The Journal of Experimental Biology 209, 78-88
Published by The Company of Biologists 2006
doi:10.1242/jeb.01972

Packaging of chemicals in the defensive secretory glands of the sea hare *Aplysia californica*

Paul M. Johnson[1,2], Cynthia E. Kicklighter[1], Manfred Schmidt[1], Michiya Kamio[1], Hsiuchin Yang[1], Dimitry Elkin[3], William C. Michel[3], Phang C. Tai[1] and Charles D. Derby[1,*]

[1]*Department of Biology, Center for Behavioral Neuroscience, and Brains and Behavior Program, Georgia State University, Atlanta, GA 30303 USA,* [2]*Department of Biology, University of Washington, Seattle, WA 98195 USA and* [3]*Department of Physiology, University of Utah School of Medicine, Salt Lake City, UT 84108 USA*

*Author for correspondence (e-mail: cderby@gsu.edu)

Accepted 7 November 2005

Summary

Sea hares protect themselves from predatory attacks with several modes of chemical defenses. One of these is inking, which is an active release of a protective fluid upon predatory attack. In many sea hares including *Aplysia californica* and *A. dactylomela*, this fluid is a mixture of two secretions from two separate glands, usually co-released: ink, a purple fluid from the ink gland; and opaline, a white viscous secretion from the opaline gland. These two secretions are mixed in the mantle cavity and directed toward the attacking predator. Some of the chemicals in these secretions and their mechanism of action have been identified. In our study, we used western blots, immunocytochemistry, amino acid analysis, and bioassays to examine the distribution of these components: (1) an L-amino acid oxidase called escapin for *A. californica* and dactylomelin-P for *A. dactylomela*, which has antimicrobial activity but we believe its main function is in defending sea hares against predators that evoke its release; and (2) escapin's major amino acid substrates – L-lysine and L-arginine. Escapin is exclusively produced in the ink gland and is not present in any other tissues or secretions. Furthermore, escapin is only sequestered in the amber vesicles of the ink glandand not in the red-purple vesicles, which contain algal-derived chromophores that give ink its distinctive purple color. The concentration of escapin and dactylomelin-P in ink, both in the gland and after its release, is as high as 2 mg ml^{-1}, or 30 μmol ml^{-1}, which is well above its antimicrobial threshold. Lysine and arginine (and other amino acids) are packaged into vesicles in the ink and opaline glands, but arginine is present in ink and opaline at <1 mmol l^{-1} and lysine is present in ink at <1 mmol l^{-1} but in opaline at 65 mmol l^{-1}. Our previous results showed that both lysine and arginine mediate escapin's bacteriostatic effects, but only lysine mediates its bactericidal effects. Given that escapin's antimicrobial effects require concentrations of lysine and/or arginine >1 mmol l^{-1}, our data lead us to conclude that lysine in opaline is the primary natural substrate for escapin in ink. Furthermore, packaging of the enzyme escapin and its substrate lysine into two separate glands and their co-release and mixing at the time of predatory attack allows for the generation of bioactive defensive compounds from innocuous precursors at the precise time they are needed. Whether lysine and/or arginine are substrates for escapin's antipredatory functions remains to be determined.

Key words: chemical defense, ink gland, opaline gland, gastropod, sea hare, escapin, L-amino acid oxidase.

Introduction

Sea hares, including species of the genus *Aplysia*, have multiple chemical defenses to deter predators. Passive chemical defenses are present in the skin, thus producing a distasteful surface to would-be predators, and many of these deterrent compounds have been identified (Kinnel et al., 1979; reviewed by Carefoot, 1987; Faulkner, 1992; Yamada and Kigoshi, 1997; Johnson and Willows, 1999; Rogers et al., 2000; Ginsburg and Paul, 2001). Active chemical defenses are released only upon predatory attack, and they include the secretions from two separate glands. One of these is ink, generally a bright purple fluid that is released from the ink gland. The other is opaline, a whitish and extremely viscous substance produced by the opaline gland. These two secretions are released into the mantle cavity – usually, but not always, simultaneously – and pumped out of the siphon toward the attacker (Walters and Erickson, 1986; reviewed by Johnson and Willows, 1999).

The ink gland of *Aplysia californica* is composed of three vesicle types: red-purple, amber and clear (Prince et al., 1998).

Most vesicles appear to have pores leading to the ventral surface of the gland. Attack by a predator stimulates motor neurons to activate muscles around individual vesicles, which squeeze the contents out of the vesicles and into the mantle cavity (Prince et al., 1998) where they are then directed by the siphon and pumped out of the mantle (Walters and Erickson, 1986).

The ink gland and its purple ink contain a diversity of molecules, only some of which have been investigated and their biological activity identified. The purple color of ink comes from red-algal derived pigments, the most abundant being phycoerythrobilin, a chromophore of the photosynthetic pigment phycoerythrin; the biological activity of these pigments is uncertain (Chapman and Fox, 1969; MacColl et al., 1990; Prince et al., 1998; Coelho et al., 1998). Ink also contains high concentrations of amino acids and related nitrogenous compounds that act against crustacean predators through phagomimicry and/or sensory disruption (Kicklighter et al., 2005). Ink of many species of sea hares has been shown to contain a protein of ~60 kDa (Yamazaki et al., 1986; Kamiya et al., 1989; Yamazaki, 1993; Nistratova et al., 1993; Melo et al., 2000; Rajaganapathi and Kathiresan, 2002; Petzelt et al., 2002; Yang et al., 2005). In *A. californica*, this protein is called escapin (Yang et al., 2005), and it has a homologue in the albumen gland, called aplysianin-A (Kamiya et al., 1986; Cummins et al., 2004). Escapin, aplysianin-A and their homologues have been shown to have antibacterial, cytolytic, haemagglutinating, and/or anti-heparin activities (e.g. Kamiya et al., 1986; Cummins et al., 2004; Yang et al., 2005), but we believe it unlikely that this is the primary function of these proteins. Rather, we believe that escapin and its homologues function as antipredatory chemical defenses, though we are not aware of any reports of such activity. Preliminary studies show that high concentrations of escapin, over a period of many hours, will lyse sea anemone's tentacle tissue and symbiotic zooxanthellae (Johnson, 2002); however, adverse effects at natural concentrations and time courses have not yet been demonstrated in sea anemones or spiny lobsters (C.E.K., unpublished data). Thus, at present, we can assess escapin's biological activity only using antimicrobial assays. Escapin's major substrates are L-lysine and L-arginine, though the products of escapin's oxidation of L-lysine and L-arginine have different antimicrobial activities. Escapin's oxidation of both L-lysine and L-arginine leads to bacteriostasis, probably due to production of hydrogen peroxide; but oxidation of only L-lysine and not L-arginine leads to bactericidal effects through mechanisms other than cytolysis (Yang et al., 2005).

Like ink, opaline contains many compounds but for only a few is the biological significance known (Kicklighter et al., 2005). High concentrations of amino acids, particularly taurine, help defend sea hares from predatory spiny lobsters through phagomimicry and/or sensory disruption. Opaline also contains unidentified chemicals that inhibit ingestion. Opaline is a source of lysine, a substrate for escapin. Additionally, given its viscous consistency, opaline has been suggested to provide substance to the secretion, to bind ink and opaline together or to adhere to the predators (for reviews, see Johnson and Willows, 1999; Kicklighter et al., 2005). Experimental evidence supports this idea: ink has phagomimetic properties, but when ink is released in the absence of opaline, it diffuses quickly and does not protect sea hares from attack by spiny lobsters (Kicklighter et al., 2005)

Given that the production of defensive chemicals in the ink–opaline secretion of sea hares requires an enzymatic reaction between escapin and its amino acid substrates, it would be interesting to know the distribution of these components within the different types of vesicles of the ink gland, in the opaline gland and in other tissues. The ink gland is known to have the chromophore phycoerythrobilin packaged in the red-purple vesicles (Prince et al., 1998). Clear vesicles, thought to be devoid of ink, are especially abundant in sea hares raised exclusively on lettuce or green algae and thus lacking the pigments to produce purple ink (Prince et al., 1998). Prince et al. (1998) argued from microscopic data that the ~60 kDa protein of ink may be packed in either the red-purple vesicles or amber vesicles.

Our study examines the location of the 60 kDa protein escapin and its substrate amino acids in the ink and opaline glands of *A. californica*. We used western blots, immunocytochemistry using antibodies against escapin and amino acids, amino acid analysis and bioassays to show that escapin is present exclusively and in high concentration in the ink gland and in no other tissues or secretions, and within the ink gland it is packaged exclusively in the amber vesicles and not the red-purple vesicles. The amino acid substrates of escapin, particularly L-lysine, are packaged in high concentration in the opaline gland and not in the ink gland.

Materials and methods

Animals and animal care

Aplysia californica Cooper 1863 sea hares were either caught in the waters off California by commercial suppliers or raised from eggs by The NIH National Resource for *Aplysia* (Miami, FL, USA). *Aplysia dactylomela* Rang 1828 sea hares were collected around the Florida Keys. Animals were maintained at Georgia State University in re-circulating, artificial seawater tanks and fed a diet of the red alga *Gracilaria ferox*.

Collection of pure secretions

Pure ink and opaline secretions were collected from *A. californica* that were cold-anaesthetized by chilling for 3–4 h at 4°C and then injected with $MgCl_2$ to prevent inking. Ink and opaline glands were removed by dissection. Ink glands were gently squeezed, releasing the ink. Opaline glands were gently blotted dry and centrifuged at 30 000 ***g*** for 30 min at 4°C to separate opaline secretion from gland tissue. These secretions were frozen at –80°C until needed. In addition, to examine separately the contents of the two types of secretory vesicles of the ink gland (red-purple vesicles and amber vesicles; Prince

et al., 1998), microdissection was used to isolate separate populations of these vesicles.

Escapin purification

Following the procedure described in Yang et al. (2005), a preparative grade Hi-Load Superdex 200 16/60 column (Amersham Pharmacia Biotech, Piscataway, NJ, USA) or an in-house prepared Sephacryl 300 HR 26/60 column was used for initial size separation of ink constituents. Fractions identified by bacterial assay to have activity (see next section) were concentrated using a Biomax 5K NMWL membrane Ultrafree Centrifugal Filter Device (Millipore: Billerica, MA, USA). Active fractions were further purified on a cation exchange Mono S HR 5/5 column (Amersham Pharmacia Biotech) to yield pure escapin, which was stored at –80°C until used.

Anti-escapin antiserum

Anti-escapin serum was obtained by injecting rabbits with denatured recombinant escapin purified from an *E. coli* expression system, as described by Yang et al. (2005).

Western blots for identifying tissues in which escapin is expressed

Thirteen tissues and secretions (albumen gland, albumen gland complex, mucus, parapodia, foot, gill, digestive gland, central nervous system, opaline gland, ink gland, ink, crop and foregut) from three field-caught adult *A. californica* (50–90 g) were analyzed by western blotting for expression of escapin. In addition, to examine differences in reproductive tissues between juvenile and adult animals, the albumen gland complex from two juveniles (5 g) was also analyzed (the albumen gland of juveniles is so small that is too difficult to dissect away only the albumen gland, so the entire albumen gland complex was removed and analyzed). Purified escapin was used as a standard. To remove tissues, animals were chilled for 3 h at 4°C and injected with isotonic $MgCl_2$ to anaesthetize them. Tissues from different individuals were combined, frozen, lyophilized and ground to a fine powder. 0.1–0.2 g of dry tissue was placed in 1.5 ml microcentrifuge tubes and saline solution containing 1% NP-40, and 2 mmol l^{-1} phenylmethylsulphonyl fluoride (PMSF) was added at a volume of 4 ml solution g^{-1} tissue. Tubes were then vortexed and spun at 25 000 ***g*** for 30 min. The supernatant was drawn off and analyzed for protein content by Bradford analysis (Bradford, 1976), except for ink, since the purple pigment in pure ink interferes with spectrophotometric readings.

Total protein (25 μg) of each tissue and escapin were separated by SDS–polyacrylamide gel (12%) electrophoresis (PAGE) in a minigel apparatus (Mighty Small II, Amersham Pharmacia Biotech) according to the method of Laemmli (1970). Electrophoresed proteins were transferred to polyvinylidene difluoride membranes (Applied Biosystems Inc., Foster City, CA USA) and analyzed by western blotting using standard procedures (Harlow and Lane, 1988). Proteins were then incubated in anti-escapin antiserum (1:5000 dilution). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Bio-Rad: Hercules, CA, USA) was used as the secondary antibody (1:3333 dilution). Antibody binding to the immunoblot was visualized by developing in nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in alkaline phosphatase buffer (2.5 mmol l^{-1} Tris-HCl, 100 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, pH 9.8).

Western blots for identifying types of ink gland vesicles in which escapin is expressed

SDS–PAGE assays were run for ink, amber vesicles and red-purple vesicles. Ink was collected as described above and purified using the procedure of Yang et al. (2005). Individual amber and red-purple vesicles were microdissected, under a light microscope, from dissected ink glands. Vesicles of each type were separately pooled into small centrifugation tubes in approximately the same frequency as they are present in the glands. Vesicles from approximately one animal were placed on each lane of each gel.

Immunocytochemical localization of escapin and amino acids in the ink and opaline glands

Vibratome sections

Ink glands were dissected from 5 g *A. californica* as described above, pinned out in a Sylgard-covered dissecting dish, and fixed for ~15 h at room temperature by immersion in 4% paraformaldehyde in 0.1 mol l^{-1} Soerensen phosphate buffer (SPB) with 15% sucrose added to adjust osmolarity. After rinsing with SPB, pieces of about 3×3 mm were cut out of uniformly thick parts of the gland that contained numerous small purple vesicles. These pieces were embedded in gelatin as described in detail previously (Schmidt et al., 1992). The gelatin blocks were hardened with 4% paraformaldehyde overnight at 4°C, rinsed briefly with SPB, and cut into 50–200 μm thick cross sections on a vibrating microtome (VT 1000 S, Leica: Wetzlar, Germany). The free-floating sections were incubated overnight at room temperature in the polyclonal anti-escapin antibody (ID-4; Yang et al., 2005) at a dilution of 1:1000 in SPB containing 0.3% Triton X-100 (TSPB). Afterwards the sections were rinsed four times for 30 min each in TSPB, then incubated for 4 h at room temperature in secondary antibody (goat-anti-rabbit CY3-labeled; Jackson ImmunoResearch, West Grove, PA, USA) diluted 1:400 in TSPB. After a further four rinses in SPB for 30 min each, the sections were covered with a solution of 1:1 glycerol:SPB containing 5% DABCO (diazabicyclol[2.2.2]octane) to prevent bleaching and a coverslip was placed over the top. Control sections, obtained from the same pieces of tissue, were treated in parallel, only they were incubated in pre-immune serum (diluted 1:1000 in TSPB) from the rabbit that had produced the ID-4 antibody instead of the antibody itself.

The sections were viewed and imaged using a fluorescence microscope (Axiophot 2, Zeiss: Jena, Germany) equipped with a high-resolution digital camera (DC 500, Leica: Wetzlar, Germany). Images of vesicles were captured under bright-field illumination with the appropriate filter settings for CY3

fluorescence. Both digital images were overlayed by a graphics program (PaintShop Pro 5, Jasc Software: Eden Prairie, MN, USA) before they were arranged into the final figures using Adobe Illustrator software (San Jose, CA, USA).

Plastic sections

Ink and opaline glands were dissected from *A. californica* as described above and fixed in 2.5% paraformaldehyde/1% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer (PB) overnight, or for up to several days at 4°C. The tissue was dehydrated through a graded methanol series (75%, 85%, 95%, 2× 100%, 30 min each solution) and two changes of absolute acetone (30 min each). The tissue was transferred to a 50:50 mixture of acetone and Eponate plastic and incubated overnight at room temperature. Following transfer through two 1-h changes of fresh Eponate, the tissue was embedded in sectioning blocks and cured overnight at 65°C. Individual, consecutive 100–500 nm thick sections, obtained using a Leica Ultracut T microtome (Leica Inc., Bannockburn, IL, USA) and diamond knife (Delaware Diamond Knifes, Wilmington, DE, USA), were transferred to water drops in separate wells of Teflon-coated spot slides (Erie Scientific, Portsmouth, NH, USA) and allowed to dry. Previously described post-embedding immunocytochemical procedures were used (Marc et al., 1990). Briefly, sections were deplasticized with 25% sodium ethoxide (one part saturated sodium hydroxide in three parts absolute ethanol, 7 min), rinsed in 100% methanol (3× 2 min each), rinsed in ultra-pure water (5 min) and dried. Individual sections were incubated in a humidified chamber overnight at room temperature with one of the following primary polyclonal rabbit antibodies (with final dilution): anti-taurine (1:4000 dilution), anti-L-alanine (1:5000 dilution), anti-L-cysteine (1:1000 dilution), anti-L-arginine (1:500 dilution), anti-L-aspartate (1:1000 dilution), anti-L-lysine (1:1000 dilution), anti-glutathione (1:100; Signature Immunologics Inc., Salt Lake City, UT, USA), anti-escapin (1:2000; ID-4; Yang et al., 2005) and pre-immune serum (1:2000) diluted in 0.1 mol l^{-1} PB containing 1% goat serum and 0.05% thiomerosal (pH 7.4). Dot blot analysis indicates that the aspartate and taurine antibodies are at least 1000-fold less cross-reactive to other structurally related antigens (Marc et al., 1990, 1995). Similarly low cross-reactivity is reported by the manufacturer for cysteine, arginine and alanine. Elimination of primary antibody, incubation of the tissues with only the secondary antibody and incubation in pre-immune serum resulted in no immunoreactivity. Following a rinse with 0.1 mol l^{-1} PB, sections were incubated in nanogold-conjugated goat anti-rabbit secondary antibody (1 nm, 1:50 dilution; Amersham Corp., Arlington Heights, IL, USA) for 1 h at room temperature, rinsed with 0.1 mol l^{-1} PB for 1 h and silver intensified for 3 min at 32°C using 0.14% silver nitrate in a hydroquinone (43 mmol l^{-1})/citrate buffer (64 mmol l^{-1}, citric acid; 141 mmol l^{-1} sodium citrate) solution (Kalloniatis and Fletcher, 1993). Following silver intensification, the slides were covered with a cover glass and Eponate plastic, then cured at 65°C overnight. Eight-bit digital images were captured on a Zeiss Axioplan 2 microscope using an Axiocam camera and Axiovision acquisition software (Carl Zeiss Microimaging Inc, Thornwood, NY, USA).

Determination of concentrations of amino acids in ink glands

The concentrations of amino acids in ink and opaline have been published already (Kicklighter et al., 2005). In that study, ink was collected by dissecting out the ink gland and squeezing the ink out of the gland into a dish. This method of collection allowed for escapin to be mixed with its major substrates, L-lysine and L-arginine (Yang et al., 2005), for a period of at least several minutes and up to 1 h, during which the concentrations of these amino acids are reduced through oxidation by escapin (Yang et al., 2005); in fact, no L-lysine or L-arginine were detected in ink collected in this way (Kicklighter et al., 2005) Since we wanted to determine the levels of these amino acids in ink before oxidation, in this study we collected ink glands in a different way. We dissected out ink glands from three animals (total mass of ink glands=3.45 g) and immediately froze whole glands in liquid nitrogen. The tissue was ground on liquid nitrogen and placed in methanol containing 1% trifluoroacetic acid (TFA) to inactivate escapin. After removal of methanol by lyophilization, a residue was dissolved in water and extracted with chloroform to remove compounds of low polarity, and then lyophilized again. For a control, we dissected out ink glands from three animals (total mass of ink glands=3.39 g) and collected ink by squeezing the glands. The collected ink and the squeezed ink glands of these controls were incubated at room temperature for 30 min to allow the amino acid oxidase activity of escapin to occur. These glands were then placed in liquid nitrogen and treated as for the whole glands. Concentrations of lysine, arginine and other free amino acids in ink and opaline were determined using an ion exchange, post-column ninhydrin detection system (Beckman Model 6300/7300 Amino Acid Analyzer, Scientific Research Consortium, Inc.: www.aminoacids.com).

Concentration of escapin in ink

We quantified the concentration of escapin in the ink of 200–300 g field-caught *A. californica* and of the homologue of escapin, dactylomelin-P, in the ink of 200–300 g field-caught *A. dactylomela*. Sea hares were kept in the lab and fed *Gracilaria ferox ad libitum* for at least 5 days before their use. Escapin levels were determined in ink collected in two ways. The first was from ink released by sea hares that are attacked by 'predators', thus representing the concentration of ink as it is expelled from the glands. Sea hares were removed from the aquarium by a glove-wearing human predator, blotted dry with a paper towel to prevent dilution of the ink when released and induced to release their ink by vigorous handling. A 200 μl pipette was used to suck up ink from the mantle cavity as it was released. Ink from each individual was placed in a separate 1.5 ml microcentrifuge tube and kept on ice. We used only animals that released ink and not opaline. For *A. californica*, we collected ink twice, with the collections separated by 1 h. Our second method of collection of ink was from ink glands dissected from anaesthetized animals, thus representing the

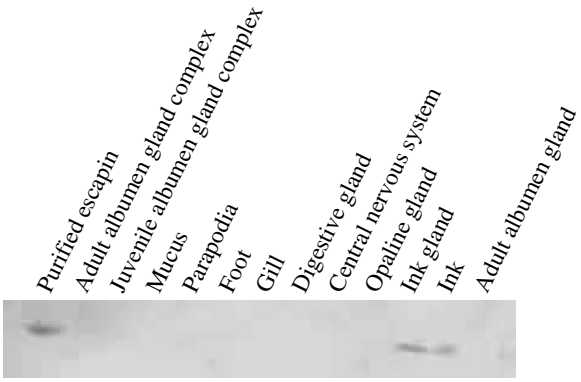

Fig. 1. Western blot shows that escapin is expressed only in the ink gland. The tissues were tested from adult *Aplysia californica*, with the addition of albumen gland complex from juvenile animals. An anti-escapin antibody labels purified protein and a 60 kDa protein in ink and the ink gland.

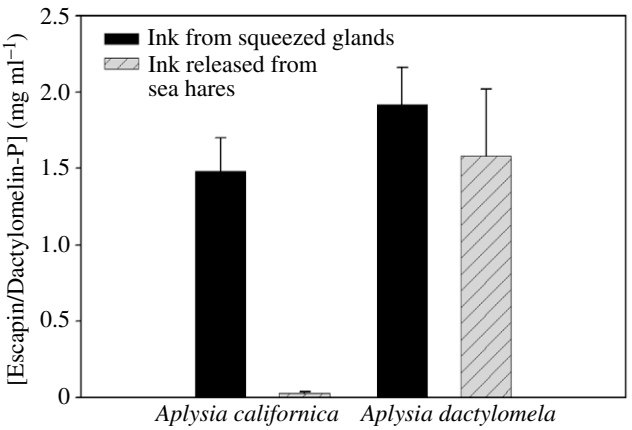

Fig. 2. Concentration of the 60 kDa protein in ink. The 60 kDa protein is escapin from *Aplysia californica* and dactylomelin-P from *Aplysia dactylomela,* which are close homologues (Yang et al., 2005). For each species, two methods of collecting ink were used. 'Ink released', ink collected from animals that were removed from the aquarium, blotted dry, handled until they inked and then ink was pipetted from the mantle cavity. 'Ink from squeezed glands', ink squeezed from dissected ink glands. Values are mean ± s.e.m., *N*=6 animals for each group.

concentration of ink as it is stored in the glands. Six animals were anaesthetized by chilling for 3–4 h at 4°C and then injected with isotonic $MgCl_2$. Ink was obtained from ink glands as described above. In total, ink was collected from 24 animals, 12 from each of the two species and 12 for each of type of ink collection. All ink samples were frozen at –20°C until their use.

The concentration of escapin and dactylomelin-P in the ink samples was quantified by western blot. Four escapin or dactylomelin-P standards (0.0001, 0.001, 0.01 and 0.1 mg) and ink samples were separated using 12% SDS–PAGE and processed as described above. Western blots were scanned, and the trace density of each standard and ink sample was determined using Quantity One software (Bio-Rad, Hercules, CA, USA). The concentration of escapin or dactylomelin-P in the ink samples was calculated from the standard curve constructed using the trace density values for the four standards.

Assay of antibacterial activity

Escherichia coli (MC4100, DH5a) was plated on Petri dishes containing LB medium. Contents of separately microdissected amber vesicles and red-purple vesicles (see the procedure for collecting these samples described above), as well as opaline and ink, were tested for antimicrobial activity. Five μl of the samples were placed on the bacterial lawn, and the dishes were incubated overnight at 37°C or room temperature. If microbial growth was inhibited, it was identifiable as a clear spot on the plate while the rest of the plate had a bacterial lawn. Antibacterial activity was also determined by co-incubation of bacteria with samples of the test substance, followed by measurement of turbidity or counting of number of viable colonies.

Results

Tissue expression pattern for escapin

Western blots using an anti-escapin antibody show that the escapin protein is expressed only in the ink gland and not in any of the other tissues examined (Fig. 1: data for crop and foregut are not shown in this figure). The high specificity of the antibody is demonstrated from the fact that there is no escapin-like immunoreactivity in the albumen gland and albumen gland complex of adults, which contains aplysianin-A precursor protein, a homologue of escapin with 60% identity to escapin (Cummins et al., 2004).

Concentration of escapin in ink

The concentration of escapin (for *A. californica*) or dactylomelin-P (for *A. dactylomela*) was measured in ink obtained in two ways: naturally released from the ink gland by disturbing the animal, and manually squeezed from dissected ink glands (Fig. 2). For *A. dactylomela*, the concentrations of dactylomelin-P are similar by these two techniques: 1.58±0.440 mg ml^{-1} and 1.92±0.241 (mean ± s.e.m.) respectively. These values are also similar to the values of escapin in ink squeezed from dissected ink glands of *A. californica*: 1.48±0.217 mg ml^{-1}. Given escapin and dactylomelin-P have a molecular mass of ~60 kDa, these levels correspond to ~25–30 μmol l^{-1}. However, the concentration of escapin in naturally released ink of *A. californica* was only 0.028±0.007 mg ml^{-1}. The escapin concentration in naturally released ink collected from these same six individual *A. californica* 1 h after the first collection of ink was similarly low (0.025±0.007 mg ml^{-1}).

Distribution of escapin within the ink gland

We used three techniques, SDS–PAGE, immuno-cytochemistry and a bioassay, to demonstrate that the expression of escapin in the ink gland is limited to only one of the two types of vesicles: the amber vesicles.

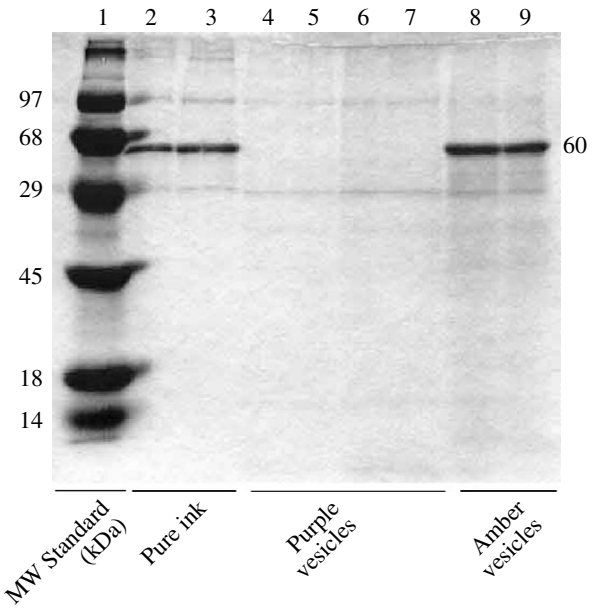

Fig. 3. Western blot shows that escapin is present only in amber vesicles of the ink gland. This SDS–PAGE of *Aplysia californica* ink and vesicles shows that the 60 kDa protein escapin is present in ink and the amber vesicles of the ink gland but not in the red-purple vesicles.

First, by microdissecting amber vesicles from red-purple vesicles and analysing them by SDS–PAGE, we found that amber vesicles but not red-purple vesicles contain the 60 kDa protein escapin (Fig. 3).

Second, immunocytochemistry with the anti-escapin antibody shows that escapin-like immunoreactivity is present in amber vesicles but not in red-purple vesicles. On cross sections through the ink gland that were at least 100 μm thick, numerous red-purple and amber vesicles were clearly identified by their color, visible with transmitted light illumination (Fig. 4A). In addition, some structures of the same size and shape but without colored contents could also be identified. Red-purple vesicles usually had a regular spherical to elliptical shape and ranged from 100 to 200 μm maximum diameter (mean diameter = 157 μm). Amber vesicles often had more irregular shapes and ranged from 150 to 260 μm in maximum diameter (mean diameter = 191 μm). The anti-escapin antibody selectively labeled the amber vesicles (Fig. 4B,C). Purple vesicles and the structures with clear contents were never labeled. Control sections incubated in pre-immune serum instead of antibody were devoid of labeling. The antibody labeling was intense only at the surface of the sections, and this lack of penetration prevented the labeling of vesicles that were not cut through in the sectioning process. We believe that this is the reason for the occurrence of a small percentage of unlabeled amber vesicles. Frequently, we observed small areas of intense labeling among the larger labeled areas that could unequivocally be attributed to the presence of amber vesicles. We interpret these structures as sections through the bottom or top of amber vesicles with the main part of the vesicle remaining in the adjacent section and thereby preventing the identification of the vesicle type based on its color.

Our third line of experimental support for escapin being present in amber vesicles comes from a bioassay showing that amber vesicles but not red-purple vesicles have antimicrobial activity, which is a feature of escapin (Fig. 5).

Immunocytochemical localization of amino acids within the ink and opaline glands

Knowing the distribution of amino acids in the ink and opaline glands is important, for two reasons. First, one might expect that the basic L-amino acids lysine and arginine, which are especially good substrates for the L-amino acid oxidase, escapin (Yang et al., 2005), would be packaged separately from escapin in order to prevent premature generation of bioactive compounds. Thus, we might expect that lysine and arginine are packaged in the ink gland in vesicles different from those of escapin or in the opaline gland. Second, given that certain amino acids (especially taurine, lysine, aspartate, alanine, glutamate and histidine) are in high concentration in

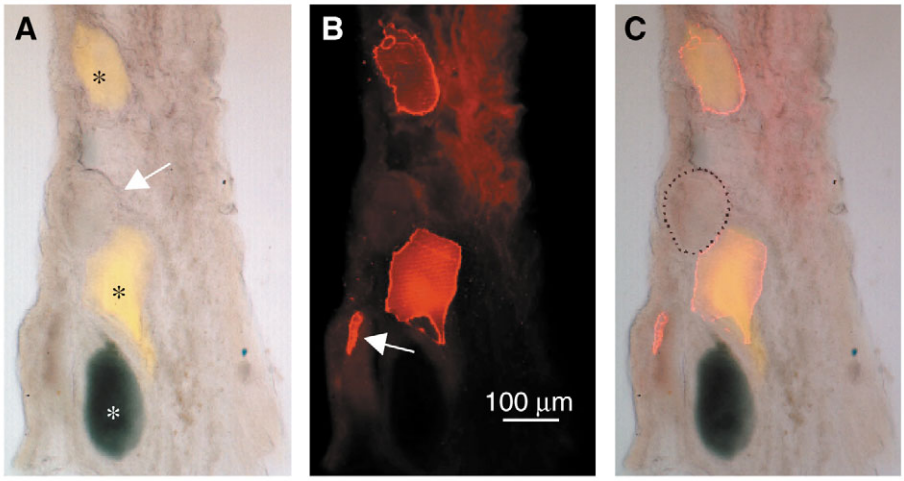

Fig. 4. Immunostaining with anti-escapin shows that escapin in the ink gland is expressed only in amber vesicles. Tissue is 100 μm thick vibrating microtome sections of ink gland of *Aplysia californica*. (A) Transmitted light illumination reveals the presence of amber (black asterisks), red-purple (white asterisk) vesicles, and an uncolored vesicle-like structure (arrow, highlighted in C). (B) Fluorescence image showing anti-escapin immunoreactivity. Note that only the amber vesicles are immunoreactive. The additional small area of immunoreactivity (arrow) might represent the cap of a vesicle whose bulk is located in the adjacent section. (C) Overlay of A and B. Note that red-purple vesicle and the uncolored vesicle-like structure (highlighted by dotted line) are unlabeled.

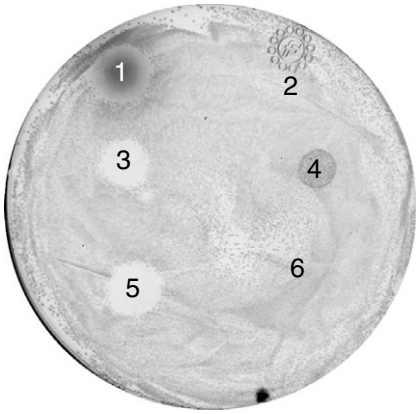

Fig. 5. Antimicrobial assay shows bioactivity only in amber vesicles of *Aplysia californica*. Clear spots on the plate represent antibacterial activity and are seen only for spots 1, 3 and 5. Spot 1, pure ink (note that this spot is free of bacteria, but appears dark because of the purple pigment of the ink); 2, buffer control; 3, red-purple and amber vesicle extracts together; 4, opaline (note that this spot is dark because it has bacterial growth greater than the control), because opaline is a very good growth medium; 5, amber vesicle extract alone; 6, red-purple vesicle extract alone.

ink and/or opaline and have an antipredatory function via phagomimicry (Kicklighter et al., 2005), it would be interesting to know their vesicular distribution in these glands.

We found that vesicles in the ink gland differ with respect to their amino acid content, and they can be divided into at least three classes. Fig. 6 shows several examples from three ink glands. One class of vesicles (labeled 'e' in Fig. 6) is intensely and specifically labeled for escapin, having low or no immunoreactivity for amino acids and including arginine, cysteine, aspartate, taurine, alanine (shown in Fig. 6) or lysine, or the tripeptide glutathione (not shown). These are probably amber vesicles, given our results described above showing that escapin is present in amber vesicles. The second class of vesicles (labeled 'a' in Fig. 6) is immunopositive for amino acids but with low or no immunoreactivity to escapin. Taurine, aspartate and alanine tend to have the highest levels of immunoreactivity and lower immunoreactivity for arginine and lysine. This is interesting since these immunoreactivity levels are closely correlated with the relatively high abundance of taurine, aspartate and alanine in ink (Kicklighter et al., 2005). We hypothesize that this second class of vesicles are the red-purple vesicles. A third class of vesicles (labeled 'c' in Fig. 6) has low or no immunoreactivity to most amino acid antibodies and for escapin, and usually similar degree of immunolabeling as preimmune controls. The immunolabeling in these vesicles is usually more intense than in the surrounding non-vesicular tissue. This suggests no or low levels of amino acids in these vesicles, without specificity. We hypothesize that these are the clear vesicles, which are devoid of ink, either because they are not filled or they have already released their ink contents.

In the opaline glands, we can distinguish at least two classes of vesicles based on amino acid immunoreactivity (Fig. 7). One class of vesicles (labeled '1' in Fig. 7) is strongly immunopositive for taurine and some, though usually less, immunoreactivity for other amino acids. A second class of vesicles (labeled '2' in Fig. 7) typically is strongly immunopositive for aspartate and alanine, moderately for lysine, and moderately to poorly for cysteine and taurine.

Concentration of amino acids in ink and opaline

Prior analysis of ink and opaline expressed from their respective glands indicated that opaline was the only source of escapin's major substrates, L-lysine and L-arginine, and that L-lysine was by far the more abundant: the concentrations of L-lysine and L-arginine in opaline were 65 190 and 340 μmol l^{-1}, respectively, while no L-lysine or L-arginine were found in ink (Kicklighter et al., 2005). However, since ink but not opaline contains escapin, it is possible that our finding that ink contained no lysine or arginine is because escapin had oxidized it all before chemical analysis. Thus, we examined the concentrations of lysine, arginine and other amino acids in ink glands frozen in liquid nitrogen immediately after dissection from an animal and before ink was released from the gland. We found lysine and arginine at concentrations of 14 and 38 μmol l^{-1}, respectively. The total amino acid concentration in this sample was approximately 10% of that in our measurements for released ink (Kicklighter et al., 2005), but this might be the result of the necessary treatment of the frozen sample with TFA, methanol and $CHCl_3$. Thus, if we assume that the loss is a general one for all amino acids, then we can correct for this by scaling up the values in the frozen sample by a constant factor. When we do this, the values for lysine and arginine are 150 and 418 μmol l^{-1} respectively, which we assume to be the upper limits to their possible concentrations in the ink gland. Thus, we believe that the concentrations of amino acids in the ink gland before escapin is mixed with them is 14–150 μmol l^{-1} for lysine and 38–418 μmol l^{-1} for arginine. If we compare these with the concentrations of lysine and arginine in opaline – 65, 190 and 340 μmol l^{-1} respectively, this leads to two conclusions: (1) lysine and arginine together are a relatively minor component of the amino acid content of ink (51–568 μmol l^{-1} out of 29 620 μmol l^{-1}); and (2) lysine is at much higher levels (over 200 times greater) than arginine in the mixture of ink and opaline. Given that only lysine and not arginine mediates the bactericidal effect, and lysine is effective only at high (>1 mmol l^{-1}) concentrations, and given that lysine and arginine mediate the bacteriostatic effect only at doses >1 mmol l^{-1}, we conclude that it is the lysine in opaline that is the primary substrate for escapin's antimicrobial effect.

Discussion

Our results demonstrate that an enzyme-based chemical defense of sea hares, i.e. the L-amino acid oxidase escapin, and its main substrates lysine and to a lesser degree arginine, are packaged to be maximally effective only when needed, i.e. when the sea hare is attacked.

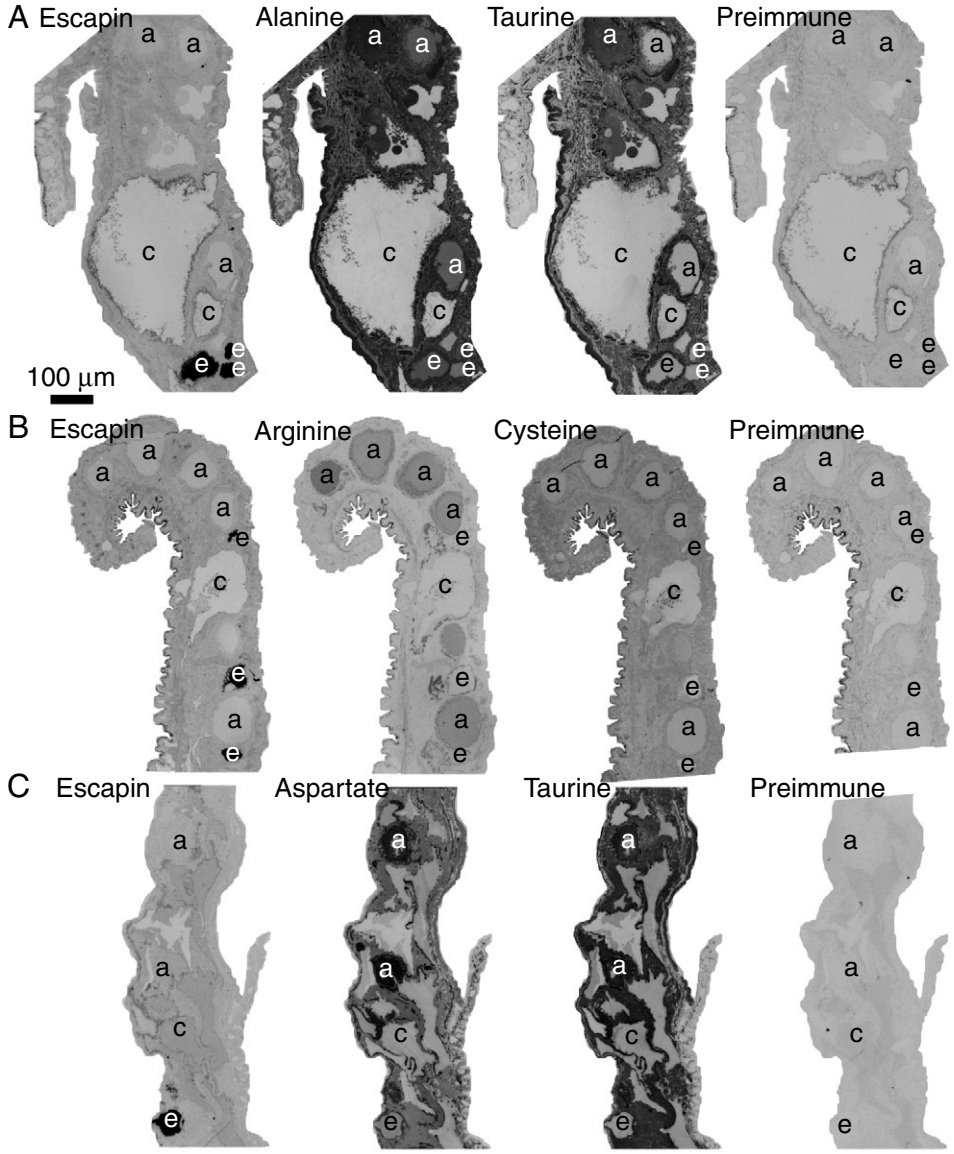

Fig. 6. Immunostaining with antisera against amino acids and escapin in ink gland of *Aplysia californica*. Tissue is 0.5 μm thick plastic sections. A, B and C are from three different ink glands, each a set of four sections from the same region of tissue labeled with different antisera, i.e. antiserum against either escapin, alanine, taurine, arginine, cysteine or aspartate. 'Preimmune' is the control, being labeled with preimmune serum. Vesicles labeled 'a' are strongly immunopositive for many amino acids but not escapin; vesicles labeled 'e' are intensely immunopositive for escapin but not for amino acids; vesicles labeled 'c' are clear and not strongly immunopositive either for amino acids or escapin.

The protein escapin has a highly restricted distribution across the tissues of the sea hare *A. californica*. The only tissue containing escapin is the ink gland; no other tissue or secretion contains escapin, not even those previously shown to have defensive chemicals, such as the opaline gland (Kicklighter et al., 2005) and skin (Kinnel et al., 1979; Fig. 1). In addition, escapin is absent from the albumen gland of both sexually mature and immature animals. This is especially interesting because it contains aplysianin-A, a protein with 60% identity to escapin (Cummins et al., 2004) and with antimicrobial function (Kamiya et al., 1986).

Within the ink gland, escapin is present only in the amber vesicles and not in the red-purple vesicles, as supported by four lines of evidence. First, SDS–PAGE identified a 60 kDa protein that had the molecular mass of escapin (Yang et al., 2005), only in amber vesicles and not in red-purple vesicles (Fig. 2). Second, using an antiserum generated against escapin, immunoreactivity occurred only in the ink gland's amber vesicles (Fig. 3). Third, an antimicrobial assay showed that amber vesicles but not red-purple vesicles have antimicrobial activity (Fig. 4), which is a property of escapin. Fourth, purified escapin is amber in color, because of the presence of FAD (flavin-adenine dinucleotide; Yang et al., 2005). Taken together, these observations lead us to conclude that escapin is present in the amber vesicles and not the red-purple vesicles.

The ink gland has high concentrations of escapin (in *A. californica*) or dactylomelin-P (in *A. dactylomela*). When squeezed from the ink gland of *A. californica* or *A. dactylomela*, the concentrations of these proteins are 1.48 and 1.92 mg ml^{-1}, respectively, or 25–30 μmol l^{-1}. The concentration of this protein in ink as it is released from an attacked sea hare is about the same as in the ink gland of *A. dactylomela* (1.58 *vs* 1.92 mg ml^{-1}) but not in *A. californica* (0.028 *vs* 1.48 mg ml^{-1}). The explanation for this difference in two closely related species is not known, but it is possible that in *A. californica* there is a disproportionately low release of escapin compared with other fluids in the ink gland. In both *Aplysia* species, multiple bouts of inking are typical (Nolen and Johnson, 2001), so the ink store of each animal is not depleted in the conditions of our experiments. The concentrations of this protein in released ink are sufficiently high to have protective effects. The concentration of released protein from both *A. californica* and *A. dactylomela* is much higher than the minimum concentration of escapin that is inhibitory against several bacterial species, which is 0.0003–0.005 mg ml^{-1} (Yang et al., 2005). Thus, the released

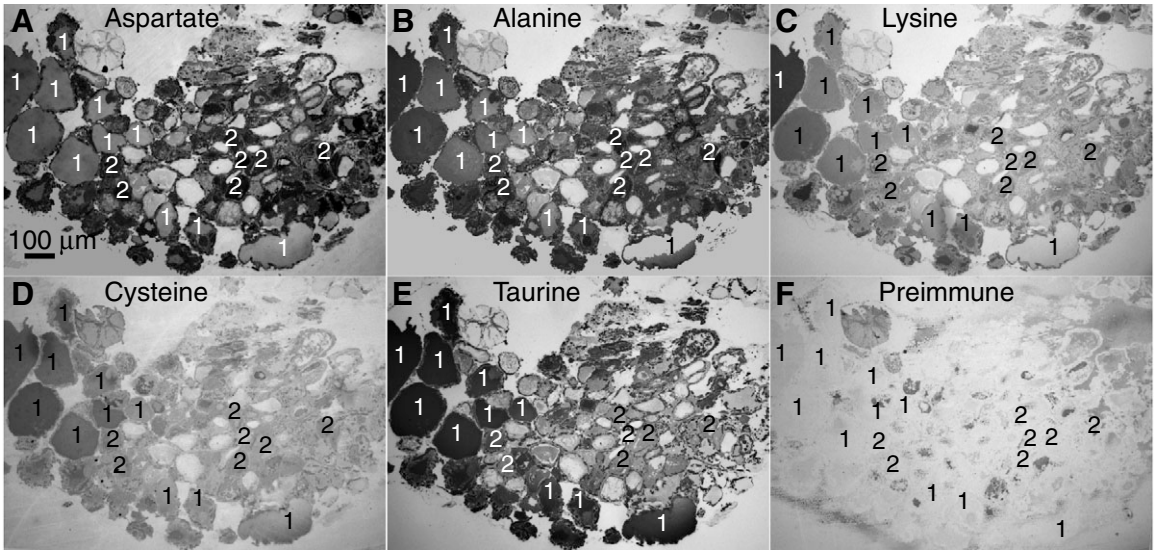

Fig. 7. Immunostaining with antisera against amino acids in an opaline gland of *Aplysia californica*. Shown is a set of 0.5 µm thick plastic sections from the same region of tissue labeled with different antisera, i.e. antiserum against (A) aspartate, (B) alanine, (C) lysine, (D) cysteine or (E) taurine, or (F) preimmune serum. Vesicles labeled '1' are strongly immunopositive for taurine and have some, though usually less, immunoreactivity for other amino acids. Vesicles labeled '2' are less immunoreactive to taurine than to most another amino acids, particularly aspartate and alanine.

protein represents a highly potent effector at its natural concentrations.

Though it is known that the ink of *A. californica* is an effective anti-feedant against the large predatory sea anemone *Anthopleura xanthogrammica* (Nolen et al., 1995), what role escapin may play has not been established. Preliminary data suggest that escapin is capable of causing direct damage to sea anemone tentacles and their symbiotic zooxanthellae (Johnson, 2002); however, the sea anemone behavioral response to ink occurs in a matter of seconds and yet it takes hours for lysis by escapin at natural concentrations (C.E.K., unpublished data). Nonetheless, it is unlikely that natural selection would maintain the production of such large quantities of a bioactive protein to be secreted in a defensive mixture that is only released under predatory attack if it did not serve an antipredatory role. It may have evolved from antimicrobial proteins, as its activity suggests, but it is unlikely that sea hares shower their predators with antibiotics after an attack in order to keep the predators microbe free. Additionally, if sea hares were releasing escapin to cover their own bodies with antibiotics, then it should be released at more regular intervals. Along with the sea anemones, several species of sea stars, crabs and fish are known to prey on sea hares (Sarver, 1978; Willan, 1979; Johnson and Willows, 1999); thus, it is plausible that escapin may deter one or more of these predators through mechanisms, as yet, unknown.

The substrates for escapin are distributed in a way to enhance their effectiveness. Lysine and arginine are the main substrates for escapin's oxidase activity (Yang et al., 2005). However, lysine probably plays a more important role in this hypothesized defense than arginine, for two reasons. First, lysine is both bacteriostatic and bactericidal, whereas, arginine is primarily only bacteriostatic (Yang et al., 2005). Secondly, lysine is present in the defensive secretion at a much higher concentration than arginine (our results; Kicklighter et al., 2005). Significantly, lysine is packaged separately from escapin: lysine is in high concentration in opaline but not ink, and escapin is only in ink. Thus, the bioactive chemicals in this pathway are only produced when ink and opaline are mixed. This occurs when the sea hare is attacked by a predator, after which the ink gland and opaline gland often co-release their contents into the mantle cavity, and then the ink-opaline mixture is squirted out *via* the siphon and directed toward the site of attack. This separate packaging of the enzyme from its substrates would aid in preventing autotoxicity within the animal before the vesicular release of the ink. Whether other L-amino acid oxidase toxins, such as those in venomous snakes (Torii et al., 2000; MacHeroux et al., 2001; Du and Clemetson, 2002; Lu et al., 2002; Stábeli et al., 2004), are packaged separately from substrates is, to our knowledge, not known.

The reason for a diversity of vesicle types in the opaline gland, specifically why one class of vesicles is relatively high in taurine and another class is relatively low in taurine (Fig. 7), is not known. The role of taurine in one type of chemical defense, phagomimicry, against predatory spiny lobsters is known (Kicklighter et al., 2005). The opaline gland is also known to have feeding deterrents (Kicklighter et al., 2005) and toxins (Flury, 1915), but whether they are distributed in different types of vesicles in this gland is not known at present.

In our laboratory studies of attacks by crustacean predators on sea hares, ink and opaline are typically both released more or less simultaneously (Kicklighter et al., 2005). Such co-release would obviously benefit the escapin-based defensive pathway by allowing mixing of the enzyme and its substrate.

However, ink and opaline contain other antipredatory compounds that do not require enzyme activity or mixing of the glandular secretions, so co-release of ink and opaline may not always be functionally necessary. In fact, the neural pathways controlling the release of ink and opaline are different, with each gland being innervated by different sets of motor neurons located in different ganglia, even though some of the sensory pathways to the motor neurons may be shared (Tritt and Byrne, 1980; Walters and Erickson, 1986). We have seen some instances in which opaline is released at least several seconds before ink (see supplemental figure in Kicklighter et al., 2005; C. E. Kicklighter, unpublished data), while others have noted that ink appears to have a lower threshold to release than opaline (Kandel, 1979; Byrne, 1980; Tritt and Byrne, 1980; Walters and Erickson, 1986; Nolen et al., 1995; Nolen and Johnson, 2001). It will be interesting to examine whether sea hares can regulate the thresholds at which the two glands are released depending on the identity of the attacking predator and thus which chemical defenses are more likely to be needed.

The ink of most sea hares has its distinctive red-purple color because of chromophores such as phycoerythrobilin. These pigments, which are derived from consumption of red algae, are also differentially packed only in the red-purple vesicles of the ink gland (Prince et al., 1998). These chromophores have not been definitively shown to have a protective function, either antipredatory or antimicrobial. It is possible that they function visually, as a chemical 'smoke-screen' (see Carefoot, 1987; Johnson and Willows, 1999) or as a visual mimic of sea hares during phagomimetic defense (Kicklighter et al., 2005). They have been speculated to be involved in chemical defenses, but the support for this is incomplete (Carlson and Nolen, 1997). Why they are packaged in ink gland vesicles separately from escapin is not known.

We thank Hsin Chien, Ying-Ju Huang, Shkelzen Shabani, Louisa Smythe, Brian Vu, Vivian Vu-Ngo and A. O. Dennis Willows for assistance and discussions. This work was supported by NSF IBN-0324435, NIH GM-34766, NSF National Science and Technology Center Grants IBN-9876754 and IBN-0322773, the Georgia Research Alliance, and GSU Research Program Enhancement Fund.

References

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*. **72**, 248-254.

Byrne, J. H. (1980). Neural circuit for inking behavior in *Aplysia californica*. *J. Neurophysiol.* **43**, 896-911.

Carefoot, T. H. (1987). *Aplysia*: its biology and ecology. *J. Exp. Biol.* **24**, 167-284.

Carlson, B. and Nolen, T. G. (1997). The effect of *Aplysia*'s defensive chemical ink on the dactyl chemoreceptors of predatory crabs (*Cancer antennarius*). *Soc. Neurosci. Abstr*. **23**, 188.

Chapman, D. J. and Fox, D. L. (1969). Bile pigment metabolism in the sea-hare *Aplysia*. *J. Exp. Mar. Biol. Ecol.* **4**, 71-78.

Coelho, L., Prince, J. and Nolen, T. G. (1998). Processing of defensive pigment in *Aplysia californica*: acquisition, modification and mobilization of the red algal pigment r-phycoerythrin by the digestive gland. *J. Exp. Biol.* **201**, 425-438.

Cummins, S. F., Nichols, A. E., Amare, A., Hummon, A. B., Sweedler, J. V. and Nagle, G. T. (2004). Characterization of *Aplysia* enticin and temptin, two novel water-borne protein pheromones that act in concert with attractin to stimulate mate attraction. *J. Biol. Chem*. **279**, 25614-25622.

Du, X. Y. and Clemetson, K. J. (2002). Snake venom L-amino acid oxidases. *Toxicon* **40**, 659-665.

Faulkner, D. J. (1992). Chemical defenses in marine mollusks. In *Ecological Roles of Marine Natural Products* (ed. V. J. Paul), pp. 119-163. Ithaca (NY): Comstock.

Flury, F. (1915). Über das Aplysiengift. *Arch. Exp. Pathol. Pharmakol.* **79**, 250-263.

Ginsburg, D. W. and Paul, V. J. (2001). Chemical defenses in the sea hare *Aplysia parvula*: importance of diet and sequestration of algal secondary metabolites. *Mar. Ecol. Prog. Series* **215**, 261-274.

Harlow, E. and Lane, D. (1988). *Antibodies: A Laboratory Manual*. New York: Cold Spring Harbor Laboratory Press.

Johnson, P. M. (2002). Multi-component chemical defense in seahares (Gastropoda: Opisthobranchia): antipredator compounds act as both honest and deceptive signals to multiple predator species. PhD dissertation, University of Washington, USA.

Johnson, P. M. and Willows, A. O. D. (1999). Defense in sea hares (Gastropoda, Opisthobranchia, Anaspidea): multiple layers of protection from egg to adult. *Mar. Fresh. Behav. Physiol.* **32**, 147-180.

Kalloniatis, M. and Fletcher, E. L. (1993). Immunocytochemical localization of the amino acid neurotransmitters in the chicken retina. *J. Comp. Neurol.* **336**, 174-193.

Kamiya, H., Muramoto, K. and Yamazaki, M. (1986). Aplysianin-A, an antibacterial and antineoplastic glycoprotein in the albumen gland of a sea hare, *Aplysia kurodai*. *Experientia* **42**, 1065-1067.

Kamiya, H., Muramoto, K., Goto, R., Sakai, M., Endo, Y. and Yamazaki, M. (1989). Purification and characterization of an antibacterial and antineoplastic protein secretion of a sea hare, *Aplysia juliana*. *Toxicon* **27**, 1269-1277.

Kandel, E. R. (1979). *Behavioral Biology of Aplysia*. San Francisco, CA, USA: Freeman and Co.

Kicklighter, C. E., Shabani, S., Johnson, P. M. and Derby, C. D. (2005). Sea hares use novel antipredatory chemical defenses. *Curr. Biol.* **15**, 549-554.

Kinnel, R. B., Dieter, R. K., Meinwald, J., Van Engen, D. C. J., Eisner, T., Stallard, M. O. and Fenical, W. H. (1979). Brasilenyne and *cis*-dihydrorhodophytin: antifeedant medium-ring haloethers from a sea hare (*Aplysia brasiliana*). *Proc. Natl. Acad. Sci. USA* **76**, 3576-3579.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

Lu, Q. M., Wei, Q., Jin, Y., Wei, J. F., Wang, W. Y. and Xiong, Y. L. (2002). L-amino acid oxidase from *Trimeresurus jerdonii* snake venom: purification, characterization, platelet aggregation-inducing and antibacterial effects. *J. Nat. Toxins* **11**, 345-352.

MacColl, R., Galivan, J., Berns, D. S., Zimec, Z., Guard-Friar, D. and Wagoner, D. (1990). The chromophore and polypeptide composition of *Aplysia* ink. *Biol. Bull*. **179**, 326-331.

MacHeroux, P., Seth, O., Bollschweiler, C., Schwarz, M., Kurfurst, M., Au, L. C. and Ghisla, S. (2001). L-Amino-acid oxidase from the Malayan pit viper *Calloselasma rhodostoma*. Comparative sequence analysis and characterization of active and inactive forms of the enzyme. *Eur. J. Biochem.* **268**, 1679-1686.

Marc, R. E., Wei-Ley, S. L., Kalloniatis, M., Raiguel, S. F. and Van Haesendonck, E. (1990). Patterns of glutamate immunoreactivity in the goldfish retina. *J. Neurosci.* **10**, 4006-4034.

Marc, R. E., Murry, R. F. and Basinger, S. F. (1995). Pattern recognition of amino acid signatures in retinal neurons. *J. Neurosci.* **15**, 5106-5129.

Melo, V. M. M., Duarte, A. B. G., Carvalho, A. F. F. U., Sebra, E. A. and Vasconcelos, I. M. (2000). Purification of a novel antibacterial and haemagglutinating protein from the purple gland of the sea hare, *Aplysia dactylomela* Rang, 1828. *Toxicon* **38**, 1415-1427.

Nistratova, S. N., Mitskevich, I. N., Mirabet-Regalado, M. E. and Josef, N. (1993). Inhibitory effect of the purple fluid produced by a mollusk *Aplysia punctata* on growth of gram-negative marine bacteria. *Microbiology* **61**, 630-632.

Nolen, T. G. and Johnson, P. M. (2001). Defensive inking in *Aplysia* spp.: multiple episodes of ink secretion and the adaptive use of a limited chemical resource. *J. Exp. Biol.* **204**, 1257-1268.

Nolen, T. G., Johnson, P. M., Kicklighter, C. E. and Capo, T. (1995). Ink

secretion by the marine snail *Aplysia californica* enhances its ability to escape from a natural predator. *J. Comp. Physiol. A* **176**, 239-254.

Petzelt, C., Joswig, G., Stammer, H. and Werner, D. (2002). Cytotoxic cyplasin of the sea hare, *Aplysia punctata*, cDNA cloning, and expression of bioactive recombinants in insect cells. *Neoplasia* **4**, 49-59.

Prince, J., Nolen, T. G. and Coelho, L. (1998). Defensive ink pigment processing and secretion in *Aplysia californica*: concentration and storage of phycoerythrobilin in the ink gland. *J. Exp. Biol.* **201**, 1595-1613.

Rajaganapathi, J. and Kathiresan, K. (2002). Heparinase in purple fluid of the sea hare, *Bursatella leachii*. *Curr. Sci.* **82**, 264-266.

Rogers, C. N., de Nys, R., Charlton, T. S. and Steinberg, P. D. (2000). Dynamics of algal secondary metabolites in two species of sea hare. *J. Chem. Ecol*. **26**, 721-744.

Sarver, D. J. (1978). The ecology and energetics of *Aplysia juliana* (Quoy and Qaimard, 1832). PhD dissertation, University of Hawaii, USA.

Schmidt, M, Van Ekeris, L. and Ache, B. W. (1992). Antennular projections to the midbrain of the spiny lobster. I. Sensory innervation of the lateral and medial antennular neuropils. *J. Comp. Neurol*. **318**, 277-290.

Stábeli, R. G., Marcussi, S., Carlos, G. B., Pietro, R. C. L. R., Selistre-de-Araújo, H. S., Giglio, J. R., Oliveira, E. B. and Soares, A. M. (2004). Platelet aggregation and antibacterial effects of an L-amino acid oxidase purified from *Bothrops alternatus* snake venom. *Bioorgan. Med. Chem*. **12**, 2881-2886.

Torii, S., Yamane, K., Mashima, T., Haga, N., Yamamoto, K., Fox. J. W., Naito, M. and Tsuruo, T. (2000). Molecular cloning and functional analysis of apoxin I, a snake venom-derived apoptosis-inducing factor with L-amino acid oxidase activity. *Biochemistry* **39**, 3197-3205.

Tritt, S. H. and Byrne, J. H. (1980). Motor controls of opaline secretion in *Aplysia californica*. *J. Neurophysiol.* **43**, 581-594.

Walters, E. T. and Erickson, M. T. (1986). Directional control and the functional organization of defensive responses in *Aplysia*. *J. Comp. Physiol. A* **159**, 339-351.

Willan, R. C. (1979). The ecology of two New Zealand opisthobranch mollusk. PhD dissertation, University of Auckland, New Zealand.

Yamada, K. and Kigoshi, H. (1997). Bioactive compounds from the sea hares of two genera: *Aplysia* and *Dolabella*. *Bull. Chem Soc. Jpn*. **70**, 1479-1489.

Yamazaki, M. (1993). Antitumor and antimicrobial glycoproteins from sea hares. *Comp. Biochem. Physiol.* **105C**, 141-146.

Yamazaki, M., Kimura, K., Kisugi, J. and Hisao, K. (1986). Purification of a cytolytic factor from purple fluid of a sea hare. *FEBS Lett.* **198**, 25-28.

Yang, H., Johnson, P. M., Ko, K.-C., Kamio, M., Germann, M. W., Derby, C. D. and Tai, P. C. (2005). Cloning, characterization and expression of escapin, a broadly antimicrobial FAD-containing L-amino acid oxidase from ink of the sea hare *Aplysia californica*. *J. Exp. Biol.* **208**, 3609-3622.