Protease in ascidian endostyle[1]

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Summary. A proteolytic activity at pH 7.6 was determined in the endostyle of the ascidian *Styela plicata.* With the use of specific inhibitors, it was demonstrated that a tryptic-like alkaline protease, TLCK-dependent, is involved.

The ascidian endostyle is a U-shaped structure, placed longitudinally in the ventral side of the branchial sac. The endostylar walls are divided into 8 zones composed of different cell types and numbered consecutively[2]. The endostyle physiomorphology has been the subject of numerous works. Particularly, much attention has been paid to zone 7 because of its capacity for binding iodine[3,4]; this characteristic has suggested a thyroid-like activity in the endostyle[5,6]. Recently the presence of a thyroglobulin-like glycoprotein has been demonstrated with immunohistochemical methods in the cells of the zones 7 and 8 of the endostyle of *Styela clava*[7].

The histochemistry[3,8,9] and ultrastructure[10-12] of glandular zones (2-4-6) have revealed cytological features that are typical of an enzymatic secretion. This observation was verified by the determination of the endostylar proteolytic activity of *S. plicata.*

Materials and methods. The specimens of *Styela plicata* (Urochordata, Ascidiacea) were gathered from the water inside the port of Genova and kept in aerated sea water, then the tunic and the underlying mantle were incised, and the endostyle was removed. The endostylar tissue was homogenized in a Mini-Potter homogenizer; the homogenizing medium was 0.15 M KCl containing 8 ml 0.02 M $KHCO_3$/l. The homogenate was centrifuged at 1000 × g for 10 min, at 4 °C, and the supernatant was used for the assay of the proteolytic activity.

A first series of assays was made with the substrate film method[13]; drops of supernatant were placed on blackened photographic plates and incubated in a moist chamber, at 37 °C, for 6, 9, 12 and 18 h. The digestion of the gelatin layer by the proteases left clear round areas on the film. Controls were made by adding to the supernatant 10^6 units/ml of Penicillin-G (Sigma Chemical Co., USA).

For a better identification of the proteases involved, the following inhibitors were used: 2 mg/ml soybean trypsin inhibitor (STI); 0.005 M TLCK trypsin inhibitor (N-a-p-tosyl-L-lysine chloromethyl ketone HCl); 0.01 M TPCK chymotrypsin inhibitor (L-1-tosylamide-2-phenyl-ethyl chloromethyl ketone).

Furthermore, the proteolytic activity was estimated by the use of casein (E. Merck, Darmstadt, FRG) suspended in 0.1 M phosphate buffer pH 7.6, as substrate[14]. The hydrolysis was made at 35 °C. Liberated amino acids were determined at 280 nm in a Unicam SP 500 spectrophotometer. The enzymatic activity was refered to the total protein content of the supernatant, determined by the method of Hartree[15], and expressed as equivalent µg/ml trypsin (Type III - Sigma Chemical Co., USA).

Results and discussion. The assays with the photographic plates demonstrated the presence of a protease in the endostylar supernatant, which was active after at least 9 h of incubation. After 18 h of incubation the drops of TLCK-supernatant revealed no lysis of the gelatin layer, but after 9 h of incubation the substrate film was digested with the drops of TPCK-supernatant. The assays made with STI inhibitor show a block of the proteolytic activity after 9 h of incubation; after longer periods the substrate is lysed.

The biochemical assay demonstrated that 1 µl of endostylar supernatant, with a total protein content of 0.44 µg/ml, shows a proteolytic activity equal to that of 0.032 µg/ml trypsin.

The hypothesis of a protease production in the ascidian endostyle was based on the intensive secretion of protein with numerous disulphide groups, demonstrated by histochemical[3,8,9] and ultrastructural[10-12] methods carried out on the glandular zones (2-4-6). Furthermore, the substrate film has shown a proteolytic activity in the endostyle of *Micro-*

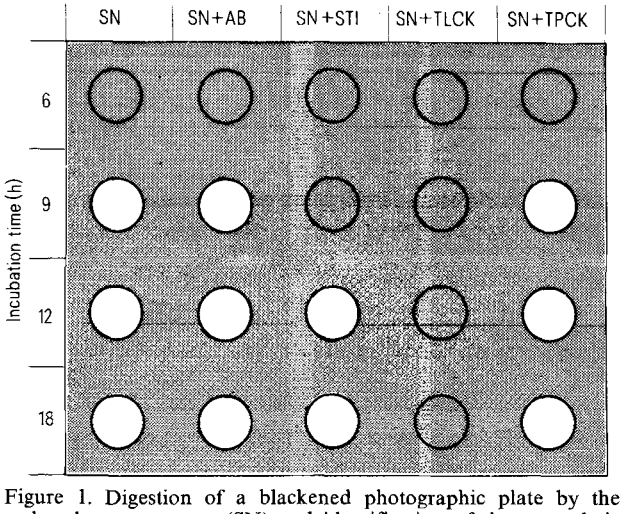

Figure 1. Digestion of a blackened photographic plate by the endostylar supernatant (SN) and identification of the proteolytic activity by the use of trypsin inhibitors (STI, TLCK) and chymotrypsin inhibitor (TPCK). Controls made with antibiotic (AB); incubation in a moist chamber at 37 °C. Empty circle: digestion of the substrate film. Dotted circle: lack of digestion.

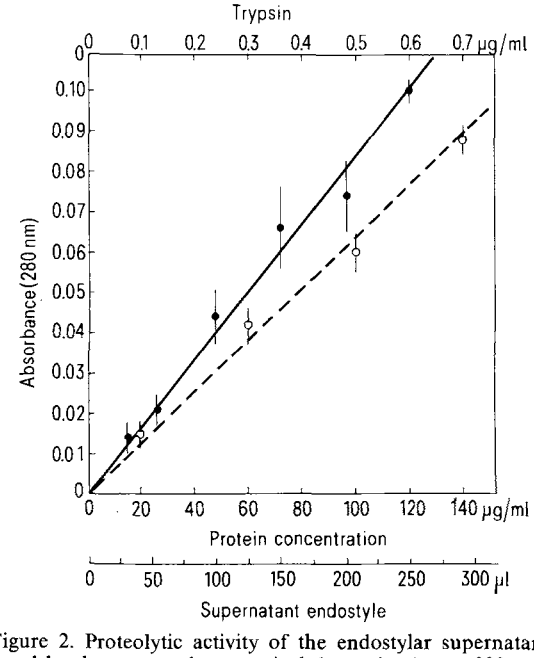

Figure 2. Proteolytic activity of the endostylar supernatant measured by the spectrophotometrical determination at 280 nm of the freed amino acids and expressed as an equivalent of µg trypsin/ml.

cosmus polymorphus[9], and the histoenzymological assays in toto have shown a chymotrpysin-like protease in the endostyle of *Ciona intestinalis* and *Phallusia mammillata*[16].
In the endostyle of the ammocoetes larvae of *Petromyzon marinus* a cathepsin-like protease, active at pH 4, was determined by biochemical assays[17].
On the basis of the above results the endostyle of *S. plicata* produces a trypsin-like enzyme, but does not show the presence of a chymotrypsin-like enzyme. This protease is likely to be mixed with the mucus secreted by the endostyle. As a result, the food particles entrapped by the mucous film begin to be digested in the pharyngeal tract, a process which appears to be rather common in the chordates.

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Cellular calcium binding state change during pentylenetetrazole-induced bursting activity in snail neurons

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Summary. According to the examination by a computer controlled electron probe X-ray microanalyzer, the calcium binding state in the cell membrane during pentylenetetrazole-induced bursting activity was different from that of normal neuronal membrane.

Pentylenetetrazole (PTZ) induces bursting activity in the D-neurons of the Japanese land snail *Euhadra peliomphala*[1]. The intracellular scattered calcium bound to the subcellular organelles was found, by an examination with a computer controlled electron probe X-ray microanalyzer (CCEPXMA)[2], to move toward the cell membrane during bursting activity. If the movement of calcium toward the cell membrane has some relation to the bursting type neuronal discharges, the calcium should be bound to the cell membrane, and the binding state of calcium would become different from the normal membrane state.
The peak X-ray spectrum of elements shifts to some extent due to binding state change[3,4], and in the case of calcium, Chun[5] described a so-called chemical shift between calcium and calcium oxide. We tried to examine whether any kind of chemical shift exists in or near the cell membrane when the normal neuron and that in the state of bursting activity are compared.
D-neurons of the Japanese land snail *Euhadra peliomphala* were used, since the D-neurons of *Euhadra* are the group most sensitive to PTZ[1]. The D-neurons were divided into 2 groups, one of which was incubated in snail Ringer solution containing PTZ (5×10^{-2} M) and the other in snail Ringer solution alone. Each group was frozen rapidly and sectioned to about 10 µm thickness by a Lipshow cryomicrotome and freeze dried by the method described previously without passing through the liquid phase[2]. Both types of freeze dried specimens were placed on a hand-made carbon disk and the chemical shift was measured successively at the same accelerating voltage (15 kV) and absorbed current (10^{-8} A). The electron beam was about 1 µm in diameter and the beam spot was placed on the cell membrane area. For the standard X-ray peak spectrum position, metallic calcium was used. According to Bragg's law ($n\lambda=2d\sin\theta$), if θ is changed slightly for some unexpected reason, an X-ray wave length shift which should be distinquished from a true chemical shift occurs. The possible origins of the slight θ change are a) movement of the specimen in the z-direction (in our system, it was within $\pm3.12\times10^{-4}$ Å), b) movement of the analyzing crystal by a temperature change (in our system, within $\pm3.12\times10^{-4}$ Å), c) electron beam diameter change (in our system, undetectable) and d) movement of the beam in the X-Y direction (in our system, 0.94×10^{-4} Å). We measured these items first and if the chemical shift of samples exceeded this range, we decided that the results were positive for a chemical shift. The error range of metallic calcium after 8 h of repeated drawing of the X-ray spectrum curve was 3.36427 ± 0.000172 Å, and at this time the error range, including all the 4 items mentioned above, was considered as constant. Under such conditions, we started to measure the chemical shift of the specimens.
The steps for measuring the chemical shift were as follows. The analysing crystal was moved in 10-µm steps successively on Roland's circle at each measuring spot. The measuring time of each step (each crystal position) was 1 sec. We drew the curve of the relative X-ray strength when the maximum X-ray strength was 10. The curve was drawn after averaging of 5 arbitrary measuring spots on the neuronal membrane of one specimen.
The figure, A, shows an example of chemical shift of normal membrane. The peak of normal neuronal membrane showed a shift to a shorter wave length by about -16×10^{-4} Å. The peak of PTZ-treated membrane was almost the same as those of metallic calcium as shown in B. C shows the summary of chemical shift of normal and PTZ-treated neuronal membrane from metallic calcium, which demonstrates a clear difference between the 2 neuronal membrane states.
The above findings demonstrate that normal and PTZ-treated neuronal membrane or the cytoplasm near the cell membrane have different calcium binding states. Our previous study suggested that PTZ induced the release of calcium bound to subcellular organelles such as lysosome-