Marked Changes in the Ribonuclease Activity of Mature and Immature Gonads of Sea Urchins *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina*

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It is generally impossible to sort male and female sea urchins before they reach maturity, i.e., while they are still in the immature stage. The ribonuclease (RNase) activity of the gonads of immature stage sea urchins consistently shows a constant activity level. Comparison of the RNase activity of the gonads of mature male and female *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* species at pH 5.0 showed that while its mean specific activity in the immature stage of female *H. pulcherrimus* increased rapidly from 7.35 to 62.79 units/mg, its activity in male *H. pulcherrimus* decreased from 7.35 to 1.90 units/mg. The same phenomenon was observed in *A. crassispina*. Based on its optimal pH, substrate specificity, and heat stability the RNase that exhibited these changes was determined to be an enzyme of the RNase T2 type. This enzyme is also thought to exert an influence on sex determination in sea urchins.

**Key words**  ribonuclease T2 type; ribonuclease activity; sea urchin; mature stage

Since in the course of investigating ribonuclease (RNase) in lower animals we learned that Fernlund and Josefsson1) had reported on the RNase of the sea urchin *Psammechinus miliaris*, we began to assess the functions of RNase in the growth process of sea urchins. The development of sea urchins can be classified into two brief stages, an immature stage and a mature stage. Immature male and female sea urchins cannot be distinguished, and it only becomes possible to differentiate between them in the mature stage. Moreover, it is uncertain whether a sea urchin of a given sex in the immature stage will be the same sex in the mature stage.2—4) If RNase activity changes during the process of maturation, some relationship between the sex of sea urchins and the enzyme would presumably exist. To determine whether any such relationship actually exists, in the present study, we measured RNase activity in the mature and immature stages of *Hemicentrotus pulcherrimus* and *Anthocidaris crassispina* and investigated the characteristics of the enzyme.

**MATERIALS AND METHODS**

**Reagents**  Sephadex G-100 was purchased from Amersham Japan Inc. Yeast RNA was supplied by Kohjin Co., Ltd. The protein Assay Kit used for the measure protein concentrations obtained from Bio-Rad. Bovine serum albumin, bovine pancreatic RNase A (Type XII), homopolynucleotide, poly-U, poly-C, poly-A, and poly-I, all supplied by SIGMA Corporation. All supplies used for SDS-PAGE were obtained from ATTO Corporation. All other reagents used were of guaranteed quality and were supplied by Wako Pure Chemical Industries, Ltd.

**Times of Sea Urchin Collection**  As shown in Table 1, the *H. pulcherrimus* and *A. crassispina* sea urchins used in this study were collected in January, March, May, July, September, and November. The *H. pulcherrimus* sea urchins were in the mature stage in March, and the *A. crassispina* sea urchins were in the mature stage in July. The methods of sorting male and female sea urchins of each species are shown in Table 1. The parts of the sea urchins that were used are shown in Fig. 1. They were homogenized and used to measure RNase activity.

**Measurement of Protein Concentrations**  The sea urchin gonads were homogenized in 0.1 M acetic acid solution, and the homogenate was centrifuged at 4 °C for 20 min at 10000 rpm. The concentrations of proteins in the supernatant were measured with a Protein Assay Kit (Bio-Rad). A calibration curve was prepared with bovine serum albumin, and the protein concentrations were determined from the

### Table 1. Colors Used to Distinguish between the Gonads of Two Species of Sea Urchins by Sex and Time of Collection

<table>
<thead>
<tr>
<th></th>
<th>Hemicentrotus pulcherrimus</th>
<th>Anthocidaris crassispina</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Immature gonad</td>
<td>Ovary (♀)</td>
</tr>
<tr>
<td><strong>Color</strong></td>
<td>Pale orange</td>
<td>Intense orange</td>
</tr>
<tr>
<td><strong>Dates of maturation</strong></td>
<td>February—March</td>
<td>January, March, May, July, September, and November</td>
</tr>
<tr>
<td><strong>Dates of collection</strong></td>
<td></td>
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Methods of Measuring RNase Activity  RNase activity in the supernatant of the sea urchin gonad homogenate was measured by Reddi’s method, with yeast RNA as the substrate. In this method non-reactive RNA is precipitated with perchloric acid and lanthanum nitrate, and the substrate solution used to measure RNase activity is prepared by dissolving RNA (2.5 mg/ml) in a 0.1 M acetic acid buffer (pH 5.0). A small amount of enzyme solution is added to the substrate, and the mixture is allowed to react at 37 °C for an appropriate amount of time. The reaction is stopped by adding 0.5 ml of 12% perchloric acid containing 20 mM lanthanum nitrate. The mixture is then centrifuged at 3000 rpm at 4 °C for 15 min, and non-reactive RNA is removed. After diluting 0.4 ml of the supernatant with 2 ml of distilled water, absorbance is measured at 260 nm. The amount of the enzyme that increases absorbance by 1.0 under these conditions is assigned a value of 1 unit (U) RNase activity.

Partial Purification of RNase from the Gonads of Sea Urchins  An attempt to partially purify RNase was made to investigate the characteristics of H. pulcherrimus and A. crassispina sea urchins RNase. The gonads of each sea urchin were homogenized, and after centrifugation at 10000 rpm, the supernatant was subjected to gel filtration on a Sephadex G-100 column (3 x 120 cm) that had been equilibrated with 0.1 M sodium acetate (pH 4.7). This process allows separation of low-molecular-weight components (Fig. 2). The enzyme obtained by partial purification in this manner was used to identify the characteristics of the enzyme, including its molecular weight, optimal pH, heat stability, and substrate specificity.

RESULTS AND DISCUSSION

There were 35 sea urchins from each H. pulcherrimus and A. crassispina, for each time investigations. As shown in Fig. 3, the RNase activity of H. pulcherrimus sea urchins underwent marked changes during the transition from the immature stage to the mature stage. Its mean specific activity in male H. pulcherrimus was always 7.35 U/mg (S.D., 1.44) in the immature stage, and it decreased to 1.90 ± 0.64 U/mg, standard deviation [S.D.] in the mature stage. By contrast, the mean specific activity in mature female H. pulcherrimus was much higher, 62.79 ± 18.05 U/mg. In the mature stage the activity level in the males was approximately 10% lower than in the females. This change is thought to occur during the change from the immature stage to the mature stage (Fig. 4a).

The mean RNase activity of male A. crassispina sea urchins decreased from 5.95 U/mg (S.D., 1.03) in the immature stage to 1.98 U/mg (S.D., 1.15) whereas in the mature stage increased to 15.62 U/mg (S.D., 2.10) in the females. The degree of the changes was slightly different from their degree in H. pulcherrimus sea urchins, but the direction of the changes in A. crassispina sea urchins were the same as in H. pulcherrimus sea urchins, a decrease in the males and an increase in the females (Fig. 4b).

The reason for these phenomena is unclear, but the indefinite sex of sea urchins in the immature stage is thought to be related to the constant RNase activity level in the immature stage, suggesting that RNase activity changes upon determination in the mature stage. A modest correlation between the activity of this enzyme and sex determination in sea urchins is also thought to exist.

The characteristics of RNase, which induce these changes, were also investigated. The optimal pH of the enzyme from both H. pulcherrimus and A. crassispina sea urchins was 5.0, i.e., similar that of the RNase of P. miliaris. The RNase of
H. pulcherrimus sea urchins was unstable at 60 °C, the same as the RNase of P. miliaris, and its activity was lost within 5 min of exposure to a temperature of 60 °C. The RNase of A. crassispina sea urchins also showed heat instability, but approximately 40% of the enzyme activity after 10 min of exposure to 60 °C (Fig. 5), indicating that A. crassispina sea urchins possess materials which promote heat stability.

The molecular mass of the RNase of H. pulcherrimus sea urchins was estimated to be approximately 40 kDa based on measurement of the molecular weight of the product of gel filtration (Figs. 2a, b) of the RNase that had been partially purified from mature stage sea urchins. The molecular mass of the RNase of A. crassispina sea urchins was clearly slightly higher than that of the RNase of H. pulcherrimus sea urchins.

Table 2 shows base specificities of the H. pulcherrimus and A. crassispina sea urchin RNases.

Despite being only partially purified, both H. pulcherrimus and A. crassispina RNases were found to be base-nonspecific. Although the data are not presented here, we demonstrated that the enzymes are not inactivated by EDTA. The characteristics of the RNase described above, which shows considerable differences between male and female H. pulcherrimus and A. crassispina sea urchins in the mature stage, is an enzyme of RNase T2 type.

Figure 6 shows the results of SDS-PAGE7) of the RNase of H. pulcherrimus sea urchins. Its molecular mass estimated from the data obtained by active staining8) was 38 kDa, a value that is consistent with the gel filtration data, but much
higher than 24 kDa, the molecular mass of RNase T2 type enzymes in general\(^9\) (RNase Rh). The molecular mass of the RNase of *A. crassispina* sea urchins is predicted to be at least 40 kDa based on the results of gel filtration with Sephadex G-100 (Fig. 2b), but its molecular mass determined by SDS-PAGE has not yet been confirmed. It is predicted that its higher molecular mass is attributable to bonds with mucopolysaccharides or polysaccharides. This observation also indicates that the heat stability of *A. crassispina* RNase, shown in Fig. 5, is superior to that of the RNase of *H. pulcherrimus* and *P. miliaris* sea urchins.\(^1\)

It was thus revealed that T2-type RNase enzyme is present in *H. pulcherrimus* and *A. crassispina* gonads and that its activity changes markedly as females and males mature.

The amino acid sequence of this enzyme, which is similar to the T2-type RNase of *H. pulcherrimus* and *A. crassispina* gonads, has not yet been determined. Data supporting the above results include the registered mRNA sequence for *Strongylocentrotus nudus* the RNase,\(^{10}\) which is akin to *H. pulcherrimus* and *A. crassispina* sea urchins, indicating that the amino acid sequences responsible for the main activity of T2-type RNase enzymes show some degree of homology. From these observations, it is assumed that the RNase that shows considerable differences in activity between male and female *H. pulcherrimus* and *A. crassispina* sea urchins in the mature stage is similar to RNase T2. Further studies will be conducted to confirm this assumption, and the primary structure will also be determined.

Figures 2a and b show the results of gel filtration of *H. pulcherrimus* and *A. crassispina* sea urchins. The presence of a previously unknown low-molecular-weight RNase was also demonstrated, and based on the molecular mass of RNase and the results of gel filtration with Sephadex G-100 and SDS-PAGE, its molecular mass is predicted to be approximately 10 kDa. The content is very low. The characteristics of this enzyme are uncertain, but its optimal pH is known to be approximately 7.5. Further study of this enzyme is needed to determine all of the roles of RNase in sea urchins.

**CONCLUSIONS**

(i) The results of this study showed that RNase activity that has an acidic optimal pH and base non-specificity rapidly increases between the immature stage in the mature stage of female *H. pulcherrimus* and *A. crassispina* sea urchins but decreases in the mature stage of male *H. pulcherrimus* and *A. crassispina* sea urchins.

(ii) The characteristics of the RNase of *H. pulcherrimus* and *A. crassispina* sea urchins strongly resemble those of the RNase of *P. miliaris*\(^9\) that is similar to RNase T2. Its RNase is also thought to be involved in sex determination.

(iii) The molecular mass of the RNase of *H. pulcherrimus* sea urchins was 38 kDa as measured by SDS-PAGE and was similar to the molecular mass of RNase T2.\(^9\) The molecular mass of *A. crassispina* RNase is known to be 40 kDa or higher when measured by gel filtration, but measurement by its molecular weight SDS-PAGE failed, probably because polysaccharides and mucopolysaccharides affect this enzyme, making measurement impossible. However, its characteristics and other observations indicate that it is also similar to RNase T2.

(iv) There is a clear correlation between the activity of *H. pulcherrimus* and *A. crassispina* RNase and the sex of sea urchins. Its activity tends to rise steadily and rapidly in female sea urchins as they mature but to decrease in male sea urchins. Its activity is the same in both immature males and females, in which sex is unknown. We will monitor changes in RNase activity before sex determination, after the change from the immature to mature stage, to study the association between the RNase of sea urchins and sex determination.

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