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Tosylarginine methylester hydrolase activity in sea urchin egg membrane

Tosylarginine methyl ester (TAME) is a substrate for several proteolytic enzymes: trypsin[1], plasmin[2], thrombin[3] and a salivary protease[4]. While testing various fractions of sonicated sea urchin eggs *(Arbacia punctulata)* for protease-like activity, it was observed that a low-speed residue (about 700 × *g*) had the capacity to hydrolyze TAME. It was also observed that the distribution of this enzyme activity changed after fertilization. The present report describes some kinetic properties of this "membrane" enzyme and its possible relationship to embryogenesis. Enzyme activity was determined by measuring the rate of radioactive methanol produced as a result of TAME hydrolysis (Fig. 1).

Approx. 0.5 ml of packed unfertilized sea urchin eggs *(A. punctulata)*, or early embryos, which had been washed 5 times with 20 vol. of filtered sea water were disrupted in 1 ml of ice-cold filtered sea water by three 30-sec bursts with a Bronson sonifer. The residue obtained after centrifuging the broken cell suspension (700 × *g*, about 1200 rev./min in an International Clinical centrifuge at room temperature for 5 min) was washed 6 times by alternately triturating it with a glass rod in 5 ml of filtered sea water and then centrifuging the suspension at 1200 rev./min. The final residue is referred to as the membrane fraction. The washings of this membrane fraction had no protease activity in the assay system described above.

A soluble preparation of the membrane fraction was prepared by sonifying the final residue for three 10-sec intervals at room temperature in 0.5-ml aliquots of a 1% detergent solution (Cutscum, Fisher). After centrifugation, the remaining residue was again sonicated in 0.5 ml of detergent solution. This process was repeated 10 times. The supernatants of each extraction were combined and dialyzed against distilled water. The dialyzed extract was saturated with $(NH_4)_2SO_4$ (100%) and left overnight at 4°. The precipitated protein was collected by centrifugation, resuspended in a minimal volume and then dialyzed against distilled water. The final protein solution was centrifuged at 12 000 × *g* to remove a slight turbidity.

The data in Fig. 1 illustrate the rates of hydrolysis of TAME with various amounts of a solubilized preparation obtained from sea urchin egg membranes. It is evident that the reaction rates are linear for extended periods of time. At the lower concentrations, linearity was observed for at least 24 h. This was important when fractions with low activity were assayed. It can also be seen that the curves in Fig. 1 do not intersect the origin. This may be due to contamination of the substrate with tritiated methanol and/or limited extraction of the labeled substrate into the toluene layer. All points on the abscissa refer to times after the initial counting rate was determined (1st reading). The data shown in Fig. 2 indicate that hydrolysis was proportional to enzyme concentration. Similar proportionality was observed with enzyme that had not been solubilized (the insoluble membrane fraction). Heating this preparation to 95° for 5 min practically eliminated hydrolysis of TAME (dashed line of Fig. 2).

To test whether the activity of this enzyme changes with development, membrane fractions were prepared from equivalent aliquots of an egg suspension before and after fertilization. In addition to the insoluble membrane fraction, the corre-

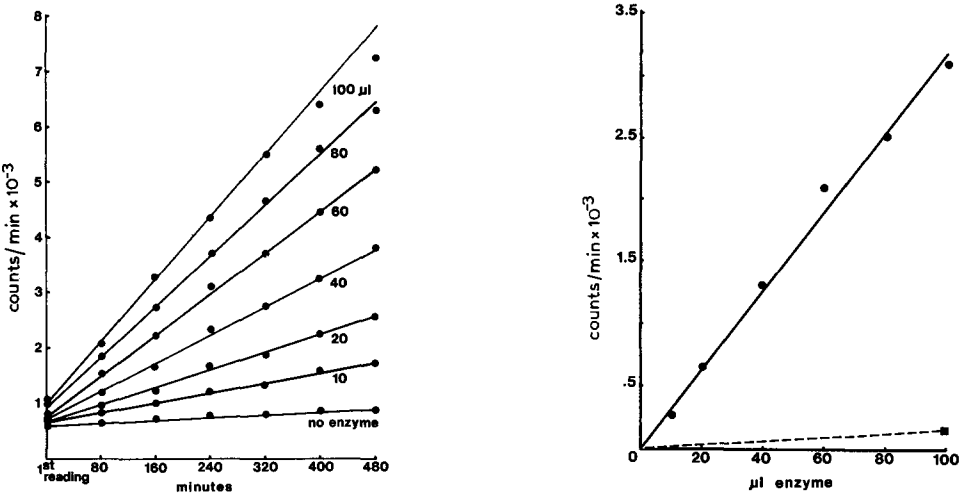

Fig. 1. Rates of ^{3}H-labeled TAME hydrolysis with various amounts of solubilized sea urchin egg membrane protein. The ^{3}H label was in the methyl ester moiety of the substrate. Since TAME is relatively insoluble in toluene, the disintegration of tritium in the aqueous phase is barely detectable. As hydrolysis proceeds, the ^{3}H-labeled methanol is extracted into the fluor-containing toluene layer and it is in this phase that ^{3}H disintegration becomes detectable[11]. The reaction was carried out in a scintillation vial containing: 20 µl 0.1 M TAME (specific activity 20 µC/mole); 20 µl 0.1 M Tris, pH 7.5; enzyme (10–100 µl of a solubilized preparation containing 10 mg protein per ml); and distilled water to a final aqueous volume of 200 µl. To this, 15 ml of a toluene solvent system (2,5-diphenyloxazole, 4 g/l; 1,4-bis-(5-phenyloxazolyl-2)benzene, 0.5 g/l) were added and the contents of the vial thoroughly shaken. Samples were counted at 80-min intervals in a scintillation counter operated at room temperature. Little difference in counting rates were observed for vials shaken prior to each counting as compared to those not shaken after the reaction was initiated. Maximal hydrolysis of TAME, determined by adding 1 mg of solid trypsin to each vial, yielded approx. 20 000 counts/min.

Fig. 2. Extent of TAME hydrolysis caused by varying amounts of solubilized sea urchin egg membrane protein (10 mg protein per ml) 240 min after reaction initiation. Each point has been corrected for zero time radioactivity. The dashed line represents residual activity following heat treatment (95° for 5 min). The specific activity of the protease preparation was approx. 60 nmoles TAME hydrolyzed per h per mg protein at pH 7.5. Details of this experiment are the same as those described in Fig. 1.

sponding supernatant fractions were tested for TAME hydrolase activity at various times during embryogenesis. The data in Fig. 3 indicate that the membrane fraction of unfertilized eggs contained more TAME hydrolase activity than the 700 × g supernatant. Following fertilization, however, there was a considerable reduction in membrane activity and a concomitant increase in supernatant activity. It is tempting to assume that the soluble activity is derived from the membrane. However, this is uncertain at present. Following the 8–16-cell stage, which occurs approx. 2 h after fertilization, TAME hydrolase activity in the membrane fraction began to increase, while the supernatant activity declined to levels observed in the unfertilized egg. Although this enzyme has not been characterized as yet, it is not likely to be trypsin since acetyllysine methyl ester is a good substrate for the membrane enzyme but not for trypsin.

As early as 1949 LUNDBLAD[5] suggested that the transient appearance of a protease soon after fertilization of sea urchin eggs was somehow related to embryo-

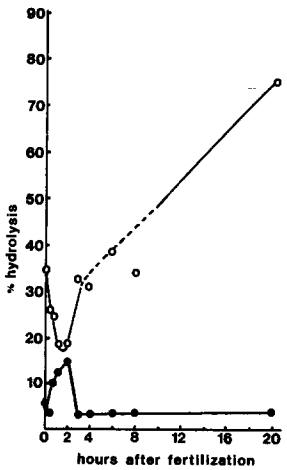

Fig. 3. Protease-like activity in the insoluble membrane and supernatant fractions of sea urchin eggs before and after fertilization. A 200-ml aliquot was taken from a suspension of sea urchin eggs (approx. 4 ml packed eggs suspended in 4 l of filtered sea water) which was being gently mixed with a mechanical stirrer. To the remaining eggs a sperm suspension was added and 200-ml aliquots were taken at 15, 30, 60, 120, 180, 240, 360, 480 and 1200 min following fertilization. Within 30 min after sperm addition about 70–90% of the egg population appeared fertilized as judged by the appearance of fertilization membranes. At each time period the sample was centrifuged, washed 3 times with filtered sea water and then sonicated in 0.5 ml filtered sea water. After centrifugation at 1200 rev./min (about 700 × *g*) an aliquot of the supernatant fraction (50 μl) was tested for protease activity using TAME as the substrate (●). The residue was washed (see text) and finally suspended in 0.5 ml filtered sea water. Aliquots (50 μl) of this insoluble membrane fraction were also tested with TAME (○). The reactions were carried out for 20 h. After this time excess trypsin (1 mg) was added to all samples and then each was counted until the counting rate was constant. That is, until all the substrate has been hydrolyzed. The cpm observed for each fraction prior to trypsin addition was then expressed as a percent of the total substrate hydrolyzed. This procedure was adopted when many samples were assayed so that pipetting errors of the labeled substrate could be readily identified. Unusually high or low counting rates after excess trypsin would have indicated nonuniform conditions for determining enzyme activity.

genesis. The transient appearance of this protease was confirmed by MANO[6], who reported such activity to be in a 8000–15 000 × *g* fraction of the cell. The present report indicates that protease-like activity appears to be associated with the membrane (700 × *g* residue) prior to fertilization. The transient appearance of this activity in the 700 × *g* supernatant following fertilization suggests that the membrane is the source of this enzyme. Although the role of proteolytic enzymes in embryogenesis is not clearly understood, it is of interest to note that specific inhibitors of proteolysis such as tosyllysine chloromethyl ketone and tosylphenylalanine chloromethyl ketone, respectively[7], inhibit sea urchin development and prevent the incorporation of labeled phenylalanine and lysine into protein[8,9]. How protease activity is involved in early development is speculative. However, the activation of such activity following fertilization may be of general significance since similar activity also appears during cotton cotyledon embryogenesis[10].

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The regulation of rat liver xanthine oxidase: Conversion of type D (dehydrogenase) into type O (oxidase) by a thermolabile factor, and reversibility by dithioerythritol

It has been reported from this laboratory that most of the "xanthine oxidase" (EC 1.2.3.2) activity of rat liver is detectable as an NAD^+-dependent dehydrogenase (called type D) in freshly prepared supernatant. The activity is converted into an oxidase (called type O) by various treatments of the supernatant, such as storage at $-20°$, treatment with proteolytic enzymes, preincubation under anaerobic conditions, and preincubation in air in the presence of particulate subcellular fractions[1–3].

The effect of the preincubation of the supernatant with other subcellular fractions (or of the preincubation of the whole homogenate) was tentatively interpreted as due to the action of proteolytic enzymes[3]. Evidence is now presented that the conversion of xanthine oxidase from type D into type O by subcellular particles is different from the conversion operated by proteolysis and is due to a thermolabile factor, apparently similar to an enzyme oxidizing thiol groups.

Rat liver was homogenized with 5 vol. of 0.1 M Tris–HCl buffer (pH 8.1) and was centrifuged at 600 × *g* for 20 min at 0°. The first supernatant so obtained was centrifuged again at 100 000 × *g* for 1 h to separate a particle-free supernatant and a sediment. The sediment from a 10-ml centrifuge tube was resuspended with 2 ml of buffer and was added to the particle-free supernatant obtained from the same tube. 2 ml of buffer were added to the supernatant in control experiments. After preincubation at 37° for 20 min the sediment was separated again by centrifugation, the supernatant was dialyzed and the activity of xanthine oxidase type D and type O was determined by following spectrophotometrically the formation of uric acid with