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Technical Report

Preparation of Medaka Hatching Enzyme

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Medaka embryos are covered by a transparent but hard chorion. The chorion makes embryonic manipulation in this species difficult. The medaka hatching emzyme is secreted from the hatching enzyme gland at hatching[1]. Dechorionation of embryos using the enzyme facilitates embryonic manipulation such as transplantation of embryonic cells between embryos, and enables removal of embryos from the chorion for detailed observation or direct treatment. Here, I introduce an easy method for preparation of the enzyme solution from medaka embryos. This method was developed by the staff and students of our laboratory by modification of the previous method reported by Yasumasu, *et al.* (1989)[2]. According to this method, 15 ml of the enzyme solution, which dissolves the chorion within 50 to 60 min, can be prepared using about 5,000 embryos.

I. Preparation

1. *Fish and breeding*: A commercially available orange-red variety of medaka is used. Healthy and mature, eight females and four males, 35 to 40 mm in body length, are placed in a 16 l tank, to obtain a total of 128 females and 64 males in 16 tanks. No materials other than fish such as aquatic plants must be present in the tanks. They are fed twice a day with pellets for carps. Temperature in the fish room is maintained at 26°C. Lighting conditions must follow a 14-hour light and ten-hour dark cycle. The fish adapt within two weeks to these conditions and begin spawning actively every day.
2. *Incubator*: Temperature is accurately adjusted to 30°C. The inside of the incubator must be completely dark.
3. A shaker
4. *Plastic containers*: 185W × 135D × 45H mm
5. 2,000 ml of distilled water
6. 100 ml of medaka balanced salt solution (BSS)[3,4]
7. Three-ml plastic transfer pipettes (Falcon)
8. Wide-mouthed transfer pipettes made by cutting the tip of three-ml plastic transfer pipettes (Falcon)
9. A plastic siphon for fish tanks with a bag made of a fine nylon mesh attatched at its outlet
10. A set of two sieves, composed of an upper sieve with 1,500 μm sieve size and a lower one with 850 μm sieve size
11. A 10-W fluorescent lamp
12. A 100-ml beaker
13. A fish net (a net for catching small-sized aquarium fish made of a fine nylon mesh)
14. Nine-cm petri dishes
15. Two forceps
16. A fine nylon mesh
17. A dissection microscope with 6 to 8 times magnification

II. Collection of embryos

1. *The day before the collection of embryos:* Clean the bottom of the fish tanks and remove sludge, such as feces and other residue, and eggs which have been dropped by females, ten hours after the beginning of the light period.
2. *The day of collection of embryos*: Females shed most of the eggs from their belly within ten hours after the beginning of the light period. Suck the eggs at the bottom of the tanks using the plastic siphon with a bag made of a fine nylon mesh attached at its outlet. The eggs are collected in the bag. If females still carry eggs on their belly, collect the eggs directly from the belly.
3. Transfer the collected eggs to a fish net. Rinse them gently with running tap water crumpling by fingers from outside the net. This treatment facilitates not only removal of sludge but also separation of collected egg clusters into single eggs.
4. Transfer the mass of eggs to the set of two sieves and rinse with running tap water. Most of contaminating materials are removed. Eggs

collect in the lower sieve. This process can be skipped, if the quantity of contaminating materials in the mass of eggs is small.
5. Transfer the eggs to nine-cm petri dishes containing distilled water. Remove the remaining sludge, and dead and contaminated older embryos other than the eggs spawned on the same day, using the transfer pipette, wide-mouthed transfer pipette, and forceps, and separate the remaining egg clusters into single eggs by removing attachment filaments using forceps under the dissection microscope.

III. Incubation of embryos

1. Transfer 2,000 single eggs in a plastic container containing distilled water, one-cm in depth, preincubated at 30°C.
2. Incubate the containers with gentle shaking (35 rpm) on the shaker in the dark room at 30°C for five days.
3. During this incubation period, remove dead and contaminated older embryos and replenish with fresh culture water preincubated at 30°C, every day. Dead embryos are easily distinguishable by the naked eye, by their whitish color. Older embryos are also distinguishable by the naked eye, by their earlier onset of eye pigmentation within two days of incubation and earlier hatching within four days of incubation.
4. On the sixth day of incubation, several hatching fry are found in the containers by five hours after the begining of the light period. This is a sign of the day of hatching.

IV. Preparation of the hatching enzyme

1. Twelve hours after the beginning of the light period on the day of hatching, place all of the embryos in the containers together in the 100-ml beaker.
2. Rinse them twice with BSS preincubated at 30°C.
3. Immerse the embryos in 15 ml of the BSS (30°C).
4. Incubate the embryos at 30°C illuminating them with the fluorescent lamp fixed five cm above it. Most of the embryos hatch in 60 min.
5. Remove the hatched fry and remaining chorions by filtration through the fine nylon mesh.
6. Centrifuge the filtered solution at 10,000 rpm for ten min at 4°C. The supernatant (hatching liquid) retains the enzyme activity for more than a month when kept on ice.
7. For long-term storage, divide the hatching liquid into small aliquots (50 to 100 μl) for each use, using microtubes, to avoid repeated thawing and freezing. Store the microtubes at –80°C after rapid freezing in liquid nitrogen.

V. Remarks

1. Feed parental fish well, especially on the day of egg collection, to protect eggs from carnibalism before collection.
2. It is possible to collect eggs directly from the females' belly. In this case, collect eggs within four hours after the beginning of light period.
3. Synchronized hatching of fry at the scheduled time is the key for obtaining an enzyme solution with high activity. To achieve this, it is important to maintain the incubation temperature, clean culture water, and sufficient oxygen supply by gentle shaking during embryonic development.
4. The hatching enzyme digests the inner layer of the two-layer chorion[1]. Holes on the outer layer of the chorion are needed to allow the enzyme to reach the inner layer. Such holes are easily made by removing the attachment filaments on the surface of the chorion using forceps, or rolling eggs between two sheets of paper towel[4].
5. Enzyme solution with higher activity than that of the hatching liquid can be prepared by homogenizing all of the embryos just before hatching at step IV-2, in five ml of BSS.
6. The enzyme in the hatching liquid can be concentrated using a cation exchange column[2].

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