HATCHING ENZYME IN SECRETORY GRANULES OF THE HATCHING GLAND OF MEDAKA

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At the time of hatching in fish, a tough egg envelope (chorion) is transformed into a sheet of thin fragile membrane by the action of the hatching enzyme, which digests away the thick inner layer of the chorion. It is well known that after synthesis, this enzyme is sequestered in secretory granules of the hatching gland cell (Bourdin, 1926; Ishida, 1944; Ouji, 1959; Yamamoto, 1963), as diagrammed in Fig. 1. A study of the intragranular hatching enzyme would aid in an understanding of the various constituent steps of the hatching process, i.e., the mechanism of formation, maturation and secretion of the enzyme, the mechanism of chorion lysis, etc. However, a method for isolating secretory granules of the hatching gland has not been well established. The present report describes the isolation of granules from homogenates of whole medaka embryos and the extraction of an enzyme having choriolytic activity from the granules.

Secretory granules were isolated from sucrose homogenates of whole embryos at 4th and 5th day stages in our culture system (Yamagami 1960; sts. 29-31 after Matui, 1949). The embryos were crushed first in cold 0.3M sucrose using a loose glass homogenizer and the preparation was filtered through a sheet of cotton gauze to remove the chorion. The filtrate was then homogenized in a Teflon pestled homogenizer, filtered through a nylon mesh (25μm) to remove cell debris, and the filtrate was centrifuged at 1,000g for 20min. at 4°C. The resulting pellet, $P_{1,000}$, contained little cell debris but numerous melanosomes in addition to the secretory granules. The pellet was suspended in a definite volume of cold 0.3M sucrose, and mixed with two volumes of sucrose-Percoll solution, which was made by dissolving sucrose in the original Percoll suspension (Pharmacia Fine Chemicals) to give a concentration of 0.3M. In order to generate a density gradient, the mixture was centrifuged at 18,000g for 4hr at 4°C in an angle rotor. A control tube, containing a mixture of the same composition as the experimental tube except that the pellet, $P_{1,000}$, was replaced by some density marker beads of various buoyant densities (Pharmacia Fine Chemicals and Clark Wilcox and Assoc.) was also centrifuged. The density gradient generated in the experimental tube after centrifugation was calibrated by analysis of control tube.

The above procedure brought about the separation of several visible layers of subcellular structures. Phase contrast microscopy as well as assays for choriolytic activity revealed that the third major layer (L_3, d=1.16-1.17) from the top of the tube consisted mostly of the secretory granules. The melanosomes which had contaminated the pellet, $P_{1,000}$ have a very high buoyant density and were all

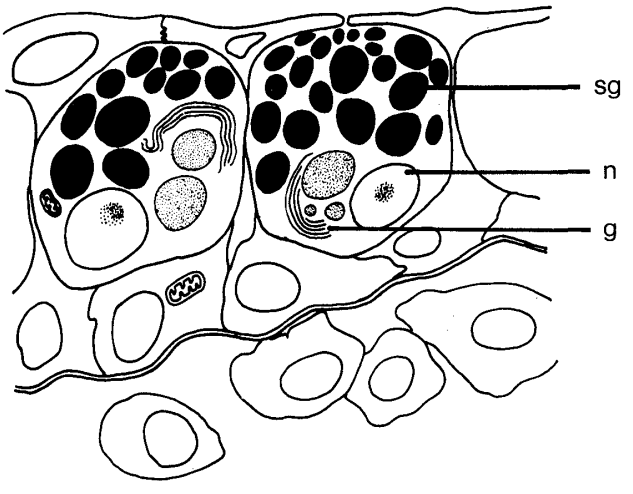

Fig. 1. A diagrammatic illustration of hatching gland cells of the prehatching stage embryo of medaka. sg, secretory granule; n, nucleus; g, Golgi body (after Yamamoto et al., 1979).

spun down on centrifugation. A fraction (Fr III) including L_3 was mixed with two volumes of the sucrose-Percoll solution and the mixture was centrifuged again at 18,000g for 4hr at 4°C. The secretory granule fraction thus obtained (Fr III_2) was mixed with a sufficient amount of 0.3M sucrose and centrifuged at 1,500g for 30min. at 4°C to obtain a pellet. The pellet consisted of secretory granules with a few melanosomes, as revealed by electron microscopy. The secretory granules were round or elliptic in shape and about 2-3μm in maximal diameter. Electron microscopy also showed that the contents of these isolated granules retained a crystalline structure with a periodicity of about 70A, similar to those of granules found in the hatching gland fixed *in situ*.

An aqueous extract of the preparation of isolated granules showed a high choriolytic activity, as determined by turbidimetry reported elsewhere (Yamagami, 1970). The specific activity of Fr III_2 was about 40 times that of the crude granule preparation, $P_{1,000}$. The choriolytic activity was very sensitive to ethylenediamine tetraacetate (EDTA) but not to soybean trypsin inhibitor (STI). This sensitivity to EDTA is one of the representative characterisitics of the hatching enzyme (Yamagami, 1973). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the aqueous extract contained primarily a single protein band with molecular weight of about 21,000. On the other hand, a hatching enzyme sample prepared from the hatching liquid (P II-0.3 enzyme, Yamagami, 1972, 1973) displayed, upon SDS-PAGE, a major and minor protein band, the major band being coincident with the 21,000 mol. wt. band of the granule extract.

These results indicate that the isolated secretory granules contained a hatching enzyme which is composed of a single polypeptide chain of about 21,000 mol. wt. and is active when dissolved in water. Moreover, these results suggest that the hatching enzyme molecule packaged in secretory granules does not undergo a "large scale modification", such as, a considerable decrease in molecular weight during the course of secretion. The buoyant density of the secretory granules (d=1.16-1.17) is higher than that of other organelles (lower than 1.12, Wolff, 1975). This fact might suggest that the enzyme protein is compacted in the secretory granule in a dehydrated state, and that the enzyme may be unable to exert its action within the granule.

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