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Physico-chemical properties of haemolymph of Chilopoda and Diplopoda (Myriapoda, Arthropoda): protein content, pH, osmolarity

Willi E. R. Xylander

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

Abstract

Different physico-chemical properties of the haemolymph of various chilopod (*Lithobius forficatus* and *Scolopendra cingulata*) and diplopod species (*Rhapidostreptus virgator*, *Chicobolus* spec.) were investigated. Haemolypmph pH ranged from 7.0 to 8.5 being slightly more alkaline in the millipede species. Protein content and osmolarity was higher in chilopods (between 296 and 371 mOsm and 65 to 73 mg protein/ml haemolymph) than in diplopods (200 to 229 mOsm and 21 to 56 mg/ml). A chromatogram-spectrophotometry of SDS-PAGEs of haemolymph samples showed 42 to 53 protein fractions in *R. virgator* and 43 in *Chicobolus* spec. (19 and 16 respectively, represented larger fractions). A haemocyte lysate only showed 12 protein fractions none of which exclusively occurred in the lysate. The results are discussed with regard to earlier investigations which partly differed significantly.

Keywords: immune response, protein spectrum, haemocytes, plasma

1. Introduction

Whereas several investigations dealt with haemocyte types and functions (Ravindranath 1973, Nevermann & Xylander 1996, Kania & Rzeski 1997, Xylander & Nevermann 2006, Xylander 2009a), antibacterial substances (Xylander & Nevermann 1990, review in Xylander 2009b) and the phenoloxidase system in Diplopoda and Chilopoda (Xylander & Bogusch 1992, 1997, Xylander 1996) only little information is available on their general haemolymph protein composition and physico-chemical properties.

Thus, Rajulu (1969, 1970, 1971, 1974) investigated the haemolymph of various diplopods, chilopods and other arthropods with regard to protein content, carbohydrates, and lipids. Helbing (1985) and Nevermann (1996) determined the haemolymph pH and total protein content, and protein pattern in the centipedes *Lithobius forficatus* and *Scolopendra cingulata*. The present study is aimed to contribute to our knowledge and understanding of physico-chemical properties of the haemolymph of Myriapoda.

2. Materials and methods

Animals investigated for obtaining haemolymph

For this study mature specimens of the following diplopod and chilopod species were used

(a) Diplopoda: *Rhapidostreptus virgator* (Silvestri, 1907), *Chicobolus* spec.

(b) Chilopoda: *Lithobius forficatus* (Linnaeus, 1958), *Scolopendra cingulata* Latreille, 1829.

Specimens were obtained and reared as described earlier (Xylander & Nevermann 1990). Haemolymph and haemocyte lysate was obtained according to the procedure description by Xylander & Bogusch (1997).

Protein content

Total protein content of haemolymph was determined photometrically according to Lowry at 750 nm. Prior to determination of the protein content haemolymph, was frozen and stored for several days at 15 °C to 18 °C. For the investigation a Protein Assay Kit by Sigma (Munich, No. P 5656) was used according to information by the supplier. As protein reference a 6 % bovine serum albumin solution was used. Prior to testing the haemolymph was diluted 1:200 with aqua dest. in acordance with the instructions.

Quantification of haemolymph proteins after SDS-PAGE

After SDS-PAGE under reducing conditions (6 % sampling gel, 10 % separation gel) and Coomassie staining, the gel was dried in cellophane and proteins bands were evaluated according to their running distance (as indicator of molecular weight, MW) and thickness (indicating amount of the specific protein) using a chromatogram (Carl Zeiss, Oberkochen) with a light source with 580 nm, a cleft breadth of 0.01 mm and a cleft width of 3.5 mm. The height of measuring device was 5 mm, the table moved 15 mm min^{-1}. A front lens was not used. For calculation a spectra calculation integrator by Physics (Darmstadt) was used.

As proteins bands only absorption maxima were counted occurring in different gels and haemolymph samples obtained from different specimens. Proteins with a very high MW (> 350–450 kD) which only had not entered the separation gel under the experimental conditions applied were not included.

Osmolarity

Investigations of osmolarity of haemolymph samples was performed using freezing-point determination by a OM 801 micro-osmometer by Fa. Vogel.

pH

For estimation of haemolymph pH punches of pH-paper were used (Merck Neuralit, Art. No. 9564, pH range: 5.5–9.0). Punches were placed on clean slides and moistened with 5 µl aqua dest. and were left for 3 min at room temperature. Then 5 µl haemolymph was added (from specimens not priorly narcotised by CO_2). The pH was estimated after 3 min referring to the pH scale from the supplier. The punches still were moist at that time. In pretests with several buffers this method had shown to have a measuring error of < 0.5. Parallel testing of haemolymph samples with other pH tests (Merck Alkalit, Art. No. 9532, pH range 7.5–14.0) came to corresponding results.

3. Results

Protein content of haemolymph

The protein content of haemolymph ranged between 21 mg ml^{-1} in *Chicobolus* spec. and 73.2 mg ml^{-1} in *Scolopendra cingulata* (Tab. 1). *Rhapidostreptus virgator* had 56 mg ml^{-1} and *Lithobius forficatus* 65.5 mg ml^{-1}.

Tab. 1 Protein content of the haemolymph of different diplopod and chilopod species.

Species	protein content [mg ml^{-1}]	n
Chicobolus spec.	21.0	9
Rhapidostreptus virgator	56.0	2
Lithobius forficatus	65.5	2
Scolopendra cingulata	73.2	2

Quantification of haemolymph proteins after SDS-PAGE

Only protein spectra of the two diplopod species were investigated which showed to differ significantly. The vast majority of detectable proteins had MW higher than 60 kD.

In the total haemolymph of *R. virgator* 42 to 53 absorption maxima were found in 4 separations from different specimens; 19 of these maxima represented larger continuously found protein fractions (Tab. 2, Figs 1 and 2). Largest protein amounts were in fractions 4 (ca. 210 kD), 6 (165 kD), 9 to 12 (110–70 kD) and 17 (18 kD) (see Figs 1 and 2). The relative protein quantity of the fractions were 3 % in fraction 4 (determined by integration the area underneath the densitogram), 13.8 % in fraction 10, 8.7 % in 11, 13.9 % in 12, 3.4 % in 17. In haemocyte lysate, 12 fractions were clearly visible. None of the bands/ fractions could be found exclusively in the haemocyte lysate (Fig. 2).

In *Chicobolus* spec., more than 40 absorption maxima were observed 16 of which could be detected regularly (Tab. 2, Fig. 1). The spectrum clearly differed from that of *R. virgator*; in *Chicobolus* spec., the more abundant proteins showed higher MWs. Larger amounts of proteins were found in the fractions 3 and 4 (> 250 kD), 6–8 (190–165 kD) and 12 (about 90 kD) (see Fig. 1).

Tab. 2 Number of detectable protein bands and larger fractions as found by chromato-spectralphotometric measurements in acrylamid gels after SDS-PAGE under reducing conditions.

	No. of bands	No. of larger protein fractions
Chicobolus spec.	43–63	16
Rhapidostreptus virgator	42–53	19

With regard to MW (retention time) and amount of proteins (area underneath the densitogram) there is little correspondence between the two species, e.g. in the fractions 11 and 12. However, the investigations on the MW of the prophenoloxidase (see Xylander & Bogusch 1997) indicate that this zymogene is one of the fractions 2 to 4.

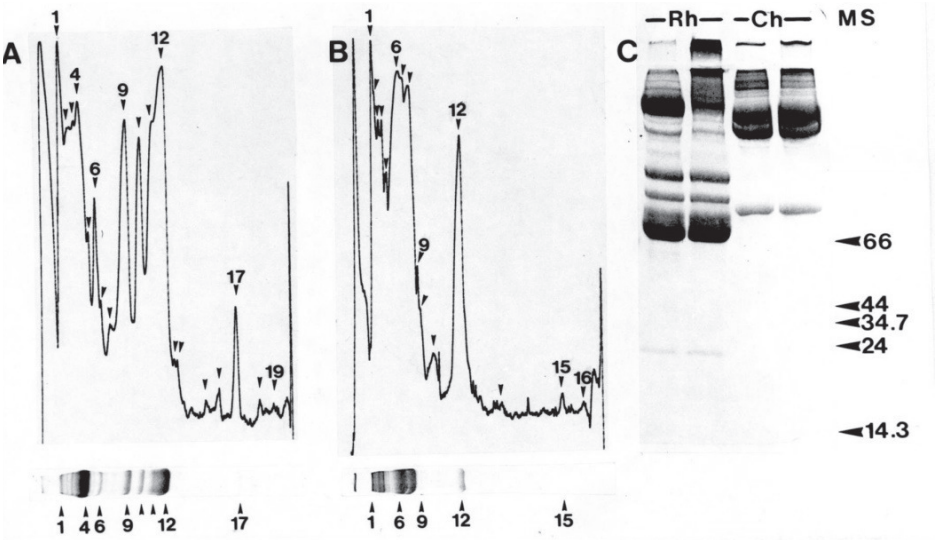

Fig. 1 Densitogram (above) and PAGE (below and right) of haemolymph of *R. virgator* (A) and *Chicobolus* spec. (B). C = PAGE with MS.

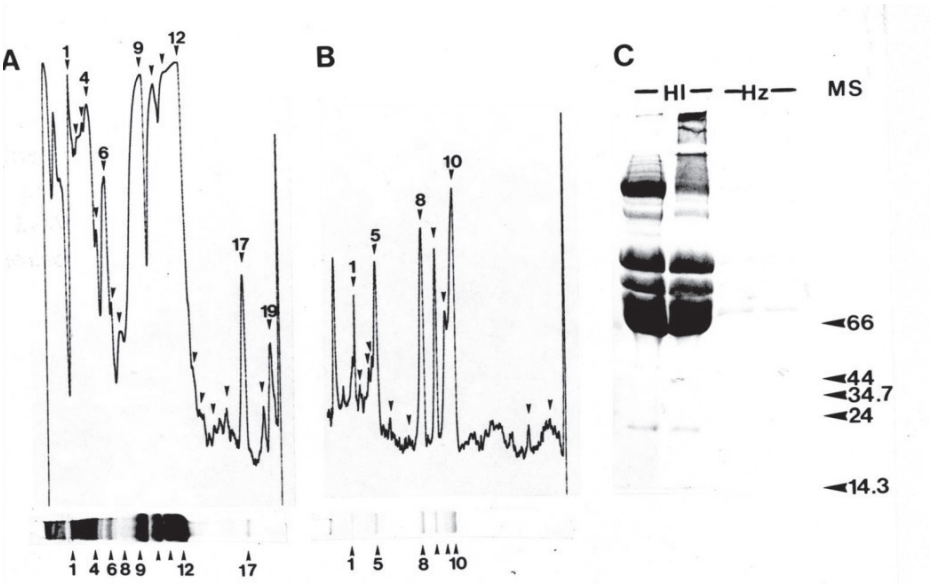

Fig. 2 Densitogram (above) and PAGE (below and right) of haemolymph (A) and haemocyte lysate of *R. virgator* (B). C = PAGE with MS.

Osmolarity

The osmolarity in the haemolymph was generally lower in the diplopod species tested (*Chicobolus* spec. 230 mOsm; *R. virgator*: 200 mOsm, Tab. 3) compared to the chilopods (*L. forficatus*: 296 mOsm; *S. cingulata*: 372 mOsm, Tab. 3). The variation between single specimens was comparably high (e.g. between 146 mOsm and 250 mOsm in *R. virgator* and 156 mOsm and 289 mOsm in *Chicobolus* spec., respectively).

Tab. 3 Osmolarity of the haemolymph of different diplopod and chilopod species.

Species	osmolarity [mOsm]	n
Chicobolus spec.	229.6 ± 47.3	12
Rhapidostreptus virgator	200.3 ± 35.4	10
Lithobius forficatus	296.0 ± 4.0	2
Scolopendra cingulata	371.5 ± 7.8	2

pH

The haemolymph pH in the both diplopod species investigated ranged from slightly to moderately alkaline (pH 8.0 to 8.5 in freshly collected haemolymph and pH 8.5 in frozen haemolymph, Tab. 4). Cell-free haemolymph plasma and total haemolymph showed no pH difference.

Tab. 4 Haemolymph pH of different diplopods and chilopods.

Chicobolus spec.	pH 8.0–8.5
Rhapidostreptus virgator	pH 8.0–8.5
Lithobius forficatus	pH 7.0–7.5*
Scolopendra cingulata	pH 8.0*

*Data by Lutz Nevermann

The haemolymph of *S. cingulata* had a pH of 8.0 and that of *L. forficatus* ranged between 7 and 7.5 in freshly collected haemolymph and around pH 8.0 after freezing (Tab. 4). Comparative tests of haemolymph of pupae of the caterpillar of *Manduca sexta* showed a pH of 6.5 to 7.0.

4. Discussion

Protein content

Rajulu (1974) mentioned a total protein content for the haemolymph of the *Cingalobolus bugnioni* Carl, 1918 of 1.975 mg ml^{-1} and for *Thereuopoda longicornis* (Fabricius, 1793) (*Scutigera longicornis*) of 1.755 mg ml^{-1} Helbing (1985) registered a haemolymph protein content between 20 to 120 mg ml^{-1} in *L. forficatus*.The protein content measured by Helbing (1985) and in this paper is significantly higher than those indicated by Rajulu (1974). Although protein content may vary between species a 10-fold to 90-fold difference may rather result from errors during measurement or calculation of protein content.Thus, protein content in Myriapoda will most probably vary between 20 to 120 mg ml^{-1} haemolymph.

Furthermore the protein content may vary seasonally as well as with regard to the status within the moulting cycle and sex (Helbing 1985, 1989, Rajulu 1974), e.g. in *L. forficatus* over the year between 20 to 120 mg ml^{-1}. In specimens of *Cingalobolus bugnioni* (body weight exceeding 20 g) between 70 % (in females) or 30 % (in males) higher protein content was found. Males and females of *T. longicornis* did not differ but their protein content also increased 10 % with age. Such variation is also known from insects (Seifert 1975).

Rajulu (1969, 1974) found 5 haemolymph proteins in *Scolopendra morsitans* Linnaeus, 1758 and 6 in *T. longicornis* as well as 6 for male and 7 for female *Cingalobolus bugnioni*. Helbing (1985) reported 15 larger protein fractions in *L. forficatus*, the proportion of which also varied seasonally. This investgations shows further proteins. A comprehensive evaluation of protein spectra of the haemolymph of Chilopoda and Diplopoda therefore is not yet possible.

Osmolarity

Osmolarity of haemolymph seems to be quite taxon specific throughout the different groups of terrestrial Arthropoda (Tab. 5). However, this parameter largely depends on environmental factors such as temperature, diet or moisture. Thus, Herzog (1983) found haemolymph of the larva of the dragonfly *Aeshna cyanea* (Müller, 1764) ranging from 339 to 405 mOsm with regard to water temperature. As Diplopoda and Chilopoda quite rapidly loose body weight and die when kept under too dry conditions (own observations) environmental or rearing conditions obviously play a crucial role for these groups. Furthermore, Wenning (1978) found slight variation of haemolymph osmolarity also in *L. forficatus* specimens reared under standardised conditions but either fed (360 mOsm) or starving (330 mOsm). Preliminary own observations on haemolymph osmolarity from diplopods kept under different moisture conditions in their artificial habitats or diets of different water content (potato or cucumber), indicate a higher relevance of the diet in this taxon. This may be due to the fact that the calcified cuticle of most diplopods provides a better short time protection against evaporation than the thinner cuticle of chilopods.

Osmolarity in the haemolymph of Diplopoda is lower than in Chilopoda; the haemolymph of the latter corresponds to the data found in insects (Tab. 3, Tab. 5). This may be related to the higher protein content of the latter group as most proteins are osmotically active. So the high values of the spider *Argiope trifasciata* (Forskäl, 1775) (haemolymph osmolarity: 400–620 mOsm, Pulz 1987, see Tab. 5) may be explained by the occurrence of haemocyanine which most tracheates lack.

pH

Haemolymph pH of Diplopoda and Chilopoda is slightly to moderately alcaline (about pH 8, see also Wennig 1989) whereas in insects it is rather slightly acidic to neutral (often ranging between 6.4 and 6.8, Buck 1953, Chapman 1969, Seifert 1975, Wyatt 1961). However, in some insects and in Crustacea alkaline haemolymph can also be found e.g. *Periplaneta americana* (Linnaeus, 1758): pH 7.5 to 8.0; *Cancer borealis*: pH 7.81; (Buck 1953, Seifert 1975).

Tab. 5 Haemolymph osmolarity in different arthropod groups..

Taxon	Osmolarity [mOsm]	Author
Insecta		
Aeschna cyanea (larva)	339–405	Herzog (1983)
Tenebrio melitor (larva)	ca. 530	Wennig (1978)
Sialis lutaria	339	Hevert (1985)
Aedes campestris	340	Hevert (1985)
Drosophila hydei	229	Hevert (1985)
Myriapoda		
Lithobius forficatus	330–360	Wennig (1978)
	296	Nevermann (this study)
Scolopendra cingulata	372	Nevermann (this study)
Rhapidostreptus virgator	200	Xylander (this study)
Chicobolus spec.	229	Xylander (this study)
Arachnida		
Argiope trifasciata	400–620	Pulz (1987)

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