Autoradiographic and morphological investigations of the defensive ecdysteroid glands in adult *Pycnogonum litorale* (Arthropoda: Pauropoda)

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**Abstract.** *Pycnogonum litorale* (Ström) is a primitive marine chelicerate. When mechanically irritated, it discharges a mixture of eight ecdysteroids (ES) that acts as a chemical defense against predatory decapod crustaceans. Injection of [3H]-20-hydroxyecdysone into the hemolymph or into the gut and subsequent autoradiography of frozen sections revealed that the majority of the ES in the pycnogonids is concentrated in epidermal glands. A novel autoradiographic technique for the localization of water soluble radioactive substances at uneven surfaces visualized the ES secretion through cuticular pores. Scanning electron microscopy shows that each pore is associated with a forked seta, which is suspected to have mechanoreceptor functions. The epidermal glands, numbering 10,000 to 20,000 in adults, are distributed on all parts of the body, except the arthrodial membranes. Thus, this system provides an effective allround defense against predators. Similar pores occur in many pycnogonid species, and it is suggested that chemical defense might be a common feature within the entire class of pycnogonids.

**INTRODUCTION**

*Pycnogonum litorale* contains the highest ecdysteroid levels ever found in any arthropod. Except in young embryos, the concentration of total ES is extremely high in all developmental stages, reaching 1965 nmol/g dry weight in juveniles (Tomaschko & Bückmann, 1993). ES in pycnogonids act as molting hormones, as they do in other arthropods (Bückmann & Tomaschko, 1992). An additional, non-hormonal function has been demonstrated recently: The ES of pycnogonids act allelochemically as powerful defensive chemicals against predatory decapod crustaceans, causing an immediate feeding inhibition (Tomaschko, 1994a). The defensive properties result from the unusually high concentrations (10^{-3}M) at which the ES are released by the pycnogonids in response to disturbance (Tomaschko, 1994b). The use of ES as a chemical defense seems to be as yet unique among animals. Furthermore, this is the first discovery of a defensive secretion in chelicerates or in any marine arthropod. The unusual function of ES as allelochemicals suggests the concentration of ES in reservoirs and the existence of exocrine glands. The aim of the present paper is to localize the reservoirs of ES and to find the defensive glands. The morphology and distribution of the glands is investigated by scanning electron microscopy. Ultrastructural details of the glands will be given in another paper.

**MATERIAL AND METHODS**

Animals

*P. litorale* came from our standard culture (Bückmann & Tomaschko, 1992).
 Autoradiography of cryosections

Juvenile and adult males and females each received an injection of 1 \mu l of water containing 0.1 \mu Ci of [\textsuperscript{3}H]20-hydroxyecdysone (20E), into the gut or into the hemolymph. Injections were made by means of a glass pipette (Clark Electronical Instruments, GC 100 F-10) pulled on a David Kopf Instruments vertical pipette puller (Model 720). For injections into the gut, the tip of the pipette was inserted approximately 5 mm through the mouth opening into the prococis. Injections into the hemolymph were performed through the dorsal arthrodial membranes of the trunk. After injection, the animals were each kept separately in 10 ml of sea water for 15 days. Embedding, cryosectioning, cold-mount autoradiography, dry-mount autoradiography, and histological staining were performed as described by Stumpf (1976) and by Bronson et al. (1991).

Surface autoradiography

Injected with [\textsuperscript{3}H]20E animals were each kept separately in 10 ml of sea water for 24 hours. Then they were removed from water, cautiously swabbed with a piece of tissue, and gently pressed in order to provoke secretion of ES. Afterwards they were immediately frozen at −30°C and lyophilized for 24 hours. A new autoradiographic method was developed in order to localize the secreted ES at the uneven surface of the cuticle without dislocating them. A ring of silver wire with an inner diameter of 20 mm was dipped in 40°C fluid nuclear emulsion. When removed slowly from the emulsion, the ring was spanned with a thin membrane of emulsion which became dry and elastic within 1 minute. When placed on the pycnogonids, the elastic membrane adhered closely to the animals' surfaces. The emulsion-coated animals were put for 90 days in cold (−30°C), desiccated (CaSO\textsubscript{4}, "Drierite") light-proof boxes for autoradiographic exposure. The autoradiographs were developed by dipping the coated animals into Kodak D-19 developer (3 min), rinsing in water (30 sec), fixing in Kodak fixer (3 min), and washing (5 min) in distilled water.

Light microscopy

Material from various parts of the trunk and appendages was fixed and rinsed with the low osmium mixed pre-fixative technique (Eisenman & Alfert, 1981), dehydrated through an ascending series of isopropanol to propylene oxide, and embedded in Epon. 200 nm sections were cut with a glass knife on a Reichert OM U3 Ultratome, stained with methylene blue and examined under a Zeiss Axioskop microscope.

Scanning electron microscopy

Animals were placed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.3, 1,000 mMNa) for 24 h, followed by 1 hour in isotonic buffer. After fixation the samples were dehydrated by a graded series of isopropanol, transferred to dimethoxypropane (DMP), critical-point dried, and coated with gold (20 nm) in a Technics Hummer V coating unit. Prepared pycnogonids were examined in a Phillips 500 scanning electron microscope.

Chemicals

[\textsuperscript{23,24-3}H\textsubscript{2}N]20-hydroxyecdysone (spec. activity unknown) was provided by Prof. K.H. Hoffmann (University of Ulm). Nuclear emulsion NTB 3 was from Eastman Kodak. All other chemicals were of reagent grade and from various suppliers (Merck, Fluka or Carlo Erba).

RESULTS

Localization of ecdysteroids within the Pycnogonids

15 days after injection of [\textsuperscript{3}H]20E into the gut or into the hemolymph, the majority of the radioactivity had accumulated in defined areas in the integument (Fig. 1a). From previous experiments (Tomaschko & Bückmann, 1990) it is known that P. litorale quickly converts 20E into 20-hydroxyecdysone 22-acetate (20E22Ac), which is the predominant ecdysteroid in all developmental stages as well as in the defensive secretions (Tomaschko, 1994b). Thus, the label on the autoradiographs mainly reflects the location of 20E22Ac within the pycnogonids. The 20E22Ac in the integument is located in cuticular pits (Figs 1b,c,d). These pits occur on all parts of the body, except the arthrodial membranes.
Fig. 1. 4 μm frozen sections of adult female pycnogonids processed for cold-mount (a) and dry-mount (b, c, d) autoradiography and stained with methylgreen pyronin show the distribution of radioactivity 15 days after injection of [¹⁴C]20E into the hemolymph. Exposure time: 49 days. a, b, d – sagittal sections; c – tangential section of the cuticle. Lettering: am – anthrodial membrane; cp – cuticular pit; cu – cuticle. Scale bars: a = 1 mm; b = 200 μm; c = 30 μm; d = 100 μm.
Fig. 2. 200 nm cross sections of a tibia of *P. litorale*, stained with methylene blue. a – cuticle with internal pitting, containing epidermal glands; b – vacuolated epidermal gland with cuticular duct. Lettering: cu – cuticle; cp – cuticular pit; gd – gland duct. Scale bars: a – 200 μm, b – 30 μm.

Micrographs of Epon sections show that the numerous cuticular pits are filled with epidermal gland cells, containing large vacuoles (Figs 2a,b). Each dermal gland has a duct with a diameter of 1 μm which leads to the outer surface of the cuticle (Fig. 2b).

Site of ES-secretion

The structure of the dorsal cuticle consists of numerous tubercles (Figs 3a,b). In the center of each tubercle is an opening of a cuticular duct, surrounded by cuticular spines (Fig. 3b,c). On the ventral side the tubercles are smaller and lack spines (Fig. 3d). All pores are associated with one or two forked setae (Figs 3c,d). The openings of the gland ducts are distributed on all parts of the body, numbering about 10,000 to 20,000 in adults. Since cuticular pits are absent in arthrodial membranes, pores are likewise absent.

The release of ES could not be recognized directly by a visible discharge of the secretion after disturbance. Therefore, an indirect method was used to visualize the secreted ES. An adult female (Fig. 4a) was pressed against a TLC plate (Merck 60F-254) that was precoated with a fluorescent indicator. Due to their UV-absorbing properties (Morgan & Poole, 1976), the secreted ES became visible when observed under UV light (254 nm). The “ecdysteroid-print” correlates with the distribution of the cuticular pores. ES are secreted by all parts of the body, except the arthrodial membranes (Fig. 4b).

In [3H]20E-injected animals, the secretion of tritiated ES through the cuticular pores is confirmed by the technique of surface autoradiography. Fig. 5 shows that ES are not secreted over the entire surface of the cuticle, but at numerous distinct locations, represented by dark spots in the surface autoradiogram. The location of these spots correlates with the location of the cuticular pores.
Fig. 3. Scanning electron micrographs of an adult female pycnogonid. a – ventral aspect, note tubercles at the cuticle surface; b – cuticular tubercles with openings of gland pores (see arrows); c – dorsal tubercle with gland pore surrounded by cuticular spines; d – surface of the ventral cuticle without pores and setae. Lettering: s – setae; d – porus of gland duct. Scale bars: a = 1 mm; b = 30 µm; c = 10 µm; d = 10 µm.
DISCUSSION

There are very few records of pycnogonids serving as food for other animals. Arnaud & Bamber (1987) suggested that pycnogonids are ingested more incidentally than actively, and that they do not constitute an appreciable part of any predator’s diet. Furthermore, the authors suggested that this is because pycnogonids have a heavily chitinized exoskeleton and little in the way of muscle content in the trunk and legs, rarely occur in great numbers and therefore probably do not offer an attractive source of food. The discovery of defensive secretion in *P. litorale* introduces a new aspect to the defensive strategies in marine arthropods.

The observation of the occurrence of defensive glands is well established in terrestrial (Blum, 1981) and freshwater arthropods (Dettner & Schwinger, 1980; Lokensgard et al., 1993). In some insects, the presence or absence, morphological peculiarities, and the chemical composition of the secretions contribute evidence for the solution of systematic questions (Deroe & Pasteels, 1982; Dettner, 1993, Pasteels, 1993). The occurrence of defensive glands in marine arthropods is as yet restricted to *P. litorale*. However, the investigation of other pycnogonid species may reveal that defensive secretion in marine arthropods is not as rare as it appears at the moment. Epidermal glands occur in all pycnogonid species (Helfer & Schloerke, 1935), and it might well be that the investigation of their morphology and chemistry contributes to our knowledge about the phylogeny of the
pycnogonids. The existing works about the function of the glands differ greatly in their conclusions. Dohrn (1881) and Loman (1907) suspected the glands to secrete mucus as a protection against microorganisms, while Tomashko (1990) found an excretory function. Davenport et al. (1987) investigated the integumental structure of the antarctic pycnogonid *Decolopoda australis* and found quite similar cuticular pitting and pores as described in the present paper for *P. litorale*. Calculating the gaseous diffusion rate through the cuticle of *D. australis*, the authors suggested a respiratory function of the pores. Thus, it seems possible that the epidermal structures described in this paper not only may have defensive functions, but are also multifunctional organs. On the other hand, the unpalatability of *P. litorale* may not rely exclusively on defensive secretion. However, it is suggested that the defensive glands are of vital importance for *P. litorale*. By secreting ES, it seems to have developed a universal feeding deterrent against decapod crustaceans. Recent experiments provide evidence that ES not only deter feeding by *C. maenas*, but have a similar effect on other decapod crustaceans, e.g. *Cancer pagurus*, *Homarus americanus*, and *Astacus astacus* (Tomashko et al., 1994). Further experiments will show whether other pycnogonid species or other marine invertebrates use ES as defensive chemicals as well.

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