Appendix II

The development of a novel histochemical method for the demonstration of bromoperoxidase in animal tissue sections

Histochemical methods for the demonstration of bromoperoxidase in animal tissue are not currently available in the literature. Consequently, a novel technique was developed to determine potential sites of Tyrian purple prochromogen bromination in the hypobranchial gland of *D. orbita*.

2. 0 Specimen collection and maintenance

Nine female *D. orbita* were employed for the development of histochemical techniques. These specimens were sampled from a population of mature *D. orbita*, held at Flinders University Marine Aquarium, Adelaide, South Australia. Animals were maintained on a diet of *Turbo undulatus*, *Mytilus edulis planulatus*, juvenile *Haliotis* spp. and *Donax deltoides* within a subtidal recirculatory aquarium at 15°C, a salinity of ~35.5ppt and a 12/12h day/night cycle. Specimens comprising the captive population were collected from the rocky intertidal and subtidal regions of the Adelaide metropolitan coast, Fleurieu and Eyre peninsulas of South Australia.

2. 1 Dissections

The shell of each live specimen was removed by cracking with a vice at the junction of the primary body whorl and spire, and the soft body removed by severing the columnar muscle. The soft body was then transferred to a dissecting tray and submersed in filtered (0.22µm) seawater to reduce osmotic stress. The dorsal mantle and pallial gonoduct were separated from the rest of the visceral mass by an incision along the lateral margins of the columnar muscle. The mantle was then folded back and pinned with the ventral surface facing up. Longitudinal and transverse incisions were made along the junction between the ctenidium and branchial hypobranchial epithelium, and the posterior gonoduct and digestive gland, respectively. Tissue was prepared for sectioning according to the specifications outlined below and examined under a compound light microscope (Olympus, BH-2).

2. 2 Tissue preparation

Tissue employed in the development of a histochemical method for bromoperoxidase was fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm and affixed to charged slides (ProSciTech, G311SF-W). Triplicate transverse serial sections from each specimen were employed in each trial. Additional serial sections were obtained from each specimen for comparative morphological staining in Modified Harris Haematoxylin and Eosin Y with Phloxine B (H&E) (Thompson 1966) and negative controls.

2. 3 Trial 1: Substrate solution adaptations

Krenn et al. (1989) and Wever et al. (1991) localized bromoperoxidase within fresh algal tissue by modifying a commonly employed bromoperoxidase assay originally developed by De Boer et al. (1987). This staining reaction is based on the bromoperoxidase catalyzed conversion of phenol red (phenolsulphonphthalien) to bromophenol blue (tetrabromophenolsulphonphthalien) in the presence of hydrogen peroxide (H_2O_2) and bromide (Br^-) (Fig. 1). As bromoperoxidase in *Murex trunculus* is also activated by incubation with H_2O_2 and KBr (Jannun and Coe, 1987), the "bromophenol-red" method may be useful for the demonstration of bromoperoxidase of within muricid hypobranchial gland sections. Consequently, the conversion of 50uM phenol red to bromophenol blue in the presence of 2mM H_2O_2 and 100mM KBr pH 6.0 (Krenn et al., 1989; Wever et al., 1991) was trialed as a substrate solution for the demonstration of bromoperoxidase in the muricid *Dicathais orbita* tissue sections.

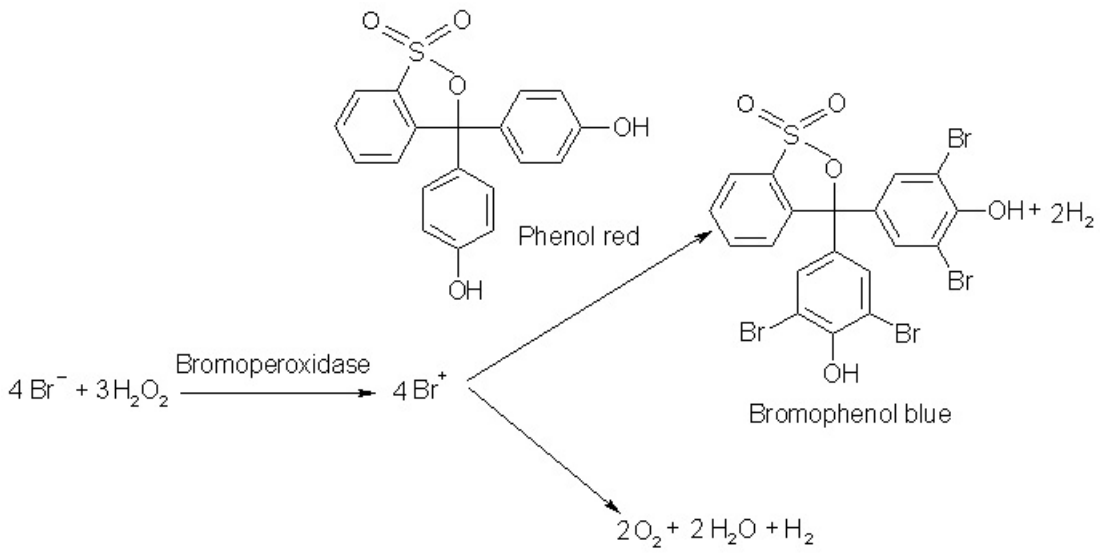

Figure 1. The proposed bromoperoxidase-assisted conversion of phenol red to bromophenol blue in the presence of hydrogen peroxide, adapted from Butler and Walker, (1993) and Sels et al. (1999).

Bromoperoxidase activity has been detected in hypobranchial gland homogenates of the muricid, *M. trunculus* (Jannun and Coe, 1987). In contrast to algal bromoperoxidase, *M. trunculus* bromoperoxidase is known to be pH sensitive and inhibited by H_2O_2 concentrations >0.2mM and the presence of various anions (Jannun and Coe, 1987). Thus, the substrate solution was adapted in accordance with the known properties of *M. trunculus* bromoperoxidase and the visible yield of bromophenol blue compared to that obtained after incubation in substrate solution for algal bromoperoxidase (Krenn et al., 1989; and Wever et al., 1991).

Fluoride (F^-) and chloride (Cl^-) ions inhibit bromination by *M. trunculus* bromoperoxidase (Jannun and Coe, 1987). Consequently, buffer solutions were prepared in distilled water and sections pre-washed to remove extracellular sodium chloride, Cl^- and F^-. Shifts in the H_2O_2 concentration optimum for maximum muricid

bromoperoxidase activity have obscured identification of the pH optimum (Jannun and Coe, 1987). However, peaks in maximum enzyme activity have been found to occur at pH 5 (125uM H_2O_2) and pH 7.4 (20uM). To minimize pH-dependent changes in cell biochemistry, a substrate solution pH close to that of seawater (pH 7.4, 20µM H_2O_2) was trialed. Bromination velocity is known to be proportional to enzyme concentration and follows a simple saturation curve below 20mM KBr at an enzyme concentration of 100µg/ml (Jannun and Coe, 1987). As the enzyme concentration within histological sections is unknown, a substrate concentration of 100mM KBr was employed, as outlined by Krenn et al. (1989) and Wever et al. (1991). Activated enzyme was found to be stable at room temperature for at least 24h (Jannun and Coe, 1987), and the assays performed on hypobranchial gland extracts (Jannun and Coe, 1987) were complete after 30min at 23-25°C. Consequently, similar enzyme stability and reaction conditions were assumed for *D. orbita* bromoperoxidase.

2. 3. 1 Substrate solution preparation

Algal substrate solution

1. 100mM Potassium Phosphate Buffer, pH 7.4. Prepare 3 batches.
    a. 100mM Monobasic potassium phosphate
        i. Monobasic potassium phosphate (KH_2PO_4) 13.61g
        ii. Distilled water Make up to 1000ml
    b. 100mM Dibasic potassium phosphate
        i. Dibasic potassium phosphate (K_2HPO_4) 17.42g
        ii. Distilled water Make up to 1000ml

c. Solution 1

i. Solution 1a 198ml

ii. Solution 1b 802ml

2. 2mM H_2O_2 Solution (1:100 serial dilution of 200mM solution)

a. 200mM H_2O_2 Solution

i. H_2O_2 6.8ml

i. Solution 1 Make up to 1000ml

b. Solution 2

i. Solution 2a 1:100 dilution

3. Substrate solution (100mM KBr, 50µM phenol red, 2mM H_2O_2 in 100mM potassium phosphate buffer, pH 7.6).

a. Potassium bromide (KBr) 1.19g

b. Phenol red ($C_{19}H_{14}O_5S$) 1.7mg

c. Solution 2 Make up to 100ml

KBr was eliminated from the substrate solution for negative controls.

Muricid substrate solution

1. 100mM Potassium Phosphate Buffer, pH 7.4 prepared as for trial 1.

2. 20µM H_2O_2 Solution (1:10000 serial dilution of 200mM solution)

d. 200mM H_2O_2 Solution

i. H_2O_2 6.8ml

ii. Solution 1 Make up to 1000ml

c. Solution 2

i. Solution 2a 1:1000 dilution

3. Substrate solution (100mM KBr, 50µM phenol red, 20µM H_2O_2 in 100mM potassium phosphate buffer) prepared as for trial 1.

KBr was eliminated from the substrate solution for negative controls.

2. 3. 2 Staining procedure

Perform the following steps in coplin jars.

1. Three changes of solution 1 Rinse
2. Incubate sections in solution 3 at room temperature 30min
3. Distilled water Rinse
4. View sections without mounting medium

2. 3. 3 Algal and muricid substrate solutions comparison

During incubation, bubbles formed within all treatment sections and microscopic examination revealed the presence of blue dye deposits. The simultaneous presence of these reaction products supports the bromoperoxidase-assisted degradation of H_2O_2 into oxygen and hydrogen gas and the generation of bromophenol blue (Fig. 1). Bromophenol blue staining was more frequent and intense in sections incubated in the muricid substrate solution (Fig. 2a) (20µM H_2O_2) in comparison to those incubated in the algal substrate solution (Fig. 2b) (2mM H_2O_2). This suggests that similar to *M. trunculus, D. orbita* bromoperoxidase activity is decreased, although not completely inhibited, by high H_2O_2 concentrations. Although dye diffusion compromised intracellular localization in both trials, discrete regions of

bromoperoxidase activity were clearly discernable within the hypobranchial gland in comparison to serial sections stained in H&E (Fig. 2). Negative control sections failed to gain any pigmentation indicative of bromophenol blue.

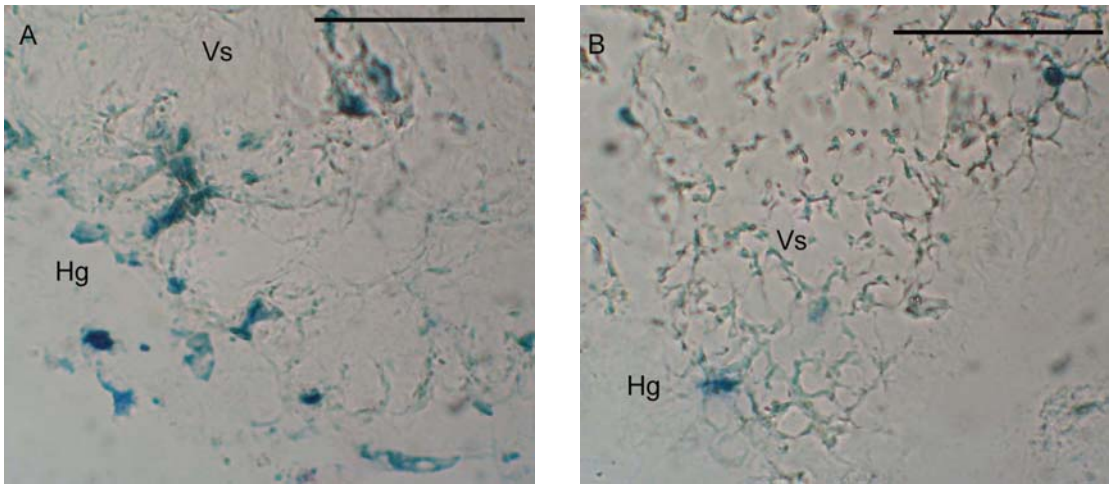

Figure 2. Increased bromophenol blue staining intensity and frequency in *D. orbita* hypobranchial gland tissue sections after (A) incubation in the muricid substrate solution and (B) the algal substrate solution. Hg, hypobranchial gland; Vs, vascular sinus. Scale bars = 100µm.

Adaptation of the algal substrate solution (Krenn et al., 1989; Wever et al., 1991) to the known properties of *M. trunculus* bromoperoxidase (Jannun and Coe, 1987) produced visibly enhanced bromophenol blue yields in *D. orbits* hypobranchial gland sections. Consequently, the muricid substrate solution was employed in the following counter staining and mounting medium trials.

2. 4 Trial 2: Counter staining

The reaction product of the bromophenol-red method is bromophenol blue. Consequently, Nuclear Fast Red (C.I. 60760) was trailed as morphological counter

stain. The effect of counter staining on enzyme localization and bromophenol blue intensity was investigated by comparison with unstained serial sections. The morphological definition provided by Nuclear Fast Red staining was determined by comparison with serial sections stained in H&E (Thompson, 1966).

2. 4. 1 Substrate solution

Prepared according to muricid substrate solution (section 2. 3. 1).

2. 4. 2 Staining procedure

Perform the following steps in coplin jars.

1.	Three changes of solution 1	Rinse
2.	Incubate sections in solution 3 at room temperature.	30min
3.	Incubate sections in Nuclear Fast Red	2min
4.	Distilled water	Rinse
5.	View sections without mounting medium	

Step 3 was eliminated for negative controls.

2. 4. 3 Counter staining comparison

Counter staining with Nuclear Fast Red provided good morphological definition of cell nuclei, nucleoli and membranes. Although bromophenol blue dye deposits remained evident after counter staining with Nuclear Fast Red, the counterstaining procedures resulted in increased dye diffusion away from active sites. Nuclear Fast Red staining also obscured the peripheral limit of bromophenol blue

staining within the hypobranchial gland of *D. orbita*. Due to these detrimental effects, localization of bromoperoxidase active sites by the already diffuse bromophenol-red method may be better achieved through comparison with serial sections stained in H&E.

2. 5 Trial 3: Mounting medium

Mounting sections prevents tissue dehydration and degradation during viewing and storage. However, as bromophenol blue is water soluble, mounting may increase diffusion away from active sites. To investigate the effect of permanent and temporary mounting on bromoperoxidase localization by the bromophenol-red method, un-mounted sections were compared to sections mounted DEPEX and glycerol, respectively.

2. 5. 1 Substrate solution

Prepared according to muricid substrate solution (section 2. 3. 1).

2. 5. 2 Staining procedure

DEPEX mounting

1.	Three changes of solution 1	Rinse
2.	Incubate sections in solution 3 at room temperature	30min
3.	Distilled water	Rinse
4.	Dehydrate in 70% ethanol	10s
5.	Dehydrate in 90% ethanol	10s
6.	Dehydrate in absolute ethanol	10s

7. Xylene 2min
8. Xylene Final
9. Mount sections in DePex mounting medium Gurr (Merck, 36125)

Steps 4-9 were eliminated for negative controls.

Glycerol mounting

1. Three changes of solution 1 Rinse
2. Incubate sections in solution 3 at room temperature 30min
3. Distilled water Rinse
4. Mount sections in glycerol

Step 4 was eliminated for negative controls.

2. 5. 3 Mounting medium comparison

Despite observations of blue pigmentation during incubation, bromophenol blue deposition was not evident in sections mounted in DePex mounting medium. As bromophenol blue is water soluble, the reaction product was most likely extracted during dehydration and xylene incubation. Although bromophenol blue was visible in sections mounted in glycerol, this medium enhanced dye diffusion and prevented localization within the *D. orbita* hypobranchial gland. As both temporary and permanent mounting mediums compromise enzyme staining, sections must be

immediately viewed without mounting medium for the accurate qualification of bromoperoxidase by the bromophenol-red method.

2. 6 The bromophenol-red staining protocol for muricid bromoperoxidase

Tissue preparation

Tissue should be fresh-frozen in O.C.T. compound (Tissue-Tek®) on a -20°C bar, cryostat sectioned at 15µm and affixed to charged slides (ProSciTech, G311SF-W).

Substrate solution

1. 100mM Potassium Phosphate Buffer, pH 7.4 prepared as for trial 1.
2. 20µM H_2O_2 Solution (1:10000 serial dilution of 200mM solution) as prepared for trial 2.
3. Substrate solution (100mM KBr, 50µM phenol red, 20µM H_2O_2 in 100mM potassium phosphate buffer) as prepared for trial 1.

Eliminate KBr from the substrate solution for negative controls.

Staining procedure

Perform the following steps in coplin jars.

1. Three changes of solution 1	Rinse
2. Incubate sections in solution 3 at room temperature.	30min
3. Distilled water	Rinse

4. View sections without mounting medium

The staining protocol provided was reproducible and allowed visualization of bromoperoxidase active sites in *D. orbita* hypobranchial gland sections. Sections should be view in conjunction with serial sections stained in H&E (Thompson, 1966) for morphological comparison.