Resilin-like protein in the clamp sclerites of the gill monogenean *Diplozoon paradoxum* Nordmann, 1832

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SUMMARY

Resilin is a soft and elastic protein, which is found in many exoskeletal structures of arthropods. Proteins with similar chemical properties have been described for other invertebrates including monogenean fish parasites. However, for the latter taxon no clear microscopic evidence for a resilin-like protein has been shown so far. Here, we present the results of detailed microscopic analyses of the clamp sclerites (attachment devices) of the monogenean *Diplozoon paradoxum*. Toluidine blue, which is known to stain resilin, selectively stained the material in the clamp sclerites. In addition, when exposed to UV light, this material exhibited an intense blue autofluorescence. The emission spectrum of this autofluorescence has its maximum at 424 nm and is nearly identical to emission spectra of blue autofluorescences observed in 2 well-studied structures containing high proportions of resilin in the exoskeleton of the locust *Schistocerca gregaria*. The results strongly indicate that the sclerite material of *D. paradoxum* contains a resilin-like protein. The presence of such a protein likely enhances the attachment efficiency of the clamp sclerites and increases their lifetime.

Key words: attachment, autofluorescence, clamp sclerite, *Diplozoon paradoxum*, resilin, *Schistocerca gregaria*.

INTRODUCTION

Resilin, an elastic protein, is well known for its superior resilience and high fatigue lifetime (Andersen and Weis-Fogh, 1964; Gosline *et al*. 2002). It consists of a network of randomly orientated coiled peptide chains, which are covalently cross-linked by the amino-acids dityrosine and trityrosine (Weis-Fogh, 1961; Andersen, 1964). When exposed to UV light, these amino acids exhibit a relatively intense blue autofluorescence, which in many studies has been used as an indication of the presence of resilin (e.g. Andersen and Weis-Fogh, 1964; Gorb, 1999; Burrows, 2009).

Until now, resilin has mainly been found in arthropod exoskeletal structures, where it is known to have different functions including (1) the generation of flexibility of wing vein joints in damsel- and dragonflies (Gorb, 1999; Appel and Gorb, 2011), (2) the enhancement of adaptability to uneven substrates in insect attachment pads (Perez Goodwyn *et al*. 2006), (3) the reduction of fatigue in folding wings of beetles (Haas *et al*. 2000) and (4) the storage of elastic energy in the jumping systems of fleas (Bennet-Clark and Lucey, 1967) and planthoppers (Burrows, 2010). In addition, the presence of resilin-like proteins containing dityrosine and trityrosine has been reported for several non-arthropod taxa such as sea urchins (Foerder and Shapiro, 1977), nematodes (Lopez-Llorca and Fry, 1989) and mussels (DeVore and Gruebel, 1978).

Clamp sclerites, which are used as attachment devices by some monogeneans (namely, *Tripathia chorinemi, Protomicrocotyle mannarensis, Pseudaxine indicana* and *Pricea multae*), very likely also contain such a resilin-like protein, as reported by Ramalingam (1973). However, until now, positive staining reactions and the emission of the typical blue autofluorescence have only been described without showing any micrographs demonstrating the exact distribution of the resilin-like protein in the clamp materials. Since such visual data are missing, it is difficult to assess the possible function of the resilin-like protein in the clamps. In addition, to the best of our knowledge, there is no information on the spectral properties of the blue autofluorescence exhibited by the clamp sclerites of monogeneans.

Diplozoon paradoxum Nordmann, 1832 (Platyhelminthes: Monogenea: Diplozoidea) is a gill parasite of freshwater fishes. Adult parasites fuse together in pairs, and each individual parasite possesses 4 pairs of posterior clamps, which are used to grasp 1 or more host secondary gill lamellae (Owen, 1963). Each clamp has 2 jaws, hinged to each other, and each jaw is supported peripherally by marginal sclerites. According to Owen (1963), each clamp is closed by an extrinsic muscle/tendon system associated with a median J-shaped sclerite. The main goal of the present study was a detailed microscopic analysis of the clamp sclerite material of *D. paradoxum* in order to obtain precise information on the presence and

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distribution of a resilin-like protein in the clamps. The study also aimed at analysing the spectral properties of this protein's blue autofluorescence and comparing these properties with those of the blue autofluorescences exhibited by well-known resilin-containing structures of locusts.

MATERIALS AND METHODS

Collection and preparation of samples

Live common bream (*Abramis brama* L.) from the Westensee (Schleswig-Holstein, Germany) were caught by a local fisherman. They were killed, and the gills were examined for the occurrence of *Diplozoon paradoxum* using a stereomicroscope. A total of 27 pairs of living adult *D. paradoxum* was collected, cleaned and identified based on the morphology of the sclerites and reproductive organs (Bovet, 1967; Bychowsky and Nagibina, 1959). Some worms were transferred to pure glycerine in a 1·5 ml microcentrifuge tube and stored at −20 °C. Five pairs of worms were fixed in a 2·5% glutaraldehyde solution (in 0·01 M phosphate buffer at pH 7·4) for 6 h at 4 °C, washed with phosphate buffer, dehydrated in a graded series of ethanols, transferred to propylene oxide and embedded in low viscosity Spurr resin. The resin blocks were polymerized at 70 °C for 24 h, and 1–2 μm thick semi-thin sections were cut using a Leica EM UC7 ultramicrotome equipped with a diamond knife.

Toluidine blue staining of whole worms and semi-thin sections through the sclerites

Whole worms and semi-thin sections were stained with toluidine blue, which is commonly used as an indicator for the presence of resilin in the structures of interest (Andersen and Weis-Fogh, 1964). For this purpose, 5 pairs of *D. paradoxum* specimens were thawed, washed with distilled water and stained in 0·005% (w/v) toluidine blue solution at 25 °C for 30 min (modified from Andersen and Weis-Fogh 1964). The worms were washed with distilled water (3 times for 3 min). Subsequently, they were transferred to pure glycerine and kept there for at least 5 days, which resulted in the removal of any excess toluidine blue in the worm tissues (see Michels *et al*. 2012). The worms were mounted in fresh glycerine under a coverslip, observed with the stereomicroscope and photographed with a Leica DFC420 camera. The semi-thin sections were transferred to a clean glass slide and stained with droplets of 0·005% (w/v) toluidine blue solution at 60 °C for 10 sec. The sections were then thoroughly washed with distilled water, dried on a hotplate, mounted in Leica CV Mount, covered with a coverslip and viewed and photographed with a Zeiss Axioplan microscope equipped with a Zeiss AxioCam MRc camera.

Confocal laser scanning microscopy imaging and spectral analyses

Five pairs of *D. paradoxum* specimens, which had been frozen in glycerine, were thawed. Subsequently, they were transferred to fresh glycerine and mounted on object slides as described elsewhere (Michels and Büntzow, 2010). The clamp sclerites were analysed with the confocal laser scanning microscope Zeiss LSM 700 equipped with the microscope Zeiss Axio Imager.M1 m and 4 solid state lasers (wavelengths: 405 nm, 488 nm, 555 nm, 639 nm). The visualization of the blue autofluorescence was performed using an excitation wavelength of 405 nm and a detected emission range of 420–480 nm. In addition, other excitation wavelengths were used to test the sclerite material for the presence of other autofluorescences. The imaging settings were chosen as described earlier (Michels and Gorb, 2012). The emission spectrum of the blue autofluorescence was analysed using the same technique and the same method as applied in a previous study (Michels *et al*. 2012).

RESULTS

All 4 pairs of clamp sclerites appear colourless when viewed in a bright-field light microscope. The material of these sclerites (median J-shaped and marginal), both in the whole worms and in the semi-thin sections, is selectively stained dark blue without any metachromasia after treatment with toluidine blue solution (Fig. 1A, B). When exposed to laser light with a wavelength of 405 nm in the confocal laser scanning microscope, both the median J-shaped and the marginal sclerites exhibited an intense blue autofluorescence (Fig. 1C). Autofluorescences excited by other wavelengths are present in the sclerites; however, their intensities are too low for a successful visualization. The spectral analyses revealed that in the case of excitation with UV laser light (wavelengths: 351 nm and 364 nm) the blue autofluorescence of the sclerites has its maximum at a wavelength of 424 nm (Fig. 2). The full width at half maximum of the emission spectrum of this autofluorescence is 74 nm (414–488 nm). In general, the emission spectrum is very similar to those of the blue autofluorescences observed in the prealar arm and the wing hinge of *Schistocerca gregaria* (see Michels *et al*. 2012) (Fig. 2). The maxima of all these 3 emission spectra are pronounced and feature a strong increase of the fluorescence intensity between 415 nm and 424 nm. They are nearly identical in the wavelength range between 415 nm and 435 nm. Compared to the other two spectra, the decrease of the fluorescence intensity above 435 nm is less pronounced in the autofluorescence spectrum of the sclerites, and this spectrum extends slightly further to longer wavelengths.

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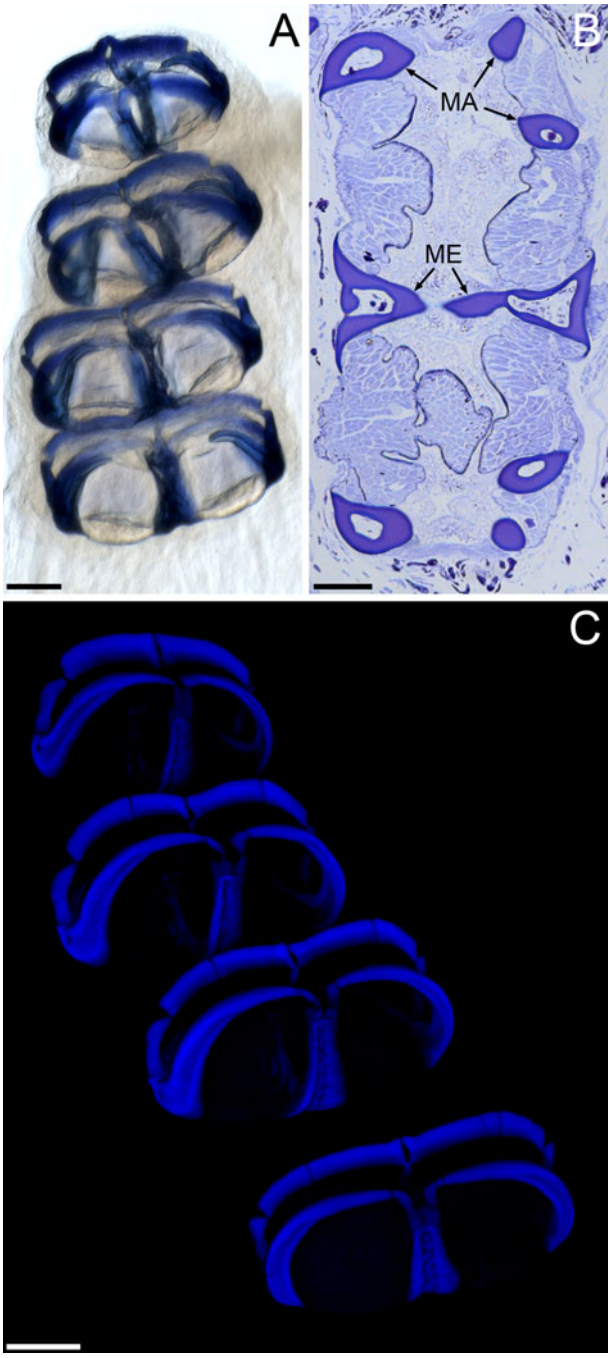

Fig. 1. Clamp sclerites of *Diplozoon paradoxum*. (A) Clamp sclerites in a whole worm, stained with toluidine blue. (B) A semi-thin section through a single clamp showing the marginal sclerites (MA) and the median sclerites (ME) stained with toluidine blue. (C) Confocal laser scanning micrograph (maximum intensity projection) showing the blue autofluorescence exhibited by the sclerites. Scale bars = 50 μm (A and C) and 25 μm (B).

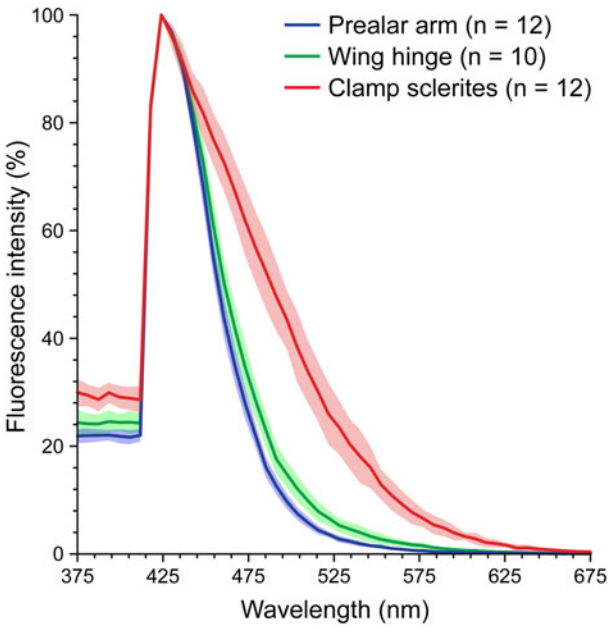

Fig. 2. Emission spectra of the blue autofluorescences exhibited by the clamp sclerites of *Diplozoon paradoxum* and the prealar arm and the wing hinge of the locust *Schistocerca gregaria*. The lines and the shaded areas represent the mean values and the standard deviations, respectively. The data from the spectral analyses of the autofluorescences in the prealar arm and the wing hinge were obtained from a previous study (Michels *et al.* 2012).

DISCUSSION

The material of the clamp sclerites of monogeneans has been shown to contain no sclerotin, elastin, reticulin, collagen and keratin (Lyons, 1966), while Milne and Avenant-Oldewage (2006) speculated that chitin is present. Our results indicate that the sclerites of *Diplozoon paradoxum* consist of a resilin-like protein. Besides the toluidine blue staining, this is strongly indicated by the emission properties of the blue autofluorescence. The prealar arm and the wing hinge of the locust *Schistocerca gregaria* were proven to contain resilin (Andersen and Weis-Fogh, 1964). The analyses of the blue autofluorescences in all these structures (prealar arm, wing hinge, clamp sclerites) yielded a pronounced conformity between their emission spectra. It is thus very likely that the sclerites contain a resilin-like protein.

The presence of such a resilin-like protein in the clamp sclerites (both median J-shaped and marginal sclerites) of *D. paradoxum* leads to the question of its function in these structures. Monogeneans use their clamps to attach to the secondary gill lamellae of fishes. Resilin has been shown to be rather flexible and elastic (Andersen and Weis-Fogh, 1964; Gosline *et al.* 2002). We suggest that the presence of such a protein in the sclerite material, in particular in the marginal sclerites, may enhance the attachment properties and the efficiency of the clamps. The reason for this might be an increased gripping capability caused by a better adaptation to the surface topography of the substrate due to the higher material flexibility. In the sclerite parts close to the hinge between the two jaws, the resilin-like protein probably provides flexibility during the opening and

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closing of the jaws. Moreover, the risk of damage of the sclerites when being exposed to external forces such as strong and constant host gill ventilating currents might be reduced by this protein, resulting in an increased life time of the clamps. Such a fatigue-preventing function of resilin has previously been discussed for various structures of insect exoskeletons, including joints in the wings of damselflies (Gorb, 1999) and beetles (Haas *et al*. 2000).

The present study indicates that resilin-like proteins are not restricted to higher taxa of invertebrates but very likely also occur in basal taxa such as monogeneans. It is conceivable that the resilin-like proteins provide the lower invertebrates with a combination of favourable properties: on the one hand they are stiffer than the relatively soft body tissues enabling the formation of efficient attachment devices, and on the other hand they are soft and flexible enough to increase the grip and fatigue resistance as described above.

In conclusion, the present study provides, to the best of our knowledge, the first detailed results from microscopic and spectral analyses of the blue autofluorescence exhibited by the clamp sclerites of monogeneans. This autofluorescence is assumed to be emitted by a resilin-like protein whose inclusion in the sclerite material might be an evolutionary adaptation increasing the efficiency of the clamps.

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