# Role of a trypsin-like protease in "informosomes" in a trigger mechanism of activation of protein synthesis by fertilization in sea urchin eggs*

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In the preceding paper (1), we showed the fact that a treatment of unfertilized eggs homogenate with trypsin resulted in the release of mt-RNA from some particles sedimenting more rapidly than microsomes. It is believed that the released mt-RNA was then transferred to ribosomes to form polyribosomes. However, this mechanism remains speculative. This paper is concerned with the next step, the activation of a protease by fertilization and the relationships between the protease and the release of mt-RNA in the process of fertilization.

The material used in the experiment was mature eggs of Hemicentrotus pulcherrimus and Anthocidaris crassispina. The change in the activity of protease before and after fertilization was examined with each subcellular fraction, and it was found that in fertilized eggs there are three pH optima with respect to protease activity in the presence of reduced glutathione, at 4.3, 6.7 and 8.0, while no activity was observed at the last pH in unfertilized eggs (Fig. 1). Thus only the pH 8 protease seems to be activated by fertilization even if reduced glutathione increased at the moment as has been pointed out by Shearer (2). The approximate intracellular pH, as measured with their homogenate immediately after homogenization, of fertilized eggs was gradually rose to about 8 in contrast to that of the outer environment after fertilization. Therefore, it can

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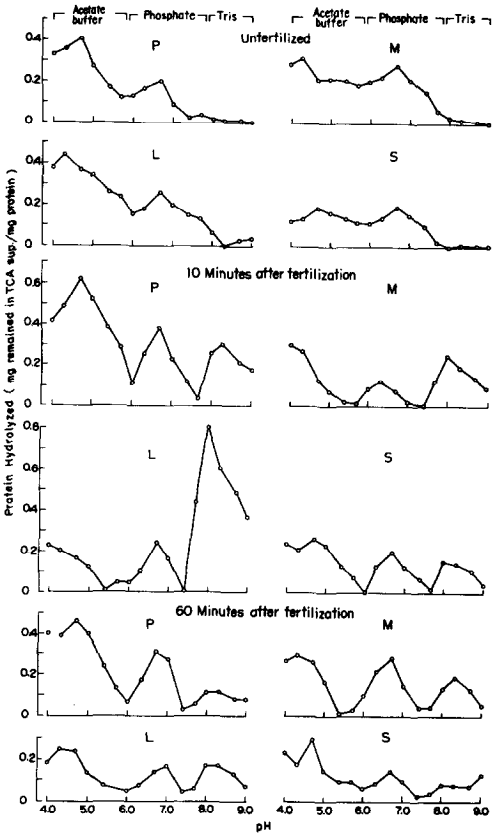


Fig. 1

Change in distribution of protease activities in subcellular fraction brought about by fertilization.

The eggs of unfertilized, and those 10 and 60 min. after fertilization from H. pulcherrimus were fractionated as in Table I. The time indicated is that of homogenization. Development was allowed at 20°. Jelly coat was removed by acidification in the case of unfertilized eggs. Each fraction obtained was subjected to the assay for protease activity according to a modification of the method of Maggio (3) in a series of buffers as indicated. The assay mixture consisted of 1 mg of heat denatured casein (4), 0.01 M $MgCl_2$, 0.1 M of the buffer, 0.01 M reduced glutathione, and the enzyme in a volume of 0.5 ml was incubated at 37° for 30 min. The values presented here are the bovine serum albumin equivalent of the amount of protein fragments soluble in 5% cold TCA after the reaction. The blank were subtracted in each series of buffers at appropriate pH.

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be said that the protease activated by fertilization is only that whose pH optimum is at 8, under physiological conditions. The pH 8 protease was activated temporarily after fertilization and its activity decreased gradually. This pattern of activity of the protease was roughly in agreement with the findings by Lundblad et al. (5-9). Thus the activation of the enzyme may be important as a trigger reaction for the activation of protein synthesis by fertilization. Since the pH 8 protease was strongly inhibited by soybean trypsin inhibitor, the decrease of the activity which follows the temporary activation may be due to such a specific inhibitor in the cells.

Table I

*Distribution of mt-RNA activity in subcellular fraction from unfertilized eggs*

| | P | L | M | S | |
|---|---|---|---|---|---|
| | | | | | cpm incorporated |
| Template RNA activity | 63 | 202 | 93 | 38 | |

Unfertilized eggs of H. pulcherrimus were fractionated in the same way as in Table I in the previous paper (1) with the following exception: the first fraction was precipitated at 8,000 x g for 10 min. and washed twice with the same medium (P). The second fraction was obtained by precipitation from the combined supernatants of the above step at 17,000 x g for 30 min. and washed once in the same way at 15,000 x g for 30 min. (L). The microsomal (M) and supernatant (S) fraction were obtained by the method of Hultin (10) from the supernatant from the foregoing step. RNA was then prepared from these fractions and the template RNA activity was assayed as in Table I in the previous paper (1) with 0.2 mg of RNA to be tested. The control without addition of RNA gave 37 cpm.

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In the previous paper (1), it was suggested that mt-RNA resides in particles sedimenting faster than microsomes. As was expected, the mt-RNA bearing particles sedimented between 8,000 and 15,000 x g in the presence of $Ca^{++}$ in unfertilized eggs (Table I). It was found in eggs 10 min. after fertilization that the pH 8 protease activity was mainly in this same fraction (Fig. 1). This fraction did not contain many ribosomes as judged by the density gradient pattern of RNA prepared from the unfertilized fraction (Fig. 2). The template RNA activity was found in polydisperse in the region from about 10 to 20S, which is roughly in accord with the findings by Nemer and Infante (11) (Fig. 2). As the fraction was still visually inhomogenous, further purification by a density gradient centrifugation was tried. The result (Fig. 3) showed the presence of RNA in a fraction corresponding to 1.05 M sucrose in the sample prepared from unfertilized eggs. The pH 8 protease activity in the sample prepared from eggs immediately after fertilization was found in almost the same fraction. On the contrary, no protease activity was found in the unfertilized sample and no RNA in the fertilized sample. Since further purification of the fractions resulted in a symmetrical

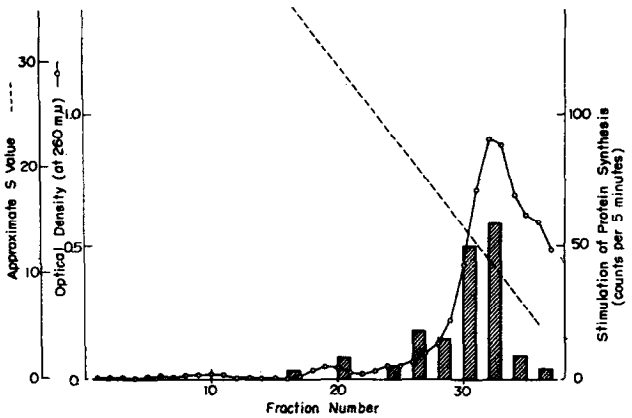


Fig. 2 Density gradient distribution of RNA from particles sedimenting between 8,000 and 15,000 x g and template RNA activity

The particles were prepared from unfertilized H. pulcherrimus eggs by the method for L fraction described in Table I, and RNA was purified from the fraction as in the previous paper (1). About 0.5 mg of RNA in 0.2 ml was developed through 4.0 ml of sucrose linear density gradient from 5 to 20% at 130,580 x g (average) for 180 min. using RPS 40A rotor of the Hitachi 40PA Preparative Ultracentrifuge. Fraction of 0.12 ml each were collected and optical density at 260 mμ was measured directly. The template RNA activity was assayed as usual (1) with various amounts of RNA as indicated after combination of neighboring two tubes of the fractions numbered, between 16 and 37. The result is expressed in histograms. The sedimentation constants were calculated (14) with 28 and 18S ribosomal RNA as reference.

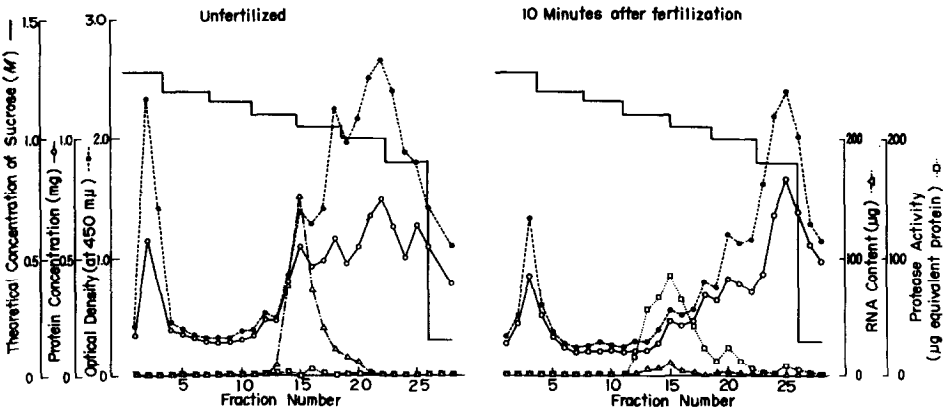


Fig. 3 Demonstration of localization of RNA and pH 8 protease activity

The L-fraction was prepared in the same way as described in Table I from unfertilized and fertilized (10 min.) eggs of H. pulcherrimus (see also Fig. 1). The fractions suspended in a medium (0.6 ml) which was used for the preparation of ribosomes (Table I in (1)) were centrifuged for 150 min. through a sucrose density gradient (12 ml) at 130,580 x g as in Fig. 2. The density gradient was composed of 1.8 ml of 1.28 M, 1.20 M, 1.16 M, 1.10 M, 1.05 M, 1.00 M and 0.90 M sucrose layers. The centrifugation was carried out 15 hr. after the preparation of the gradient at 15°. After centrifugation, fractions were collected as in Fig. 2 (0.45 ml each), and analyzed in the following way: optical density at 450 mμ directly, protein and RNA contents by the method described in the previous paper (1), and pH 8 protease activity by the method as given in Fig. 1.

distribution, this fact may indicate the possibility that the mt-RNA and the pH 8 protease are distributed in the same particles. However, simultaneous sedimentation under the same conditions as above on mixing the isolated unfertilized and fertilized fractions showed a slight difference between them with respect to the sedimentation rate, i.e. the protease peak sedimented slightly slower than the RNA peak indicating some loss of particulate weight by the release of RNA.

As shown above, the protease which is activated by fertilization seems to be particle bound at least in early development, but the enzyme could be solubilized from the lyophilized preparation of whole eggs, and partial purification could be achieved by DEAE-Sephadex A 25 chromatography (about 23-fold (12, 13). The ability to hydrolyze benzoyl-arginine amide and the strong inhibition of its activity by soybean trypsin inhibitor showed the nature of the enzyme to be trypsin-like.

In order to test the hypothesis presented in the previous paper (1), a reconstruction experiment with the purified system was done as follows. The mixture of unfertilized ribosomes and the L-particles (Fig. 3) digested with the purified pH 8 protease was treated with an excess of soybean trypsin inhibitor and the effect of the digestion on the template RNA activity was then examined. The result showed a slight activation (about 1.5 times as the control) in incorporation of amino acids into protein, but the activation was far below from that observed in fertilization. That the activation effect was low compared with that observed in normal fertilization can be interpreted as being due either to a predictably lower susceptibility of the structurally organized particles to the solubilized protease in the release of mt-RNA, or, to a species specificity, since the protease and the particles used originated from different species. In any case, it is reasonable to conclude that, in actual fertilization, even a slight activation of the protease would be sufficient to release the mt-RNA effectively because of their structural proximity.

The nature of the particles still remains to be investigated; Brachet (15) has suggested that the cortical granules might play such a role. This possibility is supported by the observation that polyribosomes are found at the periphery of the egg (16). A preliminary experiment has excluded the possibility that they are lysosomes. The facts presented here are believed to show the presence of another kind of informosomes in unfertilized egg besides those proposed by Spirin and Nemer (17).

In summary: it is proposed that the trigger mechanism leading to the rapid stimulation of protein synthesis after fertilization in sea urchin eggs consists in the release of mt-RNA from certain particles by the activity of a trypsin-like protease. The protease is activated at the moment of fertilization; it is believed to occur in the same particles as those which contain the mt-RNA.

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A part of this work has been reported in the meeting of the Japan Society for Cellular Chemistry held on December 14, 1965 (18).

References

1) Mano, Y., Nagano, H., Biochem. Biophys. Res. Commun., in press.
2) Shearer, C., Proc. Roy. Soc. B, 93, 213 (1922)
3) Maggio, R., J. Cell. Comp. Physiol., 50, 135 (1957)
4) Kunitz, M., J. Gen. Physiol., 30, 291 (1947)
5) Lundblad, G., Nature, 163, 643 (1949)
6) Lundblad, G., Exp. Cell Res., 1, 264 (1950)
7) Lundblad, G., Arkiv Kemi, 4, 537 (1952)
8) Lundblad, G., Lundblad, I., Arkiv Kemi, 6, 397 (1953)
9) Lundblad, G., Arkiv Kemi, 7, 127 (1955)
10) Hultin, T., Exp. Cell Res., 25, 405 (1961)
11) Nemer, M., Infante, A. A., Science, 150, 217 (1965)
12) Lundblad, G., Runnström, J., Exp. Cell Res., 27, 328 (1962)
13) Lundblad, G., Lundblad, M., Arkiv Kemi, 20, 137 (1963)
14) Martin, R. G., Ames, B. N., J. Biol. Chem., 236, 1372 (1961)
15) Brachet, J., Bull. Classe Sci. Acad. Roy. Belg., 49, 682 (1963)
16) Stafford, D. W., Sofer, W. H., Iverson, R. M., Proc. Natl. Acad. Sci. U. S., 52, 313 (1964)
17) Spirin, A. S., Nemer, M., Science, 150, 214 (1965)
18) Mano, Y., Symposium for Cellular Chemistry, 17, in press.