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STUDIES ON THE CHEMICAL STRUCTURE OF COD EGG CHORION

BY

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ABSTRACT

The chemical structure of the cod egg chorion has been investigated in preparations of chorion obtained after decompression rupture of cod eggs. The chorion was purified free of non-integral components by sucrose gradient centrifugation or by membrane filtration. Both preparations of cod egg chorion were shown to be almost entirely proteinaceous in nature, with only minor amounts of carbohydrates present. The protein units of the chorion displayed molecular sizes and amino acid composition akin to the keratin class of proteins. The modifications and developmental fate of chorion keratins are discussed.

INTRODUCTION

The succesful fertilization of fish eggs and the survival of the embryo until hatching is dependent upon the structural properties of the egg chorion. At the time of hatching the egg chorion has become modified to allow the entrance of the fish larva into its natural environment. The molecular mechanisms underlying these processes are not well understood (B. Shapiro et al., 1981), and there is particularly scanty information concerning such processes in marine fishes (K. Yamagami, 1981).
The present study concerns experiments undertaken to delineate the chemical components of cod egg chorion, and our first attempts to explain in molecular terms the rigidity changes of the chorion upon fertilization and the flaccidity of the chorion at the time of hatching.

MATERIALS AND METHODS

The fish species studied was cod (Gadus Morhua). Unfertilized eggs were obtained by dissection of fish during the spawning season. Fertilization of eggs was allowed to take place in the laboratory, and samples of fertilized eggs were collected 1hour and 24hours after fertilization and also just prior to and after hatching.

Preparation of ruptured egg chorion. A French Press instrument was modified such that the orifice of the drain valve allowed passage of intact cod eggs without clogging of the drain valve. Samples of cod eggs (15ml packed volume) were diluted with equal volumes of buffer, and compressed to 6000psi. Upon rapid decompression almost quantitative rupture of the eggs was achieved. The buffers employed were 10mM Tris-HCl at pH 7.2 containing 150mM NaCl, with or without 1% SDS (sodium dodecyl sulfate). The ruptured eggs were layered on top of a discontinuous sucrose gradient containing two equal portions of respectively 15% and 30 % sucrose in the above buffers. Upon centrifugation for 10mins in a Sorvall GLC-2 centrifuge at 1000xG, the egg chorion constituted the pellet. Upon double recentrifugation no further material was found in the supernatant. As separation was most easily achieved with SDS in the buffer, this buffer was adopted as the standard buffer. The pelleted material was dialyzed for 72 hours against buffer changes of decreasing osmolarity, until a final dialysate was obtained in distilled water. Initial dialysis was at room temperature, with the final dialysis carried out at $4^{o}C$.

- Subsequently we have employed an alternative method which allows rapid isolation of large quantities of pure chorion. Instead of centrifugation and dialysis after decompression cleavage of the eggs, the ruptured material is applied to a Nylon cloth (25 micron pores) and washed repeated with buffers of decreasing osmolarity. Smaller pores such as Millipore filters (0.45micron) are less suitable due to clogging propensities. The pure chorion preparation may simply be removed from the filters by gentle scraping at the end of the washing procedure.

Chemical methods. Amino acid composition of isolated chorion material was obtained upon hydrolysis in 6N HCl for 16hours at 110^{o}C under a nitrogen atmosphere.The amino acids were quantitated after post-column reaction of ninhydrin and amino acids eluted separately after standard ion exchange column chromatography. Neutral sugars, amino sugars and uronic acids were analyzed by standard methods (N.Tietz,1980).

Electrophoresis. Molecular analysis of proteins integral to the chorion membrane was performed by SDS polyacrylamide gel electrophoresis (PAGE) according to the method of Shapiro et al., 1967. Running gels of 7.5% acrylamide were found to be suitable for the separation of proteins extracted from the chorion by various chemical treatments. Proteins were visualized in the PAGE by Coommassie Blue or by silver staining of glutaraldehyde-treated gels.

RESULTS

Initial experiments with intact cod eggs demonstrated that such eggs were uniquely inert to a series of rather harsh chemical treatments. Thus, the eggs ,- whether fertilized, hardened or unfertilized, are resistant to degradation by high concentrations of proteases such as bovine trypsin , chymotrypsin, or elastase, or bacterial collagenase, even after extended periods of exposure, or sequential treatments. Furthermore, the eggs also remain intact after treatment with 1 M HCl or 1 M NaOH, and to prolonged boiling in 1% SDS. Even extraction with 10 excess volumes of chloroform: methanol (= 2:1) to remove lipids similarly appears not to disrupt the chorion.

When purified chorion was subjected to compositional analysis, it was found to be predominantly composed of proteins, with only trace amounts of sugars present. Hence, the inertness to proteolyttic

destruction exhibited by chorion in intact eggs must be due to protein modifications or crosslinking, or to a protective cover of a non-protein envelope exterior to the bulk of the protein in the chorion (Iwasaki et al., 1984)

The polymerized state of the proteins in the chorion is further demonstrated by PAGE of extracted chorion proteins. Various depolymerizing strategies were employed, such as high salt (0.5 M NaCl); treatments with chelators (1mM EDTA or EGTA); treatment with chaotropic agents such as 8M urea; treatment with 1% 2-mercapto-ethanol to break disulfide linkages ; or boiling in denaturing agents such as SDS (1%). None of these protocols provided for quantitative solubilization of chorion proteins either employed alone or in sequence or in combination. Some material was extracted but less from hardened fertilized egg chorions than from unhardened fertilized egg chorions. The chorions from hatched eggs appear to contain less total protein than chorion from eggs at the time of fertilization. The solubilized material appeared to be of rather low molecular weight, and also consisted of a limited number of molecular species. The major components exhibited molecular weights of 55000Dalton and 45000Dalton respectively. Two other prominent molecular species exhibited molecular weights of approximately 30000 D and 20000 D . The first two components appear to be of molecular sizes reminiscent of the molecular weights typical of those reported for the two classes of keratin molecules.

The keratin-like nature of the chorion proteins was further substantiated by amino acid analysis of these proteins. Only small differences were observed in amino acid composition between fertilized and unfertilized chorion proteins, while both exhibit an overall composition strikingly similar to the keratin class of protein molecules. However , both preparations are low in cystein contents (Fuchs et al., 1981 ; Kraighn ,1964)

In view of the marked differences in mechanical rigidity between unfertilized and fertilized eggs, it is noteworthy that the chorions from these two stages exhibit rather similar chemical inertness. Hence , while chemical bonds may further polymerize the chorion proteins upon fertilization, the main bondings in the chorion protein polymer already exist in the unfertilized egg. When the chorion preparation was tested in the presence of strongly alkaline ammoniacal copper hydroxide, the lammellar chorion dissolved and left behind,after removal of the reagent, thin and "ghost-like" shells. Thus, non-hydration or redox-sensitive crosslinkages of chorion

proteins endow the chorion with its unique chemical and structural inertness. (Smithberg,M. 1966; Hagenmaier et al., 1976)

DISCUSSION AND CONCLUSIONS

The cod chorion is primarily a protein aggregate already polymerized in the unfertilized state, and it is further polymerized upon fertilization. The experiments indicate that cod chorions possess an external protective cover which seems to be non-protein or perhaps conjugated proteins (proteoglycan or mucin) in nature, and which renders the chorion insensitive to many proteolyttic enzymes. The proteins of the chorion apparently are partly removed towards the time of hatching of the egg, but our experiments give no information as to the biological fate of such proteins. The chorion proteins appear to be keratin-like in structure, and the asymmetrical sensitivity towards degradation by external and internal enzymatic processes, suggests a mechanism by which cod larvae may safely digest away the chorion at the time of hatching without at the same time autolyzing the fish embryo . Clearly fine control mechanisms must operate which allow this selective keratin digestion in one structure (chorion) but not in another adjacent structure (the fish skin). Potentially,excess skin keratin digestion at the time of hatching may cause damage to the embryos that may affect their viability after hatching (Huse and Jensen, 1981). We have initiated experiments to characterize chorion-degrading (hatching) enzyme(s) in cod eggs in order to evaluate this possibility.

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FIGURE I: Scanning Electron Micrographs of decompression-cleaved, isolated chorion

FIGURE II: SDS-PAGE (7.5%) of the proteins extracted from isolated chorions.

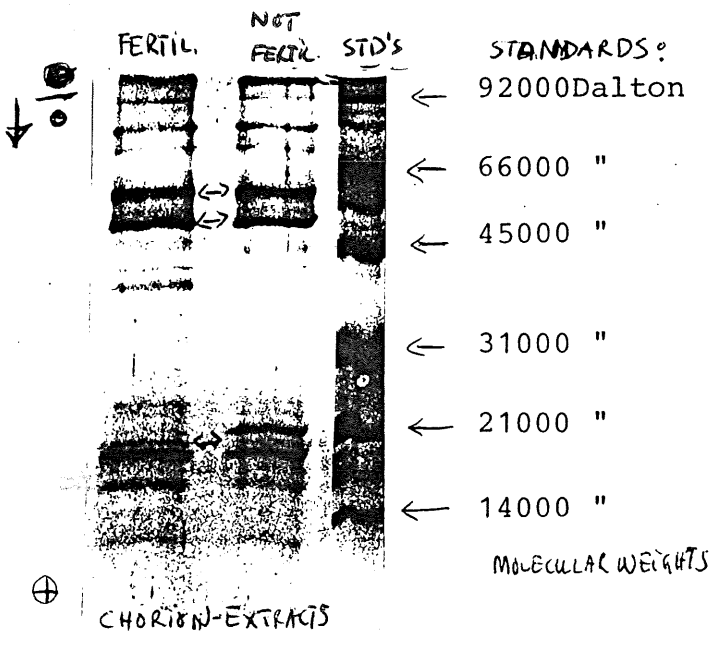

TABLE I: Amino Acid Contents of purified cod chorions

AA:	CHORION from COD	C.L.*	Keratin MAN
Lys	7.0	5.7	5.5
Arg	7.1	6.4	4.5
His	5.4	4.3	1.0
Asp	7.2	9.4	9.2
Thre	7.4	6.1	4.0
Ser	2.3	7.3	9.1
Glx	9.3	13.0	13.0
Pro	n.d	n.d.	2.7
Gly	12.5	8.9	18.5
Ala	7.5	9.1	5.1
1/2 Cys	0.4	.5	1.4
Val	4.6	7.5	4.9
Meth	.2	.3	1.2
Ileu	4.8	4.1	4.9
Leu	9.0	8.9	9.8
Tyr	2.5	4.5	2.3
Phe	4.2	4.2	4.3

Chorion from unfertilized eggs.
Values from Cyclopterus lumpus (C.L.) listed for comparison
n.d.= not determined