the lysis assay (1), and later of the "inhibition zone assay", a standardized version of the spot test (29), similar to those that have been in use for a long time for the assay of antibiotics, (30, 31). In this assay, the diameters for the inhibition zones were related to antibacterial activity (2, 4).

An advantage of the lysis assay is that results are obtained directly. However, only antibacterial factors that actually lyse the bacteria can be detected. In addition, variable end-points in the lysis were obtained with purified cecropins, due to the presence of light-scattering "ghosts", presumably consisting of undegraded peptidoglycan from the cell wall. These difficulties were overcome in the inhibition zone assay. Large numbers of samples could easily be processed with both assays, a prerequisite for the fractionation studies.

In the inhibition zone assay, we first estimated the activity by comparison with standard curves, prepared with different dilutions of a reference hemolymph (2). Difficulties were encountered, however, when we found that dilution series of different preparations gave standard curves that were not parallel, sometimes even crossing each other. Similar problems appeared when we wanted to compare the sensitivity of different bacteria. For this reason I investigated the theory of the inhibition assay (4). In connection with the assay of antibiotics, expressions for the relation between zone size and the concentration of antibiotic have previously been derived, using equations for diffusion in two dimensions from a point-like source (32, 33), or equations for diffusion in one dimension from a reservoir with a constant concentration of antibacterial
substance (34). Starting with a two-dimensional approach, I devised methods to calculate the "lethal concentration" of a substance (the lowest concentration that will just inhibit bacterial growth in the agar plate) from the zone diameters. The varying slopes of the standard curves could also be explained (4).

The very small size of the cecropins made SDS-electrophoresis less suitable as an analytical tool. A good resolution was instead achieved in 15 percent polyacrylamide gels under non-denaturing conditions, using a discontinuous acidic buffer system (1). This system had the additional advantage that it could easily be combined with a biological detection technique in which the gels were overlaid with live bacteria in nutrient agar. The position of antibacterial substances was visualized as bands without bacterial growth (1). In this way, at least four different cecropins could be detected also in very crude fractions such as whole hemolymph. However, acid polyacrylamide gel electrophoresis does not resolve the different forms of the protein P5. For this purpose analytical isoelectric focusing has so far been the method of choice. Also this technique could be combined with the bioassay to detect antibacterial bands. In addition, it could be combined with immunofixation to detect specific proteins against which antisera were available (5).

Preparative methods

A time-consuming part of this investigation was the development of techniques for the isolation and purification of the immune proteins. Two major technical difficulties were encountered in this work. One was the tendency of whole hemolymph to form a clot as a result of various manipulations, notably after contact with the chromatographic materials used in our columns. A way to avoid this was to introduce a brief boiling at pH 4 as a first step. This caused most proteins in the hemolymph to form a clot, while surprisingly the antibacterial activity was retained in the supernatant (Hultmark, unpublished). This technique was abandoned, however, as it was felt that it might introduce minor modifications in the antibacterial proteins. Instead, gel filtration was used as a milder method to separate the large clot-forming proteins from the antibacterial factors. It was important to perform this step rapidly, using high flow rates, coarse Sephadex beads, and not too long columns, as otherwise clotting occurs in the column. Neutral pH and low ionic strength was avoided in this step to prolong clotting time. When more is known about the clotting system of insect hemolymph it may be possible to find conditions where this reaction can be inhibited altogether.

Another problem was the low yield of antibacterial activity obtained. The purified cecropins had a high and reproducible specific activity (4). However, the
combined antibacterial activity in the different isolated forms accounted for only 5% of the activity against *E. coli* D31 originally present in the hemolymph, in spite of the fact that cecropins appear to be the major colilysic factors. One possible reason for the loss of activity would be that the cecropins act more efficiently in the hemolymph where they are present together, and where other synergistic factors may occur. Another reason could be loss of material due to adsorption. Because of their amphipatic nature, the cecropins should have a high affinity both to hydrophobic and to hydrophilic surfaces, especially when the latter are negatively charged. It was noted that the cecropins were usually lost if the preparations were dialysed, not because the small molecules pass through the membrane, but because they bind to it. The loss of activity in the P5 preparations is discussed later.

To limit the loss of material, the number of steps in the purification procedures was kept small. Methods were used that as far as possible were based on independent physical properties of the protein molecules, such as size (gel filtration), charge (ion exchange chromatography, chromatofocusing) and hydrophobic affinity (hydrophobic interaction chromatography, ammonium sulphate precipitation). Volatile buffers (ammonium formate and acetate) were used in most of the work. Desalting could then easily be performed by freeze-drying, without the need for dialysis.

**INDUCTION OF THE ANTIBACTERIAL PROTEINS**

Hemolymph from untreated pupae contain no detectable antibacterial activity. The induction of antibacterial factors after an injection of bacteria was followed in detail by Faye et al. (24) in pupae of *Samia cynthia*. After a lag period of about 10-12 hours, antibacterial activity appeared, and it increased until a maximum was reached 2-4 days later. We found a similar lag period in *H. cecropia* (1), but in this species, the maximum is not reached until 7-9 days after the vaccination (M. Ressner, personal communication).

The appearance of antibacterial activity in the hemolymph could in principle be due to either an activation of inactive precursors, a release of active material from cellular depots, or *de novo* synthesis of antibacterial factors. That RNA and protein synthesis are indeed required for the expression of antibacterial activity in *S. cynthia* was shown by Faye et al. (24) in experiments with inhibitors of RNA synthesis (actinomycin D) and protein synthesis (cycloheximide). Later, Croizier (18) got similar results for the induction of lysozyme activity in *G. mellonella*. In contrast, Kamp (35),
Pawning & Davidson (20) and Jarosz (36), working with larvae of *G. mellonella*, obtained only minor effects with actinomycin D.

We therefore reinvestigated this question, both in *H. cecropia* (1) and in *G. mellonella* (2). In both species we were able to block the appearance of lytic factors with actinomycin D. By injecting the inhibitor at various times after vaccination, we could determine a lag period for RNA synthesis. For *H. cecropia*, Boman et al. (37) found the lag period to be 5 hours, while in *G. mellonella* we found it to be about 3 hours (2). These results, together with the fact that radioactive amino acids are actively incorporated into the immune proteins (1, 24), make us favour the conclusion that the immune response in these insects is brought about by de novo synthesis of antibacterial proteins.

In larvae of *G. mellonella*, we also found a variable constitutive level of lysozyme activity (2). The cecropin-like factors were, however, only observed in immunized animals. Similarly, unvaccinated larvae of *H. cecropia* contained lysozyme in the hemolymph, corresponding to about 10% of the maximally induced level (Hultmark, unpublished).

### ISOLATION OF THE ANTIBACTERIAL PROTEINS

When hemolymph from immunized pupae of *H. cecropia* is electrophoresed in an acidic gel system, at least five antibacterial bands can be discerned, using *E. coli* as indicator organism (see e.g. (2), Fig. 2). In addition, a band with lysozyme-like activity can be detected by its action on *Micrococcus luteus* ((2), Fig. 7). During the course of the isolation of the antibacterial factors an even greater heterogeneity became apparent, and this work finally led to a scheme for the purification of 14 antibacterial factors (Fig. 1). They are all proteins or polypeptides, and can be grouped into three major classes: the cecropins, the P5 proteins, and the lysozymes. In addition, a minor antibacterial peptide of uncertain identity, the "factor G", was isolated (4).

### THE CECROPINS

The cecropins, first referred to as the P9 proteins (1, 2), constitute a group of small bacteriolytic proteins with some rather extreme properties. They have molecular weights of about 4,000, and they are very basic ($p_I > 9.5$). They are heat-stable, resisting even prolonged periods of boiling (1, 2). The term cecropins was introduced by us as a generic name for all antibacterial proteins that show sequence homology to the first discovered P9 proteins (3).
Fig. 1. Purification scheme for the antibacterial factors in hemolymph from immunized pupae of H. cecropia
Chemical structure

So far six cecropins have been isolated from *H. cecropia*. Their primary structures were determined in a collaboration with Hans Bennich's laboratory in Uppsala (3, 4). The sequence of the amino terminal 28-37 residues, constituting the major part of the polypeptide chains, were obtained directly by automatic Edman degradation. The C-terminal part of the sequences of cecropins A, B and D were determined by carboxypeptidase degradation, and for cecropins A and D also by sequencing of tryptic digests. In the latter case, the C-terminals are found in the largest tryptic fragments, and thus the presence of other fragments does not interfere with C-terminal sequencing. Furthermore, to improve the yields in the final steps of the Edman degradation, the terminal lysine residues were made more hydrophilic by chemical modification (4).

The three major cecropins, A, B and D, were found to have different, but closely related, amino acid sequences (Fig. 2). As they are present together in most, probably all, investigated individuals, it was concluded that they are not allelic forms. Thus, there are at least three cecropin loci in the genome of *H. cecropia*. All major cecropins have their carboxy terminals blocked by uncharged groups, which can be removed by mild acid hydrolysis. Possibly they are amidated, as has been found for a large number of other biologically active peptides (38-40).

Too little material was obtained of the minor cecropins for a complete characterization. It can be inferred, however, from their partially determined primary structure, that they are not independent gene products. The 37 residues determined for cecropin C are identical to the complete sequence of the major cecropin A. The difference between the forms in their electrophoretic mobility may be due to the absence of a blocking group in C. A similar relation may exist between cecropin E and the major cecropin D. Cecropin C and E may thus be precursors or degradation products of the major cecropins. In the bee venom toxin melittin (41), the amidated C-terminal is formed in a reaction where a terminal glycine is cleaved off (42). Similarly, in calcitonin, the C-terminal -Pro-NH$_2$ may be derived from a -Pro-Gly- structure (43). Interestingly, the amino acid compositions of both C and E allow for an extra glycine, compared to the corresponding major cecropins. The C-terminal blocking groups do not appear to be crucial for the antibacterial activity. The activity of these unblocked minor cecropins are similar to those of the major forms.

The minor cecropin F has one amino acid substitution (17 Asp $\rightarrow$ Asn), compared to cecropin D. Though deamidation is a common event, amidation is not. Thus, it is unlikely that cecropin F represents a form that has been post-translationally modified before or during the purification procedure. As this
The primary structures of the cecropins A, B, and D (adapted from (3) and (4)).

The four C-terminal residues in B are tentative (3). The international one-letter code for the amino acids was used, heavy letters denoting polar residues and thin letters nonpolar ones:

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Code</th>
</tr>
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<tbody>
<tr>
<td>Cysteine</td>
<td>C</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>D</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>E</td>
</tr>
<tr>
<td>Histidine</td>
<td>H</td>
</tr>
<tr>
<td>Lysine</td>
<td>K</td>
</tr>
<tr>
<td>Asparagine</td>
<td>N</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Q</td>
</tr>
<tr>
<td>Arginine</td>
<td>R</td>
</tr>
<tr>
<td>Serine</td>
<td>S</td>
</tr>
<tr>
<td>Threonine</td>
<td>T</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Y</td>
</tr>
<tr>
<td>Leucine</td>
<td>L</td>
</tr>
<tr>
<td>Alanine</td>
<td>A</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>F</td>
</tr>
<tr>
<td>Proline</td>
<td>P</td>
</tr>
<tr>
<td>Valine</td>
<td>V</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>I</td>
</tr>
<tr>
<td>Methionine</td>
<td>M</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>W</td>
</tr>
</tbody>
</table>

The letters a and r denote the N-terminal amino groups and the C-terminal blocking groups, respectively.

cecropin was isolated from a batch of hemolymph taken from about 400 genetically heterogeneous pupae, it may be a product of a modified cecropin D allele that is present in a low frequency in the population.

A cecropin, containing all but the four C-terminal residues of the cecropin A sequence, has also been chemically synthesized (44). This synthetic substance, cecropin A(1-33), was very similar in its properties to the naturally occurring form, though the antibacterial activity was slightly lower. These results constitute an indirect confirmation of the primary structure that we have determined, and they preclude the possibility that the activity of the cecropins is due to impurities in the preparations.

The primary structures of the major cecropins allow some generalizations. They all have a highly charged "head" region from the N-terminal to residue 17. Then follows a hydrophobic region between residues 18-30. Finally, the C-terminal seven amino acids again contain several polar groups. Empirical methods to predict secondary structure from amino acid sequences have been applied on cecropin A and B (44, 45). Residues 1-11 within the charged head, as well as the C-terminal polar region had strong potentials for α-helix formation. Similar conclusions were also reached for cecropin D (4), though in this molecule a third
centre for α-helix formation was predicted between residues 14-19. In the "head", the charged residues are interspersed with hydrophobic residues in a regularly repeating pattern (Fig. 2). Model building studies (44, 45) show that when this structure is folded into an α-helix, all charged residues will be found on one side of the helix, and all hydrophobic residues on the other. Such a strong polarisation of the helix, and indeed of the whole molecule into highly charged and highly hydrophobic regions should give the cecropins an amphipathic character that fits well with the hypothesis that their major target is the bacterial membrane (see below).

The structural predictions were also tested experimentally by Steiner (45), who made an estimate of helical content by CD and NMR measurements. He found that in aqueous solutions, cecropins A and B assumed a random coil configuration. In a hydrophobic environment, however, there was a structural change, and for cecropin A, a conformation with more than 80% α-helix could be obtained. It may be assumed that this highly ordered configuration is adopted by the cecropin at the contact with the bacterial membrane. The open, random, conformation found in solution would explain the heat-stability of the cecropins.

In gel filtration, the cecropins elute together with lysozyme (3, 4), the latter protein having a molecular weight of 15,000 (1). Identical results were obtained under conditions ranging from 0.1 M ammonium formate, pH 6.5 to 2 M ammonium acetate, pH 4.5 (Hultmark, unpublished). Apparently this implies an oligomeric conformation, as the monomer molecular weight of the cecropins is around 4,000. However, equilibrium centrifugation of cecropin A in 0.2 M ammonium formate, pH 6.5, gave a molecular weight of 4,200 ± 300, indicating free monomers only (3). Neither did crosslinking studies with dimethylsuberimidate give any evidence for oligomers. The anomalous mobility of the cecropins in gel filtration may be caused by exceptionally large Stoke's radii of these molecules in solution due to their open, random conformation.

**Biological activity**

Addition of cecropin to a suspension of *E. coli* cells causes death of the bacteria within a few minutes. Following similar kinetics, there is also a partial reduction of the turbidity of the suspension (1). When observed in the phase-contrast microscope, the cells retain their form, but lose in contrast. An increase in the absorbance at 260 nm in the extracellular medium indicates that there is a leakage of cell contents out of the cell (Hultmark, unpublished, c.f. (3)). The ghosts produced apparently rely on the peptidoglycan of the cell wall for their structural integrity, as the suspension is rapidly cleared with impure, lysozyme-containing, cecropin preparations. Externally added lysozyme has the same effect (Hultmark, unpublished). The rapid onset of lysis (1)
indicates that this is a primary effect of cecropin action, not a secondary phenomenon following cell death. Furthermore, the target must be the cell membrane, rather than the cell wall.

The specificity of the cecropins has been investigated on several occasions. A potent bactericidal effect was first demonstrated for cecropin A and B on some Gram negative strains, using the killing assay (1). These results were later extended in an experiment with 14 Gram positive and Gram negative strains, using the inhibition zone assay (4). Of the tested strains, only Bacillus thuringiensis, a well-known insect pathogen, was totally resistant to cecropin action. Most sensitive was Escherichia coli D31, the organism used in our standard assays.

In contrast to its broad effect on different bacteria, cecropin A was unable to lyse mammalian cells in culture (3). This is also in agreement with the lack of effect of immune hemolymph on sheep erythrocytes (27). The cecropins have some structural similarities to another lytic peptide, melittin, a toxin from bee venom (41). This peptide of 26 amino acid residues also has a positively charged "head", and a hydrophobic "tail", though here the charged region is found in the carboxy terminal of the molecule. In contrast to the cecropins, however, melittin attacks membranes of bacteria and mammalian cells indiscriminately (3).

Boman et al. (46) found an increased sensitivity to hemolymph in E. coli strains that have defects in the lipopolysaccharide of their outer membrane. An increased sensitivity was also recently found in some E. coli mutants that have defects in their outer membrane proteins (I. Sidén, personal communication). Thus, an intact outer membrane appears to give Gram negative bacteria a somewhat increased resistance. The differences in the effects on the outer membrane mutants were most pronounced with cecropin D, which was relatively inefficient against E. coli wildtype. The high positive charge of cecropins A and B, compared to cecropin D, probably make them less hindered by the outer membrane, which is negatively charged.

The strain differences found by Sidén were, however, only evident when calcium ions were present in the assays. As it is usually performed, the agar in the inhibition zone assay contains a rich medium with citrate, a known calcium chelator. Under these conditions, the wild-type strains were found to be as sensitive as those with a defect outer membrane, also to cecropin D. Possibly the outer membrane is rendered leaky in the presence of citrate. Ethylenediaminetetraacetic acid (EDTA), another chelator of divalent cations is known to seriously affect the integrity of the outer membrane (47). The presence of citrate in the assay may also be the explanation to the unexpectedly small differences observed (4) between three Serratia strains, that differ greatly in sensitivity in vivo and in killing assays (48).
THE P5 FAMILY

Compared to the detailed molecular and biological data that have been gathered for the cecropins, the study of the P5 proteins is still incomplete. P5 was originally defined as a 24,000 molecular weight band in SDS electrophoresis of hemolymph, appearing after immunization. In its purified form it was found to lack activity against E. coli in the killing assay (26).

During the development of the first step in the purification of the cecropins, I found a peak of antibacterial activity that was separated from the cecropins by gel filtration (4). It corresponded to an antibacterial band of low mobility in acid gel electrophoresis. In an attempt to purify the active material, it was found to co-purify with material that was reactive to antibodies against P5. This work led to the purification of P5 in an antibacterially active form (5).

A heterogeneity of the protein P5 was indicated already in the work of Pye & Boman (26). In addition to the major peak, electrofocusing at a neutral pH, they found P5-like material also in a more basic peak. An even greater heterogeneity was apparent in the present investigation, when material reacting with anti-P5 antibodies was found to be distributed in no less than six bands in analytical electrofocusing, all of them also showing antibacterial activity (5). Using chromatofocusing, these bands were separately isolated. Electrofocusing and chromatofocusing may sometimes give an artefactual heterogeneity, caused by the formation of different aggregates in the low ionic strength used in these techniques. The P5 heterogeneity was, however, also observed in ion exchange chromatography, performed at a higher ionic strength (Hultmark, unpublished).

The amino acid composition was determined for the six forms (5). The results show that all forms must be related to each other. The two acidic forms, P5 E and P5 F, were virtually identical, F possibly being a degradation product of E. In hemolymph, P5 F is present only in small amounts, and its antibacterial activity appears to be lower than that of the other forms. Similarly, the differences between the four basic P5 proteins, P5 A-E, were within the limits of experimental error. It was suggested that the six forms may arise as a result of post-translational modification of only two primary gene products (5). The amino terminal amino acid sequence of P5 F was also determined. It bears no resemblance to the cecropin sequences.

The P5 proteins had a potent antibacterial activity against E. coli. The effect on this organism was bactericidal rather than bacteriostatic (5). During the purification of the P5 proteins a loss of activity was often encountered. To see if this loss of antibacterial activity is accompanied by a change in the
primary structure, two preparations, one with a high, and one with a low specific activity, were compared in their electrophoretic behavior. They both gave single bands at the same position, both in SDS electrophoresis and in electrofocusing. A loss of activity due to conformational change is also less likely, since the activity is stable during heating, even to 100°C. At present it can not be excluded that the antibacterial activity of P5 is caused by the association of a cofactor. Loss of activity is probably also the reason why Pye & Boman (26) failed to detect the antibacterial properties of P5. They did however obtain a synergistic effect when P5 was combined with two antibacterial ammonium sulphate fractions.

LYSOZYME

A protein with lysozyme-like activity was isolated from the immune hemolymph (1). Its molecular weight (15,000) and amino acid composition were found to be similar to other lysozymes, especially those from lepidopteran sources. The N-terminal amino acid sequence of the H. cecropia lysozyme is compared in Fig. 3 with previously known insect lysozyme sequences, all of them from the order Lepidoptera, and with hen egg white lysozyme. The lysozymes clearly constitute a homogenous group of enzymes.

In addition to the major lysozyme peak, varying amounts of a second peak are usually observed (Hultmark, unpublished). This peak corresponds to a band of slightly lower mobility in acid electrophoresis. The reason for this heterogeneity has not been further investigated.

The purified lepidopteran lysozyme efficiently lysed cells of some Gram positive bacteria, like Micrococcus lysodeicticus, Bacillus megaterium and, more slowly, B. subtilis (1). It had, however, no effect on the Gram negative bacteria Escherichia coli and Pseudomonas aeruginosa (1, 2). Obviously, lysozyme can account for only a minor part of the bactericidal activity in immune hemolymph. A possible synergistic effect of this enzyme with low concentrations of cecropins and P5 proteins remains to be investigated.
Fig. 3. Preliminary amino terminal structure of lysozyme from H. cecropia, compared to other lysozymes. Automatic Edman degradation was performed by Ake Engström, University of Uppsala, Sweden, following previously described procedures (4). Residues 19 and 41 are tentative. The sequence is compared to previously published data for insect lysozymes from *Bombyx mori*, *Spodoptera littoralis* and *Galleria mellonella*, all Lepidopterans (23), and to hen egg white lysozyme (49). Dashes denote undetermined residues, and the empty space between residues 22-24 represents a deletion in the insect lysozymes. The international one-letter code for the amino acids was used:

<table>
<thead>
<tr>
<th>Amino Acid</th>
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<tr>
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<tr>
<td>Cysteine</td>
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<tr>
<td>Aspartic acid</td>
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<td>E</td>
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**OCCURRENCE OF SIMILAR SYSTEMS IN OTHER INSECTS**

To see whether humoral antibacterial factors similar to those found in *H. cecropia* were also present in other species, hemolymph from immunized larvae or pupae from seven other butterflies and moths were screened with the acid electrophoresis technique (2). Our results show that cecropin-like factors and probably also P5 are wide-spread within the order Lepidoptera. Furthermore, we were able to partially purify two cecropin-like proteins and lysozyme from the greater wax moth, *Galleria mellonella* (2). Later, two cecropins have also been purified from the Chinese oak silkworm, *Antheraea pernyi* (50). Their primary structures were found to resemble those of the *cecropia* cecropins B and D. Boman et al. (51) demonstrated the presence of an inducible antibacterial activity in the hemolymph of *Drosophila melanogaster*, a dipteran. Experiments by C. Flyg in this laboratory (personal communication) indicate that this activity may also be due to cecropin-like factors.
DISCUSSION

From our results it appears that the humoral bactericidal response in *H. cecropia* is largely the effect of three classes of antibacterial proteins, the cecropins, the P5 proteins, and lysozyme. Similar factors were probably also the active principles in most of the antibacterial fractions described in previous works (summarized in Table 1). The results of Gingrich (12), Hink & Briggs (13), Kawarabata (14), Bakula (15), and Natori (16) are compatible with the presence of cecropins, if one keeps in mind that the molecular weights obtained by gel filtration are unreliable, and that heat treatment under some circumstances may cause co-precipitation of the cecropins with heat sensitive proteins present in crude preparations. Some of these workers concluded that the active factors were of non-protein nature, but this conclusion was based on unreliable criteria like heat-sensitivity, and precipitability with trichloroacetic acid. Croizier (18) recently isolated an antibacterial protein, "P 20", from the hemolymph of *Spodoptera littoralis*. It was retained by an anion exchanger, and had a molecular weight of 20,000. It may be closely related to the acidic forms of P5.

Other factors may, however, remain to be discovered. Stephens & Marshall (11) isolated an antibacterial fraction from *Galleria mellonella*, that passed dialysis membranes, was weakly acidic, and was unaffected by trypsin. Moreover, Kinoshita & Inoue (17) reported a factor from *Bombyx mori*, that had no activity in itself, but acted like a cofactor to lysozyme, rendering this enzyme active against *E. coli*. These results are not readily explained by the properties of cecropins or P5. Furthermore, in *H. cecropia* an activity against *Bacillus cereus* and *B. thuringiensis* has been demonstrated, the "Bt killing factor". It can be dissociated from the cecropin- and P5-like activities against *E. coli* by selective effects of different inhibitors (27). A similar activity has also been demonstrated in *Locusta migratoria*, an insect that appears to lack cecropins and P5. It was due to a heat-stable and protease-resistant factor of molecular weight less than 5,000 (52).

The humoral immune response in insects appears to lack the high degree of specificity that is characteristic for immunity in vertebrates. It only shows the most fundamental degree of specificity necessary for an immune system - the ability to discriminate between "self" and "non-self". Nevertheless, a distant relationship should exist also between the immune systems of insects and vertebrates. Both systems must have evolved from a primordial immune system that was present in their common ancestor. Even this very primitive animal must have been able to defend itself against microbes.
It is reasonable to assume that the primordial immune system was mainly a cellular one, and that it had the capacity for specific recognition (53). Indeed, histocompatibility and graft rejection, with a specific memory component, have been demonstrated even in the most primitive of present-day metazoans; in Cnidaria (54), and in sponges (55). Specific recognition at the cellular level is also present in higher protostomates such as annelids (56) and probably in insects (57), and may be involved in the primary induction of the immune response we have investigated. The humoral immunoglobulins in vertebrates may later have been developed from membrane-bound immunorecognition molecules of this cellular defence, though Warr (58) has warned that we still have too little data to decide whether invertebrate and vertebrate immunity can be considered homologous. An interesting case of specific humoral antitoxins in cockroaches has also been reported (59). This response also showed a specific memory effect (60).

In addition to the capacity for recognition, the primitive immune system must have possessed effector molecules, perhaps largely intracellular (lysosomal), for the destruction of invading microbes. The humoral factors we have found in insects may be derived from such bactericidal components. Interestingly, cationic antibacterial proteins with characteristics similar to the insect proteins have also been demonstrated in the lysosomes of mammalian phagocytic cells. Several such proteins with molecular weights ranging from 4,000 to 59,000 have been found in polymorphonuclear leucocytes of rabbits and humans (61-63). Basic bactericidal proteins of low molecular weight, the so-called beta-lysin, have also been demonstrated in serum of different mammals. Beta-lysin of platelet origin was described as a heat-stable, cationic protein with a molecular weight of approximately 6,000 (64), or 1,800 (65-67). Finally, a basic antimicrobial protein with a molecular weight between 8,000 and 20,000 has been purified from bovine seminal plasm (68).

There are not yet enough solid data at hand to decide whether there is any true homology between these vertebrate antibacterial proteins and the ones we have found in insects, but at least this is an exciting possibility. Insect immunity may then provide a good model system for non-specific, innate immunity in general.
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Hans Bennich and Åke Engström determined all amino acid sequences.

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