Hybridization of Sea Urchins, *Hemicentrotus Pulcherrimus* and *Glyptocidaris Crenularis*

Kenzi Osanai

Department of Biology, University of Iwate, Ueda, Morioka, Japan

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In combinations of certain sea urchin species the cross fertilization is very hard or impossible to occur. It has been known that the percentage of cross fertilization is increased by treating the eggs with several methods, such as heavy insemination, standing in sea water, egg water, hyperalkalinity, removing vitelline membrane with trypsin or urea (Cf. Harvey 1956) and calcium-free sea water (Okada 1953). Hultin (1948) observed that the cross fertilization capacity was increased to a level equal to or somewhat less than that in the corresponding homologous fertilization, when the vitelline membrane was removed by treating with ion-free urea' solution or by digestion with trypsin, and he concluded that the first protective mechanism in the sea urchin egg against heterologous sperm seems to be the species specificity of the delicate vitelline membrane. Aketa and Onitake (1969) suggested also that species specificity of sperm binding system residing in this membrane plays a primary role in preventing cross fertilization.

In the combinations of *Hemicentrotus pulcherrimus* and *Glyptocidaris crenularis*, used in the present observation, only a few or none of the eggs is fertilizable by heterologous sperm. Therefore, an attempt was carried out to test whether or not the cross fertilization rate in these combinations is increased by digesting the vitelline membrane with pronase. As the results, the hybridization was promoted by the pronase-treatment in both combinations, *Hemicentrotus* eggs \times *Glyptocidaris* sperm and *Glyptocidaris* eggs \times *Hemicentrotus* sperm. This paper deals with the method of pronase-treatment and with the early development of hybrids obtained by this method.

**Material and Method**

The materials used were *Hemicentrotus pulcherrimus* (A. Agassiz) and *Glyptocidaris crenularis* A. Agassiz, collected at Asamushi, Aomori. Breeding seasons at Asamushi are from December to early May in *Hemicentrotus* and from late February to April in *Glyptocidaris* (Hirai 1963).

The gamates were obtained by the potassium chloride method. The eggs were washed with clear sea water prior to use. The unfertilized eggs were suspended in 0.1 per cent sea water solution of pronase P (Kaken Chemical Co.) for two hours and then transferred to normal sea water. After the eggs were washed by exchanging sea water several times, they were inseminated with homologous or heterologous sperm. The previous observation in
Strongylocentrotus nudus showed that fertilization was remarkably inhibited after treating the egg with pronase for a few minutes, but fertilization was increased again in prolonged treatment (OSANAI and TAMURA 1970). From this fact the duration of pronase-treatment in the present experiments was determined to be two hours.

In many series of experiments one drop of dry sperm was diluted with one ml of sea water and one drop of the diluted sperm suspension was added to the dish containing 10 ml of sea water and one drop of eggs. The final concentration of sperm was about $2.5 \times 10^{-4}$.

**Observation**

1. Cross fertilizability of non-treated eggs.

Hybridization was very hard in the combination of Hemicentrotus eggs and Glyptocidaris sperm. In one of the three cases only 1.5 per cent of the Hemicentrotus eggs could be fertilized with Glyptocidaris sperm and in two cases no fertilized embryos were obtained. The heterologous fertilization was able to be detected by membrane elevation and cleavage. In the cross of Glyptocidaris eggs and Hemicentrotus sperm no egg was fertilized (Table 1). These results show that the heterologous crossing of these species is very difficult under normal condition.

2. Fertilizability of pronase-treated eggs.

Even if Hemicentrotus eggs were pretreated with 0.1 per cent pronase sea water solution for two hours and then inseminated with Hemicentrotus sperm, high per cent of them was fertilizable. In the pronase-treated eggs the fertilization membrane was not elevated after fertilization, because the vitelline membrane, one of membrane precursors, was digested by the enzyme. Therefore, fertilization percentage was estimated by counting cleaving eggs. The pronase-treated eggs were fertilized in high per cent with homologous spermatozoa and began

<table>
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<th>Combination</th>
<th>Fertilization (%)</th>
<th>Average (%)</th>
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<tr>
<td>Non-treated</td>
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<tr>
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<td>G x G</td>
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<tr>
<td>Pronase-treated</td>
<td></td>
<td></td>
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<tr>
<td>H x H</td>
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<td>69.7</td>
</tr>
<tr>
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<tr>
<td>G x G</td>
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<td>74.6</td>
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to develop. This shows that sperm-accepting activity remains in the egg surface though the vitelline membrane was apparently removed by the pronase-treatment. When the Hemicentrotus eggs pretreated with pronase were inseminated with Glyptocidaris sperm, considerable numbers of the eggs were fertilized with heterologous spermatozoa and cleaved. The percent of the heterologously fertilized eggs (average 32.6%) was generally lower than that of the homologous ones pretreated with pronase (69.7%), but markedly higher than that of the non-treated eggs inseminated with heterologous sperm (0.5%).

The Glyptocidaris eggs were also fertilizable after pronase-treatment. When the pronase-treated eggs were inseminated with Glyptocidaris or Hemicentrotus sperm, the average percent of fertilization was 74.6 in the homologous combination and 39.0 in the heterologous (Table 1). From these results, it is ascertained that cross fertilization is promoted or induced by pre-treating the eggs with pronase.

3. Early development of hybrids.

When the Hemicentrotus eggs were inseminated with Glyptocidaris sperm without pronase-treatment, the small numbers of the eggs began to develop and arrived at the early gastrula stage 48 hours after insemination. At the same time, the control Hemicentrotus embryos (Hemicentrotus eggs × Hemicentrotus sperm) developed to mid-gastrulae and the Glyptocidaris embryos (Glyptocidaris eggs × Glyptocidaris sperm) were early gastrulae. The developmental progress of hybrids was later than that of the normal Hemicentrotus embryos, but was similar to normal Glyptocidaris embryos. The Hemicentrotus gastrulae were spherical in shape, while the Glyptocidaris gastrulae were ellipsoidal, lengthening somewhat along the animal-vegetal axis. The shape of hybrids resembled that of the Glyptocidaris (Fig. 1). These facts show that developmental rate and embryonic form in the gastrula stage are influenced by the paternal genome.

![Fig. 1. Normal and hybrid gastrulae, 48 hours after insemination. A, Hemicentrotus; B, hybrid, Hemicentrotus egg, non-treated, × Glyptocidaris sperm; C, Glyptocidaris.](image)

![Fig. 2. Normal gastrulae (A) and vegetalised hybrids (B). The Hemicentrotus eggs were pretreated with pronase and then inseminated with Hemicentrotus (A) or Glyptocidaris sperm (B).](image)
When *Hemicentrotus* eggs were pretreated with pronase and then inseminated with *Glyptocidaris* sperm, considerable numbers of them began to develop. In this case the fertilization membrane was not elevated, but the hyaline layer was formed. For the blastomeres were connected each other with hyaline coat, thier arrangement in hybrids was normal. The development of hybrids delayed somewhat in comparison with that of the control embryos (pronase-treated *Hemicentrotus* eggs × *Hemicentrotus* sperm). The majority of hybrids developed into the fully grown pultei (Fig. 3), but some of them developed often into the abnormal pultei with the extruded gut (Fig. 4).

![Fig. 3. Plutei developed from non- or pronase-treated *Hemicentrotus* eggs inseminated with homologous or heterologous sperm, 4 days after insemination. a, *Hemicentrotus* egg, non-treated, × *Hemicentrotus* sperm; b, *Hemicentrotus* egg, non-treated, × *Glyptocidaris* sperm; c, *Hemicentrotus* egg, pronase-treated × *Hemicentrotus* sperm; d, *Hemicentrotus* egg, pronase-treated, × *Glyptocidaris* sperm. (×70)](image)

The gastrulae which developed from the pronase-treated *Hemicentrotus* eggs inseminated with *Hemicentrotus* or *Glyptocidaris* sperm, 45 hours after insemination, were shown in Fig. 2. In the heterologous combination, the gut was not invaginated and the mesenchyme cells were gathering into clusters at intermediate site between the animal and the vegetal poles, while in the control the mesenchyme cells formed the clusters around the base of the invaginating gut. The elongation of the embryonic shape and the alternation of the gathering site of the mesenchyme cluster seemed to show that hybrids might be vegitalized by the hybridization...
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procedure, pronase-treatment and heterologous fertilization.

The pronase-treated *Glyptocidaris* egg fertilized with *Hemicentrotus* sperm cleaved in apparently normal manner. The *Glyptocidaris* eggs were rather sensitive to a harmful effect of pronase or temperature rise, differentiating into abnormal larvae. The abnormality was more conspicuous in the heterologous combination than in the homologous one. The *Glyptocidaris* eggs, pretreated with pronase and fertilized with homologous sperm, developed often into the deformed larvae with the extruded gut. When the pronase-treated *Glyptocidaris* eggs were fertilized with *Hemicentrotus* sperm, typical exogastrulae were formed in considerably high frequency (Fig. 4). This seems to show that the vegetalization in the pronase-treated embryos is strengthened by the presence of the heterologous genome.

![Abnormal larvae developed from pronase-treated *Glyptocidaris* eggs inseminated with homologous or heterologous sperm, 4 days after insemination. a-c, *Glyptocidaris* egg, pronase-treated, × *Glyptocidaris* sperm; d-g, *Glyptocidaris* egg, pronase-treated, × *Hemicentrotus* sperm.](image)

**4. Characteristics of hybrid pultei.**

The *Hemicentrotus* pultei differ markedly in shape and skeletal structure from the *Glyptocidaris*. The skeleton system is schematically shown in Fig. 5. The hybrids reflected partly maternal or paternal characters. By comparing hybrid characters with the normal, the influence of maternal or paternal genome on embryonic characters was made clear.

*Hemicentrotus pultei.* The *Hemicentrotus* zygotes developed into pultei about four days after fertilization and survived for a week without feeding. In fully grown pultei the vertex was acutely angular in shape and the anal arms were tapered off to a point. The body rods were club-like in form and thicker in vertical region, in which the pair of them crossed
Fig. 5. Schematic figure representing skeletons in sea urchin pluteus. A, anal side view; B, lateral view. a, oral rod; b, anal rod; c, oral vertex rod; d, transverse rod; e, anal vertex rod or body rod.

Fig. 6. Plutei developed from pronase-treated eggs inseminated with homologous or heterologous sperm. 6 days and 18 hours after insemination a, *Hemicentrotus* egg, pronase-treated × *Hemicentrotus* sperm; b, *Glyptocidaris* egg, pronase-treated, × *Glyptocidaris* sperm; c and d, *Hemicentrotus* egg, pronase-treated, × *Glyptocidaris* sperm.
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with an acute angle. Each rod running into the arms was constituted with a single spicule. The transverse rods were also spicular and separated each from the other. The oral vertex rod was not observed (Figs. 3 a, c; 6 a; 7 a, b).

*Glyptocidaris pultei*. The anal arm was round at the tip. The angle between a pair of the anal arms was larger than that of the *Hemicentrotus pulex*. The anal rod constituting the anal arm was not solid, but fenstrate. The fenstrate rod was constituted with three or four long spicules, which formed the ladder-like structure by being connected each to the others with small projections. The transverse rods were thicker and connected in anal side. The anal vertex rods curved in vertical region and stretched out many branches, which connected each to the others and formed a network of apical skeleton. There was a pair of the oral vertex rods, which extended from the oral rod towards the vertex (Figs. 6 b; 7 c, d).

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Fig. 7. Pultei of *Hemicentrotus* and *Glyptocidaris*. a and b, *Hemicentrotus* egg, pronase-treated, × *Hemicentrotus* sperm; c and d, *Glyptocidaris* egg, non-treated, ×*Glyptocidaris* sperm. 4 days after insemination. (×155)
Hybrids, *Hemicentrotus* eggs × *Glyptocidaris* sperm. When non- or pronase-treated *Hemicentrotus* eggs were fertilized with *Glyptocidaris* sperm, they could develop into the fully grown pultei. The hybrid pulteas had the thicker anal arms, whose tip was round. The anal rod consisted of the two or three spicules, but did not form fenstrate structure. The transverse rods were thick and connected each to the other as in the normal *Glyptocidaris* pultei. The anal vertex rod was thicker than that of the normal *Hemicentrotus* pultei and stretched out some small branches, which were not connected into a network. The vertex was rather acute because the anal vertex rod did not curved. The oral vertex rod was formed.

![Fig. 8. Hybrids of *Hemicentrotus* and *Glyptocidaris*. a-c; *Hemicentrotus* egg, pronase-treated, × *Glyptocidaris* sperm, 4 days after insemination; d-f, *Glyptocidaris* egg, pronase-treated, × *Hemicentrotus* sperm, 5 days after insemination. (×155)](image)

Hybrids, *Glyptocidaris* egg × *Hemicentrotus* sperm. As mentioned above, the hybrid larvae, which were developed from the pronase-treated *Glyptocidaris* eggs fertilized with *Hemicentrotus* sperm, were much or less abnormal. The typical exogastrulae or the larvae with the extruded gut were frequently observed. In the latter some of skeletons differentiated. The anal rod consisted of the spicules more than three, though the fenstrate structure was
not formed (Figs. 4 d-g; 8 d-e).

The characteristics of the normal pultei and the hybrids were summarized in Table 2. In the reciprocal crosses of *Hemicentrotus* and *Glyptocidaris*, the anal rod consisted of the axial spicules more than two, but did not differentiate fenestrate structure. Each spicule was similar in shape to that of *Hemicentrotus*. The pattern of the anal rod expressed fundamentally that of the *Glyptocidaris* pultei, but the branching and fenestration were repressed by the presence of *Hemicentrotus* cytoplasm or nuclei.

Table 2. Skeletal character in the pultei of *Hemicentrotus*, *Glyptocidaris* and their hybrids

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<thead>
<tr>
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<th>H × H</th>
<th>G × G</th>
<th>H × G</th>
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<tbody>
<tr>
<td>Anal rod</td>
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<tr>
<td>Axial skeleton</td>
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<td>more than 2</td>
<td>more than 2</td>
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<tr>
<td>Fenestration</td>
<td>-</td>
<td>+</td>
<td>-</td>
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</tr>
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<td>separate</td>
<td>fuse</td>
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<td>Oral vertex rod</td>
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<td>?</td>
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<tr>
<td>Branching of anal vertex rod</td>
<td>-</td>
<td>++</td>
<td>+</td>
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</table>


In the combination of *Hemicentrotus* eggs and *Glyptocidaris* sperm, the oral vertex rod, which was absent in the *Hemicentrotus* pultei, differentiated as in the *Glyptocidaris* and the transverse rods were also *Glyptocidaris* type. The branching and curving of the anal vertex rods were regressive. It is not ascertained, however, whether this inhibition is attributed to the repress of *Glyptocidaris* gene expression by the *Hemicentrotus* genome or the maternal cytoplasm, because the fully grown pultei were not obtained in the reverse crossing.

**Discussion**

The hybrids developed from the pronase-treated eggs had some of paternal characters. Therefore, the development of the pronase-treated eggs was not attributed to the parthenogenetic effect of pronase or the contamination of homologous sperm, but true cross fertilization.

When the eggs were inseminated with homologous sperm after the pronase-treatment, they developed often into the gut-extruded larvae. This seems to show that pronase is one of vegetalizing agents. In the combinations of the pronase-treated eggs and heterologous sperm, vegetalized larvae increased remarkably. This suggests that the presence of the heterologous genome strengthens the vegetalization effect of pronase. The role of the paternal genome in vegetalization is obscure. The strengthened vegetalization, however, may be attributed to an incompatibility between the paternal nucleus and the maternal cytoplasm or nucleus.

In sea urchins it was reported that cross-fertilization was promoted by treating eggs with trypsin (Bohus Jensen 1953, Hultin 1948a, b). Hultin (1948a) observed that the cross fertilization capacity is increased to a level equal to or somewhat less than that in the
corresponding homologous fertilization, if the vitelline membrane is removed by digestion with trypsin. BOHUS JENSEN (1953) reported also that a treatment of the Lytechinus variegatus eggs with crystalline trypsin made them cross fertilizable by sperm from Mellita sexiesperforata or Echinometra lucunter. These reports suggest that a protein factor located in the vitelline membrane may be a species specific barrier against heterologous sperm. AKETA, ONITAKE and TSUZUKI (1972) observed with the electron microscope that the vitelline membrane is broken up by treating the egg with trypsin. AKETA (1967) isolated sperm-egg bonding substance from the vitelline membrane and estimated that this substance has the role in the sperm-egg bonding at fertilization. AKETA and ONITAKE (1969) observed that the sea urchin eggs pretreated with antiserum against sperm-bonding protein of homologous species could not bind sperm, and heterologous antisera did not inhibit the sperm binding, but block sperm penetration. From this observation, they presumed that two systems, one relating to sperm-binding and the other to sperm penetrating, reside in the vitelline membrane and species specificity of the sperm-binding system play a primary role in preventing cross fertilization.

In the pronase-pretreated eggs the cross fertilization was promoted, but the formation of the fertilization membrane was inhibited. This result supported the opinion that species specific protein preventing cross fertilization resides in the vitelline membrane. The eggs were, however, fertilizable after being placed in pronase solution for two hours. This seems to suggest that the vitelline membrane is not indispensable for sperm-egg binding or sperm penetration, though it may play an important role in the intact egg.

Summary

The cross fertilization is very difficult in the combination of Hemicentrotus pulcherrimus and Glyptocidaris crocularis. If the eggs are treated with 0.1 per cent sea water solution of pronase P for two hours and then inseminated with heterologous sperm, the cross is induced or increased. This may be attributed to removing the species specific barrier against heterologous sperm from the egg surface by pronase-treatment. The hybrids of Hemicentrotus eggs and Glyptocidaris sperm develop into the fully grown pulte, while the hybrids of the pronase-treated Glyptocidaris eggs and Hemicentrotus sperm develop into the abnormal larvae, such as exogastrulae. The hybrids have not only maternal, but also paternal characters. These cross combinations may be useful to analyse species specificity in fertilization and the effect of heterologous genome in gene expression during early developmental process.

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