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Stabilization and sclerotization of *Raja erinacea* egg capsule proteins

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Synopsis

Sclerotization of skate egg capsule occurs after secretion of capsule precursors from the shell gland and involves a form of quinone tanning in which catechols are introduced in utero and subsequently oxidized to quinones by catechol oxidase. A latent form of enzyme is incorporated in the capsular matrix during secretion. Oxidase activity increases concomitantly with increasing catechol and quinone contents. Six major proteins ranging in size from 95 kDa to 20 kDa comprise the skate egg capsule, all of which contain elevated levels of glycine, serine, proline and tyrosine. Hydroxyproline occurs in all but one protein, however, none has an amino acid composition typical of collagen. Solubilization of two proteins from pre-tanned capsule requires reducing agents indicating that an early event leading to matrix stabilization is mediated by disulfide bonds. Stabilization of the other proteins along with the disulfide bonded proteins directly correlates with increasing catechol content, catechol oxidase activity and quinone formation.

Introduction

Low angle X-ray diffraction analyses of elasmobranch egg capsules have provided evidence for an ordered fibrillar component weakly resembling collagen fibrils (Bear 1952, Knight & Hunt 1974, Rusaouen et al. 1978). Ultrastructural studies of the fibrillar sheets of *Scyliorhinus canicula* egg capsule have provided similar evidence showing closely packed fibrils characterized by a centrosymmetrical axial banding pattern with a 37 nm repeat period (Knight & Hunt 1974, 1976). This observation has similarly been interpreted as evidence for collagen-like fibrils, despite the difference from the typical 64–67 nm banding pattern of fibrillar collagens.

Characterization of the proteins that comprise elasmobranch egg capsules has been hindered by the exceptional insolubility of the capsular material at oviposition. Only strong mineral acids and hot alkali dissolve the tanned capsule (Hussakof & Welker 1911). Therefore, acid hydrolysis has been employed to obtain overall amino acid composition. In previous reports, amino acid compositions of hydrolyzed, whole capsular material resemble collagen in having elevated glycine contents, substantial proline levels and significant amounts of hydroxyproline (Krishnan 1959, Gross et al. 1958, Knight & Hunt 1974, Cox et al. 1987). However, there are significant differences. Glycine, proline and hydroxyproline levels are generally lower than those of interstitial collagens. Rather high levels of tyrosine are also characteristic of these capsules leading to the hypothesis that another protein rich in tyrosine is also present (Gross et al. 1958, Knight & Hunt 1974). Beyond amino acid analyses of capsule hydrolyzates, and histochemical studies of shell glands discussed later, nothing is known about the proteins that comprise elasmobranch egg capsules.

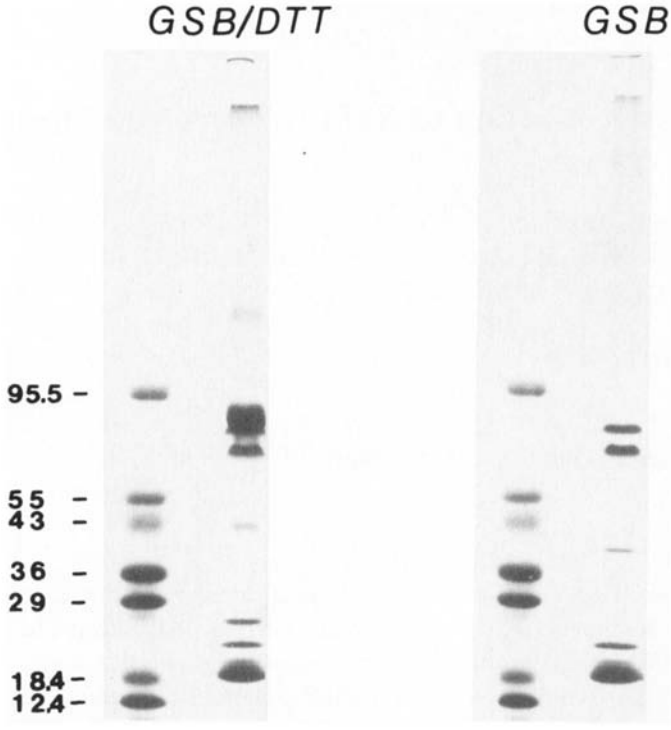

Fig. 1. Proteins solubilized from untanned egg capsule with gel sample buffer (GSB: 0.1M Tris-HCl, 3.3% sodium lauryl sulfate, 15% glycerol, pH6.8) with or without 50mM dithiothreitol (DTT). The newly secreted, untanned portion of a partially formed egg capsule was excised, split into two equivalent samples. One sample was placed in GSB without DTT and the other sample was placed in GSB containing 50mM DTT. Extraction was performed at 25°C for at least 24h. Egg capsule extracts were electrophoresed on 4–20% linear gradient SDS-PAGE.

We report on the identification and partial characterization of six major proteins that form the egg capsule of the little skate, *Raja erinacea.*

Materials and methods

Partially formed capsules were obtained from spawning little skates, *Raja erinacea.* The recently secreted, untanned portions of capsules were extracted with 3.3% sodium lauryl sulfate, 15% glycerol, 0.1M Tris-HCl, pH6.8 containing 50mM dithiothreitol (gel sample buffer; GSB/DTT). Untanned capsular material was solubilized after 24h of nutation at room temperature. Extracts were electrophoresed directly on 4–20% linear gradient polyacrylamide gels.

For amino acid compositional analyses of capsule proteins, extracts were electrophoresed as described above and then electroeluted onto a PVDF (polyvinylidene difluoride) membrane (Immobilon Transfer, Millipore). After electrophoresis the gels were soaked for 5min in two changes of transfer buffer (10mM 3-[cyclohexylamino]-1-propanesulfonic acid, 10% methanol, pH11). Proteins were then transferred to a prewashed PVDF membrane by electroelution at 0.3A for 5h at 4°C. Transferred proteins were localized by rapid staining with Ponceau-S; the blot was destained in 0.1% acetic acid, dried, and then protein bands were excised and analyzed for amino acid composition.

Amino acid analyses were performed using a modification of the phenylisothiocyanate pre-column derivatization method described by Bidlingmeyer et al. (1984). Lyophilized protein or Pierce H amino acids as standards were subjected to gas-phase hydrolysis for 20h at 110°C using constant boiling 6N HCl with 1% phenol. PVDF blots were likewise derivatized and hydrolyzed. The phenylthiocarbamyl derivatives were separated by chromatography on a Waters Pico-Tag column (3.9mm×15cm) maintained at 38°C using a Beck-

man System Gold HPLC. Our standard hydrolysis conditions destroy tryptophan, cysteine and to some extent methionine.

In order to determine whether stabilization of the egg capsule proteins correlates with the tanning process, a partially tanned capsule was dissected into consecutive strips down the length of the capsule from the untanned newly secreted material to the formed and tanned posterior end. Each strip was then dissected into three equivalent specimens. One specimen was extracted directly in gel sample buffer containing DTT. Another specimen was extracted with gel sample buffer lacking DTT. These extracts were directly electrophoresed on 4–20% PAGE. The third specimen was weighed after blotting dry and hydrolyzed in nitrogen purged 6N HCl at 108° C for 24 h. Catechol contents were measured in diluted aliquots of these hydrolyzates by the Arnow (1937) method. This method specifically detects *o*-diphenols that are un- or mono-substituted with the side chain *meta* or *para* to the first hydroxyl group; catechols substituted with more than one side chain are largely unreactive (Waite & Tanzer 1981).

Results

Electrophoresis on 4–20% linear gradient polyacrylamide gels of GSB/DTT extracts from newly secreted, pre-tanned capsular material revealed six major proteins ranging in apparent molecular weight from 95 kDa to 20 kDa (Fig. 1). There were no major protein bands migrating with an apparent molecular weight between 100 kDa and 400 kDa. However, there was material that did not enter the top (4%) of the resolving gel. Several additional proteins appearing in minor amounts were also detected.

Amino acid compositions of the six major capsule proteins are shown in Table 1. All of these proteins contain elevated levels of glycine, serine, proline and tyrosine. Glycine content varied from just under 200 residues to over 500 residues per thousand (RPT). Serine content averaged approximately 100 RPT with the 70 kDa protein being especially serine rich at 152 RPT. Significant amounts of proline were found in the 90, 70, 38 and 27 kDa proteins. Hydroxyproline was present in significant amounts in the large proteins. Tyrosine levels rang-

Table I. Amino acid composition of *Raja erinacea* egg capsule proteins. Extracts of untanned capsule in GSB/DTT were electrophoresed on linear 4–20% gradient SDS/PAGE. Proteins were then electroeluted onto PVDF membrane, hydrolyzed and their amino acid compositions determined. Values presented are in residues per 1000 residues. Tryptophan and cysteine were not determined.

Amino acid	Proteins					
	90 kDa	70 kDa	38 kDa	27 kDa	23 kDa	20 kDa
ASX	111	173	115	106	53	47
GLX	56	24	50	49	9	5
HYP	19	2	11	14	1	0
SER	106	152	108	94	81	85
GLY	258	196	229	279	516	522
HIS	2	11	19	12	28	39
ARG	59	11	42	36	4	3
THR	51	62	46	39	9	0
ALA	58	29	53	47	5	0
PRO	79	65	72	69	25	23
TYR	86	177	110	115	204	218
VAL	25	6	24	24	3	9
MET	2	10	8	13	1	1
ILE	14	5	22	22	6	3
LEU	15	6	24	24	7	3
PHE	10	5	21	21	2	1
LYS	35	65	45	38	45	41

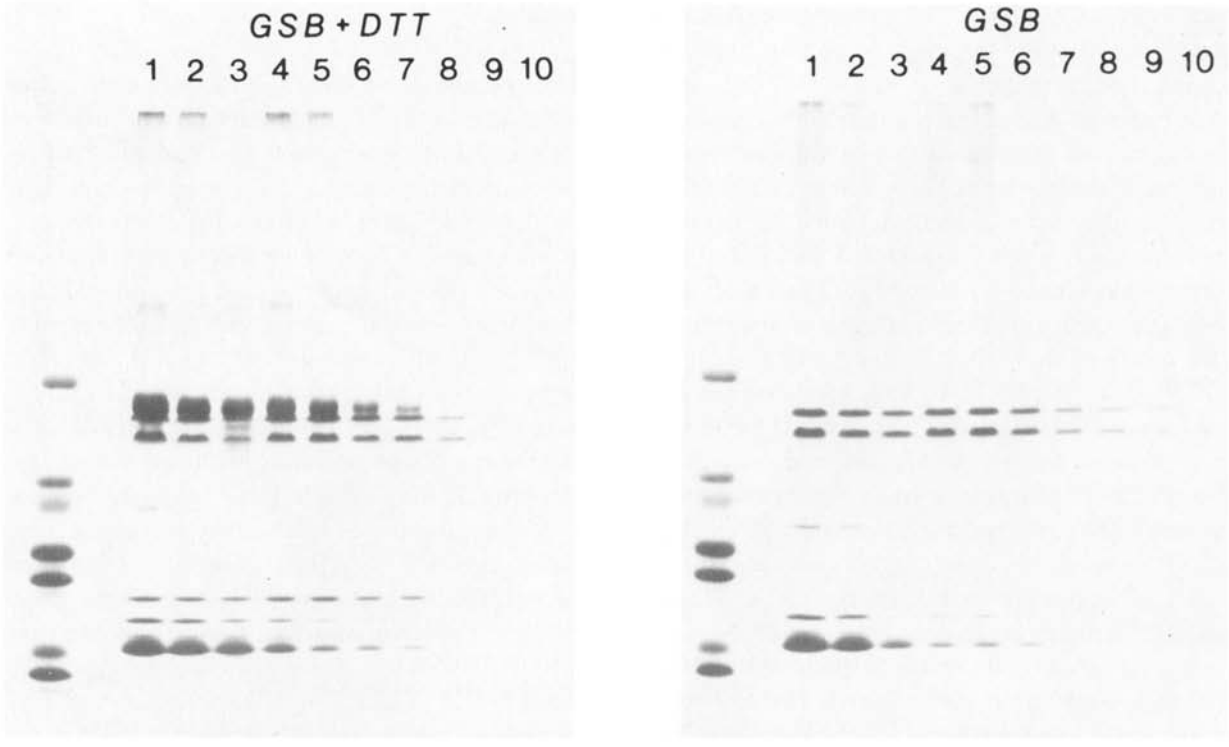

Fig. 2. Solubility in GSB/DTT of egg capsule proteins from sequential specimens of a partially tanned capsule. The #1 specimen was from the untanned portion of the capsule still within the lumen of the shell gland. The #10 specimen was from the oldest, tanned portion of the capsule. The intervening specimens showed intermediate levels of tanning that increased from the #1 to #10 specimen.

ed from 86 to over 200 RPT. Highest levels of tyrosine were found in the 23 and 20 kDa proteins that also contained unusually high levels of glycine. Approximately three quarters of the amino acids in these proteins are glycine and tyrosine.

Solubility of these egg capsule proteins in gel sample buffer coordinately declined with time after secretion from the shell gland. Figure 2 shows the amount of each protein solubilized by GSB/DTT in successive specimens from a partially formed capsule. Most protein was solubilized from the untanned material still within the lumen of the shell gland. Little protein solubilization occurred in the oldest, most tanned specimen. When DTT was omitted from the GSB extraction, the 95 and 27 kDa proteins were not solubilized. Omission of DTT did not affect solubilization of the other proteins or their electrophoretic migration.

Decreasing solubility of these proteins in gel sample buffer directly correlated with increasing catechol contents (Fig. 3). Greatest solubilization occurred in specimens with no catechol. Little solubilization occurred in specimens with greatest catechol contents. All proteins coordinately declined in solubility with increasing catechol contents.

Discussion

The egg capsule of *Raja erinacea* is composed of six major proteins ranging in apparent molecular weight from 95 kDa to 20 kDa. The greater number of proteins than that postulated from previous biochemical analyses corresponds with histochemical observations, since Rusaouen (1976, 1978) distinguished six zones with discrete secretory activity in nidamental glands of *Scyliorhinus canicula*. Whether collagen is present in egg capsule proteins remains an open question for the little skate. Although none of the major proteins identified here have an amino acid composition typical of interstitial collagens, we can not rule out the possibility that

one or more of the larger proteins is an elasmobranch minor collagen. Moreover, since the large capsule proteins contain elevated levels of glycine, proline and hydroxyproline, they may contain collagenous domains. Further study will be necessary in order to identify collagens, if present, in skate egg capsules. Nevertheless, our results clearly show that the egg capsule of the little skate contains proteins other than collagen and that these proteins are important for capsule matrix formation and stabilization.

Two of the small capsule proteins, the 23 kDa and 20 kDa proteins, are most unusual in that glycine accounts for over half of each protein, and tyrosine accounts for almost one quarter. These proteins are likely to be homologous with the so called polyphenolic and tyrosine-rich proteins detected histochemically in shell glands of other oviparous elasmobranch species (Brown 1955, Threadgold 1957, Krishnan 1959, Rusaouen 1976). Their amino acid composition resembles egg capsule proteins from several invertebrates that are likewise enriched in glycine and tyrosine. Among the insects these include a family of oothecal proteins from the cockroach, *Periplaneta americana* (Pau 1987), chorion proteins from three species of *Drosophila* (Martinez-Cruzado et al. 1988), silkmoth, *Bombyx mori,* chorion proteins (Spoerel et al. 1986), and eggshell proteins of the polyphemus moth, *Antheraea polyphemus* (Jones et al. 1979). Several eggshell proteins from two species of parasitic helminths, *Schistosoma mansoni* (Bobek et al. 1986) and *Fasciola hepatica* (Waite & Rice-Ficht 1989) are also glycine-enriched, although tyrosine levels are lower, probably as a result of conversion to 3,4-dihydroxyphenylalanine prior to secretion. Why glycine-tyrosine rich proteins are abundant in eggshells of organisms as diverse as invertebrates and elasmobranchs is unknown.

An early event in capsule stabilization appears to rely on the formation of disulfide bonds since the 95 kDa and 27 kDa proteins were solubilized only when DTT was included in the extraction buffer. This observation is in accord with histochemical tests of shell glands documenting the presence of sulfhydryls in granule-bound capsule precursors (Vovelle 1967, Rusaouen-Innocent 1991). Whether

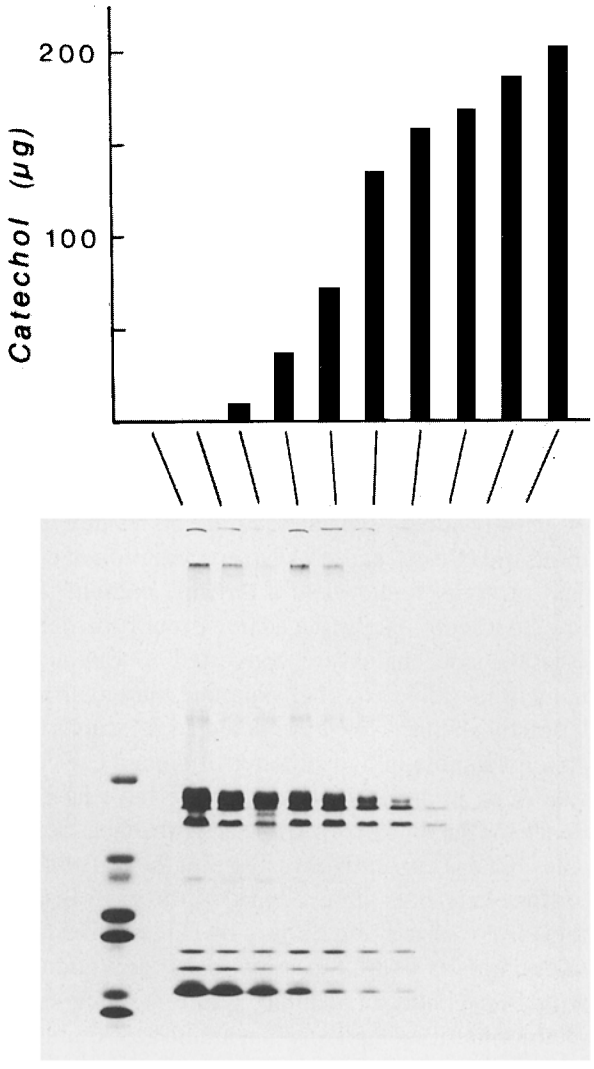

Fig. 3. Catechol content (µg per specimen) and capsule protein solubility in GSB/DTT in sequential specimens from a partially tanned capsule.

disulfide bonds form between these two proteins is not known. Clearly, disulfide bonds are not exclusively intramolecular nor do they mediate simple monomer-dimer associations. Similarly, we don't know when disulfide bond formation occurs: whether during synthesis and processing of these proteins within gland cells or during assembly of secreted capsule precursors.

Tanning of the capsule matrix following secretion and assembly of capsule precursors relies on the introduction and oxidation of catechols as shown here and in a previous report (Koob & Cox 1990).

We found here an inverse relationship between the solubility of capsule proteins and matrix catechol content. Newly secreted capsular material contains little or no catechol, however, it contains unusually high levels of tyrosine. Following secretion of capsule proteins, catechol content in the capsular matrix increases. Two mechanisms would account for the increase in catechol after secretion from the shell gland. The uterus could provide catechols, or, alternatively, phenolic groups in capsule precursors could be converted to catechols by hydroxylation. The data presented here together with previous amino acid analyses provide support for the latter hypothesis. Capsule precursors contain tyrosine at 86 and 220 residues per thousand, yet tyrosine levels in fully tanned capsules average 50 residues per thousand (Cox et al. 1987). No combination of capsule proteins would yield a tyrosine content that low. So it seems likely that a large proportion of the tyrosine side chains are converted to catechols, namely 3,4-dihydroxyphenylalanine, via a hydroxylation mechanism. We have detected 3,4-dihydroxyphenylalanine in hydrolyzates of tanned egg capsule (Cox et al. 1987). However, we have no evidence for tyrosine hydroxylase activity in these capsules but are currently investigating the possibility.

Once catechols are produced in the egg capsule they are oxidized to quinones by catechol oxidase (Koob & Cox 1990). Since quinones form adducts with side chains of alanine, lysine, cysteine and methionine, it seems likely that amino acid-quinone condensation products readily form during the tanning process. If the reactive quinones are themselves side chains of structural proteins produced from tyrosine via 3,4-dihydroxyphenylalanine, then quinone condensation would result in a highly crosslinked protein polymer. Whether this pathway or another operates during sclerotization of skate egg capsule is currently being explored.

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