CATECHOL OXIDASE IN THE LITTLE SKATE (RAJA ERINACEA) EGG CAPSULE: ENZYME LATENCY INDUCED BY AN ENDOGENOUS FACTOR

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The leathery egg capsule of the little skate (Raja erinacea) is composed of proteins secreted by the nidamental gland. The durability of the egg capsule is due in part to a tanning process that involves conversion, by catechol oxidase, of secreted catechols to quinones which sclerotize the capsular matrix. Catechol oxidase activity has been demonstrated in both egg capsule and shell gland extracts and the enzyme has been partially characterized with respect to substrate specificity, pH optima, and response to salinity (Koob & Cox, Biol. Bull. 175, 202-211, 1988). Catechol oxidation was generally preceded by a brief latent period following substrate addition to nidamental gland extracts. Enzyme latency was shown to be diminished after treatment with chymotrypsin and enhanced after treatment with proteinase inhibitors (Cox & Koob, Comp. Biochem. Physiol. 95B, 767-771, 1990). Proteinase and proteinase inhibitor treatments were also observed to increase and decrease initial rates of enzyme activity respectively. These observations indicated that some sort of proteinase sensitive factor was involved in the activation of glandular catechol oxidase. Furthermore, partial purification of the glandular enzyme by precipitation in ammonium sulfate resulted in a substantial reduction of latency, suggesting that either the enzyme was activated by purification or that a latency inducing factor was being removed. Accordingly, we began to sytematically investigate the character of enzyme latency and to examine glandular extracts for the presence of endogenous factors which affect the duration of latency.

Nidamental gland extracts were prepared by disruption with a ground glass homogenizer in 0.05 M NaH_2PO_4, 1.0 M NaCl, pH 7.0 (2 ml/g wet weight) and were clarified by centrifugation as previously outlined (Koob & Cox, op. cit.). Aliquots of supernatant were stored frozen at -20° C and used immediately after thawing. Heat denatured extracts were prepared by boiling for 10 min, subsequent chilling on ice, and 10 min centrifugation at 15,000 x g to clarify. Denatured extracts were stored at -20° C prior to use. Oxidase activity was measured as previously described (Koob & Cox, op. cit.). Specifically, 0.03 ml aliquots of enzyme were diluted into 14 volumes of extraction buffer at 0.5 M NaCl and the reactions were initiated by addition of 0.15 ml 4 mM 4-methylcatechol (final concentration 1 mM). The production of quinone was monitored spectrophotometrically at 400 nm.

Catachol oxidase activity in a crude nidamental gland extract is shown in Fig. 1A. The extract exhibited a brief period of latency (1.8 min) followed by a rapid transition to a catalytically active state. The latent period remained consistent between fresh extract and stored frozen samples, but was diminished when extracts were stored at 4° C for 1-2 weeks. Latency varied between different glandular extracts. For example, extracts from the right and left glands of the same animal exhibited respective latent periods of 1.8 ± 0.2 min (± S.E., n = 6) and 4.5 ± 0.2 min (± S.E., n = 6). The duration of the latent period was highly sensitive to substrate concentration. In a typical assay with 4-methylcatechol concentrations of 1, 2, 4 and 8 mM, an extract exhibited respective latencies of 5.7, 3.2, 2.3, and 1.7 min.

Prior results demonstrated that purification of crude nidamental gland extract by ammonium sulfate precipitation diminished the latency of catechol oxidase. Accordingly, samples of crude extract were centrifugally filtered (400 x g for 1 min) through a 2 ml bed of Sephadex G-25 equilibrated with extraction buffer. The filtrate was immediately assayed for catechol oxidase

activity (a 0.15 ml aliquot of filtrate was used in each assay to compensate for the approximate five-fold dilution of enzyme which occurred during filtration). While crude extract exhibited a latent period of 1.8 ± 0.2 min. (± S.E., n = 3), gel filtered extract had a latency of 0.3 ± 0.1 min. (± S.E., n = 3), a five to six fold decrease. These results indicate that the crude extract contained a latency factor which was retained by G-25 Sephadex resin.

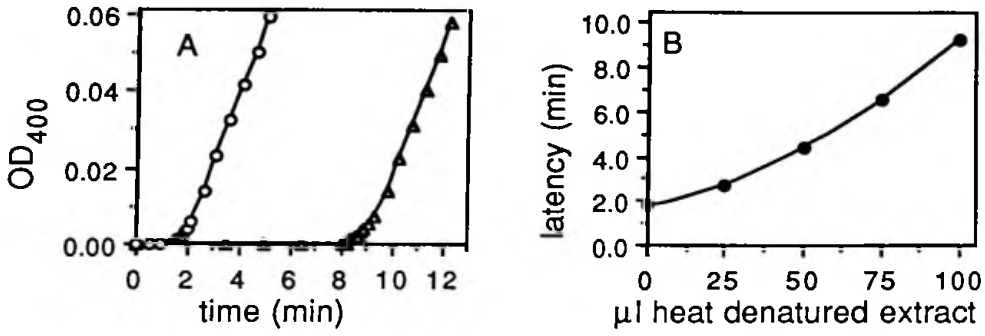

Fig. 1. A) Aliquots of shell gland extract (0.03 ml) were preincubated for 3 min. with (Δ) or without (O) 0.1 ml heat denatured extract in a final volume of 0.45 ml buffer. Reactions were initiated by the addition of substrate. B) Aliquots of native extract were incubated with indicated volumes of denatured extract and assayed as above.

Samples of crude extract were heat denatured to destroy enzyme activity. Aliquots of denatured extract were then added to native extract to test for the presence of a latency factor. As shown in Fig 1A, the addition of denatured extract caused a substantial increase in latency while having no effect on the initial rate of enzymatic oxidation once the reaction began. The effect of denatured extract on native extract latency was concentration dependent in a curiously non-linear manner (Fig. 1B). The duration of the latent period was not affected by altering the preincubation of enzyme with heat denatured extract from 3 to 20 min prior to substrate addition.

Heat denatured extract was dialyzed against extraction buffer (4 x 1 liter volumes) using Spectrapor dialysis membrane with a 12,000 - 14,000 molecular weight cutoff. When native extract was assayed in the presence or absence of 0.1 ml of dialyzed denatured extract the respective latent periods were 1.8 ± 0.1 min (± S.E., n = 3) and 1.8 ± 0.2 (± S.E., n = 6), indicating that the latency factor was removed by dialysis.

Heat denatured extract (0.5 ml) was hydrolyzed in 6 N HCl at 110° C for 24 h, and neutralized by three cycles of lyophilization and water washes. The freeze-dried material was then dissolved in 0.5 ml of extraction buffer and the pH adjusted to 7.0. When 0.1 ml of hydrolyzed material was added to native extract the latent period was unchanged (1.7 ± 0.2 min, ± S.E., n = 3), indicating that the latency factor was destroyed by hydrolysis.

These results demonstrate that crude nidamental gland extracts contain an endogenous factor which prolongs catechol oxidase latency without affecting final rates of enzyme activity. This factor has a relatively low molecular weight, based on the observations that it is removed by gel filtration and by dialysis. The factor is labile under hydrolytic conditions, indicating that it is organic rather than mineral in composition. The factor appears to function only temporarily and might be depleted or altered by the presence of substrate. Further studies on the character and structure of the latency factor will be essential to determine its mechanism of action and physiological significance.

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