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Antibacterial substances and characteristics of the haemolymph of Chilopoda and Diplopoda (Myriapoda, Arthropoda)

Willi E. R. Xylander

Senckenberg Museum für Naturkunde Görlitz, P.O. Box 300154, 02806 Görlitz, Germany;
e-mail: willi.xylander@senckenberg.de

Abstract

The antibacterial characteristics of the haemolymph of Chilopoda and Diplopoda are reviewed and new results are presented. Substances acting against gram-negative as well as gram-positive bacteria and haemolysins have been found in the haemolymph (in haemocytes and in the plasma) of various species. These substances are sensitive against heating. In general, the chilopods tested showed higher antibacterial activity than the diplopods. Some substances occur permanently in the haemolymph but their amount can be increased by immunisation (with bacteria or cell-wall components). Age had no effect on antibacterial activity. One of these substances is lysozyme, the MWs of which range from 15.5 to 16.5 kD with regard to the species tested. Although substances in *Triaenostreptus* spec. showed similarity with cecropins in PAGE under acidic conditions, antibodies against cecropin A from *Hyalophora cecropia* Linnaeus, 1758 did not react with substances in the haemolymph of the myriapods tested.

Keywords: immunity, inducibility, lysozyme, haemolysins

1. Introduction

Two strategies of immune defence have been described from arthropods: (a) the cellular immune response by haemocytes and other tissues resulting in phagocytosis of smaller foreign bodies and nodule or capsule formation around larger xenografts and (b) the humoral defence, which occurs extracellularly but is normally cell-mediated. Both types of reactions have also been described for Diplopoda and Chilopoda (e.g. Xylander & Nevermann 1990, Nevermann et al. 1991, 1996, Xylander 1992, Nevermann & Xylander 1996).

Antibacterial defence relies on both systems: During cellular immune reactions, bacteria and other microbes can be phagocytosed and destroyed intracellularly by lysis and metazoan parasites are encapsulated by numerous haemocytes that isolate the parasites from their nutrient resources. On the other hand, melanin and the intermediate products of its formation may be deposited on the surface of microorganisms and act bactericidal. Furthermore, specific antibacterial substances can be found, which may occur in the haemolymph permanently or are induced after an infection or injury. Additionally, haemolysins can kill bacteria.

Antibacterial substances (ABS) have been found in nearly all larger groups of arthropods and other invertebrates (e.g. Boman 1986, Xylander & Nevermann 1990, Xylander et al. 1997). Over the last 25 years, several classes of ABS were characterised including lysozyme, cecropin, attacin, diptericin, abaecin, apidaecin, and coleoptericin (e.g. Mohrig & Messner

1968, Okada & Natori 1983, 1985, Boman 1986, Dunn 1986, Keppi et al. 1986, Boman & Hultmark 1987, Casteels et al. 1989, 1990, Bulet et al. 1991, Samakovlis et al. 1991). Whereas many investigations focussed on insects, only a few have dealt with other invertebrate groups. Kawano et al. (1990) found lectins and other substances with antibacterial capabilities in *Limulus* spec., and Fenouil & Roch (1991) and Xylander et al. (1997) showed that lysozyme and other ABS occur in the haemolymph of crustaceans.

Antibacterial substances against gram-positive and gram-negative bacteria have also been described from the haemolymph of Diplopoda and Chilopoda (Xylander 1989, 1992, van der Walt et al. 1990, Xylander & Nevermann 1990, Jarosz et al. 1991, Nevermann et al. 1996). However, little is known about the nature of these substances:

(a) Xylander & Nevermann (1990) showed that one of the substances is a lysozyme;

(b) van der Walt et al. (1990) as well as Xylander & Nevermann (1990) showed inducible antibacterial activity against gram-negative bacteria;

(c) van der Walt et al. (1990) presented an acidic PAGE of diplopod haemolymph with microbial overlay where an inhibition zone was visible;

(d) in a study on crustacean antibacterial haemolymph substances, Xylander et al. (1997) used the haemolymph of a diplopod as a reference and showed that its lysozyme had a MW of about 14.5 kD;

(e) Nevermann (1996) showed that lysozyme is formed and stored in haemocytes in chilopods.

In this paper new results are presented on the antibacterial substances in Diplopoda and Chilopoda.

2. Materials and methods

Animals

The investigations described were performed with specimens of the following species: *Rhapidostreptus virgator* (Silvestri, 1907)*, Chicobolus* spec., *Lithobius forficatus* (Linnaeus, 1758) and *Scolopendra cingulata* Latreille, 1789. The specimens were obtained and reared as described previously (Nevermann & Xylander 1996).

Immunisation

For investigation of antibacterial activity, specimens were ‘immunised’ by inoculation with a suspension of bacteria or bacterial cell-wall components as described previously (Xylander & Nevermann 1990, Xylander et al. 1997). Specimens inoculated with ringer only or untreated specimens served as control.

Preparation of agar plates

Bacteria were obtained and prepared as described previously (Xylander & Nevermann 1990). All manipulations except inoculation were performed in a clean bench.

SDS-PAGE with murein (ML-lyo gels)

For investigation of the molecular weight (MW) of lysozyme in various Diplopoda and Chilopoda, we developed a procedure for a native SDS-PAGE that has been briefly described previously (Xylander et al. 1997). The gel buffer contained an autoclaved suspension of

lyophilised *Micrococcus lysodeikticus* (Schröter, 1872), resulting in an opaque gel after polymerisation. After PAGE the proteins were 'activated' by incubation in Triton X 100, and lysozyme activity caused a lysis zone that could be correlated to the MW of the proteins of a molecular standard applied to the same gel.

Acidic SDS-PAGE

For detection of molecular similarities of ABS acting against gram-negative bacteria, an acidic native gel-electrophoresis according to Hultmark et al. (1980) was performed. Subsequently, proteins were blotted to nitrocellulose and incubated with rabbit antibodies against cecropin A from *Hyalophora cecropia* Linnaeus, 1758.

The 15 % acrylamide gel used consisted of 7.5 ml of an acrylamid stock solution (40 g acryamid, 0.53 g bis-acrylamid in 100 ml aq. bidest.), 3 ml gel buffer (7.15 ml conc. acetic acid, 40 ml aq. bidest. adjusted to pH 4 with 40 % KOH and subsequently filled to 50 ml with aq. bidest.), 9 ml aq. bidest., 280 µl APS and 100 µl TEMED. Electrophoresis was performed in a Biorad-electrophoresis-apparatus at 4 °C (cooling with circulation) with a constant current (50–60 V) and a specific buffer (1.6 g acetic acid conc., 62.5 g ß-alanine adjusted to pH 4 with HCl and filled to 2 l with aq. bidest; this buffer was diluted 1:10 with aq. bidest prior to use); the buffer was permanently exchanged during PAGE. Ten µl haemolymph samples from immunised *Hyalophora cecropia*, *Galleria mellonella* (Linnaeus, 1758), *Chicobolus* spec. and *R. virgator* each were applied to the gel.

After electrophoresis the gel was transferred to a Biorad Western-Blot-apparatus. Proteins were blotted from the gel to a nitrocellulose membrane with 0.8 mA cm^{-2} for 2 h at room temperature. The membrane was incubated under permanent movement overnight in a 5 % milk powder suspension in Tris-buffer (2.42 g Tris, 29.22 g NaCl in 1 l aq. bidest.). Subsequently, buffer was removed and the membrane washed twice in Tris-buffer. Rabbit antibodies against cecropin A from *Hyalophora cecropia* were diluted 1:250 in bovine serum albumin and the membrane was incubated in this antibody suspension for 3 h under moderate movement. Then the membrane was washed four times for 10 min in a Tris solution (2 l Tris-buffer, 0.1 % bovine serum albumin, 0.05 % Tween 20) and subsequently incubated in a suspension of antibodies (swine against rabbit) coupled with alkaline phosphatase (11 ml Tris-buffer with bovine serum albumin, 14 µl antibodies). The membrane was then washed in reaction buffer without substrate (50 mM Tris + 1 mM Mg^{2+}, pH 8.3). The substrate of the alkaline phosphatases (20 mg alpha-naphtylphosphate + a spatula tip Fast Blue B) was diluted in reaction buffer and the nitrocellulose membrane incubated until the staining reaction was clearly visible. Staining was stopped by transferring the membrane to another container filled with aq. bidest. The membrane was dried between two layers of filter paper and stored protected from light.

Haemolysins

For testing myriapod haemolymph for the occurrence of haemolysins, 5 to 10 µl haemolymph were applied (as in the ABS tests) to agar plates containing sheep erythrocytes. 1 % agar solutions (Merck, blood agar Basis, Purch. No. 10886) was sterilised and 5 % sterile sheep blood was added after the agar solution cooled down to 45 °C. Agar plates were stored in the refrigerator until use. Immunisation of arthropods prior to application was performed with sterile *M. lysodeikticus* suspension (see above). Lysis zone diameters were determined after storing the plates for 24 h at room temperature.

3. Results

Occurrence of lysozyme

Within the haemolymph of non-immunised diplopods and chilopods, substances inhibiting the growth of living *Micrococcus luteus* (Schröter, 1872) and lysing the cell wall of gram-positive bacteria were present. Haemolymph of *R. virgator*, *L. forficatus* and *Scolopendra oraniensis* H. Lucas, 1846, but not *Chicobolus* spec. formed transparent lysis zones in agar with suspended lyophilised *Micrococcus lysodeikticus* by depolymerising murein (Fig. 1). The diameter of the zones decreased from *S. oraniensis* to *R. virgator* and *L. forficatus* Tab. 1). The diameter of the inhibition zones on living *Micrococcus* was generally larger in Chilopoda than in Diplopoda (Tab. 1 and Figs 1 and 2, see also Xylander & Nevermann 1990).

Lysis and inhibition zones could also be found after application of homogenated accessory genital glands taken from female *Scolopendra cingulata* during its brood-care period. A homogenate of its salivary glands had no antibacterial effect.

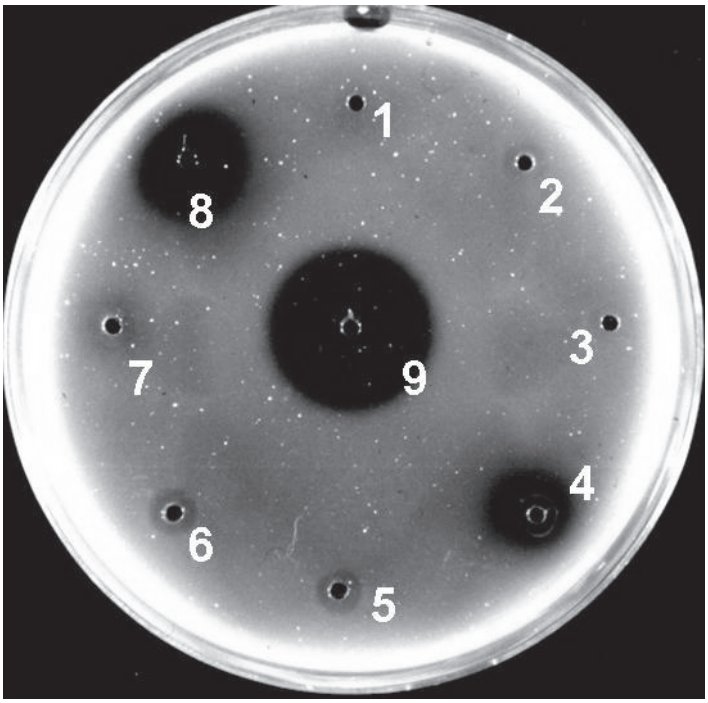

Fig. 1 Lysis of murein by lysozyme from the haemolymph of various myriapods as indicated by transparent zones in agar plates containing lyophilised *Micrococcus lysodeikticus*. Samples applied: 1–3: *Chicobolus* spec.; 4–6: *Rhapidostreptus virgator*; 7: *Scolopendra cingulata*; 8: *Manduca sexta* (Lepidoptera); 9: lysozyme standard.

Tab. 1 Inhibition-zone and lysis-zone diameter [% of a lysozyme standard solution] in haemolymph samples of untreated diplopod and chilopod species against living and lyophilised gram-positive bacteria (*Micrococcus* spec.). n. d. = not detected.

	lyoph. *Micrococcus lysodeikticus*	living *Micrococcus luteus*
R. virgator	22 %	27 %
Chicobolus spec.	n. d.	38 %
L. forficatus	35 %	50 %
S. oraniensis	21 %	42 %

Other antibacterial substances

Xylander & Nevermann (1990) reported that no inhibition zones were found in agar with suspended *Escherichia coli* (Migula, 1895) after application of 4 µl of haemolymph of different chilopod and diplopod species, whereas the growth of another gram-negative bacterium, *Enterobacter cloacae* (Jordan, 1890), was inhibited after application of the same volume of haemolymph. This inhibition was found with and without stimulation of the immune system. Zone diameter was larger in the chilopod species (*Lithobius*: 62 % of a standard, *Scolopendra oraniensis*: 45 %) than in Diplopoda.

Small inhibition zones against *E. coli* were found in a few specimens of *S. cingulata*, *L. forficatus*, *R. virgator* and *Chicobolus* spec. (10–20 % of the specimens investigated) after application of 10–15 µl of haemolymph, whereas haemolymph from the majority of specimens treated in the same way had no inhibitory effect. Equivalent results were found with another gram-negative bacterium (*Pseudomonas flavescens*). The reason for this variation is unknown. Inhibitory effects were observed more often in immunised specimens (80 % of the observations). In all cases the inhibition zones were significantly smaller than those caused by haemolymph from immunised L5-instar larvae of *Manduca sexta* (Linnaeus, 1763) used as reference (see Xylander & Seifert 1990 for the antibacterial effect of different stages of this lepidopteran).

Effect of age/body weight on the antibacterial effect

The antibacterial effect showed no correlation with weight (as an indication of age) in the Diplopoda tested. Haemolymph of juveniles of *R. virgator* (body weight: 2.3 to 4.5 g) had the same inhibitory capabilities on *Micrococcus* as mature specimens (correlation coefficient $r = 0.175$, $n = 9$). The same was found in *Chicobolus* spec. ($r = 0.098$, $n = 16$). However, in *Chicobolus* only semiadult specimens (body weight: 1.75 to 2.35 g) were used for a reference, as not enough haemolymph could be obtained from smaller individuals.

Location of antibacterial substances in *R. virgator*

Comparative tests on antibacterial activity against gram-positive bacteria with whole haemolymph, plasma or haemocytes lysate in *R. virgator* showed significant antibacterial effect in all three, whereas the cacodylate buffer (in which the haemocytes had been resuspended prior to sonification) applied here as a control had no effect (Fig. 2). Haemocyte lysate had about the same effect as the plasma. Effects were also found against the gram-negative bacterium *Bacillus subtilis*, but here the effect of the haemocyte lysate was lower. In

tests on agar with *Pseudomonas flavescens* haemocytes lysate as well as total haemolymph showed growth inhibition, whereas plasma resulted in such weak effects that they could not be quantified. Effects against *E. coli* could not be found in any test or haemolymph fraction.

Obviously, antibacterial substances are located in the haemocytes as well as freely in the plasma.

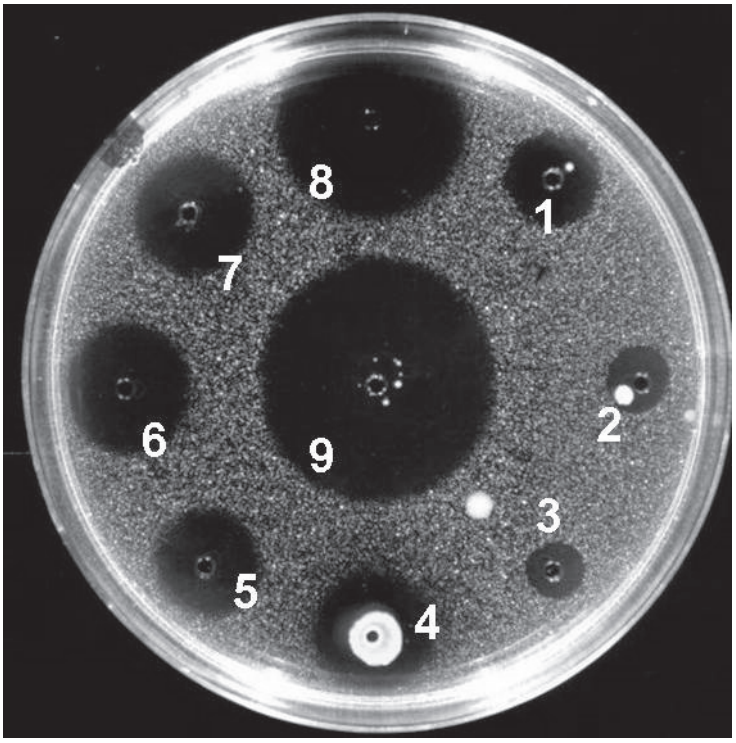

Fig. 2 Inhibition of bacterial growth by haemolymph of various myriapods as indicated by inhibition zones on agar plates containing living *Micrococcus luteus*. Samples applied as in Fig. 1.

Inducibility of antibacterial substances: a review

After inoculation with *E. cloacae* ß-12, which induces antibacterial immune responses in insects, the bacteriostatic effectivity of diplopod haemolymph increases (Xylander & Nevermann 1990). In *Chicobolus* spec. the inhibition zones increased from initially 35.2 % to a maximum of 54.5 % of a lysozyme standard after inoculation. The antibacterial effect decreased again after a few days. Wounding also induced antibacterial effectivity in this species. After immunisation the effect increased in *R. virgator* from 23.7 % to 37.6 % on agar with living *Micrococcus* (inhibitory effect) and from 21.7 % to 28.7 % on agar with lyophilised *Micrococcus* (murein lysis). These reactions are weak when compared to the immune responses of insects.

In the spirostreptid diplopod *Triaenostreptus triodus* (Attems, 1909), immunisation with very high doses of *E. coli* and applying extraordinarily large amounts of haemolymph (30 µl) on test agar with suspended *E. coli* resulted in clear inhibition zones (van der Walt et al. 1990). Untreated specimens of the same species had no effect against the bacterium. Xylander & Nevermann (1990) could not find effects against *E. coli*: All specimens tested died within 24 h after application of about 10^5 bacteria g^{-1} body weight (this bacterial load was significantly lower than that applied by van der Walt et al. 1990). Immunisation with higher concentrations of *E. cloacae* (3–6 10^7 g^{-1} body weight) caused death within 1 to 2 days. Within this period, no significant formation of ABS against *E. coli* could be found.

After application of autoclaved suspensions of lyophilised *Micrococcus lysodeikticus* for immunisation, ABS against *E. coli* were found in *R. virgator* und *Chicobolus* spec. However, larger amounts of haemolymph had to be applied to the agar plates to see an effect (Xylander 1992).

Heat resistance

ABS in the diplopod species investigated were sensitive to heating. Mean inhibition zone diameter of untreated *Chicobolus* decreased from 41.1 % to 17 % (n = 7) of a lysozyme standard (Xylander & Nevermann 1990); a decrease was also found in *S. oraniensis*. In *R. virgator* the antibacterial effect vanished completely after such treatment (n = 6).

Acidic PAGE and Western blot

In Western blot, after separating haemolymph samples of two immunised lepidopterans, *Chicobolus* spec. and *R. virgator* on an acidic PAGE, the antibody against cecropin A from *Hyalophora cecropia* reacted exclusively with the cecropin of this species, showing one clear and a faint second band (the second probably resulted from another cecropin with a higher MW). No staining reactions occurred with haemolymph samples of the two diplopod species nor with that of *Galleria mellonella* (Fig. 3). Obviously, the antibody did not recognise ABS of the other species, which are not identical with cecropin A from *H. cecropia*. Therefore, from these results, the occurrence of cecropin or cecropin-like substances in Diplopoda cannot be excluded.

MW of myriapod lysozyme: Results from the native SDS-PAGE with a ML-lyo gel

After incubation of the gels in Triton X 100, lysis zones became visible after 2 h (egg-white lysozyme) to 3 h (Diplopoda). Zones from egg-white became fully transparent within 24 h, whereas those from diplopods remained greyish. Lysis zones from egg-white lysozyme and *M. sexta* haemolymph showed a corresponding running distance of about 14 kD (and a second, less significant one in egg-white lysozyme probably representing lysozyme g, see Audy et al. 1989). Lysis zones in samples from *R. virgator* and the chilopods showed more distance to the SDS-running front and were estimated to be 15.5 kD (*R. virgator*), 16 kD (*L. forficatus*) and 16.5 kD (*S. cingulata*) according to the rf-calculation of the molecular standard (Fig. 4). The sample with haemolymph of *Chicobolus* spec. showed no visible lysis zone.

Fig. 3 Western blot of haemolymph samples from different insects and diplopods onto nitrocellulose after PAGE run under acidic conditions and incubated with rabbit antibodies against cecropin A from *Hyalophora cecropia* (Lepidoptera). For details of the preparation procedure, see Materials and methods section. Samples applied: 1: *Hyalophora cecropia*; 2: *Rhapidostreptus virgator*; 3: *Chicobolus* spec.; 4: *Galleria mellonella* (Lepidoptera).

A rough estimation of lysozyme activity (according to Audy et al. 1989) resulted in a 200-fold lower lysozyme activity in the samples from *R. virgator* and the chilopods than the lysozyme standard and a 20-fold lower activity compared to *M. sexta*. Referring to the information of the supplier of the lysozyme standard, lysozyme activity of *R. virgator* and *L. forficatus* ranges around 50 U ml^{-1} haemolymph. The activity in *Chicobolus* seems to be significantly lower.

Haemolysins

Haemolymph from *R. virgator* and *Chicobolus* spec. caused erythrocyte lysis on the blood agar plates, indicating the occurrence of haemolysins, whereas haemolymph of *L. forficatus* did not (Fig. 5). Lysis zones diameter with haemolymph samples from immunised diplopods did not differ from those of untreated specimens. Lysis zones caused by diplopods were somewhat smaller than those from *M. sexta*.

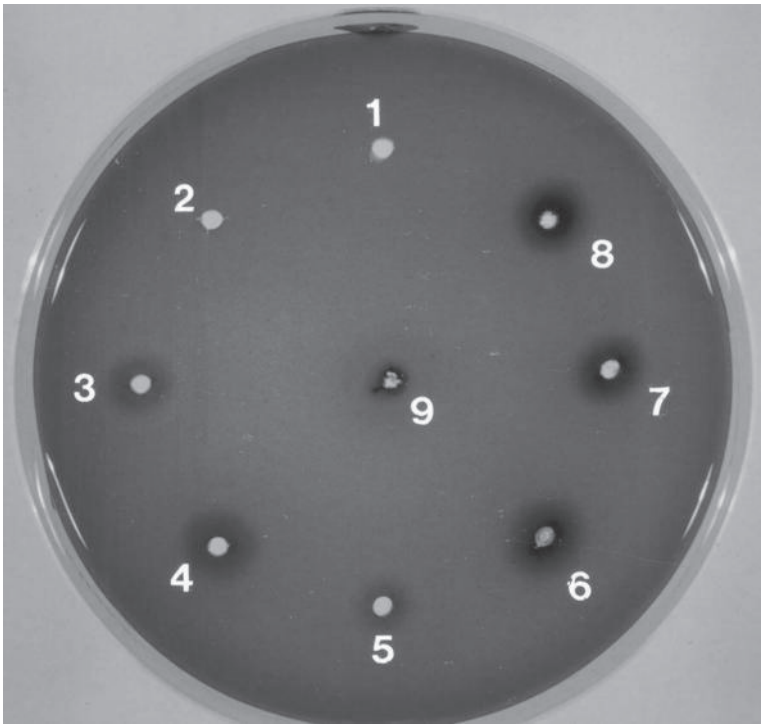

Fig. 5 Lysis zone in sheep-erythrocyte-agar, indicating haemolysin activity in the haemolymph of various myriapods and *Manduca sexta*. For details of the preparation procedure, see Materials and methods section. Samples applied: 1–2: *Lithobius forficatus* (not immunised, n.i.); 3: *Chicobolus* spec. (n. i., 5 µl); 4: *Chicobolus* spec. (n. i., 10 µl); 5: *Rhapidostreptus virgator* (n.i., 5 µl); 6: *Rhapidostreptus virgator* (n. i., 10 µl); 7: *Rhapidostreptus virgator* (immunised, 5 µl); 8: *Rhapidostreptus virgator* (immunised, 10 µl); 9: *Manduca sexta* (immunised, 5 µl).

4. Discussion

In arthropods, numerous different antibacterial substances have been described and characterised (see overview listed in Xylander 1994), which differ in regard to their mode of action, activity, tissue of formation and chemical composition. These substances can be divided into those that show effects against gram-negative bacteria (which are often also effective against gram-positive) and those that act preferentially (or exclusively) against gram-positive bacteria (e.g. lysozyme).

Lysozyme

Lysozyme is the major ABS against gram-positive bacteria found in the haemolymph of arthropods (Mohrig & Messner 1968); it enzymatically depolymerises the multi-layered murein sacculus of these bacteria between N-acetylglucosamine and N-acetyl-muraminic acid (Schlegel 1985). Lysozyme is described as being heat resistant; it is composed of about 120 amino acids and its MW ranges between 13.8 to 15.5 kD (Engström et al. 1984, Boman 1986, Boman & Hultmark 1987, Götz 1988, Carlsson et al. 1991). The major functions of lysozyme are (1) to destabilise the cell by depolymerising the cell wall and thus to make the cell wall more easily accessible for other ABS and (2) to enhance lysis of bacteria killed by the ABS (Boman 1986).

The lysozyme of insects is similar to that of egg-white (see Engstöm et al. 1985, Boman 1986, Dunn 1986, Rosenthal & Dahlmann 1991). However, in *Trichoplusia ni*, Andersons et al. (1990) found a lysozyme with somewhat different characteristics.

The lysozyme of Diplopoda and Chilopoda had a slightly higher MW than that of insects and crustaceans (Xylander et al. 1997). Furthermore, the lysozyme activity in Myriapoda is significantly lower than in insects. This is rather due to a lower quantity of lysozyme per haemolymph volume than from a lower enzymatic activity.

Chilopods showed a higher average activity than diplopods (Xylander & Nevermann 1990, this paper). Most likely, the almost ten-fold higher number of haemocytes in chilopods compared to diplopods (see Xylander & Nevermann 2006, Xylander 2009), which are the location of lysozyme formation and storage (see Nevermann 1996), results in a higher quantity of lysozyme. However, the individual activity variation was high in both groups. Similar variation between tested specimens has also been described for decapod crustaceans by Fenouil & Roch (1991).

Other ABS

The most important ABS in insects are the cecropins (Hultmark et al. 1982, Boman et al. 1986, Morishima et al. 1990). They form amphipatic alpha-helices with different charges on each side of the molecule (Boman et al. 1986, see also Jaynes 1989, Xylander 1994). Single cecropin molecules aggregate and build clusters, which form pores in the membranes of their target bacteria, destabilising the cell/outside ion gradient (Okada & Natori 1985, Steiner et al. 1988, Jaynes 1989) and thus destroying many gram-negative as well as gram-positive bacteria. Other ABS found in insects are attacins (Boman 1986), diptericins (Keppi et al. 1986, 1989), apidaecins (Casteels et al. 1989), coleoptericins (Bulet et al. 1991) and andropins (Samakovlis et al. 1991). From Xiphosura different tachyplesins and polyphemusins are known that have fungistatic and bacteriostatic effects on gram-negative and gram-positive bacteria (Murakami et al. 1991).

Besides lysozyme activity, some weak growth inhibition against gram-negative *E. cloacae* and *P. flavescens* as well as *E. coli* has been observed in diplopods and chilopods (Xylander & Nevermann 1990, Xylander 1992). This effect could even be found in untreated animals; however, the number of specimens presenting an effect increased significantly after immunisation. It seems probable that these ABS are present permanently in the population investigated, e.g. due to contamination of the diet with bacteria. Therefore, feeding on the diet may induce a weak immune reaction permanently and the effect found was a result of 'immunisation' via the food supply (see Samakovlis et al. 1990).

A substance with antibacterial effects against *E. coli* had been described from the diplopod *T. triodus* by van der Walt et al. (1990). It is a protein/oligopeptide, relatively small and strongly negatively charged. With regard to these characters as well as the migration pattern in the acidic SDS-PAGE (according to Hultmark et al. 1980), this protein resembles the cecropins. However, van der Walt et al. (l.c.) did not try any comparative tests with, e.g., ABS from lepidopterans. As a speciality of *T. triodus*, ABS in this species could only be found after immunisation with very high doses of *E. coli*. Also other experiments such as Western blotting with subsequent application of antibodies against cecropin as well as gel filtration chromatography (data not shown) did not show any results that could help to answer the question of the similarity or even homology of ABS against gram-negative bacteria in millipedes and insects.

Nonetheless, results indicate that the factor from the haemolymph of myriapoda tested here inhibiting the growth of gram-negative bacteria was not lysozyme: Even the haemolymph of species that did not cause Lysis zones on agar with lyophilised *M. lysodeikticus* had clear bacteriostatic effects against living *M. luteus*. These were even more effective in *Chicobolus* spec. than in *R. virgator*.

The growth of gram-negative *Enterobacter cloacae* was inhibited by haemolymph from *Chicobolus* spec., *R. virgator*, *L. forficatus* and *Unciger foetidus* (C. L. Koch, 1838) (Xylander & Nevermann 1990, Jarosz et al. 1991). Egg-white lysozyme showed no inhibitory effect on the same plates.

After application of higher doses of haemolymph, a bacteriostatic effect could also be found against *E. coli* and *P. fluorescens*.

Therefore, it is highly probable that the antibacterial substances found in *T. triodus* (or very similar ones) can also be found in other diplopod species (and in Chilopoda).

The ABS found in the genital glands in *S. cingulata* may have a protective function for the genital tract (as shown for andropin in insects); however, their major task is considered to be the protection of eggs and early juvenile stages against bacterial infections during brood care. These substances, which occur permanently and do not have to be induced by immunisation, increase in effectivity during egg laying and brood care (Radl pers. comm.). This is another similarity to andropin.

Inducibility

Lysozyme occurs permanently in the haemolymph and other tissues of arthropods (e.g. Dunn 1986, Mohrig & Messner 1968, Xylander & Nevermann 1990, Xylander et al. 1997), but its titre normally increases after infection, injury or immunisation, reaching many times the normal level (Mohrig & Messner 1968, Hultmark et al. 1980, Dunn et al. 1985, Trenczek 1988). Such effects have also been shown for myriapods (Xylander & Nevermann 1990). However, the period from the trigger event to a measurable effect is much shorter in insects (6–15 h, see Hultmark et al. 1980, Hoffmann et al. 1981, Keppi et al. 1986) than in Diplopoda (about 72 h, see Xylander & Nevermann 1990), and also the time span for reaching the maximum effect is longer in Myriapoda.

In contrast to lysozyme, the formation of other ABS needs a more or less specific trigger (Hoffmann 1980, Spiess et al. 1986, Boman 1986, Dunn 1986, Postlethwait et al. 1988, Carlsson et al. 1991). A few observations indicate that gene expression for ABS can occasionally be found without an observable trigger (Samakovlis et al. 1991). Also other proteins may be induced by infections, such as haemolin, another protein of the immunoglobulin super-family (Faye 1990, Sun et al. 1990, Schmidt 1991).

For *T. triodus*, van der Walt et al. (1990) also found that ABS against *E. coli* occurs only after immunisation. In contrast, ABS from the haemolymph of Chilopoda and other Diplopoda against other gram-negative bacteria were found also without immunisation (Xylander 1989, Xylander & Nevermann 1990, Jarosz 1991).

Sites of formation and storage of ABS

In insects, the fat body seems to be the most important site of ABS formation; ABS are later discharged into the haemolymph (Faye & Wyatt 1980, Dunn et al. 1985, Dunn 1986, Keppi et al. 1986, Trenczek & Faye 1988, Russell & Dunn 1990). Furthermore, haemocytes may synthesise (or at least set free) various ABS (Zachary & Hoffmann 1984, Dimarcq et al. 1990, Samakovlis et al. 1990, Fenouil & Roch 1991, Murakami et al. 1991, Toh et al. 1991, Nevermann 1996, Xylander et al. 1997).

In diplopods, haemocytes have also been shown to build ABS, e.g. lysozyme. The ABS was found to be equally distributed in the plasma and the corresponding haemocyte lysate, whereas in *Lithobius forficatus* most lysozym was found in the haemocyte lysate and little in the plasma (Nevermann, pers. comm.). ABS were considered to be synthesised and stored in the haemocytes and discharged e.g. after bacterial infection, as shown in other arthropods without an elaborated fat body, e.g. Xiphosura or Crustacea (Fenouil & Roch 1991, Murakami et al. 1991, Nakamura et al. 1988, Toh et al. 1991, Xylander et al. 1997). Probably, the formation of the majority of ABS in haemocytes (rather than other tissues as in insects) may explain the comparatively low titers and slower formation in Xiphosura, Crustacea and Myriapoda when compared with insects.

Various ABS can act synergistically to kill and degrade bacteria phagocytised by single haemocytes or haemocyte nodules (e.g. Götz 1988, Russell & Dunn 1990, Nevermann et al. 1996, Nevermann & Xylander 1996). Their localisation inside the haemocytes appears to be a prerequisite for a successful cellular immune defence. Furthermore, quick intracellular degradation, e.g. by lysozyme, accelerates the regeneration of haemocytes for future immune responses (Russell & Dunn 1990).

Various ABS of arthropods have, however, been found in other tissues than haemocytes and fat body, such as in the genital tract of Scolopendra (this paper), the pericardial complex of *Manduca sexta* (Russell & Dunn 1990), and the gut epithelia, hind gut, male genital tract or salivary glands of dipterans (Lemos & Terra 1991, Samakovlis et al. 1991, Tryselius et al. 1992). It is not absolutely clear, however, whether the ABS found in these tissues are all synthesised there or in haemocytes or the fat body and are subsequently transferred to the target tissues via the haemolymph.

Haemolysins

Haemolysins in the body fluids have been described from various groups of invertebrates. They are considered to destroy gram-negative bacteria, protozoa, metazoan parasites and pathogen-modified body cells or tissues (Kauschke & Mohrig 1987a, b, Canicatti 1990, Roch et al. 1991). They may also help to opsonise foreign material for other elements of the immune system (Canicatti 1990).

Haemolysins recognise molecules on the surface of foreign cells and tissues (carbohydrates or lipids), aggregate, thereby often change their conformation and subsequently integrate into the cell membrane of the pathogen forming a transmembraneous channel or pore (Valembois et al. 1986, Roch et al. 1989, Canicatti 1990, 1991, Ojcius & Young 1990, Raghunathan et al. 1990). The pore significantly disturbs the ion equilibrium between the pathogen and its surroundings, often causing its death.

In various invertebrates an increase in the haemolysins titre was found after immunisation (Kauschke & Mohrig 1987b, Canicatti et al. 1988, Phipps et al. 1989). This was not the case in *R. virgator* in this study. In other invertebrates, haemolysins are synthesised and stored in haemocytes (Valembois et al. 1986, Canicatti & Ciulla 1987, Canicatti et al. 1988, Leippe & Renwrantz 1988a, b). Presently, there is no indication of the tissue where haemolysin is formed in Myriapoda, but it can be presumed that also haemocytes are involved.

Whether the haemolysins of Myriapoda described are identical with the ABS (against gram-negative bacteria) has to be investigated in the future.

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