PROTEASE ACTIVITY IN ALBUMEN DURING DEVELOPMENT OF THE LITTLE SKATE *RAJA ERINACEA*

J. William Straus[1] and Thomas J. Koob[2]
[1]Department of Biology, Vassar College, Poughkeepsie, NY 12604
[2]Skeletal Biology Section, Shriners Hospital for Children, Tampa, FL 33612

The fertilized egg in little skates (*Raja erinacea)* is packaged in a proteinaceous capsule together with a gelatinous albumen. The function of this albumenous material is presently unknown, but presumably it provides mechanical support or protection for the early embryo and it might represent a source of nourishment. The albumen disappears by the time about one third of development is completed and the embryo then becomes bathed in sea water that enters and exits through slits in the four horns (Koob & Summers, Bulletin MDIBL 35, 108-111, 1996). Ouang examined albumen disappearance in elasmobranch eggs (*Scylliorhinus canicula*) in 1931 (Annales de L'Institut Oceanographique 10, 285-370) and proposed that digestive enzymes secreted by the embryo might dissolve the albumen. We herein present evidence for the presence of proteases in skate eggs and their role in the dissolution of the egg albumen.

At least two distinct gel phases make up the albumen inside the egg capsule at oviposition. A viscous jelly like albumen comprises the bulk of material in the egg lumen surrounding the yolky embryo. A relatively solid gel fills the horns and is present at the anterior and posterior seams. The composition of these gels remains unknown and incubation in heated electrophoresis sample buffer with detergent and reducing agent (2% SDS and 2% β mecaptoethanol, pH 6.8 at 90°C for 10 min) did not yield any solubilized proteins that could be detected after SDS-PAGE with Coomassie Blue staining. As embryonic growth proceeds, the albumen liquefies, first near the embryo and then progressing outward. Concomitantly, the embryo can be seen to exhibit considerable undulatory behavior, indicating that albumen liquefaction may serve in part to create space for embryonic movement.

The precise mechanism of albumen liquefaction is currently unknown, but digestion by proteases and/or glycosidases seems likely. Accordingly, we tested the albumenous fractions for the presence of proteases by SDS polyacrylamide gel electrophoresis with porcine gelatin substrate co-polymerized in the gel to detect protease activity (Heussen & Dowdle, Anal. Biochem. 102, 196-202, 1980). The liquefied albumen was collected from egg capsules at various times after oviposition, mixed with equal volumes of electrophoresis loading buffer (0.5 M Tris, pH 6.8, 20 % glycerol, 10% SDS) and subjected to electrophoresis on "zymogram" gelatin/polyacrylamide gels (Novex, San Diego) to separate and detect proteolytic enzymes. Following electrophoresis, zymogram gels were incubated to promote substrate digestion and then stained with Coomassie Blue. Protease activity was revealed by the absence of staining where gelatin digestion had occurred (Fig 1).

The approximate molecular weights and relative activity of albumen proteases (Fig. 1) are summarized in Table 1. There was no apparent protease activity in any albumen fractions from freshly laid eggs (not shown). The activity of a high molecular weight protease was barely detectable in the liquefied albumen of a 33 d old egg that had been incubated in flowing sea water at ambient temperature (12° - 13° C). The liquid albumen from 46 d old eggs exhibited two strong bands of protease activity (apparent Mr ≥125 kDa and 118 kDa) and three minor bands with apparent molecular weights of 115, kDa, 104 kDa and 77 kDa. Albumen from 54 d old eggs exhibited additional bands of protease activity, including a strong band of approximately 100 kDa and several smaller weakly resolving bands. By 64 d, the number of detected proteases increased to at least eight, while their apparent sizes were diminished. The strongly staining bands of the 46 d and 54 d eggs were absent or diminished in the 64 d eggs, and several new lower molecular weight bands appeared, including a

strongly staining band at about 37 kDa (Fig. 1, Table 1). There is a possibility that low levels of additional proteases are present in all of the fractions, either masked by proximity to strongly resolving bands, or unresolved because of the incubation period. Future study must include longer zymogram incubations and alternate substrates to determine whether additional proteases are present.

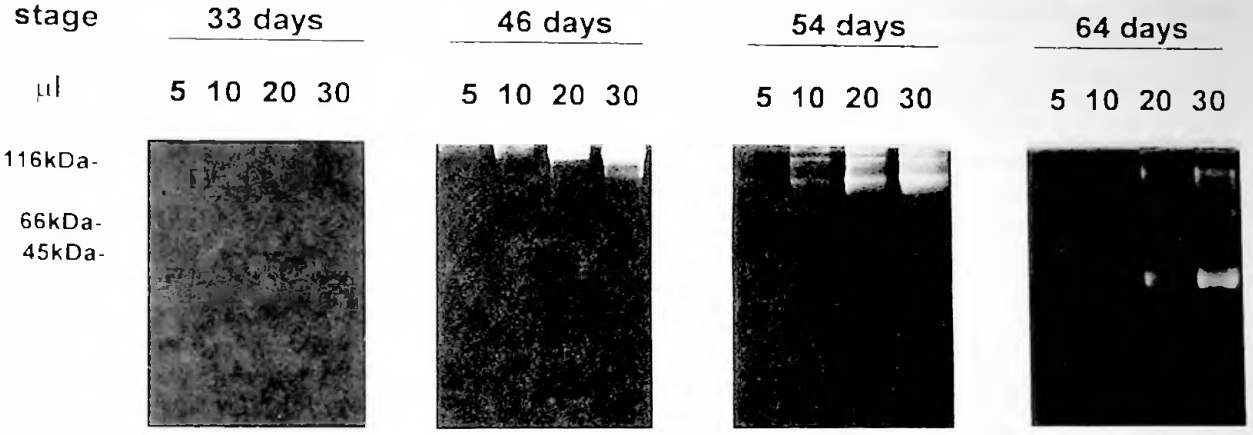

Fig. 1. Zymograms of protease activity in albumen during development. Aliquots of liquid albumen from eggs of the indicated ages were mixed with equal volumes of electrophoresis sample buffer (10% SDS, 20 % glycerol, 0.5 M Tris, 0.1% bromophenol blue, pH 6.8). The indicated volumes of albumen/loading buffer were applied to 10% polyacrylamide zymogram gels and run at 100 V. The gels were then equilibrated in 50 ml 2.5% Triton X-100 for 30 min, followed by a 30 min wash in zymogram developing buffer (Novex; 10 mM Tris base, 40 mM Tris-HCl, 0.2 M NaCl, 5 mM $CaCl_2$, 0.02% Brij 35), and then incubated in fresh developing buffer for 24 h at ambient sea water temperature (12° - 13° C) prior to staining. Resolved molecular weight standards were β galactosidase, bovine serum albumen, and chicken ovalbumin.

Table 1. Summary of changes in skate egg albumen proteases during development (see Fig. 1).

Age	33 d	46 d	54 d	64 d
apparent mol. weight	*relative activity**			
≥ 125 kDa	+	++++++	++++++	+++
118 kDa	-	++++++	++++++	±
115 kDa	-	+++	+++	+
104 kDa	-	+++	±	+++
99 kDa	-	±	++++++	±
97 kDa	-	-	±	+++
77 kDa	-	+	+	+++
37 kDa	-	±	+	++++++
28 kDa	-	-	-	+
22 kDa	-	-	-	+

* relative activity based on visual evaluation of band intensities: (-) absence of proteolytic activity; (±) indeterminant activity, difficult to distinguish from background staining; (+) low activity, (+++) moderate activity, (++++++) strong activity

Attempts to characterize the albumen proteases with various proteinase inhibitors met with limited success. Aliquots of albumen from 54 d and 64 d eggs were treated with the following inhibitors; 1 mM diisochlorocoumarin (DIC), 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.3 mM E-64, 1 mM tosyl-lysyl cholormethyl ketone (TLCK), 1 mM tosyl-phenyl chloromethyl ketone (TPCK), 0.15 mM pepstatin, 5 mM EDTA, or 1 mM phenanthroline. After incubation for 30 min at ambient temperature (≈ 22°C), one volume of zymogram electrophoresis sample buffer was added to each sample. Subsequent activity gel electrophoresis revealed no differences between control and inhibitor treated specimens (data not shown) indicating that no inhibition occurred. If these data are accurate, then the albumen proteases do not interact with broad spectrum inhibitors of serine proteases (DIC, PMSF, TLCK, TPCK), inhibitors of cysteine proteases (E-64, PMSF, TLCK, TPCK), chelating inhibitors of metalloproteases (EDTA, phenantholine), or an inhibitor of aspartic proteases (pepstatin) (Benyon & Bond, Proteolytic Enzymes, IRL Press, 1989). If the proteases in skate albumen are serine, cysteine, or metallo- proteases, then they are either highly unusual with respect to inhibitor interactions, or some unknown factor interfered with inhibition. Most of these inhibitors are hydrophobic (they require dissolution in dimethylsulfoxide or other solvents prior to incubation with enzyme in aqueous solution) and might have absorbed to the egg albumen or other material. Pepstatin is a reversible inhibitor of aspartate proteases and it is possible that inhibitory effects were not observed due to diffusion during and following electrophoresis. Clearly, the proteases will have to be purified and subjected to a more systematic analysis to characterize their catalytic properties.

The presence of proteases in liquefied albumen is consistent with a role for proteases in liquefaction. The virtual absence of protease activity in the albumen of younger eggs has two possible explanations. Maternally produced proteases might be stored in the albumen as inactive zymogens and activated at a later time, possibly by secretions from the embryo. Alternately, the embyros might secrete the proteases as development progresses. Both schemes are supported by the observation that liquefaction begins proximal to the embryo and progresses distally. Ouang (*op cit*) observed a similar phenomenon in developing elasmobranch eggs and proposed that the embryo secretes digestive enzymes from an eclosion gland located at the anterior end. The age dependent increase in protease species with an accompanying decrease in molecular weights has several possible explanations. First, the embryo might secrete or activate a single protease or small number of proteases that autodegrade to yield multiple forms of smaller but active enzymes. Alternately, the embryo might secrete distinct proteinases over time. Further study is essential to answer these questions.

Funded by grants from the Shriners of North America to TJK and a Blum/Halsey Scholar Award and MDIBL Fellowship to JWS