OXIDATIVE ENZYMES AND THE FORMATION OF EGG CAPSULES IN THE LITTLE SKATE (RAJA ERINACEA)

J. William Straus[1], Nicole C. Rabidou[1], Jennifer K. Bogdany[1], and David L. Cox[2]
[1]Department of Biology, Vassar College, Poughkeepsie, NY 12601
[2]Department of Biology, University of Oregon, Eugene, OR 97403

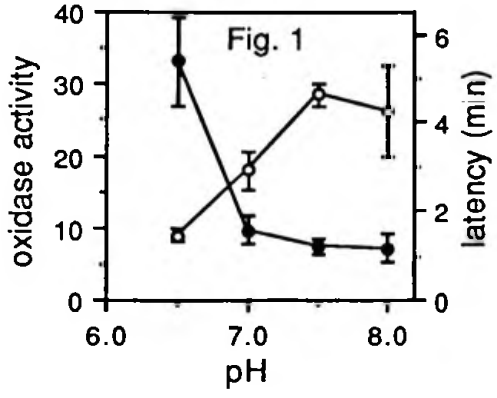
The little skate (Raja erinacea) produces leathery proteinaceous egg capsules which afford protection to embryos for periods often exceeding a year in duration. Egg capsule formation begins with the secretion and assembly of capsular proteins in the nidamental gland of the anterior oviduct. Newly formed capsules undergo sclerotization and tanning as they pass into the uterus. Sclerotization appears to involve the oxidation of catechol residues to colored quinones, which are thought to derivatize with capsular proteins. Catechol oxidation in egg capsules is enzymatically catalyzed by a catechol oxidase, which is secreted from the nidamental gland and incorporated into the capsular matrix (Koob & Cox, Biol. Bull. 175, 202-211, 1988). To better understand the mechanism and regulation of catechol oxidation in skate egg capsule, we have engaged in the purification and characterization of catechol oxidase from nidamental gland extracts. This report presents a new approach to the initial purification of nidamental gland catechol oxidase. Additionally, we have begun to examine egg capsules for the presence of other oxidative enzymes that might play a role in capsular sclerotization.

Spawning female skates were maintained in running seawater and fed frozen shrimp on a daily basis. Nidamental gland lysates were prepared by homogenization as previously described (Koob & Cox, 1988, op cit.). Egg capsules were collected shortly after oviposition and maintained in running seawater prior to assays. For assays, the dorsal wall of each capsule was bisected and cut transversely into paired 1 x 0.5 cm sections. Enzyme activity was assessed by following the oxidation of 4-methyl catechol spectrophotometrically at 400 nm (Straus & Cox, Bulletin MDIBL 30, 23-24, 1991).

A major problem associated with the characterization of nidamental gland catechol oxidase has been the presence of proteinaceous aggregates that cause viscosity and turbidity in crude nidamental gland extracts. Efficient separation of catechol oxidase from this proteinaceous material has proven to be difficult. The proteinaceous aggregates did not sediment out of solution after crude nidamental gland extracts were centrifuged for 12 h at 70,000 x g (4° C). Purification procedures, such as ammonium sulfate precipitation, resulted in significant yield reductions, while chromatographic separation of catechol oxidase from crude extracts has been hampered due to clogging of chromatographic media by protein aggregates. While examining the effects of pH on catechol oxidase activity in crude nidamental gland extracts, we discovered that incubation of extracts at pH 5.5 (0.05 M sodium phosphate, 0.5 M NaCl) for 10 min at 4° C resulted in the precipitation of protein aggregates. Acid precipitates were then removed by centrifugation at 16,000 x g for 10

min. The levels of catechol oxidase activity in three representative nidamental gland extracts were not affected by acid precipitation, indicating that purification by acid precipitation gave an approximate 100% yield of enzyme. For example, when 0.03 ml aliquots of a representative extract were assayed before and after acid precipitation, the respective activities were 0.025 ± 0.002 $O.D._{400}$ units/min and 0.026 ± 0.002 $O.D._{400}$ units/min (n = 3 ± SD). Moreover, the specific activity in one extract increased fourteen fold, from 67 ± 3.4 $\mu mol^{-1}mg^{-1}$ protein to 942 ± 22 $\mu mol^{-1}mg^{-1}$ protein. Specific activity increases in other nidamental gland extracts were comparable.

We found that the effect of pH on catechol oxidase activity in crude extract and partially purified extract (by acid precipitation) was slightly different than in extracts prepared by ammonium sulfate precipitation. In the latter case, partially purified catechol oxidase was found to have optimal activity over a range of pH 6.5 to 7.0 (Koob & Cox, 1988, op cit.). As shown in Fig. 1, the optimal activity for catechol oxidase in crude nidamental gland extract was somewhat higher, in the range of pH 7.5. A comparable pH optimum (about 7.5) was observed with extracts that had been purified by acid precipitation (not shown). This disparity in pH optima probably resulted from differences in enzyme purity. Catechol oxidase in crude nidamental



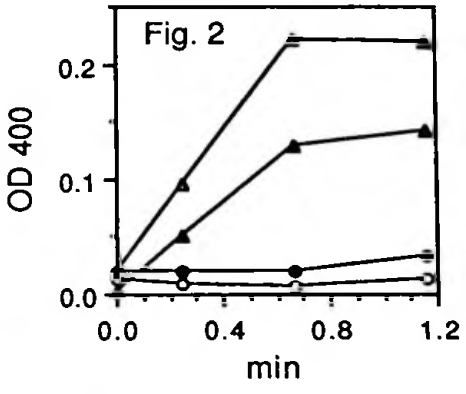


Fig. 1. Effect of pH on catechol oxidase activity (O) and latency (●). Activity measurements represent the change in $O.D._{400}$/min. Assays were performed in 0.05 M sodium phosphate, 0.5 M NaCl at the indicated pH's. Each point represents the average of three determinations ± SD.

Fig. 2. Effect of hydrogen peroxide on 4-methylcatechol oxidation by representative skate egg capsule sections. Oxidation of 4-methyl-catechol by egg capsule sections was monitored spectrophotometrically at 400 nm in the presence (Δ) and absence (▲) of 10 mM H_2O_2. Control measurements of 4-methyl catechol oxidation (in the absence of egg capsule) with (O) and without (●) 10 mM H_2O_2.

gland extracts exhibits a characteristic latent period between the addition of substrate and the onset of oxidation, due to the presence of a low molecular weight latency factor. Latency typically decreases as enzyme activity increases (Straus, Monian & Cox, Comp. Biochem. Physiol., in press, 1993). As shown in Fig. 1, the latent period decreased as enzyme activity increased. Thus, changes in pH did not appear to affect the inverse relationship between latency and enzyme activity.

Enzymes other than catechol oxidase have the potential to contribute to the oxidation of catechols in skate egg capsule formation. For example, peroxidases are nearly ubiquitous in vertebrate cells. Accordingly, we have attempted to determine whether peroxidases are present in the skate egg capsule. Briefly, paired sections of dorsal egg capsule were incubated for 2 min in the presence or absence of hydrogen peroxide, a peroxidase co-substrate, and 2,2'dipyridyl, a putative peroxidase inhibitor. As shown in Fig. 2, hydrogen peroxide stimulated oxidation of 4-methyl catechol. Conversely, 2,2'dipyridyl lowered the rate of 4-methyl catechol oxidation in a concentration dependent manner, by 0%, 16%, 27%, and 29% when capsule sections were incubated for 2 min and then assayed in the presence of 1 mM, 4 mM, 8 mM, and 16 mM 2,2'dipyridyl respectively. These results are consistent with the hypothesis that a peroxidase is present in the skate egg capsule, but must be interpreted with caution since the observed effects could have resulted from redox interactions with the reagents, or from reagent interactions with catechol oxidase. (Supported by a Blum-Halsey Scholar Award from the MDI Biological Laboratory to JWS).