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MORPHOLOGICAL AND CYTOCHEMICAL STUDIES ON THE SECRETORY GRANULES OF THE PYLORIC CAECA OF THE STARFISH, *ASTERIAS AMURENSIS*

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Recently, some extensive biochemical studies on the properties of starfish proteases have been reported. The results have shown that the pyloric caeca of various species of starfish contain some trypsin-like enzymes (Camacho, Brown and Kitto, 1970; Winter and Neurath, 1970; Bundy and Gustafson, 1973; Kozlovskaya and Elyakova, 1974, 1975; Elyakova and Kozlovskaya, 1975). These purified enzymes, having a molecular weight of about 25,000, are inhibited by N-a-tosyl-L-lysyl-chloromethane or diisopropylphosphofluoridate and show substrate specificities similar to bovine trypsin. The cleavage specificity and amino acid composition are also strikingly similar to that of bovine trypsin (Gilliam and Kitto, 1976; Camacho, Brown and Kitto, 1976). It is thus highly probable that starfish tryptic enzymes are homologous to vertebrate trypsins.

The starfish proteases have been assumed to be contained in the iron hematoxylin- or azocarmine-positive granules in the secretory cells (Anderson, 1953, 1966; Chia, 1969). However, as far as the authors are aware, there is no positive proof for this assumption. In addition, the ultrastructure of these granules has not been reported. Therefore, the present investigations have been carried out to study the fine structure of the granules and also to clarify whether these granules actually possess proteolytic enzymes in a zymogen form.

Materials and Methods

Starfish, *Asterias amurensis*, were collected in the vicinity of Oshoro Biological Station during the period of January to December, 1975.

Electron microscopy

Fresh pyloric caeca and the isolated secretory granules were fixed in Karnovsky's glutaraldehyde-formaldehyde fixative (Karnovsky, 1965), post-fixed in 1% OsO_4 buffered with 0.1 M cacodylate, pH 7.4, dehydrated through graded ethanol and embedded in Epon (Luft, 1961) as usual. Ultrathin sections were cut with a Porter-Blum MT-2 ultramicrotome, stained with uranyl acetate (Watson, 1958) and lead citrate (Reynolds, 1963) and examined under a Hitachi HU-12 electron microscope.

Staining of the secretory granules

The one μ thick Epon sections were stained with 0.1% azocarmine-5% acetic acid overnight at room temperature, differentiated with 5% phosphotungstic acid for one hour, rinsed in water and mounted in synthetic resin after drying. With

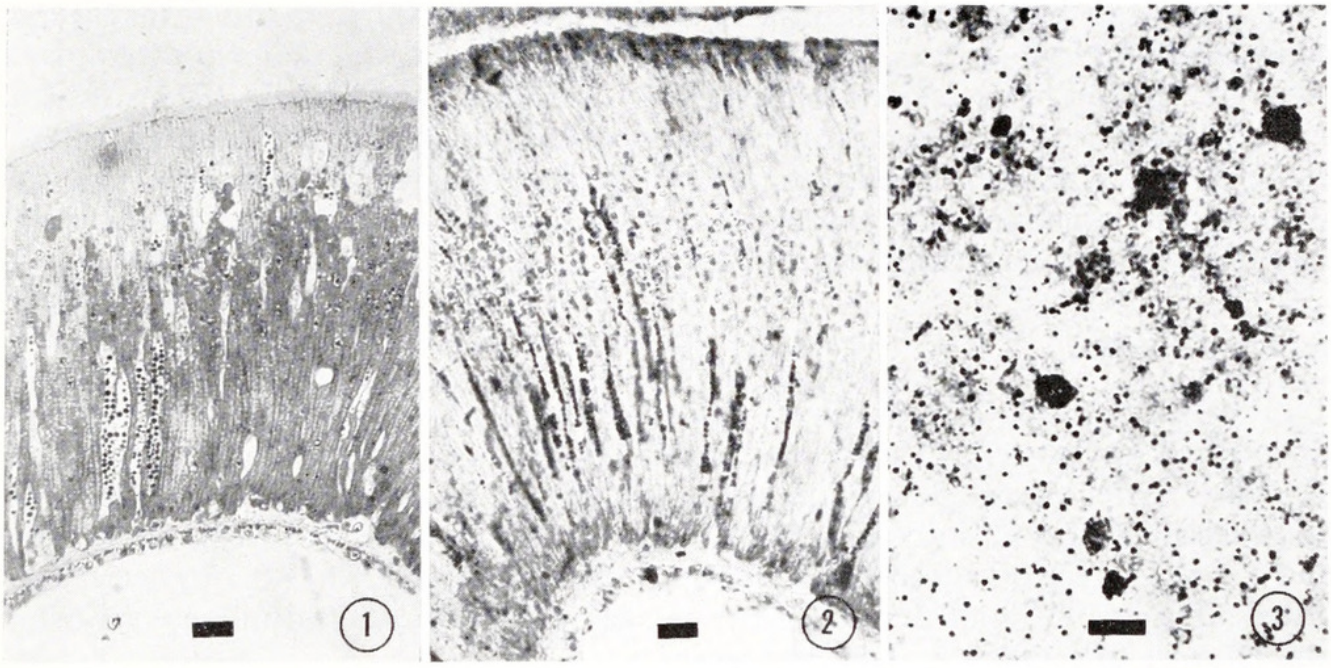

FIGURE 1. Thick Epon section of the pyloric caeca stained with azocarmine, showing the positive reaction of secretory granules. Scale bar is 10 μ.

FIGURE 2. Formalin-fixed, frozen section of the pyloric caeca stained with Baker's acid hematein, showing the positive reaction of secretory granules. Scale bar is 10 μ.

FIGURE 3. Azocarmine-methylene blue staining of the secretory granule-rich (1.7 M) fraction obtained by sucrose density gradient centrifugation of pyloric caeca homogenates. Scale bar is 10 μ.

this technique, only the secretory granules in the secretory cells were stained red (Fig. 1).

Besides the azocarmine method described above, the acid hematein test for phospholipids (Baker, 1946; Hori, 1963) was also found to be specific for the secretory granules (Fig. 2). However, this method was more time-consuming than the azocarmine method.

The fractions obtained during isolation of the secretory granules were smeared on microscope slides, fixed in ethanol-formalin-acetic acid (85 : 10 : 5) for ten minutes, rinsed in water and stained with 0.1% azocarmine-5% acetic acid for one hour. The slides were then rinsed in water, differentiated with 5% phosphotungstic acid for fifteen minutes, and stained in a formic acid-sodium acetate buffer containing 0.2% methylene blue (Dempsey and Singer, 1946). After washing in water, the slides were dehydrated and mounted in synthetic resin as usual. With this method, the secretory granules were stained brilliant red, the nuclei pink, and other components blue (Fig. 3).

Protease assay

Samples up to 0.1 ml were incubated in 2 ml of 1% casein in 0.1 M borate buffer, pH 8.5, for fifteen minutes at 30° C. The reaction was stopped by adding 4 ml of 5% trichloroacetic acid (TCA). The mixture was then filtered and optical density of the filtrate was measured at 280 nm (Kunitz, 1947). The blanks were prepared by first mixing casein solution with TCA and then adding enzyme. One unit of casein-hydrolyzing activity was defined as the amount of enzyme which

gave rise to an increase in absorbancy at 280 nm of 1.000 per minute under the conditions of assay.

This method was devised according to previous data (Hori, 1975), which indicated that the partially purified proteases had an optimum pH at about 8.5 and that under the above assay conditions, the rate of casein hydrolysis was linear with both time and enzyme concentration when optical density changes of less than 0.05 per minute were measured.

Electrophoresis

Proteases, about 0.02 units, were electrophoresed on polyacrylamide gels by the method of Ornstein (1964) and Davis (1964) using plastic columns, $3 \times 12 \times 100$ mm. After electrophoresis, the gels were sliced into strips of 1.5 mm thickness and placed on the substrate-agar film prepared as follows: 1 g casein, 10 g urea and 1 g agar were dissolved in 100 ml of 0.1 M borate buffer, pH 8.5 by warming. After filtering, 1.5 ml of this solution was spread over a standard microscope slide and allowed to solidify at room temperature.

The slides carrying polyacrylamide gels were incubated in a moist chamber at 30° C for thirty minutes and immersed in acetic ethanol (50% ethanol containing 5% acetic acid). After removal of gels, the substrate-film was air-dried, stained in 0.2% Amido black in acetic ethanol, rinsed in acetic ethanol and air-dried. This is a modification of Uriel's method (1960).

Histochemical detection of the protease activity

The pyloric caeca were fixed in 10% formalin containing 1% $CaCl_2$ for six hours at 4° C, impregnated with 20% sucrose-20% gum acacia overnight and frozen-sectioned in a cryostat. Sections were then washed with cold 95% ethanol, mounted on gelatin film and air-dried. The gelatin film was prepared as follows: 3.5 g gelatin was dissolved in 100 ml of distilled water by warming. Of this solution 0.4 ml was spread over a microscope slide, air-dried, polymerized in 25% formalin overnight and washed thoroughly with running tap water.

Sections mounted on gelatin film were incubated in 50 mM borate buffer, pH 8.5 at 30° C for five to fifteen minutes. After incubation, sections were flashed off the film, and the film was stained first in 1% light green SF for five minutes and then in 0.5% basic fuchsin for twenty minutes. This is a modification of Cunningham's method (1967).

Isolation of the secretory granules

With the azocarmine staining as the marker, the secretory granules were isolated from epithelial cells of the pyloric caeca. Fresh pyloric caeca, 8 g in each experiment, were placed in 70 ml of 1 M sucrose (10 mM $CaCl_2$ was added to all sucrose solutions used in this study). They were dissociated by mincing with scissors and by gentle pipetting. The samples were then filtered through a platinum mesh (150 mesh) and the volume of the filtrate was adjusted to 80 ml with 1 M sucrose. The filtrate was then centrifuged at $4300 \times g$ for ten minutes. The precipitate was washed with 1 M sucrose, suspended in 15 ml of 1.3 M sucrose,

sonicated for five seconds and subjected to sucrose density gradient centrifugation; each centrifugation tube contained from bottom to top 1 ml of 1.8 M sucrose, 1.3 ml of 1.7 M sucrose and 2.5 ml of sample in 1.3 M sucrose. The samples were centrifuged at 40,000 rpm (maximum, 175,000 $\times$ g; minimum, 84,000 $\times$ g) for one hour using a Hitachi swinging bucket rotor RPS 50. After centrifuging, the content of each tube was divided into supernatant, 1.3 M (interphase between 1.3 M and 1.7 M layers), 1.7 M and precipitate fractions. The 1.7 M fraction contained the majority of secretory granules (Fig. 3).

For protease assay and electrophoresis, each fraction was homogenized and placed at 37° C for one hour in order to activate proteases. For protein assay, each fraction was diluted with an appropriate amount of water, and treated with 5% TCA. The TCA precipitate was taken up with 0.1 N NaOH and assayed by the method of Lowry, Rosebrough, Farr and Randall (1951).

Results

Ultrastructure of the secretory granules

The azocarmine-positive granules, measuring 0.5–2.0 μ in diameter, are limited by a single membrane measuring about 60 Å in thickness and may be classified into three types according to the morphology of their contents: first, dense granules which contain dense, homogeneous amorphous substances; secondly granules which contain a dense, homogeneous core imbedded in amorphous substances, and thirdly, light granules which contain amorphous substances only (Figs. 4–7). The size and shape of the core in the second-type granules varied greatly in different granules. This seems to suggest that these granules might be transitory forms from the light to dense granules (Fig. 7). The dense granules may be considered the matured forms.

The light granules were much more numerous than the dense granules in the specimens collected during the pre-breeding season (January to May), while the dense granules were predominant in the specimens collected during the post-breeding season (August to December). All types of granules were positively stained with azocarmine.

The granules were often seen abutted on rough endoplasmic reticulum, but the membrane of granules was in no case continuous with the membrane of rough endoplasmic reticulum. Golgi apparatuses were not numerous.

The clear vacuole, usually associated with the secretory granule clump (Anderson, 1953, 1966), appeared to be either an empty space formed by an extensive outgrowth of the outer nuclear membrane or a large space which was limited by a single membrane, but contained little or no electron-dense material (Fig. 7). The cytoplasm of the secretory cell was rather scanty in comparison with the adjacent storage cells, but the rough endoplasmic reticulum was well-developed, particularly in the middle portion of the cell.

Histochemical detection of the protease activity

With the substrate-film method, the protease activity was demonstrated in the glandular epithelium as well as in the lumen. Because of the poor resolution inherent to such methods, it was impossible to ascertain if the intraepithelial activity was actually located specificially in the secretory granules.

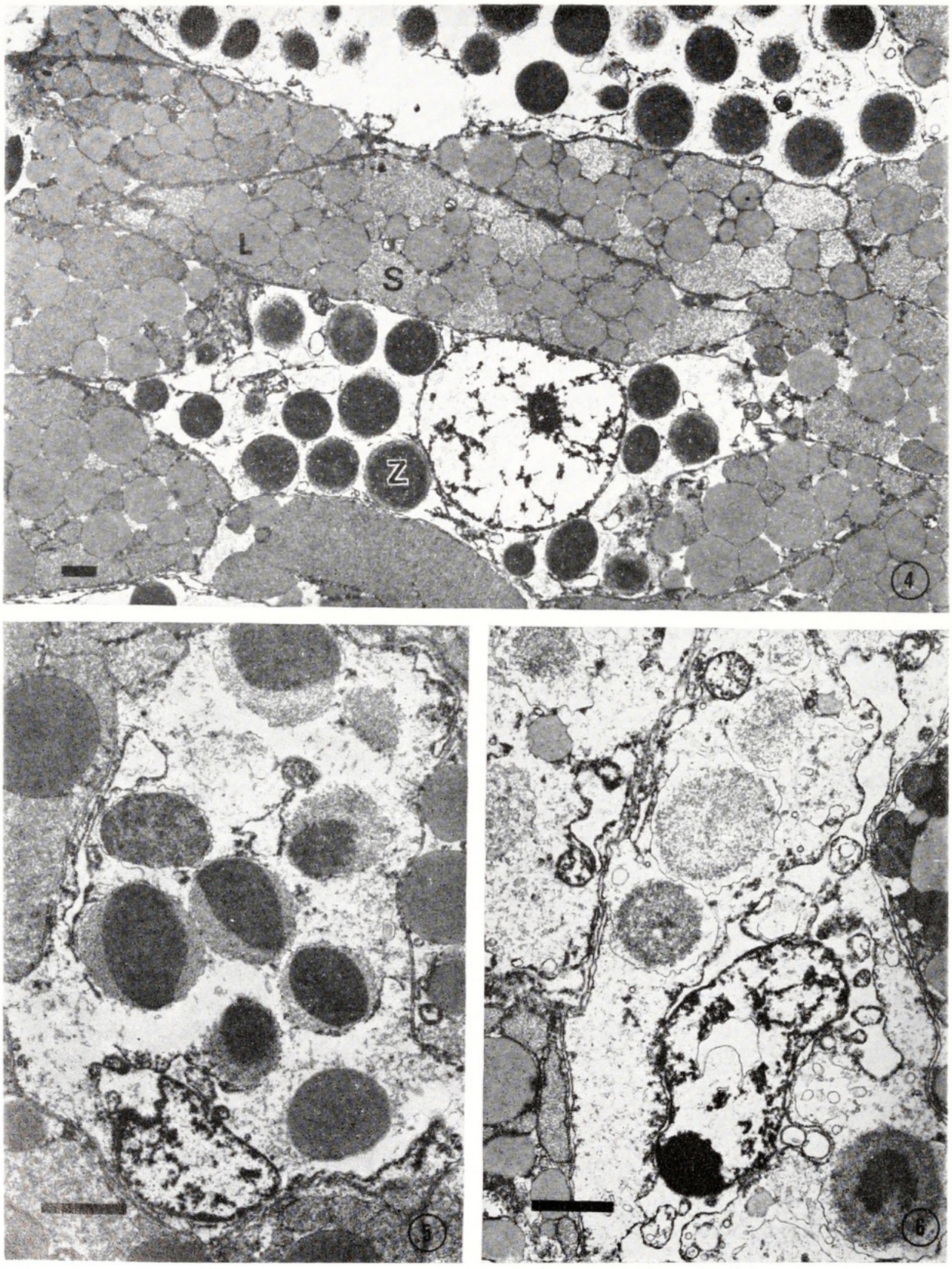

FIGURE 4. Fine structure of the secretory cells, showing dense secretory granules (Z); L and S, lipid and storage granules in the adjacent storage cell. Scale bar is 1 μ.

FIGURE 5. Fine structure of the secretory granules which have a dense core surrounded by light amorphous substance. Scale bar is 1 μ.

FIGURE 6. Fine structure of the secretory granules which are characterized by light amorphous content. Note an extensive outgrowth of the outer nuclear membrane studded with ribosomes. Scale bar is 1 μ.

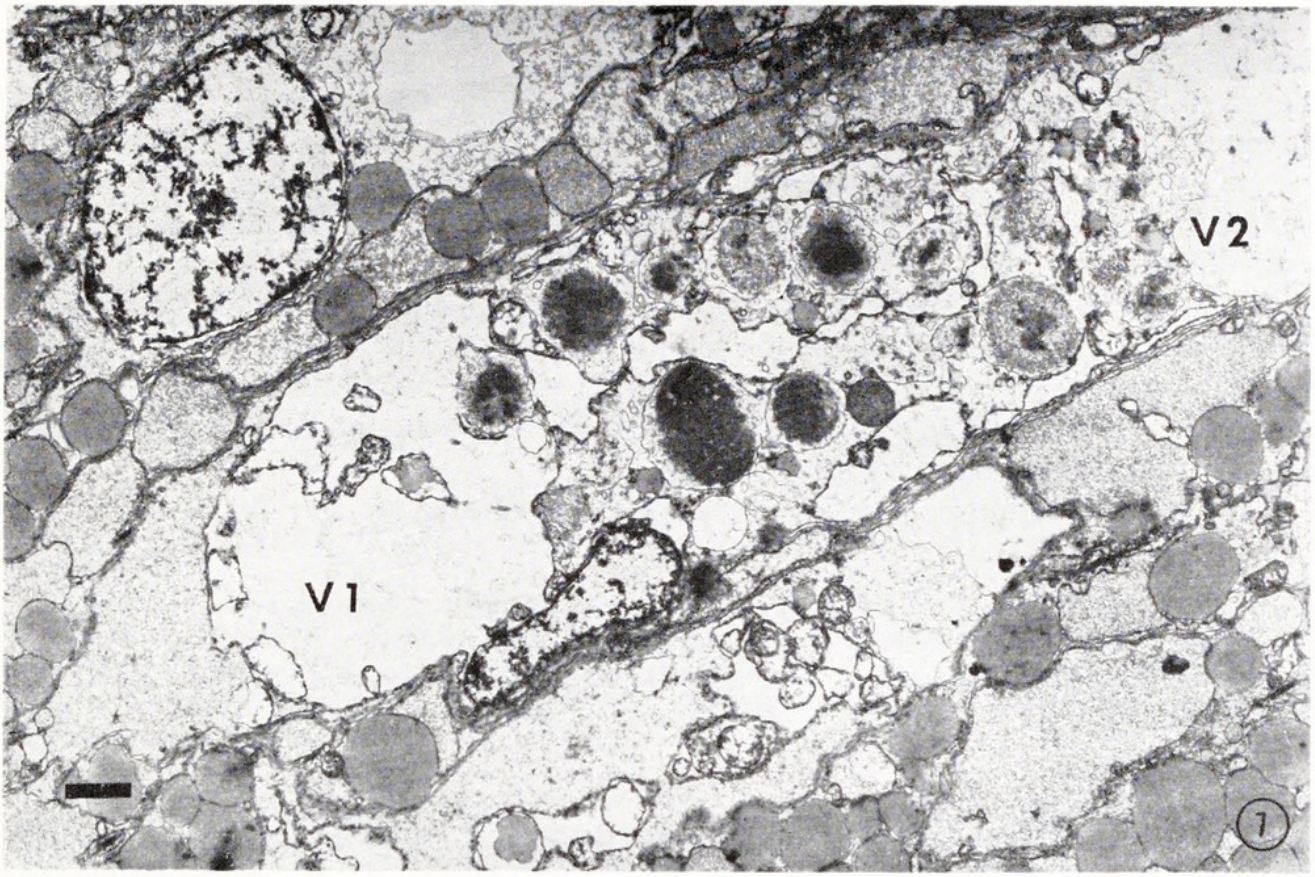

FIGURE 7. Fine structure of a secretory cell containing several secretory granules of different types, in addition to the associated vacuoles, V_1 and V_2. Scale bar is 1 μ.

Properties of the isolated secretory granules

The results of the enzyme and protein assays of the various fractions obtained during one purification procedure are given in Table I. The isolation was performed six times with six different animals, and the results were essentially the same.

TABLE I

Separation of secretory granules from pyloric caeca.

	Tissue homogenate	4300 × *g* sediment	Density gradient				
			Supernatant	1.3 M	1.7 M	Sediment	Total
Protease activity (units/g tissue)	13.04	6.07	0.76	1.27	2.29 *s 1.78 *p 0.51	0.44	4.76
Protein (mg/g tissue)	84.97	11.78	0.85	5.00	1.70 *s 0.34 *p 1.36	3.78	11.33
Specific activity (units/mg protein)	0.15	0.52	0.89	0.25	1.35 *s 5.24 *p 0.38	0.12	

*s and *p, 27,000 × *g* supernatant and sediment of the homogenized 1.7 M fraction, respectively.

The amount of azocarmine-positive granules estimated by histological inspection was in agreement with the quantitative data on the protease activity; *i.e.*, the granules were much more abundant in the 1.7 M fraction than in the other fractions (Table I). About 50% of the total enzyme activity was recovered in the 1.7 M fraction. The relatively high specific activity of proteases in the supernatant fraction would be due to the enzyme released from the granules during centrifugation.

Attempts to further purify the granules by subjecting the 1.7 M fraction to the second sucrose density gradient centrifugation were fruitless, probably because of the fragility of granules. In fact, more than 50% of the activity became unsedimentable, and the latency of the protease activity in the sedimented granules was lost after the second centrifugation.

The 1.7 M fraction exhibited a very low level of enzyme activity, if assay was

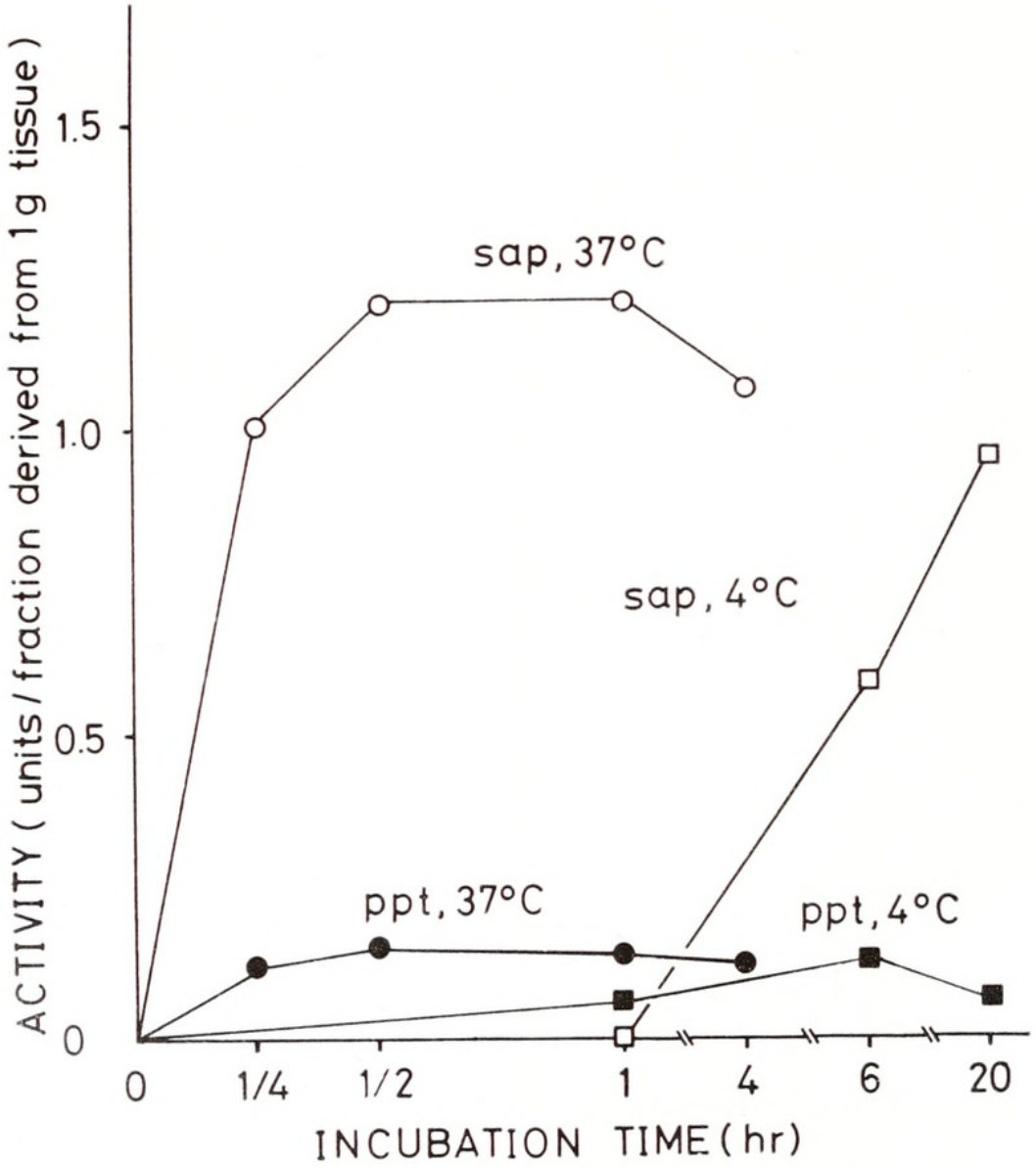

FIGURE 8. Activation of proteases by incubation. The 1.7 M fraction was homogenized, separated into supernatant (sap) and sediment (ppt) by centrifuging at 27,000 × *g* for five minutes, and incubated at 4° C or at 37° C. Protease activity is expressed as units per fraction derived from g tissue.

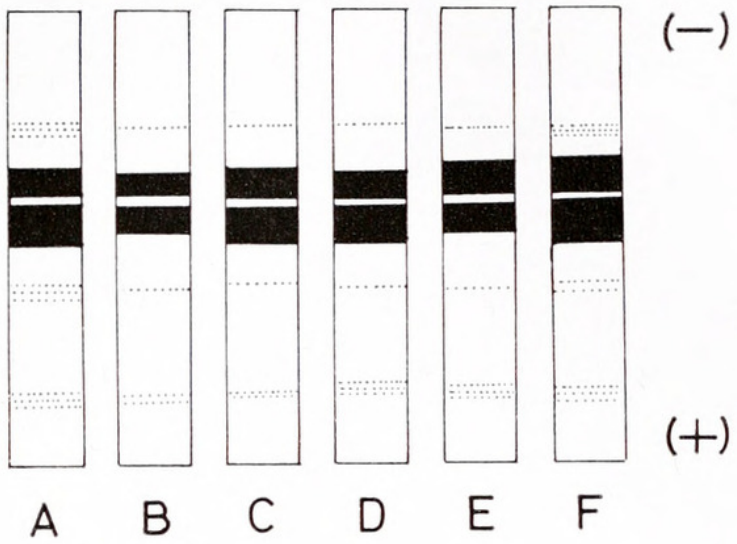

FIGURE 9. Electrophoretic separation of the casein-hydrolyzing activities: a, pyloric caeca homogenates; b, 4300 × *g* sediment of the homogenates; c-f, supernatant, 1.3 M, 1.7 M and sediment fractions obtained by sucrose density gradient centrifugation of the 4300 × *g* sediment, respectively. No significant change in the electrophoretic pattern occurred during separation of the secretory granules.

performed with the freshly prepared fraction. On the other hand, the enzyme was activated rapidly by incubation at 37° C. The activation also occurred at 4° C, though it required longer duration (Fig. 8). This suggests that the enzyme activation may be physiologically possible in poikilothermal animals. The results presented in Figure 8 also show that solubilization of the enzymes by homogenization alone would not cause their instantaneous activation, that the activation might be an enzyme-catalyzed reaction, and that the enzymes exist in easily-solubilized forms and are not bound to the granular membranes.

The specific activity of the 1.7 M fraction was nine times, and that of the supernatant obtained from the 1.7 M fraction was thirty-five times greater than that of the original tissue homogenates (Table I). Electrophoretic patterns of the activated enzymes were almost the same in all fractions obtained during purification (Fig. 9). The data excluded the possibility that particular forms of the enzymes were selectively isolated during purification.

By electron microscopy, the 1.7 M fraction contained numerous granules, the morphology of which were the same as those of the secretory granules in tissues. Some contaminated elements, such as smooth-surfaced vesicles, mitochondria and storage granules usually appeared in these fractionated specimens. Many secretory granules were present separately, but some were isolated in the form of clumps, surrounded by dense cytoplasm rich in rough endoplasmic reticulum (Fig. 10). Mitochondria were often observed between such clumps of granules.

DISCUSSION

The ultrastructure of secretory granules in the starfish pyloric caeca has been described probably for the first time in the present paper. In a report on the ultrastructure of the pyloric caeca of *Asterias rubens*, Bargmann and Behrens (1968) have mentioned that the so-called zymogen granules have not been observed.

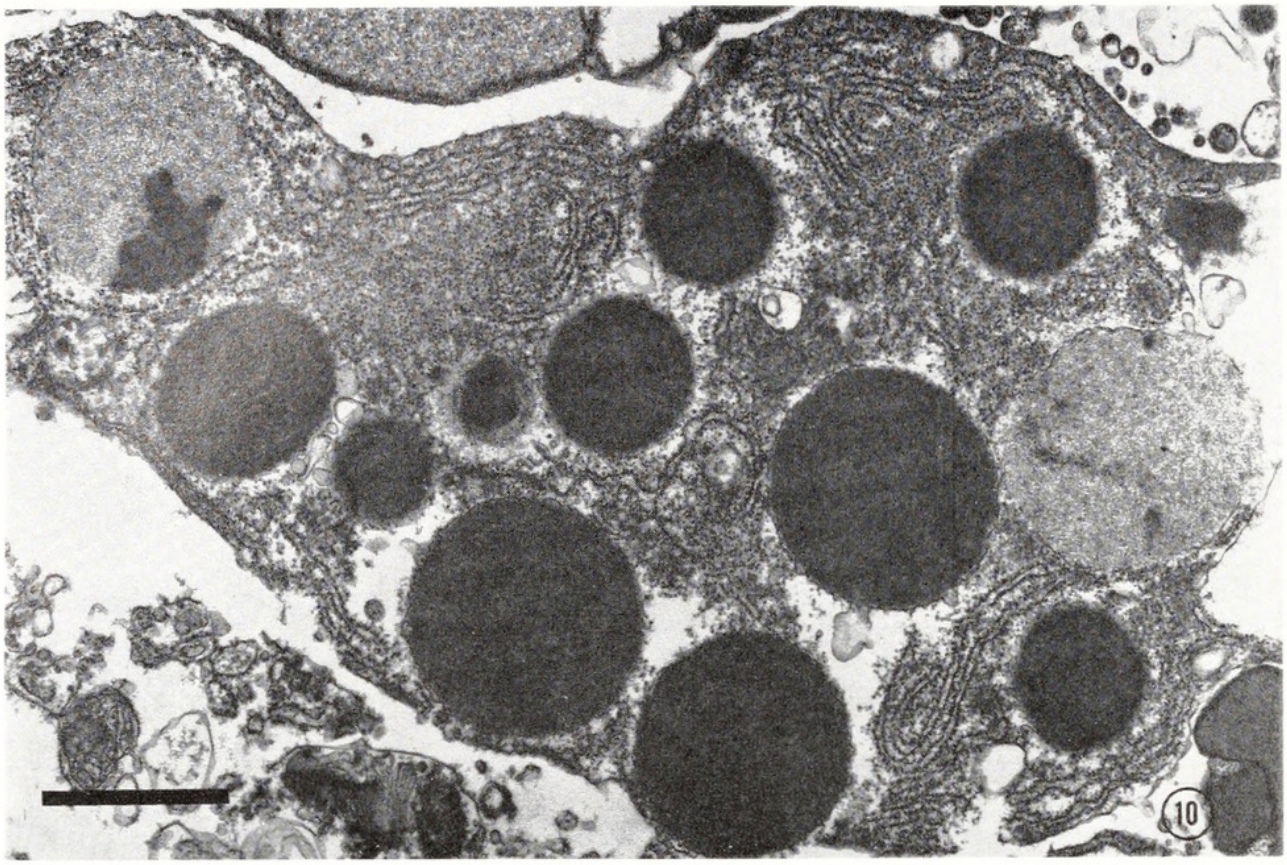

FIGURE 10. Fine structure of an aggregate of the secretory granules found in the 1.7 M fraction obtained by sucrose density gradient centrifugation, showing the secretory granules of dense, light and core-bearing types as well as the surrounding rough-surfaced endoplasmic reticulum. Scale bar is 1 μ.

Their failure in identifying the granules might be related to the morphological variation of the ultrastructure of granules, because secretory granules in starfish pyloric caeca do not always appear as dense as those in mammalian pancreatic acini. As described above, some granules (light granules) are characterized by amorphous contents which (like the dense granules) are also azocarmine-positive. Therefore, if the specimens from the pre-breeding season were used for study, then the light granules could predominate. It would be difficult to identify the granules as zymogen morphologically, unless a careful comparison was made from light- and electron microscopic preparations.

Morphologically, the dense granules appear to be the mature form and the granules having a dense core appear to be transitory forms between the light and dense granules. However, it is also possible that some core-bearing granules might have already been matured. Possibility also exists that the morphological heterogeneity of the granules might reflect their biochemical heterogeneity (*i.e.*, different types of granules contain different sets of hydrolytic enzymes). The granular fraction obtained by sucrose density gradient centrifugation exhibited the same electrophoretic pattern of protease activity as that of the original tissue homogenates. However, this does not necessarily mean that each isolated granule is biochemically equivalent.

The dense granules in starfish pyloric caeca were morphologically quite similar to the secretory granules described by Holland and Lauritis (1968) in gastric exocrine cells of the purple sea urchin, *Strongylocentrotus purpuratus*. It is un-

certain, however, if the gastric exocrine cells also contain granules homologous to the light and core-bearing granules described in the present paper.

It was extremely difficult to obtain a pure fraction of the secretory granules, because of the wide spectrum of the density of the granules. The same was true with other cellular organelles, such as mitochondria and rough and smooth endoplasmic reticulum. Our attempts to obtain pure fractions of these organelles by modifications of the methods commonly employed for fractionation of vertebrate tissues have so far been unsuccessful. Obviously, an entirely different technique is required for such works with starfish pyloric caeca.

It has been reported that the proteases in starfish pyloric caeca are stored as inactive zymogens (Camacho, Brown and Kitto, 1970; Bundy and Gustafson, 1973), and that the zymogen may be contained in granules (Peng and Williams, 1973; Tillinghast and Levasseur, 1975). Such reports are based on the findings that the protease activity of crude homogenates or of an extract of acetone powder increases upon incubation at 20° C or above, and that the activity in crude homogenates can be easily sedimented by relatively low centrifugal force. The present data not only are consistent with these observations, but also have clearly demonstrated that the enzymes are present in the so-called zymogen granules in an inactive form. These enzymes may be activated by self-catalyzed hydrolysis or contact with some activators or removal of inhibitors. The present data do not agree with the findings of Tillinghast and Levasseur (1975), who state that when the enzyme is spontaneously released from the granules, further activation does not occur. Presumably, in the pyloric caeca, the content of the secretory granules is released into the glandular lumen, where activation of proteases may take place as with mammalian enzymes.

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Summary

A fraction rich in secretory granules was prepared from the pyloric caeca of *Asterias amurensis* by sucrose density gradient centrifugation. The freshly prepared fraction exhibited no casein-hydrolyzing activity, but showed nine times as much specific activity as that of tissue homogenates after incubation at 37° C for thirty minutes. Electron microscopy showed that the secretory granules were membrane-bound granules measuring 0.5–2.0 μ in diameter and contained dense and/or light amorphous substances.

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