Acid phosphatase in eggs of the zebrafish, *Brachydanio rerio*

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Summary. 2 isozymes of acid phosphatase have been identified by polyacrylamide disc gel electrophoresis in the ovary and mature, unfertilized eggs of *B. rerio.* Histochemically, the enzyme appears to be localized in preyolk bodies of previtellogenic oocytes and in yolk platelets of vitellogenic and postvitellogenic oocytes. The contents of the cortical granules at all stages of oocyte differentiation were acid phosphatase negative.

Acid phosphatase (E.C. 3.1.3.1) is an important lysosomal enzyme that has been identified in adult as well as in embryonic tissues[1-3]. Since hydrolytic enzymes, particularly acid phosphatase, have been correlated in several embryonic systems with localized cell and tissue degeneration[4-6], it has been postulated that acid hydrolases might function shortly after fertilization in the rupture of cortical granules from the cytoplasm. Cortical granules are membrane-bound subcellular organelles found in the peripheral cytoplasm of the ripe eggs of many animal species. These granules fuse with the plasma membrane upon activation of the egg, rupture, and typically discharge their contents at the egg surface[7-9]. The contents of the cortical granules have been implicated in the elevation of the vitelline membrane and in the late block to polyspermy[10]. Since the discharge of cortical granules can be slowed by low temperature, Vacquier[11] has implicated an enzymatic mechanism in this secretory process.

Little is known about the chemical and enzymatic constituents of eggs and their cortical granules. The enzyme β-1,3 glucanase has been detected in the eggs of the sea urchin, *Strongylocentrotus*[12]. Histochemical attempts to demonstrate the presence of acid phosphatase in the cortical granules of sea urchins have produced contradictory data[13,14]. Cortical granule fractions obtained by zonal centrifugation of *Strongylocentrotus* eggs were rich in acid phosphatase, but the precise organelle localization of the enzyme could not be established[15]. Varute and Patil[16] claim to have localized acid phosphatase and β-glucuronidase in the cortical granules of oocytes of selected amphibians, reptiles and birds. Anderson[17] could detect no acid phosphatase activity in the cortical granules of the guinea-pig and rabbit.

The objectives of this study were to: a) determine by electrophoresis the activity of acid phosphatase in the adult ovary and in ripe, unfertilized eggs of *B. rerio,* and b) demonstrate histochemically the localization of this enzyme in oocytes of the ovary.

Materials and methods. For electrophoretic studies, ovary tissue was dissected from mature female fish, homogenized in 5% Triton X-100 and centrifuged for 60 min at 4 °C (25,000 × g). Samples of 40 unfertilized eggs were collected from gravid females[18], homogenized in 5% Triton X-100 and centrifuged as above. 1 ml of either ovary of unfertilized egg supernatant was combined with 0.3 ml of a saturated sucrose solution and subjected to electrophoresis using 10% polyacrylamide gels, 0.04 M histidine-NaOH (pH 7.5) as the gel buffer and 0.01 M histidine-NaOH (pH 7.5) as the electrode buffer[19]. Electrophoresis was carried out for 65 min at a constant current supply of 1.5 mA/gel tube. Acid phosphatase bands were stained according to Barka[20] and Barka and Anderson[21] using either α-naphthyl acid phosphate and Fast Garnet GBC or naphthol AS-TR phosphate and hexazonium pararosanilin (Sigma Chemical Co.).

For histochemical studies, ovaries were fixed in 10% formalin containing 1% $CaCl_2$ (pH 7.2) for 24 h (4 °C). Fixed tissue was frozen, embedded in Ames OCT compound (Fischer Sci. Co.) and sectioned at 12 µm in an International Harris cryostat (−20 °C). Other ovaries were fixed overnight in Bouin's fixative and routinely processed to obtain sections for staining with either Alcian blue (pH 2.5) or periodic acid Schiff reagent (PAS).

Results and discussion. 2 distinct forms of acid phosphatase (AP 1 and AP 2) were resolved by electrophoresis of ovary tissue (figure 1A). The slower-moving band (AP 2) always appeared more intensely stained than the faster-moving band (AP 1) irrespective of the substrate used in the staining mixture (figure 1B). 2 zones of acid phosphatase with similar electrophoretic and staining properties were separated from homogenates of unfertilized eggs (figure 1C), thus indicating that both isozymes of this enzyme are sequestered in the egg during oogenesis. The existence of 2 forms of acid phosphatase using polyacrylamide gels and a similar system of electrophoresis has been reported for other vertebrate tissues[22]. Where 3 or 4 forms of the enzyme have been reported in the literature[3,6] the differences in position and number of bands are probably related to differences in conditions for electrophoretic separation. Both AP 1 and AP 2 hydrolyzed α-naphthyl phosphate and napththol AS-TR phosphate, thus confirming other studies which have demonstrated the nonspecificity of this enzyme toward its substrates. The adult ovary of *Brachydanio* showed oocytes in all stages of differentiation and maturation (figure 2A). These oocytes have been staged according to size (measured in diameters) and other salient cytological features using a modification of the scheme proposed by Malone and Hisaoka[23]. Stage 1 (10–20 µm) and stage 2 (25–90 µm) oocytes were characterized by large nuclei in a small volume of cytoplasm; they were distinguished from each other on the basis of the number of nucleoli in their nuclei. Small, discrete vesicles were visible in the cytoplasm between nucleus and plasma membrane in stage 3 oocytes (100–150 µm). Their positive staining responses to Alcian blue and PAS and their topographical location indicated that these vesicles were the early stages

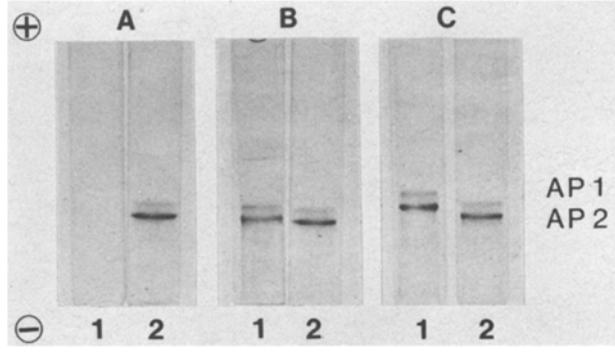

Fig. 1. Electrophoretic separation of isozymes of acid phosphatase in *B. rerio. A* Control gel (1) and ovary (2). *B* Isozymes of ovary stained with AS-TR phosphate-hexazonium pararosanilin (1) and α-naphthyl phosphate-Fast Garnet GBC (2). *C* Ovary (1) and unfertilized egg (2) homogenates. Migration of isozymes is toward the anode.

in the formation of the cortical granules. All oocytes assigned to stage 4 (150–275 µm) were characterized by intense cortical granule formation throughout the cytoplasm. The stage 5 or vitellogenic oocyte (300–450 µm) showed a heterogenous population of variously-sized yolk vesicles and yolk platelets around the nucleus. The transformation of yolk vesicles into definitive crystalline yolk platelets was accompanied by movement of the cortical granules to the periphery of the egg. The mature or stage 6 oocyte showed a central yolk mass and a thin rim of peripheral cytoplasm dominated by Alcian blue and PAS positive cortical granules.

The histochemical reaction localizing acid phosphate activity in ovarian oocytes appeared as distinct, particulate deposits of reddish-brown dye (figure 2B). Control sections showed an absence of coloration except for a nonspecific reddish cast to the cytoplasm of stage 1 and stage 2 oocytes. No acid phosphatase activity was detected in either stage 1 or stage 2 oocytes irrespective of the length of time in the staining solution (figure 2B). Acid phosphatase positive sites were initially localized in the vicinity of the nucleus and beneath the plasma membrane in stage 3 oocytes (figure 2B). Maximum intensity of the staining reaction was evident after 20 min of staining. The contents of the developing cortical granules showed no evidence of staining reaction.

There was a noticeable increase in the number of enzyme staining sites throughout the cytoplasm of the stage 4 oocyte, particularly between cortical granules (figure 2C). The general level of the staining reaction at each site was comparable to that observed in the stage 3 oocyte. Hence, there appears to be an increase in acid phosphatase activity through previtellogenesis (stages 3 and 4) as indicated by an augmentation in the number of enzyme positive staining sites. The general level of acid phosphatase activity decreased sharply in vitellogenic and postvitellogenic oo-

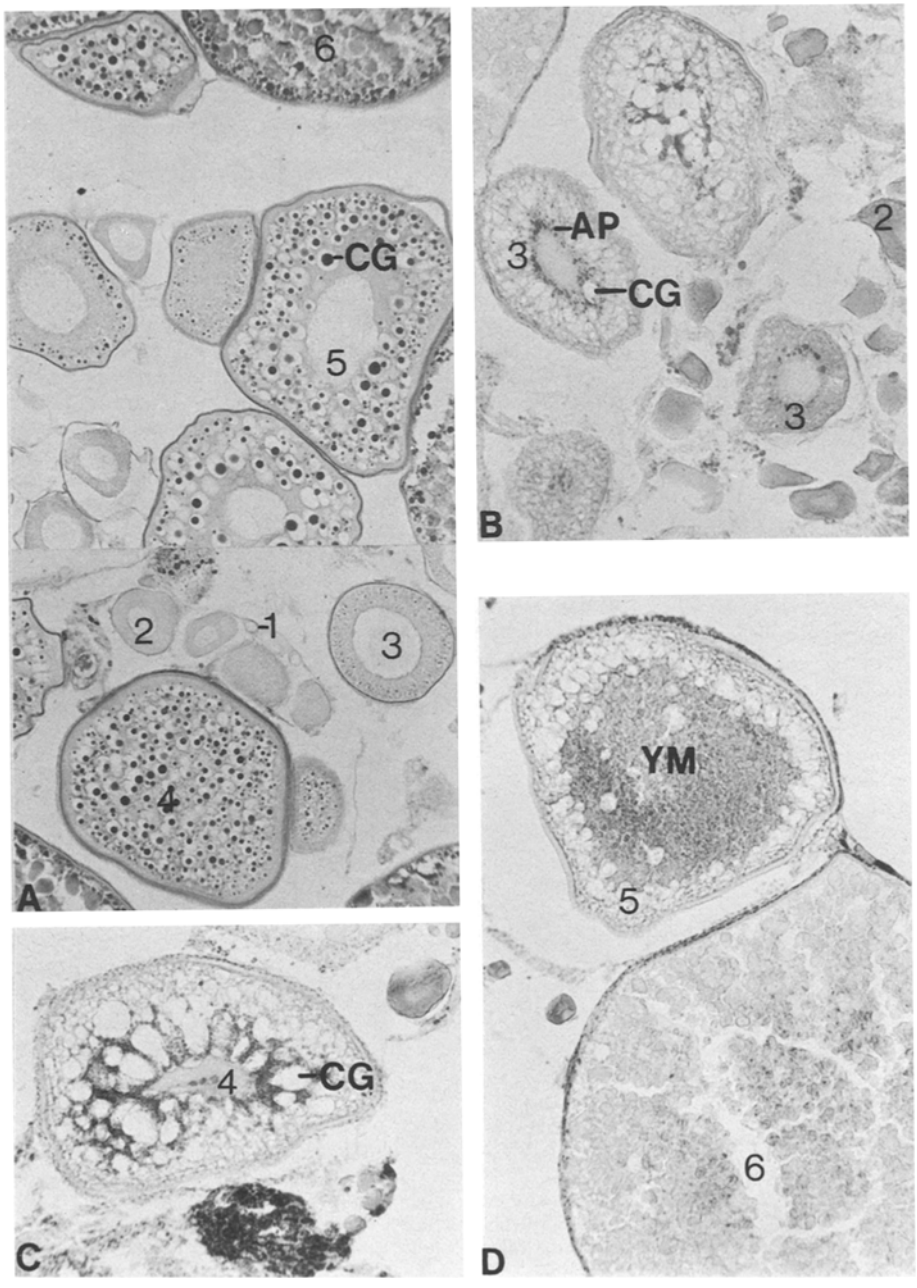

Fig. 2. *A* Paraplast section through adult ovary showing oocytes in various stages (1–6) of development. Cortical granules (CG) are PAS-positive. × 125. *B* Frozen section through a portion of the ovary showing localization of acid phosphatase activity (AP) in young and advanced stage 3 oocytes. Younger oocytes show no activity. × 160. *C* Intense AP activity between cortical granules in a stage 4 oocyte. × 120. *D* AP activity in the stage 5 oocyte is confined to the developing yolk vesicles and yolk platelets of the yolk mass (YM). Note the enzyme activity in the central yolk platelets of the stage 6 oocyte. × 96.

cytes. The intensity of the staining reaction was reduced and the reaction product confined to the central, perinuclear population of yolk bodies in the vitellogenic oocyte (stage 5) (figure 2D). The enzyme was uniformly distributed throughout the smaller yolk vesicles. With the transformation of the larger yolk vesicles into yolk platelets, acid phosphatase activity was further diminished and restricted in distribution to the noncrystalline portions of these inclusions. The enzyme in the postvitellogenic or stage 6 oocyte was detectable in trace amounts in the yolk platelets in the center of these cells (figure 2D). Again, reactive material was never observed in the cortical granules of stage 5 and stage 6 oocytes.

The present study shows that acid phosphatase is highly localized in previtellogenic, vitellogenic and postvitellogenic oocytes. The same enzyme has been previously detected histochemically in the follicle cells surrounding vitellogenic and postvitellogenic oocytes[24]. The absence of detectable acid phosphatase activity in the cortical granules of the zebrafish egg conforms with observations made on eggs of echinoderms[14] and mammals[17]. Although Schuel et al.[15] found acid phosphatase in cortical granule fractions prepared from centrifuged sea urchin eggs, yolk platelets were contaminants of these fractions. Yolk platelets stain positively for this enzyme (see below). Other studies[16] claiming to have demonstrated the presence of acid phosphatase in cortical granules must be viewed with reservation when staining reactions are not clearly shown. The absence of acid phosphatase in the cortical granules or adjacent cortical cytoplasm in *Brachydanio* strongly suggests that this enzyme plays no role in their exocytosis upon activation. Acid phosphatase in the developing oocyte of the zebrafish appears to be largely localized in yolk vesicles, yolk platelets and their precursors. Yolk platelet acid phosphatase has been identified in the eggs of other animal species[25]. The reduction in acid phosphatase reactivity which accompanies the transformation of the yolk vesicle into the yolk platelet in the zebrafish may be related to the enzyme becoming membrane-bound[25]. We suggest that acid phosphatase is stored in the yolk platelets of *Brachydanio* to subsequently be used to initiate and/or promote the utilization of yolk during embryogenesis.

The sites of acid phosphatase activity in the previtellogenic oocyte (stages 3 and 4) are clearly preyolk bodies, but they cannot be precisely correlated with any subcellular structure on the basis of this study. There appears to be a dual origin for yolk vesicles and yolk platelets in the zebrafish. Yolk platelets have been described as being assembled from modified mitochondria[26] or from the fusion of pinocytotic vesicles[27]. Presumably, the source of most of the yolk proteins of these platelets is the liver. The sites of acid phosphatase activity detected in previtellogenic oocytes appear to topographically overlap the distribution of mitochondria as described in studies by Malone and Hisaoka[23] and Ulrich[27]. Temporally, pinocytotic vesicles do not appear in the developing zebrafish oocyte until stage 5[27] or well after the time when acid phosphatase can be initially localized.

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Mitochondrial calcium efflux and porcine stress-susceptibility

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Summary. Mitochondrial Ca^{2+} efflux rates of *M. longissimus dorsi* correlate very closely with parameters associated with porcine stress-susceptibility. Experimental data support the measurement of mitochondrial Ca^{2+} efflux to be a very sensitive and reliable method for differentiating porcine stress-susceptibility.

Two well known stress syndromes exist in certain breeds of pigs, particularly those developing leaner carcasses. The first is malignant hyperthermia which could be induced by various agents such as halothane[2-7] and suxamethonium[8]. The predominant clinical symptoms for this syndrome are gross muscular rigidity, rapid rise in body temperature, tachycardia, hyperventilation, severe metabolic acidosis and elevated levels of serum metabolites[9,10]. (The manifestations of porcine malignant hyperthermia are also similar to those described for human malignant hyperpyrexia, but it is not clear to what extent the porcine data are applicable to humans. The frequency of occurrence in anaesthetic deaths in apparently healthy patients is about 1 in 15,000 anaesthesia[11]). The 2nd type of porcine stress syndrome is