

ARTICLES

Halide Peroxidase in Tissues That Interact With Bacteria in the Host Squid *Euprymna scolopes*

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Abstract An enzyme with similarities to myeloperoxidase, the antimicrobial halide peroxidase in mammalian neutrophils, occurs abundantly in the light organ tissue of *Euprymna scolopes*, a squid that maintains a beneficial association with the luminous bacterium *Vibrio fischeri*. Using three independent assays typically applied to the analysis of halide peroxidase enzymes, we directly compared the activity of the squid enzyme with that of human myeloperoxidase. One of these methods, the diethanolamine assay, confirmed that the squid peroxidase requires halide ions for its activity. The identification of a halide peroxidase in a cooperative bacterial association suggested that this type of enzyme can function not only to control pathogens, but also to modulate the interactions of host animals with their beneficial partners. To determine whether the squid peroxidase functions under both circumstances, we examined its distribution in a variety of host tissues, including those that typically interact with bacteria and those that do not. Tissues interacting with bacteria included those that have specific cooperative associations with bacteria (i.e., the light organ and accessory nidamental gland) and those that have transient nonspecific interactions with bacteria (i.e., the gills, which clear the cephalopod circulatory system of invading microorganisms). These bacteria-associated tissues were compared with the eye, digestive gland, white body, and ink-producing tissues, which do not typically interact directly with bacteria. Peroxidase enzyme assays, immunocytochemical localization, and DNA–RNA hybridizations showed that the halide-dependent peroxidase is consistently expressed in high concentration in tissues that interact bacteria. Elevated levels of the peroxidase were also found in the ink-producing tissues, which are known to have enzymatic pathways associated with antimicrobial activity. Taken together, these data suggest that the host uses a common biochemical response to the variety of types of associations that it forms with microorganisms. *J. Cell Biochem.* 72:445–457, 1999. © 1999 Wiley-Liss, Inc.

Key words: *Vibrio fischeri*; myeloperoxidase; antimicrobial activity; symbiosis; accessory nidamental gland; invertebrate immunity; light organ; gill cyst

All animals have a range of interactions with bacteria, from beneficial to pathogenic. In each type of relationship, the host and bacteria have evolved strategies by which to promote or deter the persistence of the association. However, while the molecular and biochemical mechanisms regulating these various types of associations must be differently controlled, the nature of these differences is poorly understood. A challenge to researchers that study animal–bacterial interactions has been finding tractable

model systems in which to identify and describe these differences.

The sepiolid squid *Euprymna scolopes* has demonstrated usefulness as a model for the study of animal–bacterial associations [McFall-Ngai and Ruby, 1991, 1998; Ruby, 1996]. This host establishes and maintains stable, beneficial associations with bacteria in two tissue types, the light organ and the accessory nidamental gland. In the light organ, the host houses symbiosis-competent strains of the luminous bacterium *Vibrio fischeri*, which grows extracellularly within epithelia-lined crypts of the adult light organ [McFall-Ngai and Montgomery, 1990]. The organ is surrounded by accessory tissues, including diverticula of the ink sac, a reflector, and a muscle-derived lens [McFall-Ngai and Montgomery, 1990], that serve to modify the bacterial luminescence for use by

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the host in its nocturnal, antipredatory behavior [Moynihan, 1983]. Similar to several other squid species [Bloodgood, 1977; Kaufman et al., 1998], adult females of *E. scolopes* also maintain an extracellular bacterial consortium in the accessory nidamental gland (ANG), a set of tissues associated with the female reproductive system. In addition to these stable cooperative associations, *E. scolopes* can also have bacteria associated with gill tissues (A.L. Small and M.J. Ngai, personal observation). When exogenous pathogenic or indigenous opportunistic bacteria invade the circulatory system of cephalopods, they are transported to the gills, where they are concentrated and sequestered in cysts [Bayne, 1973, 1974]. The occurrence of such a variety of host-bacterial relationships within a single host offers the opportunity to determine whether the same genes, and their corresponding products are involved in the control of these different forms of symbiosis.

Previous studies of the light organ of *E. scolopes* demonstrated high levels of an mRNA that encodes a protein with significant sequence identity to a specific halide peroxidase, mammalian myeloperoxidase (MPO) [Tomarev et al., 1993; Weis et al., 1996]. The original descriptions of the activity of MPO in human neutrophils showed that it catalyzes the production of potent microbicides in direct response to pathogens [Klebanoff, 1991]. Specifically, hypohalous acids are produced from halide ions and H_2O_2 , which is generated by the respiratory burst activity of the cell [Klebanoff, 1967a,b]. MPO-like enzymes have also been reported in a variety of invertebrate species [Nelson et al., 1994; Johansson et al., 1995], where they are presumed to play a role in respiratory-burst mediated host-defense responses [Dikkeboom et al., 1988; Adema et al., 1991; Noel et al., 1993; Anderson, 1994; Song and Hsieh, 1994; Anderson et al., 1995; Greger et al., 1995].

Because both MPO and MPO-like enzymes, in vertebrates and invertebrates, have been presumed to function only in the defense of the host against pathogens, the discovery of an MPO-like enzyme in a beneficial association of *E. scolopes* presented an apparent contradiction and suggested that this enzyme may have a broader function than previously described. In the present study, we first determined that the squid enzyme is a member of the family of halide-dependent peroxidases, which typically produce antimicrobial compounds. Then, tak-

ing advantage of the array of symbiotic associations that occur in *E. scolopes*, we asked: What is the distribution of this enzyme in host tissues, and how does this distribution relate to the presence or absence of bacterial interactions?

MATERIALS AND METHODS

General Procedures

Adult *E. scolopes* were collected on Oahu, Hawaii, and transported to the University of Southern California in Los Angeles, where they were maintained in a recirculating aquarium system as previously described [McFall-Ngai and Montgomery, 1990].

All reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise noted. Protein concentrations were determined spectrophotometrically [Whitaker and Gram, 1980]. These and other spectrophotometric determinations were performed on a Kontron 930 Uvicon spectrophotometer.

Biochemical Characterization of the Squid Peroxidase Enzyme

We compared the characteristics of the peroxidase activity in the squid symbiotic light organ, which had been previously reported to have high activity [Weis et al., 1996], with those of purified human MPO (CalBiochem, San Diego, CA) under three assay conditions that typically have been used to describe halide peroxidases: the halide-dependent diethanolamine (DEA) assay [Andrews and Krinsky, 1982; Schlenk et al., 1991] and the halide-independent dianisidine [Krawisz et al., 1984] and guaiacol assays [Desser et al., 1972; Tien and Kirk, 1988; Taurrog et al., 1992; Koduri and Tien, 1995]. The squid eye was used as a negative control tissue in all three assays. Determinations of peroxidase activity in both the squid light organ and eye tissues were made after the removal of their lenses, which were found to contain no activity. Initial experiments were performed for each assay to optimize pH and substrate concentrations for the squid light organ peroxidase. In the halide-independent assays, the squid peroxidase and human MPO had the same optima for pH and substrate concentrations. However, in the halide-dependent DEA assay, the pH optimum of the squid peroxidase was 3.5, whereas the pH optimum of human MPO was 4.4–5.0, as previously reported [Andrews and Krinsky,

1982; Andrews et al., 1984]. Thus, in the DEA assays, we compared the squid and human peroxidases at their respective optima.

Halide-dependent peroxidase activity. Squid were anesthetized by cooling, and the tissues were either assayed immediately or frozen on liquid nitrogen until the time of assay. Freezing had no detectable effects on the squid peroxidase activity. Symbiotic light organ and eye tissues were homogenized on ice in 0.2 M sodium acetate buffer, pH 3.5, and the homogenates were centrifuged at 9,000*g* for 20 min. For each assay of squid tissue extracts, 15 μ l of the resultant supernatant fluid was added to 485 μ l of an assay buffer consisting of 1 mM H_2O_2 , 50 mM DEA, and 100 mM NaCl in 0.2 M sodium acetate buffer, pH 3.5. For each assay of human MPO, 15 μ l of the purified protein was added to 485 μ l of an assay buffer consisting of 1 mM H_2O_2 , 100 mM DEA, and 50 mM NaCl in 0.2 M sodium acetate buffer, pH 4.5. Activity was expressed as the change in absorbance at 280 nm \cdot min⁻¹ \cdot mg protein⁻¹ at 25°C. The dependency of the activity on each substrate was verified by determining the activity in the absence of NaCl, H_2O_2 and DEA, respectively. To further confirm any halide dependency, the tissue extracts were dialyzed overnight against homogenization buffer at 4°C to remove halides that may have been present in the tissue homogenate, and then were assayed in the presence and absence of added NaCl. In addition, to determine whether any portion of the activity in the reaction was due to non-protein-based chemistry (e.g., Fenton reactions) [Hill and Allen, 1978], extracts were boiled for 10 min and centrifuged at 9,000*g* for 15 min, and the supernatant fluid was tested for activity.

Halide-independent peroxidase activity. The following assays, typically referred to as the guaiacol and dianisidine peroxidase assays, have also been used to detect halide peroxidases. However, they are referred to as halide-independent assays because the chromophores in these assays are reduced not only by the reactive-halide products of the enzymatic reaction, but also by direct enzymatic reduction of the chromophores.

Tissue samples were prepared for the guaiacol assay by homogenizing them in 50 mM sodium phosphate buffer, pH 6.0, and centrifuging the homogenate at 9,000*g* for 20 min. Aliquots of the supernatant fluids were added to a reaction mixture containing 0.33 mM H_2O_2

and 12 mM guaiacol in 50 mM sodium phosphate, pH 6.0. The increase in $A_{470 \text{ nm}}$ was recorded for 1 min at 25°C, and the specific activity was calculated as the number of mmol $\text{H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$, using a molar extinction coefficient of 26.6 cm² \cdot mmol⁻¹ for the product tetraguaiacol [Desser et al., 1972; Tien and Kirk, 1988; Koduri and Tien, 1995]. In addition, the guaiacol assay was used to determine whether a heme group, characteristic of MPO, is present in the active site of the squid peroxidase [Taurog et al., 1992]. In these experiments, the percentage of the activity lost was determined when sodium cyanide (0.1 mM) or sodium azide (0.5 mM) was added to the reaction buffer.

Dianisidine peroxidase activity in tissue extracts was detected spectrophotometrically using a method modified from Krawisz et al. [1984], as previously described [Weis et al., 1996]. Squid tissues were homogenized on ice in 50 mM sodium phosphate buffer, pH 6.0, and the homogenates were centrifuged at 9,000*g* for 20 min. Fifteen microliters of supernatant fluid were added to 485 μ l of reaction buffer containing 1 mM H_2O_2 and 5 μ M O-dianisidine-HCl in 50 mM sodium phosphate buffer, pH 6.0. The increase in absorbance at 460 nm was monitored for 1 min at 25°C, and the specific activity was expressed as mmoles of O-dianisidine produced \cdot min⁻¹ \cdot mg soluble protein⁻¹, using a molar extinction coefficient of 11.3 cm² \cdot mmol⁻¹ for the absorbing product [Hurst et al., 1984]. Peroxidase activity was also measured in the presence of 1 mM salicylhydroxamic acid (SHAM), a specific inhibitor of halide peroxidases that is often used in conjunction with the dianisidine assay. SHAM blocks the heme group in the active site of halide peroxidase enzymes and reacts with a histidine residue in the binding pocket of these enzymes [Ikeda-Saito, 1991; Hori et al., 1994].

Anatomical Patterns of Peroxidase Occurrence

We performed enzyme assays, immunocytochemistry, and DNA-RNA hybridizations (Northern blots and quantitative slot-blots) to define the distribution of the halide peroxidase in various squid tissues (Fig. 1). We measured the specific activity of the peroxidase in these tissues using the dianisidine-based halide-independent method described above. For light organ samples, we also calculated the total activity in the whole light organ and compared

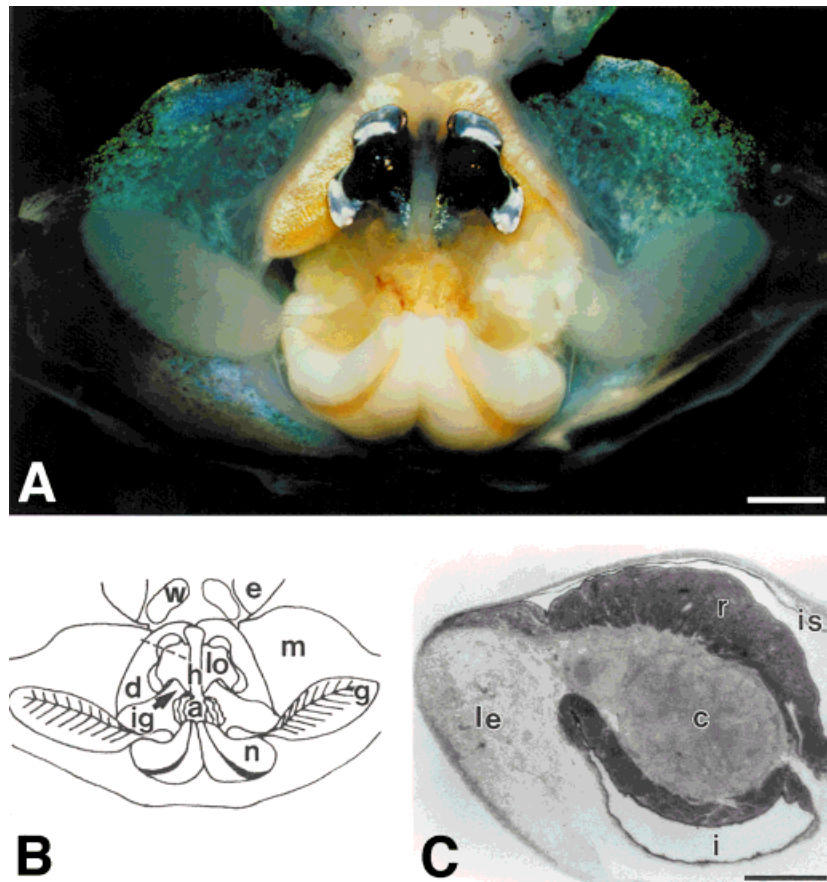


Fig. 1. Tissue types of *Euprymna scolopes* used in peroxidase localization. **A:** Ventral dissection of the squid exposes the tissues included in the comparison. Scale bar = 5 mm. **B:** Line drawing corresponding to the photograph in A, labeled to indicate the organs used in the tissue comparisons. Dashed line through the light organ (lo) shows the location of the cross section in C. a, accessory nidamental gland; d, digestive gland; e, eye; g, gill; ig (arrow toward dorsal surface), ink gland; and w,

white body. The hindgut (h), the mantle (m), and the nidamental gland (n) were labeled in the drawing to aid in orientation, but they were not used in the tissue comparison. **C:** Cross section of the light organ illustrates the components used for comparison within the symbiotic light organ. Scale bar = 500 μm . c, light organ core; i, ink sac diverticula; is, ink sac; le, lens; r, reflector tissue.

it with the total activity of the component tissues (the core and the remaining constituents) measured separately. For immunocytochemistry, all materials and supplies specific to microscopy were purchased from Ted Pella (Redding, CA). The tissues were fixed and embedded as previously described [Weis et al., 1993], sectioned onto nickel grids and blocked overnight at 4°C with sheep serum diluted 1:50 in 50 mM sodium phosphate with 100 mM NaCl, pH 7.4 phosphate-buffered saline (PBS), and 1% bovine serum albumin (BSA). The tissue sections were then incubated overnight at 4°C in the primary antibody at a dilution of 1:200 in 1% BSA in PBS. The polyclonal antiserum used in these experiments had been generated against the expressed gene product of a squid peroxidase cDNA clone [Weis et al., 1996]. Two differ-

ent control incubations were performed at this step, specifically, either a 1:200 dilution of pre-immune serum in place of the primary antibody, or no antibody added at this incubation step. After a 60-s rinse of the grids with PBS, a 15-min block in 1% BSA in PBS was performed before incubation with the secondary antibody. A 1:50 dilution of sheep anti-rabbit IgG complexed to biotin was used as the secondary antibody. The grids were then rinsed, incubated for 30 min with a 1:50 dilution of streptavidin, which was conjugated to 15-nm gold spheres, in PBS, washed in PBS to remove excess reagents, and finally washed with nanopure water. The sections were analyzed using a JEOL 100 CX transmission electron microscope. The relative concentration of immune cross-reactive sites was quantified by counting the total number of

gold spheres within 8- μm^2 areas of tissue. A two-tailed Student's *t*-test was used to determine the statistical significance of the differences obtained in the comparisons.

To prepare the samples for extraction of total RNA, all tissues were frozen in liquid nitrogen immediately following dissection from the anesthetized squid. Two component tissues of the light organ were separated from the rest of the organ: the bacteria-containing core of host epithelial tissue and the light organ lens. The core material and the remaining light organ tissue were frozen separately. Total RNA was extracted according to the method of Chomczynski and Sacchi [1987], using RNASSTAT-60 reagent (TEL-TEST, Friendswood, TX). Northern blots were performed as previously described [Tomarev et al., 1993]. Briefly, 10 μg of total RNA from each tissue were separated on a 1% agarose gel containing 1.8% formaldehyde, and then transferred to nylon membrane. These membranes were hybridized sequentially with two ^{32}P -labeled probes: first a 780-base pair (bp) fragment of a peroxidase cDNA clone from *E. scolopes*, and then a 650-bp fragment of an actin cDNA clone from *E. scolopes*, which was used to control for loading levels. The peroxidase and actin probes used in this study were generated using the same methods of Tomarev et al. [1993].

The same probes, as well as procedures, used to detect the squid peroxidase and actin mRNA in Northern blots were used in quantitative slot blot analyses. In each slot, 5 μg of total RNA was applied by vacuum to a nylon membrane. Quantification of radiolabel was performed using a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA) in conjunction with the ImageQuaNT software package provided with

the system. The peroxidase probe counts were normalized for loading conditions by dividing the peroxidase counts by the actin counts for the corresponding tissue on the blot after it was reprobed for actin mRNA levels. The relative intensities of samples in each of the four blots were compared by setting a slot containing a known amount of the peroxidase DNA fragment equal to 100 U, and scaling the intensity of the other slots on each blot to this value. The average and standard deviation values for each tissue were calculated on the basis of the scaled values for the peroxidase probe, divided by the values for the actin probe of that tissue in each blot. A two-tailed Student's *t*-test was used to determine whether the mRNA values in each sample were above the background.

RESULTS

Peroxidase Activity Assays

The three methods used for detecting peroxidase activity showed measurable activity in extracts of the whole light organ. By contrast, eye tissue had no detectable activity (Table I). In the diethanolamine (DEA) assays, total soluble extracts of whole squid light organs had approximately 10-fold less activity than that of purified human MPO when each sample was measured under conditions that were optimal for that tissue. The specific activity of the peroxidase enzyme in the total soluble extracts of the squid light organ as determined by the guaiacol assay was 1,000-fold less, and by the dianisidine assay was nearly 200-fold less as compared with that of purified human MPO (Table I). The magnitude of the differences in specific activity measured in squid tissues when compared with purified human MPO were not unexpected;

TABLE I. Peroxidase Activity Measured by Three Methods

| | Mean spec act (\pm SD) | | |
|--------------------------|--|--|--|
| | Diethanolamine ^a (n = 6) | Guaiacol ^b (n = 6) | Dianisidine ^c (n = 6) |
| Extracts of squid tissue | | | |
| Light organ | 3.09 (\pm 0.6) | 64.8 (\pm 9.2) | 85.6 (\pm 14) |
| Eye | <0.4 ^d | <0.3 ^d | <0.6 ^d |
| Human MPO | 33.5 (\pm 5.1) | 5.93×10^4 (\pm 0.3 $\times 10^4$) | 1.50×10^4 (\pm 1.6 $\times 10^2$) |

MPO, myeloperoxidase; SD, standard deviation.

^a $\Delta A_{280\text{nm}} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

^bmmoles guaiacol reduced $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

^cmmoles O-dianisidine reduced $\cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$.

^dNot above baseline for the assays.

MPO represents less than 5% of the total soluble protein even in cells where it is most abundant [Johnson and Nauseef, 1991], and the squid tissue extracts may contain a variety of inhibiting factors.

To determine the nature of the active site of the squid enzyme, we used pharmacological agents that act as inhibitors of halide peroxidases. The presence of sodium cyanide, sodium azide and SHAM inhibited the activity in light organ extracts 95%, 82%, and 96%, respectively, and human MPO 93%, 86%, and 92%, respectively. Therefore, both activities appeared to involve heme-containing active sites with ligand binding pockets of similar conformation.

Analyses of activity with the DEA assay showed that the enzyme is halide dependent (Table II). When chloride ions were not included in the reaction mixture, or when samples were dialyzed to remove chloride ions, the activity was at baseline levels for all samples. When chloride ions were added back to the dialyzed samples, 75% of the activity was restored in squid tissue extracts and 86% in the human MPO samples. The loss of activity in the squid peroxidase and human MPO was typical of a decline in activity that accompanied the overnight dialysis procedure required in these experiments. In the absence of the substrates diethanolamine and hydrogen peroxide, 98–99% of the squid peroxidase activity was lost. Boiled samples showed baseline levels of activity, indicating that Fenton-type reactions were not responsible for the observed activity.

TABLE II. Halide-Dependent Peroxidase Activity

| Assay conditions | Sample treatment | Mean spec act (\pm SD) (n = 6) | |
|---|-------------------|-----------------------------------|-------------------|
| | | Squid light organ | Human MPO |
| Standard ^a No added Cl ⁻ | Undialyzed | 3.09 (\pm 0.6) | 33.5 (\pm 5.1) |
| Standard No added Cl ⁻ | Dialyzed | n.d. ^b | n.d. |
| Standard No added Cl ⁻ | Dialyzed | 2.34 (\pm 0.9) | 28.7 (\pm 5.3) |
| No DEA | Undialyzed | n.d. | n.d. |
| No H ₂ O ₂ | Undialyzed | n.d. | n.d. |
| Standard | Boiled/undialyzed | n.d. | n.d. |

DEA, diethanolamine; MPO, myeloperoxidase; n.d., not detectable; SD, standard deviation.

^aSee Materials and Methods for assay conditions.

^bNot detectable above baseline for the assay.

Anatomical Patterns of Peroxidase Occurrence

A variety of squid tissues (Fig. 1) were compared to determine whether a correlation existed between the presence of the peroxidase in a particular tissue and that tissue's level of interaction with microorganisms. We analyzed tissues that have persistent associations with bacteria, including the light organ, accessory nidamental gland (ANG), and gills with bacteria-containing cysts. For each of these tissues and control samples, i.e., an array of tissues that typically do not interact with bacteria, we determined the dianisidine peroxidase activity, localized the peroxidase within the tissues using immunocytochemistry with an antibody to the squid peroxidase, and quantified peroxidase mRNA levels using DNA–RNA hybridizations.

Enzyme assay-based tissue comparisons.

Specific activity of the peroxidase (Table III) was highest in two types of squid tissues: those with sustained bacterial interactions (e.g., the light organ and gills with cysts) and those associated with ink production (e.g., the ink gland and ink sac). However, the light organ core by itself had relatively low specific activity, although it showed activity that was approximately 10-fold higher than baseline levels. Specific activities of the peroxidase were at base-

TABLE III. Distribution of Peroxidase Activity in Squid Tissues

| | Mean spec act (\pm SD) ^a | Total activity (%) | % Inhibition with 1 mM SHAM |
|----------------------------|--|--------------------|-----------------------------|
| Whole light organ | 85.6 (\pm 14) | 100 | 95 |
| Light organ without core | 144 (\pm 23) | 179 | 99 |
| Light organ core | 5.48 (\pm 1.3) | 1.30 | 92 |
| Ink gland | 92.0 (\pm 6.7) | 139 | 98 |
| Ink sac | 28.9 (\pm 3.2) | 33.9 | 94 |
| Accessory nidamental gland | 6.73 (\pm 1.9) | — | 95 |
| Gill with cyst | 25.8 (\pm 6.1) | — | 93 |
| Gill without cyst | n.d. ^b | — | — |
| White body | n.d. | — | — |
| Eye | n.d. | — | — |
| Digestive gland | n.d. | — | — |

n.d., not detectable; SD, standard deviation.

^ammoles of O-dianisidine reduced \cdot min⁻¹ \cdot mg protein⁻¹.

^bNot detectable above baseline for the assay.

line levels in gills without bacterial cysts, the white body, and metabolically active tissues without sustained bacterial interactions, such as the eye and the digestive gland. To compare the total activity of the whole light organ compared with its components, we set the whole organ values to 100% and reported the percentage of that value for each component (Table III). The total activity in the light organ minus the core and in the ink gland alone were higher than either of these tissues samples when the organ was treated as a whole. Because the core of the light organ was the only tissue missing from this extract, this high activity was contributed by other light organ tissues (i.e., the ink gland, the ink sac, and the reflector). Assays performed with the inhibitor SHAM showed that most of the measurable activity was attributable to the activity of halide peroxidases.

Immunocytochemistry-based tissue comparisons. Antibody labeling of the various squid tissues (Fig. 2) mirrored the peroxidase activity levels with one notable exception, the core tissue within the light organ. The core showed low levels of specific activity in enzyme assays (Table III), but relatively high and statistically significant ($P \leq 1.7 \times 10^{-8}$) immune cross-reactivity against the peroxidase antibody in immunocytochemistry. Other tissues with persistent interactions with bacteria, i.e., the ANG ($P \leq 9.8 \times 10^{-5}$) and gills with cysts ($P \leq 1.1 \times 10^{-8}$), both showed significant immune cross-reactivity with the squid halide

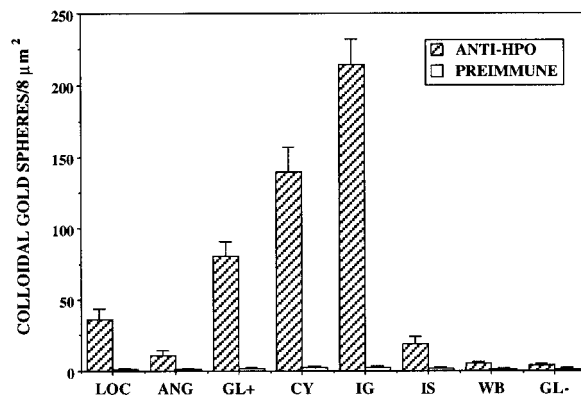


Fig. 2. Quantification of the peroxidase in various squid tissues by immunolocalization. The mean ($n = 5$) was reported \pm SD for both the immune and preimmune treatments of each tissue. Tissue types compared include LOC, light organ core; ANG, accessory nidamental gland; GL+, gills infected with bacteria; CY, the bacteria-containing cysts themselves (within the gills); IG, ink gland; IS, ink sac; WB, white bodies; and GL-, gills without bacterial cysts.

peroxidase antibody. By contrast, gills without cysts showed a much lower but significant ($P \leq 4.7 \times 10^{-4}$) cross-reactivity. The ink gland associated with the light organ had the greatest level of immune cross-reactivity of any tissue analyzed. Tissues that generally do not interact directly with bacteria, such as those within the eye, the white body, the digestive gland, and the reflector and lens of the light organ, had low or insignificant ($P > 0.05$) immune cross-reactivity compared with preimmune controls.

Two different patterns of peroxidase localization emerged when examining immune cross reactivity at the subcellular level (Table IV). In a previous study of the squid light organ [Weis et al., 1996], the peroxidase antibody localized to the apical portions of the epithelial cells that border the bacteria-containing lumina. In the present study, we have found that this pattern extends to other tissues that interact with bacteria, specifically, the ANG, and the gill with bacterial cysts. In examining the luminal spaces containing the bacteria of the light organ, ANG, and encysted gills, we found that immune reactive sites were also abundant on the surfaces of the bacterial cells in these tissues (Fig. 3). In contrast with this apical localization in bacteria-associated tissues, tissues involved in ink production, specifically the ink gland, showed high densities of immune cross-reactive sites dispersed throughout the cytoplasm of the cells (Table IV).

The presence of bacteria in the gills (Fig. 4) significantly altered the pattern of immunolocalization of the peroxidase. In gills with cysts, not only did the antibody localize to the apical

TABLE IV. Abundance of Peroxidase Immune Reactive Sites in Apical and Basal Regions of Epithelial Cells

| | Mean no. of colloidal gold spheres/8 μm^2 (\pm SD) ($n = 6$) | |
|----------------------------|--|-------------------|
| | Apical region | Basal region |
| Light organ core | 36.2 (\pm 7.4) | n.d. ^a |
| Accessory nidamental gland | 10.8 (\pm 3.6) | n.d. |
| Epithelia of gill | | |
| With cyst | 80.7 (\pm 10) | n.d. |
| Without cyst | 4.30 (\pm 1.1) | n.d. |
| Ink gland | 214 (\pm 18) | 229 (\pm 20) |

n.d., not detectable; SD, standard deviation.

^aNot detectable above preimmune controls of the same regions (<1 colloidal gold spheres/8 μm^2).

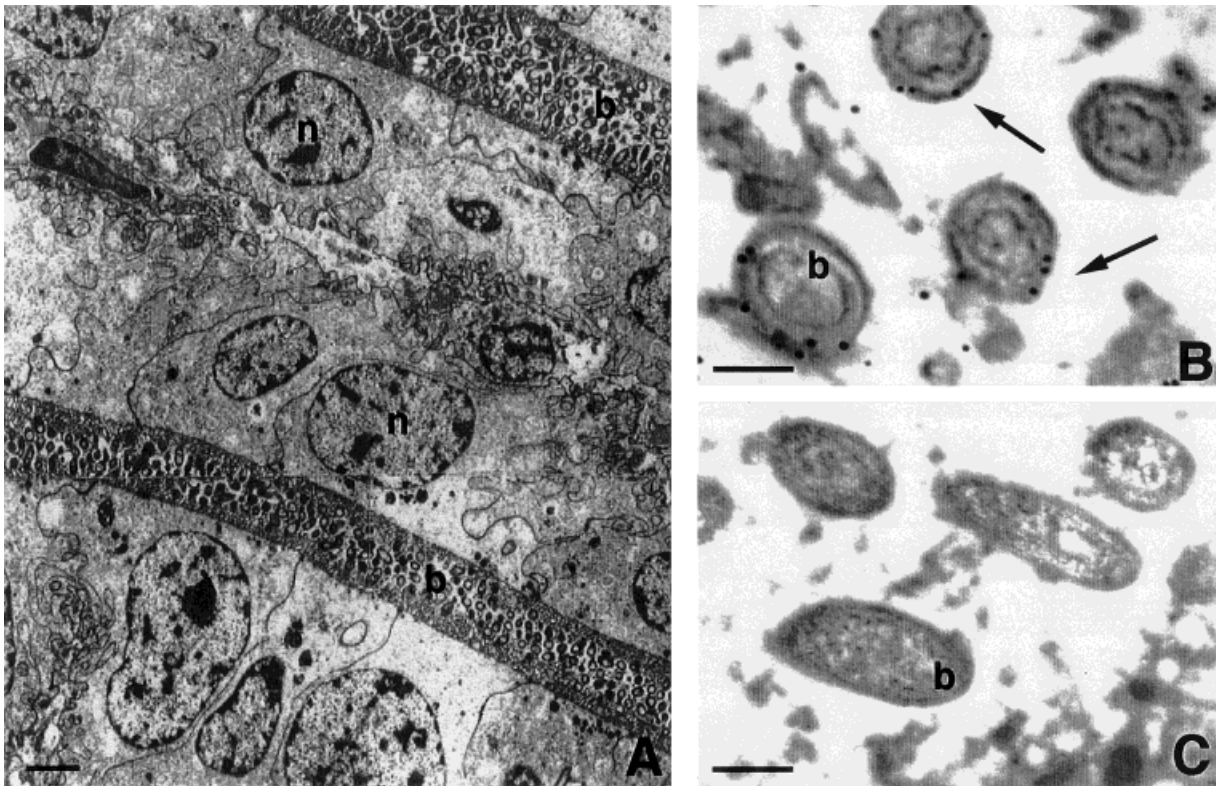


Fig. 3. Immunolocalization of the peroxidase at the surface of the bacterial cells. **A:** Transmission electron micrograph showing an overview of the central core region of the light organ, which contains the extracellular bacterial symbionts. Scale bar = 3.0 μm . b, bacterial cell; n, host cell nucleus. **B:** Abundant immune cross-reactive sites were demonstrated in the extracellular crypt space surrounding the bacteria and at the bacterial cell surfaces (arrows). Scale bar = 0.35 μm . **C:** Preimmune control showing a corresponding view of the bacterial symbionts within the light organ. Scale bar = 0.5 μm .

portions of cells interfacing with bacteria (Fig. 4B), but also to the apical portions of gill epithelia interacting with the environment (Fig. 4C), i.e., not in direct contact with cyst bacteria. In apices of both these types of cells, the immune-reactive sites coincided with abundant electron-dense vesicles (arrows), similar to those that had been previously described in the light organ epithelia that directly interface with the bacterial symbionts [Weis et al., 1996]. In gills without cysts, not only were there few sites that were immune reactive against the peroxidase antibody, but also electron-dense vesicles were not in abundance (Fig. 4D).

mRNA-based tissue comparisons. Northern blot analyses were performed with total RNA obtained from the whole light organ, the core, the light organ minus the core, and the eye to confirm that the cDNA probes reacted specifically with the peroxidase mRNA within each of the sample tissues of the squid, as previously described [Tomarev et al., 1993]. In

all tissues except the eye, which was negative, the 780-bp peroxidase cDNA probe cross-reacted as a single band at 3.1 kb. This blot was reprobed with a 650-bp actin cDNA probe, which cross-reacted as a single band at 1.9 kb for all the tissues tested (data not shown).

Quantitative slot-blot analysis using these probes revealed that peroxidase mRNA was detected in tissues with sustained bacterial interactions and those involved in ink production (Fig. 5). Specifically, the peroxidase mRNA levels in the core of the light organ showed a pattern similar to that observed by immunocytochemistry, but different from that observed in activity assays; i.e., the peroxidase mRNA levels in the rest of the light organ without the core, composed of ink sac, ink gland, and reflector, were approximately four times that in the core. The gill tissue with bacterial cysts also showed high levels of peroxidase mRNA, whereas only low levels of peroxidase mRNA were found in gill tissue without cysts. The

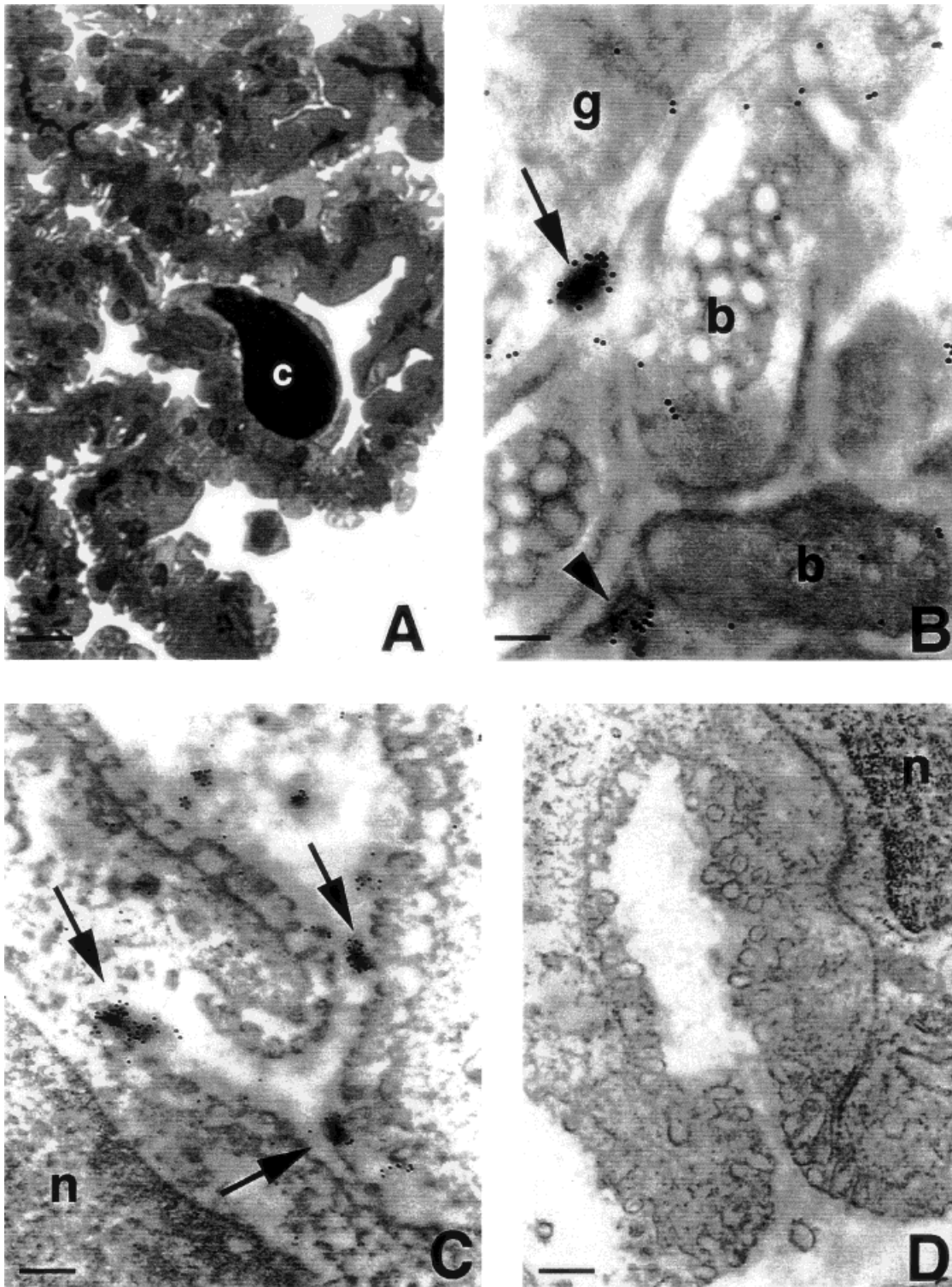


Fig. 4. Gill tissue infected with bacterial cysts showing high immune cross-reactivity to peroxidase antibody relative to gills without bacterial cysts. **A:** Light micrograph illustrating the anatomical form of a bacterial cyst (c) within the epithelia of the gill lamellae. Scale bar = 0.4 mm. **B:** High-magnification transmission electron micrograph of the gill tissue (g), showing the area at the interface between a host epithelial cell and a bacteria-containing cyst demonstrating the strong immunopositivity of the electron-dense, vesicle-like areas in the cytoplasm (arrow). There were also abundant immune cross-reactive sites

near bacterial cells (b) within the bacteria-containing cysts themselves (arrowhead). Scale bar = 100 nm. **C:** In gills with bacteria-containing cysts, immune cross-reactive sites were associated with electron dense, vesicle-like areas in the cytoplasm near the apical surfaces of gill epithelial cells (arrows) that interface with the environment. Scale bar = 0.5 μ m. n, host cell nucleus. **D:** In gills without bacteria-containing cysts, there was lower immune cross-reactivity with the peroxidase antibody and there were no electron dense, vesicle-like areas observed in the cytoplasm. Scale bar = 0.5 μ m.

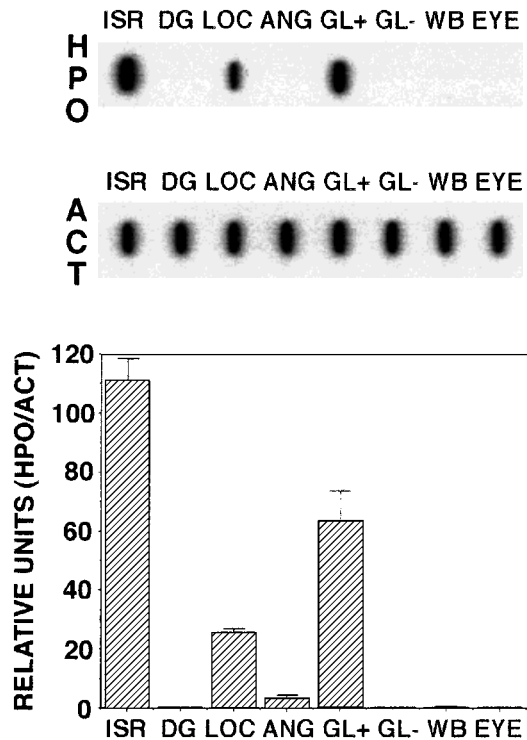


Fig. 5. Squid peroxidase mRNA levels were highest in tissues with sustained bacterial interactions and in ink-producing tissues. HPO, Representative slot-blot that was probed for the squid peroxidase mRNA. ACT, The same representative blot that was stripped and then probed for actin mRNA. Results of the phosphoimager analysis of the blots ($n = 4$) depicted graphically at the bottom. Units are reported as the relative counts obtained from the HPO-probed blots normalized to that for the ACT-probed blots. ISR, light organ without the core, but including the ink gland, ink sac, and reflector tissues; DG, digestive gland; LOC, light organ core; ANG, accessory nidamental gland; GL⁺, gill with bacteria-containing cysts; GL⁻, gill without bacteria-containing cysts; WB, white body; and EYE, eye. Order of tissue placement was chosen such that suspected positives would be dispersed.

ANG showed low but significant ($P \leq 0.03$) peroxidase mRNA levels as compared with the background. The white body, the eye, and the digestive gland all showed insignificant ($P > 0.05$) levels of peroxidase mRNA above the background under the conditions of exposure of these blots.

DISCUSSION

The results of this paper provide evidence to support the classification of the squid peroxidase as a member of the family of halide-dependent peroxidases. In addition, the tissue comparisons support a correlation between sustained interactions with bacteria in the squid tissues and high levels of the peroxidase activ-

ity, immune cross-reactivity with an antibody to the peroxidase, and peroxidase mRNA.

The classification of the squid peroxidase as a member of the halide peroxidase family has important implications for the hypothesized role of this protein in *E. scolopes*. The data presented here, as well as previous reports on the nature of the squid enzyme [Tomarev et al., 1993; Weis et al., 1996], indicate that it is most similar to one specific member of this family (i.e., MPO), a key component of microbicidal pathways [Klebanoff, 1970, 1991]. The strongest evidence that the squid enzyme is involved in antimicrobial activity is provided by the comparative studies of gill tissue. Specifically, the recruitment of this enzyme into gills with bacteria-containing cysts, and the nearly undetectable levels of the enzyme in gills without these cysts, suggests that the peroxidase is an inducible component of the system of nonspecific immunity in the squid.

Although increases in peroxidase levels in the gill tissue correlated with the presence of bacteria, we also have evidence for abundant peroxidase gene expression, protein production and/or activity in two other tissues with bacterial interactions (i.e., the light organ and the ANG), which maintain sustained associations with beneficial bacterial symbionts. The finding of this peroxidase in both beneficial and pathogenic/opportunistic animal-bacterial associations suggests that the protein may be involved in the control of both types of symbioses, although the peroxidase must somehow be regulated differently in these types of relationships to generate the different outcomes. For example, whereas in a pathogenesis the peroxidase may function to rid the tissues of invading microorganisms, in beneficial associations the peroxidase may play a role in specificity, impose limits on symbiont number, or both. In addition, the bacterial partners may respond differently in beneficial associations, which are most likely to be the result of extensive coevolution between the host and symbiont that will be reflected in the biochemistry of the association [Nishiguchi et al., 1998]. Our data on the levels of the peroxidase in the light organ suggest that gene expression and protein production in the core are relatively high, although the measured activity of this enzyme in extracts was low. One possible explanation for these observations is that the bacteria-associated component of the light organ has an inhibitory effect on the per-

oxidase activity. Recently, Visick and Ruby [1998] reported that *V. fischeri* has a highly active catalase. Perhaps this and other constituents of extracts of the light organ core, of host and/or symbiont origin, may be involved in the biochemical interplay controlling the symbiosis.

In addition to squid tissues that have interactions with bacteria, ink-producing tissues also had comparatively high levels of the halide peroxidase. In our study, the subcellular distribution of the peroxidase was different between ink-producing tissues and bacteria-associated tissues, but the distribution in the ink-producing tissues was similar to that reported by Palumbo et al. [1997b] for the ink-gland cells of the cuttlefish *Sepia officinalis*. These investigators provided evidence for the involvement of a similar peroxidase in ink production in *S. officinalis*. Using immunocytochemistry, Palumbo and coworkers found peroxidase at detectable levels only in the ink-producing tissues. However, this study did not examine the ANG nor infected gills of this species, tissues that in our study of *E. scolopes* contained high levels of the peroxidase. (Light organs are not present in *S. officinalis*.)

The distribution of the peroxidase in both the ink-producing and light organ tissues may reflect evolutionary constraints on the formation of the light organ. In contrast with fish bacterial light organs, which are distributed in a variety of anatomical locations depending on the host species [McFall-Ngai and Toller, 1991], bacterial light organs of cephalopods are always associated with the ink sac, although they have evolved independently several times [Nesis, 1987]. High levels of peroxidase in ink sac epithelia may have preadapted those tissues to the formation of controlled bacterial interactions. Thus, the presence of the peroxidase in ink-producing tissues of both squids that do and do not have light organs would suggest that ink-production may be the primitive function of the enzyme. By contrast, the presence of the myeloperoxidase-like enzyme both in the gills of *E. scolopes* and the hemocytes of other molluscs [Torreilles et al., 1997], argues that the primitive function of the peroxidase may be for the modulation of the interactions of host tissues with microbes.

A third possibility is that the peroxidase and other enzymes of the pathway, such as tyrosinases, have evolved dual functions (i.e., for both

pigment production and immune defense), but the regulation of the same enzyme(s) differs depending on the function. The evidence that the peroxidase in *S. officinalis* is involved in melanogenesis in conjunction with tyrosinase enzymes [Gesualdo et al., 1997; Palumbo et al., 1997a] provides support for a possible role of these peroxidases in host immunity. In other invertebrates, melanogenesis has been demonstrated to function in both immunity and pigment deposition [Charalambidis et al., 1994a; Nappi et al., 1995; Qiu et al., 1998]. For example, the defense response of encapsulation of microorganisms in some dipteran species requires the formation of eumelanin deposits [Rizki and Rizki, 1984; Nappi et al., 1992; Nappi and Vass, 1993]. Further, many of the same enzymes and enzyme products of the eumelanin pathway in the cuttlefish that result in ink production [Palumbo et al., 1997a] are the same as those involved in the formation of melanotic bodies in insects [Marmaras and Charalambidis, 1992; Marmaras et al., 1993, 1994, 1996; Charalambidis et al., 1994a,b, 1995, 1996a,b; Qui et al., 1998]. Thus, this peroxidase may serve multiple functions within a given species.

The finding of a halide peroxidase in squid tissues associated with a variety of bacterial interactions within *E. scolopes* suggests that the modulation of the oxidative environment is a key component in the control of both pathogenic and nonpathogenic associations. The mechanisms by which the peroxidase, and other proteins associated with the oxidative state of the tissues, may be regulated differently should be best studied in experiments with newly hatched squid, where exposure to bacterial symbionts of all types can be manipulated. Studies of regulation of host biochemistry in the light organ will also be advanced by the application of molecular genetic approaches with *V. fischeri* [Graf et al., 1994; Visick and Ruby, 1996].

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