STUDIES ON SKATE (*RAJA ERINACEA*) EGG CAPSULE FORMATION. IV. CATECHOL OXIDASE ACTIVATION

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The egg capsule of the little skate (*Raja erinacea*) is a complex proteinaceous tertiary membrane that protects the enclosed embryo during incubations of nearly two years in the Gulf of Maine. The chemical stability of this extraorganismal material results from a catechol oxidase catalyzed process of sclerotization that begins during secretion of capsule precursors in the female's shell gland. These precursors are soft and white when secreted and undergo parallel increases in hardness, color, catechol content and catechol oxidase activity while the capsules are *in utero* (Koob & Cox, *The Bulletin 26:* 109-112, 1986; Koob, *J. Exp. Mar. Biol. Ecol. 113:* 155-166, 1987).

We have reported that oxidation of catechols by shell gland extracts does not begin immediately with substrate addition but occurs only after a brief delay (*The Bulletin 25:* 132-134, 1985). Treatment of extracts with α-chymotrypsin significantly reduced latency periods following substrate addition and increased initial rates of substrate oxidation. The aims of the present study were to explore the effects of selected inhibitors and α-chymotrypsin on shell gland extracts to gain insight into the process of procatechol oxidase activation during egg capsule formation.

Raja erinacea shell glands actively forming egg capsules were salt extracted as previously described (Koob & Cox, *Biol. Bull. 175:* 202-211, 1988). To examine the effects of various enzyme inhibitors on catechol oxidase paired glands were excised on ice and divided into equivalent sections for immediate homogenization in extraction buffer containing the following reagents: (1) no added reagents (control) ; (2) 1mM Phenylmethylsulfonylfluoride (PMSF); or (3) proteinase inhibitor cocktail (PIC) containing 1mM PMSF, 25mM EDTA, 10mM *N*-ethylmaleimide, and 5mM benzamidine-HCl. Homogenates were clarified and directly assayed for catechol oxidase activity (Koob & Cox, *op. cit.*). To determine whether the effects of these inhibitors might be reversible, 5ml of each gland extract was separately dialyzed against 3 x 2L of 0.5M NaCl, 50mM NaH_2PO_4, pH 7.0 at 4°C for 36h and then assayed. Chymotrypsin (40μg/ml), and PMSF (1mM) were added to assay mixtures in some experiments and were pre-incubated up to 120 minutes prior to assays.

Extracts containing the PIC or PMSF alone displayed initial rates of substrate oxidation 12% those of control values. The duration of the initial lag phase following substrate addition was increased over 10-fold in these extracts. Oxidase activity was not restored by dialysis nor could it be activated by chymotrypsin treatment following dialysis. Treatment of control extracts with chymotrypsin for 1 min. eliminated the lag time preceding substrate oxidation and doubled the initial oxidation rate. Chymotrypsin treatment of PIC extracts for periods up to two hours had minimal effect on lag phase duration (it decreased from 17.5 to 13.8 min.) and did not increase oxidation rates. Control extracts incubated with PMSF for up to two hours showed no change in duration of the delay in oxidation following substrate addition. PMSF treatment of these extracts for ten or more minutes reduced oxidation rates by half, indicating that some of the PMSF-sensitive factor may have already participated in the activation process.

These experiments establish that PMSF included during shell gland extraction irreversibly inhibits a serine proteinase that is necessary for catechol oxidase activation. Chymotrypsin does not induce catechol oxidase activity in extracts containing either PMSF or PIC, demonstrating that its ability to play a role in catechol oxidase activation depends specifically upon its active site. Chymotrypsin is also unable to activate catechol oxidase in dialyzed PMSF or PIC extracts suggesting it does not act directly on catechol oxidase but rather upon some other factor(s). Taken together these observations indicate that a multistep catechol oxidase activation cascade occurs during skate egg capsule formation.

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