Korean Journal of Malacology, Vol. 15 (2) : 81 - 92. December, 1999

Cytochemical and Immunocytochemical Study on the Cellulase Activity in the Accessory Glands of the Digestive System of the Oriental Land Snail, *Nesiohelix samarangae*

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ABSTRACT

The histochemical, cytochemical, and immmuno-cytochemical investigations were conducted to find out the cellulase activity in the accessory glands of the digestive system of the oriental land snail *Nesiohelix samarangae* under the LM, SEM, and TEM.

The cellulase activity was shown in the epithelium of the digestive gland by labelling with the immunogold (protein-A gold) particles. The epithelial cells showing the cellulase activity were Type 1 and Type 3 cells out of five types of the epithelial cells of the digestive gland.

None of epithelial cells of the mucus gland and the salivary gland were not labeled with the immunogold particles.

Keywords : Gastropod, Snail, Digestive gland, Cellulase, Immunogold

INTRODUCTION

Since an anatomical and morphological study on the molluscan stomach was conducted by Purchon(1956), Owen (1956), Morton (1960), Smith (1967) and Rudman (1972a, b) have studied morphologically on the stomach, the alimentary tracts, and the digestive gland. Otherwise, a histological study was carried out on the *Marisa cornuarietis* by Lufty and Demian (1967).

Demian and Michelson (1971) reported histochemical study on the mucin secreted in the alimentary tracts. Roldan and Garcia (1988) had an anatomical study on the alimentary tract of *Theba pisana*. Alba *et al.* (1988) studied on endocrine cells in the *Helix aspersa*. Recently Boer and Kits (1990) reported ultrastructures of the alimentary tracts of *Lymnaea stagnalis* and Jeong *et al.* (1993) reported ultrastructures of the alimentary tracts of two freshwater snails, *Parafossarulus manchouricus* and *Radix auricularia coreana*. Physiological and chemical studies on the digestive organs of the mollusks are very rare until present time. Yonge (1932) mentioned cellulase secretion from the Pterocrea, and Newell (1953) also reported cellulolytic activity in the crystalline style of Lamellibranch. Maeda *et al.* (1996) purified cellulase with activity as a cellobiohydrolase from the intestinal juice of the giant land snail *Achatina fulica*. All of the enzymatic studies in the mollusks were carried out with the extracts of the mollusks examined. Recently, Jeong and Lee (1997a, b) and Jeong *et al.* (1998) reported cellulase activity in the epithelial cells of the stomach and intestine of the oriental snail, *Nesiohelix samarangae* by cytochemical and immunocytochemical methods.

Present study was designed to find out the cellulase activity in the accessory glands of the digestive organ such as mucus, salivary, and digestive glands of the

Received October 13, 1999; Accepted November 21, 1999
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1225-3480/15202

snail, *N. samarangae.*

MATERIALS AND METHODS

1. MATERIALS

The adult specimens of the oriental land snail *N. samarangae* were collected from the Kaeuido, an island located in the yellow sea belonging to the Chungnam province, Korea. The collected snails were feed on green lettuce and carrots with the calcium powder until the experiments in the laboratory.

2. METHODS

The snails were dissected in 0.2 M phosphate buffer (pH 7.4) to isolate the accessory glands such as the mucus gland, salivary gland and digestive gland under the stereoscope.

1) Histochemical Study

The specimens were fixed with buffered neutral formalin and embedded in paraffin for three hours, washed three times with 0.1M phosphate buffer(pH 7.4), dehydrated in the graded concentrations of alcohol series, embedded in Epon-812 mixture in routine method. The Epon mixture with the embedded specimens were incubated for 12 hours at each temperature of 35℃ and 45℃, and finally incubated for 48 hours at 65℃. The semithin sections(3 μm in thickness) obtained with ultramicrotome were etched with saturated NaOH solution. After the etching, the specimen sections were stained with toluidine blue and double stains of PAS-Alcian blue and methylene blue-basic fuchsin. The stained specimens were observed under the light microscope, Nikon Optiphot-2.

2) Ultrastructure Observations

The specimens were fixed with 3% glutaraldehyde buffered with 0.1M phosphate buffer (pH 7.4) for 2 hours and washed with the buffer.

The specimens for the scanning electron microscopy were dehydrated in the alcohol-amylacetate series, dried with critical point dryer, gold coated 100Å in thickness, and observed with JSM-5410 LV scanning electron microscope.

The specimens for transmission electron microscopy were cut into proper sizes in the fixative during the prefixation and postfixed with buffered 1% OsO_4 for 2 hours. The fixed specimens were dehydrated in a series of graded ethyl alcohol concentrations, embedded in Spurr mixture, and incubated for polymerization for 3 days at 70℃.

The thin sections obtained by ultramicrotome were double stained with uranyl acetate and lead citrate, and observed with JEM-1010 transmission electron microscope.

3) Cytochemical Examination

To confirm the cellulase activity in the ultrastructural level, the specimens firstly fixed were washed three times with the buffer and were incubated in 0.01 M carboxymethyl cellulose (C.M.C.) solution for 20 minutes. After this incubation, the specimens were transferred to the Benedict's reagent and boiled for 10 minutes at 80-90℃. But, the control specimens were not boiled at this stage. The whole specimens were post fixed with 1% OsO_4 for one and half hours and washed the buffer. The specimens were dehydrated in a graded series of alcohol concentrations and were embedded in Spurr mixture. The thin sections obtained from the ultramicrotome were stained with uranyl acetate and lead citrate, and observed with JEM-1010 transmission electron microscope.

4) Antibody Production

The antibody, necessary for the immunocytochemical study to confirm the cellulase activity, was produced by following procedures.

The visceral masses of the several snails were homogenized in 1M phosphate buffer (pH 6.0) at 3,000 rpm for 15 minutes in the cold room at 4℃ and the superficial layer of the body fluid of homogenized snail was isolated for next procedure.

To examine the cellulase activity, 0.1 g of carboxymethyl cellulose and 1g of agar were mixed in a beaker with saline solution and the final volume of the mixture adjusted to 100 ml was boiled and transferred into a petridish and become cool in the room temperature. On this 1% agar medium the above snail body fluid was spotted and kept for 1 hour at 36℃, stained with Congo Red for 1-2 minutes and finally destained with 1 M NaCl solution. Extraction of the cellulase from the snail body fluid was performed by

the non-SDS PAGE.

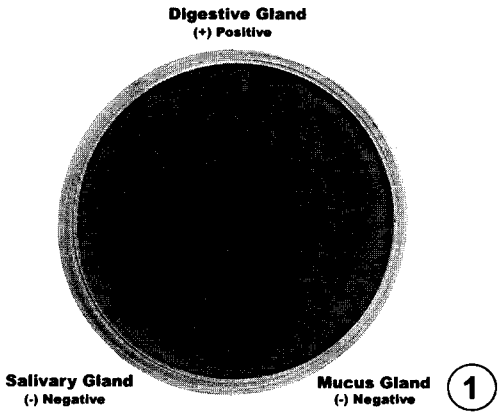
Antibody against the cellulase of the snail was made in the experimental rabbits by injecting the cellulase with adjuvant. The confirmation of antibody production in the rabbits was conducted with the double diffusion method on the 1% agar medium. The IgG was separated from the serum of the rabbit blood, obtained by cardiac puncture, through a process of DEAE sephacell column chromatography.

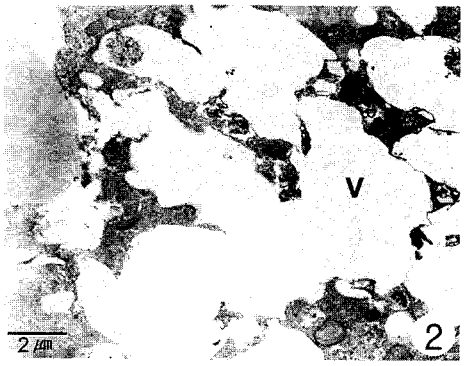
5) Confirmation of Cellulase by the Screening Method for Carboxymethyl Cellulase

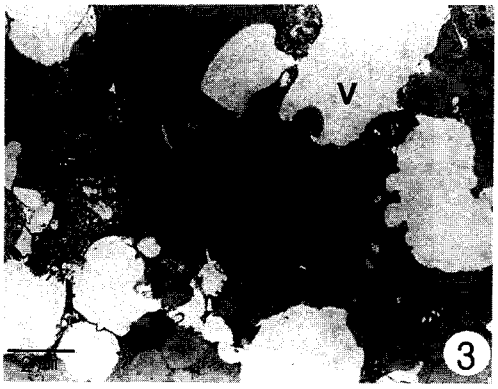
The mucus gland, salivary gland and digestive gland dissected out from the snail were homogenized in each test tube by species containing phosphate buffer (pH 6.0) and centrifuged in 3,000 rpm for 15 minutes. The superficial layers of the tissue fluids were sampled out and spotted in the 1% agar medium containing 0.1% carboxymethyl cellulase. The agar medium, spotted with the tissue fluids from the accessory glands was incubated at 36℃ for 1 hour for reaction, stained with Congo Red for 1-2 minutes, and destained with 1M NaCl solution.

6) TEM Tissue Preparation for Immunocytochemical Study

For the observation of immunogold labeled tissues with the transmission electron microscope, the specimens were prefixed with phosphate buffered 2% formaldehyde (pH 6.0) for 2 hours and postfixed with







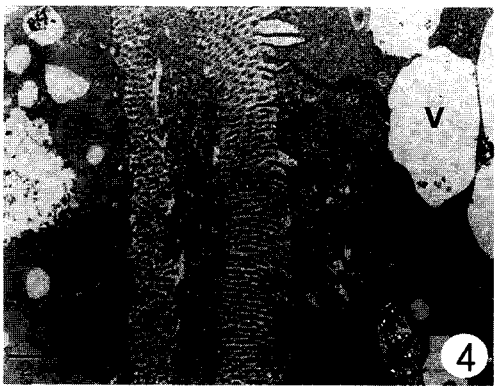


Fig. 1. Screening for carboxymethyl cellulase activity.
Figs. 2-4. Epithelial cells of the digestive gland not showing any cellulase activity in the Benedict's reactions. MV; microvilli, Sc; secretory granule, V; vacuole

2% OsO_4 for 30 minutes. Dehydration was performed in 30% alcohol for 30 minutes at 0℃, embedded in Spurr mixture in routine method, and incubated for 4 days at relatively low temperature (55℃) to prevent enzyme denaturation. The thin-sections (70 nm) obtained with ultramicrotome were loaded on the nickel grids.

7) Immunogold (protein-A gold) labelling and Observation

To eliminate nonspecific antigen-antibody reaction, the specimens were treated with 3% BSA (bovine serum albumen). The each of the nickel grids with specimen was floated on the top of a drop of phosphate buffered primary anti-cellulase (pH 6.0) in upside down position for primary reaction, incubated in the humidity chamber for overnight at 4℃, and washed three times with PBS for 5 minutes per each time. For the immunogold labelling, the specimens were exposed to the secondary antibody (20 nm protein-A gold-conjugated anti-rabbit IgG, Sigma) diluted with PBS (pH 7.4) in the ratio of 100:1, incubated in the humidity chamber for 3 hours at room temperature, washed three times with PBS for 5 minutes per each time by the floating method mentioned above, and finally washed with secondary distilled water by dipping method.

The immunostained specimens were double stained with uranyl acetate(for 10 minutes) and lead citrate (for 2 minutes), and observed with JEM-1010 transmission electron microscope.

RESULTS

Through a series of examinations conducted to confirm the cellulase activities in the epithelia of the accessory digestive glands (the mucus gland, the salivary gland, and the digestive gland), the following results were obtained.

1. Cellulase Activity Confirmation by the Screening Method

The digestive gland was the only accessory gland which showed cellulase activity in the screening method for carboxymethyl cellulase. The 1% agar medium containing 0.1% carboxymethyl cellulose was partly hydrolyzed at the place spotted with the tissue fluid of the digestive gland and was not stained with Congo Red. But the other media spotted with the tissue fluids of the mucus gland and the salivary gland were stained with the dye Congo Red (Fig. 1).

2. Cytochemical Examination

The digestive gland epithelium which was experienced Benedict's reagent did not show any product of the Benedict's reaction under the transmission electron microscopy (Figs. 2-4).

3. Histochemical Study

Total three cell types were classified according to the results of the histochemical examination which was carried out under the light microscope (LM).

Type 1 (T1) cell was majority in number and was stained blue in the upper part of the cytoplasm but the lower part of the cytoplasm was stained red in the PAS-Alcian blue stain (Figs. 5-7). Overall cytoplasm of this type cell was positive to the PAS, and stained blue in a double stain with methylene blue-basic fuchsin (Fig. 8).

Type 2 (T2) cell was rather oval in shape with wide basal part. This cell possessed cytoplasm stained purple and nucleus was stained deep purple in the PAS-Alcian blue stain. The overall cytoplasm of this type cell was stained red in PAS (Figs. 6, 7).

Type 3 (T3) cell showed the same reaction to T1 cell to the stains applied, but the T3 cell possessed cilia on the apical surface of the cell (Figs. 5-7).

4. Ultrastructure Observation

Types of the epithelial cells of the digestive gland were classified into five in the observations under the transmission electron microscope (TEM).

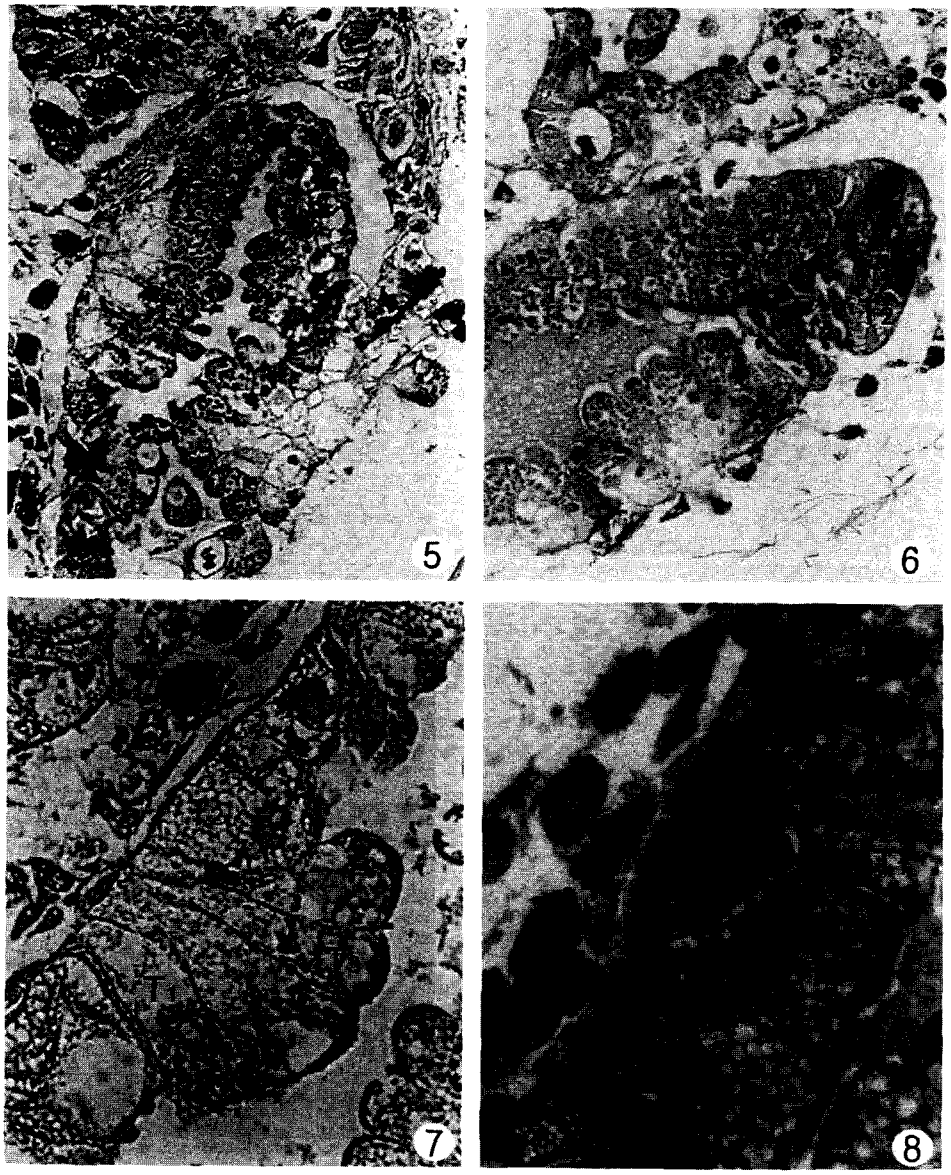
Type 1 (T1) cell was columnar cell covered with microvilli on the apical surface. The microvilli were 0.8 - 0.9 μm in length according to scanning electron microscopy (Figs. 11, 12).

The cytoplasm of T1 cell showed low electron density and its nucleus was situated in the middle part of the cell (Fig. 15). T1 cell contained a lot of mitochondria in the apical cytoplasm, and numerous secretory granules in the upper part of the cytoplasm. The secretory granules in the T1 cell cytoplasm showed high and low two grades in electron density (Figs. 10, 16). The

epithelial cells were connected with neighboring cells with tight junctions and zonula adherens.

Type 2 (T2) cell was rather oval in shape due to its smaller and wider feature than the T1 cell. This cell possessed microvilli on the luminal surface and its nucleus was situated in the middle part of the cytoplasm showing moderate electron density. The T2 cell contained numerous secretory granules in the cytoplasm (Fig. 13).

Type 3 (T3) cell was generally same to the T1 except possession of cilia on the apical surface of the cell. The cilia observed under LM, SEM, and TEM were 10 μm in length. The T3 cell also contained numerous secretory granules in the cytoplasm (Figs. 13).



Figs. 5-7. The digestive gland epithelium stained with PAS-Alcian blue. The apical cytoplasm of Type 1 (T_1)and 3 (T_3) cells are stained blue. But, the lower part of the cytoplasm of these cells are stained red. Type 2 cells (T_2) are stained red.

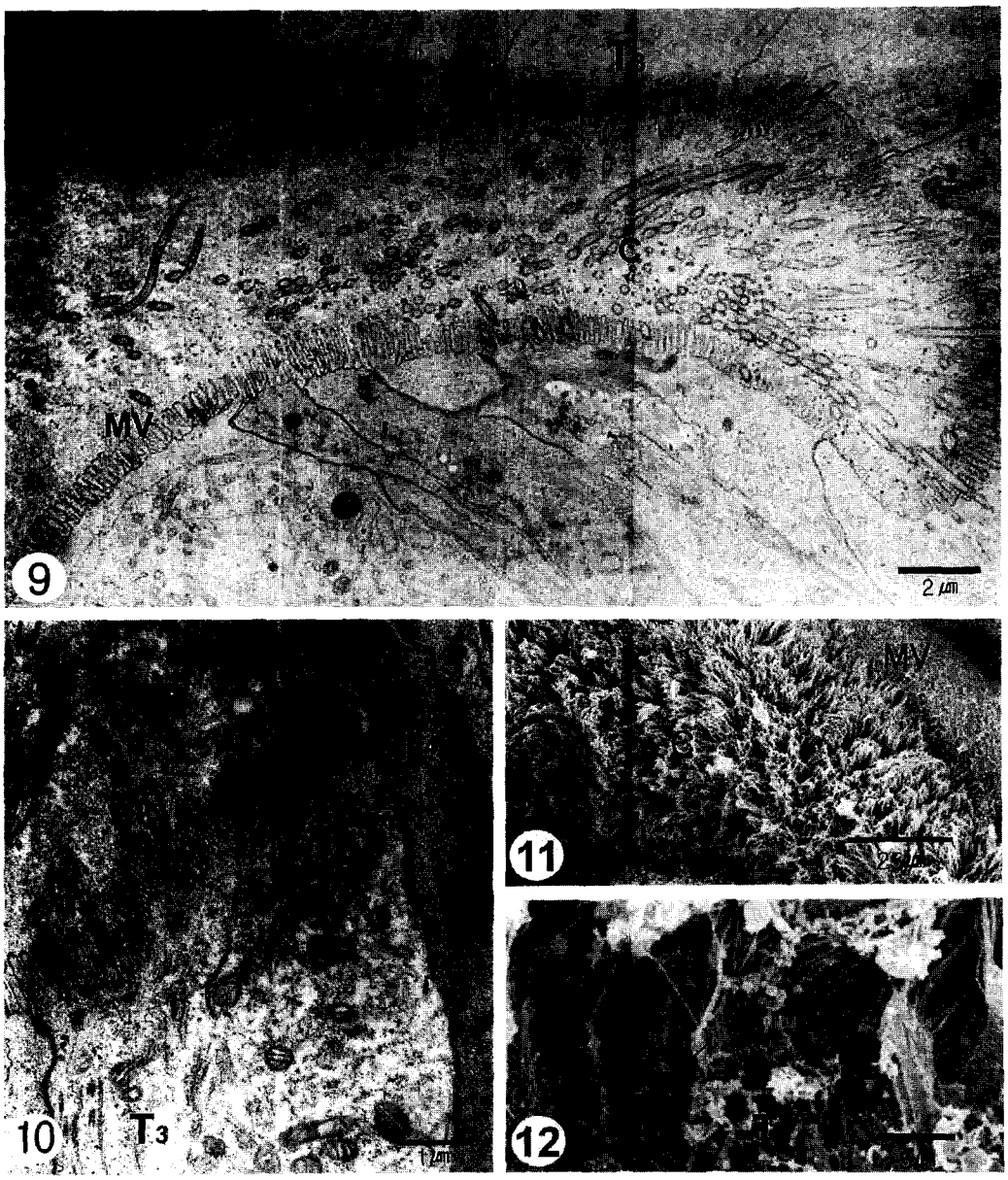
Fig. 8. The digestive gland epithelium stained with methylene blue-basic fuchsin. The digestive gland epithelium stained blue.

Type 4 (T4) cell possessed cytoplasm in high electron density, and it contained electron dense granules in the apical cytoplasm. The organelles of this type cell were not observed well (Fig. 10).

Type 5 (T5) cell in slender form possessed microvilli and cilia on the apical surface, and contained numerous secretory granules in the cytoplasm showing relatively high electron density (Fig. 14). The T4 and the T5 were not found under the LM observations.

5. Immunogold (protein-A gold) Labelling

The protein-A gold particles were labeled in the T1 and T3 cells. They were selectively labeled on the



Figs. 9-10. Type 1 (T_1) and 4 (T_4) cells possess microvilli on the apical surfaces microvilli (MV) . Type 3 cell (T_3) possesses microvilli and cilia (C) on the apical surfaces.

Figs. 11-12. Scanning electron microscopic view of the digestive gland epithelium showing microvilli (MV) and cilia (C).

cytoplasm around the rough endoplasmic reticula(rER), on the membrane of the rER. The gold particles also were labeled on the secretory granules in lower electron density. The gold particles were not labeled on either of mitochondria, electron dense secretory granules, and the lumen of the digestive gland (Figs. 17-21).

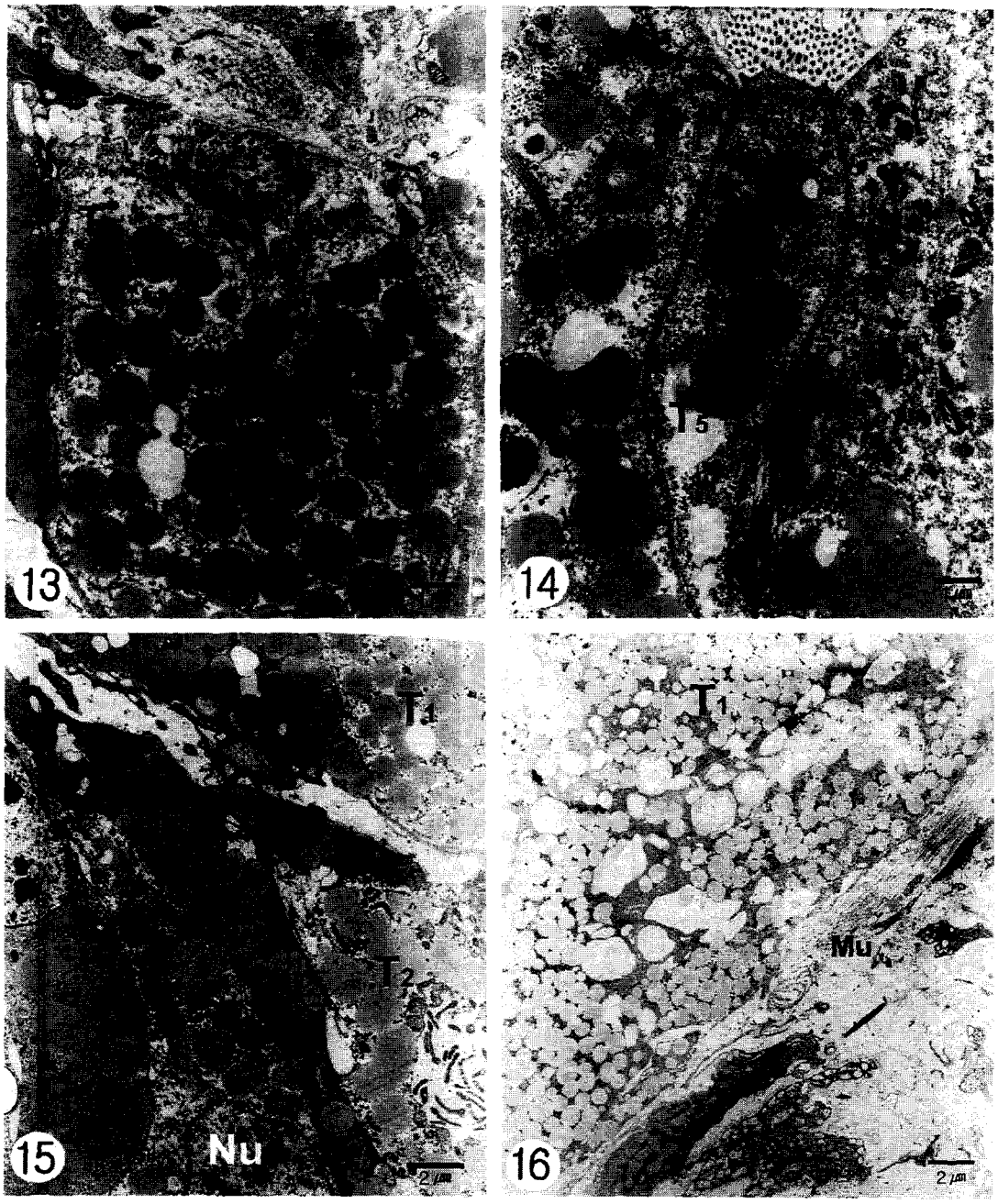


Fig. 13. Type 2 cell (T_2) containing numerous secretory granules (SG).
Fig. 14. Type 5 cell (T_5) in slender form in shape shows secretory granules contained.
Fig. 15. Type 5 cell (T_5) situated between the neighbouring Type 1 (T_1) and Type 2 (T_2) cells. Nu; nucleus
Fig. 16. Type 1 cell (T_1) containing numerous secretory granules. Mu; muscle

DISCUSSION

The each class or order of the Mollusca has big variation in feeding habits and digestion processes. The digestive processes differ in the various molluscan classes, and it is not possible to make a generalized statement on digestion in the Mollusca as a whole.

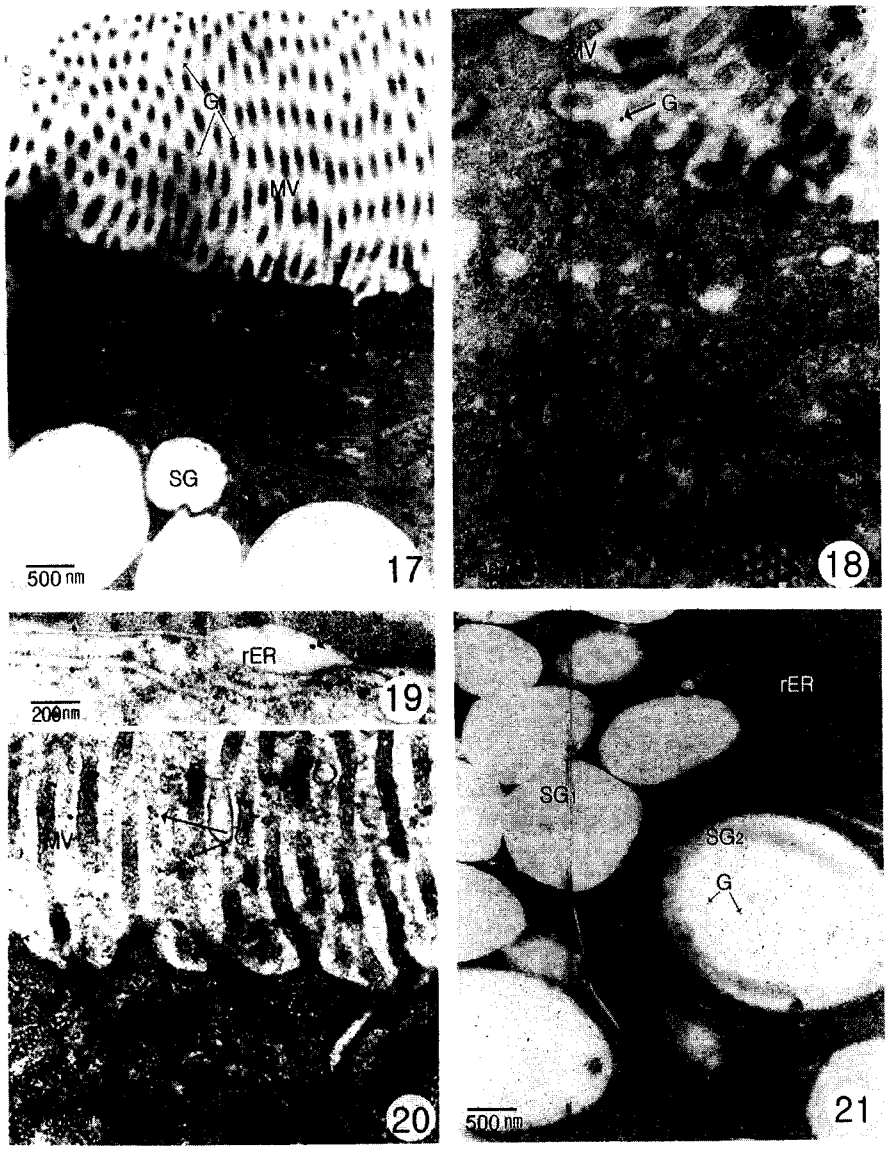


Fig. 17. Apical portion of Type 1 cell labeled with the protein-A gold particles (G). MV; microvilli, SG; secretory granule

Fig. 18. Apical portion of Type 1 cell labeled with the protein-A gold particles (G).

Fig. 19. The gold particles (G) are labelled on the rER.

Fig. 20. High magnification of the apical part of the Type 1 cell labeled with the protein A-gold particles (G). MV; microvilli

Fig. 21. The gold particles (G) are labeled on the one type (SG_2) of the secretory granules. SG_1; Secretory granules in moderate electron density. SG_2; Secretory granules in lower electron density. These granules are selectively labelled with the protein-A gold particles.

Even the Bivalvia, which may appear rather uniform in their habit of feeding on small particle, are not a homogeneous as regards their digestive processes.

In the Bivalvia, the crystalline style is involved in digestive function in certain patterns by species. This is an elongated organ hyaline rod lying in a separate diverticulum, the style sac, with an intermittent rotary motion. In the earlier study, Coupin (1900) found an amylase and Berkeley (1923) found an oxidase associated with the crystalline style. They mentioned that style-bearing organisms, and lamellibranchs are considered to be capable of extra cellular digestion only of starch or glycogen. Yonge (1931) stated that the remainder of digestion by the two enzymes is carried out by wandering phagocytes. In spite of the fact that the bivalves feed on organic detritus containing large amount of cellulose, no cellulase was detected. In this connection Boswell (1941) observed that the breakdown of cellulose by living fungi appeared to involve an oxidase as well as a cellulase with the production of uronic acid. In the oyster, *Ostrea edulis* (Yonge, 1926), the style showed none of proteolytic or lipolytic activity. Lavine (1946) presented an evidence that the crystalline styles of the clams *Mya arenaria* and *Mactra solidissima* are incapable of digesting pure cellulose, but regenerated cellulose. There for he stated that the style contains a cellulose, and presumably a β-glucosidase active towards β-glucosidic linkage as well as amylase system which degrade the α-glycosidic linkages of starch and glycogen. Owen (1956) observed the stomach and digestive glands of 4 species belonging to Nuclidae (Lamellibranchia) and reported that the stomach is relatively large and possesses a well-developed style sac, although a rod-like crystalline style is absent. He also suggested here that while some mechanical trituration occurs in the stomach, enzymes are also present, according to the fact that he could not find any diatom or protozoan skeleton in the stomach. He found out that the epithelium of the style sac secretes an amylase and a lipase, and the extracts of the digestive gland contains an amylase, a lipase, and a protease.

In the Gastropoda, every type of feeding habit is exhibited. There are herbivores, carnivores, scavengers, deposit feeders, suspension feeders, and parasites. It is easily supposed that the gastropods have remarkable variation in digestion process.

The herbivorous gastropods include some marine prosobranchs, the freshwater prosobranchs, the operculate land snails, a variety of ophistobranchs, and the majority of the pulmonates. The most marine species feed on fine algae that can be rasped from a rock or other surfaces, or on large algae, such as kelps, that can carry the weight of the snail (Steneck and Walting, 1982). The freshwater and landforms also consume the tender parts of aquatic and terrestrial vascular plants. A few terrestrial snails and slugs are serious agricultural pests. The giant African land snail *Achatina fulica* which is introduced to other countries as an edible snail even in Korea, is known as a destructive species. The oriental land snail *Nesiohelix samarangae*, used in the present study, also is known as the most destructive land snails in Korea.

The digestion process in the herbivorous gastropods has been one of interesting subjects for investigators. The most interesting subject has been the cellulase, which is the one of several hydrolases that may be present in certain part of the intestinal tracts.

Cellulases have been found in a number of marine form: in the abalone, a archaeogastropod, *Haliotis giganteus*; in the sea hare, *Dolabella scapula* (Hashimoto and Onoma, 1949); in *Cryptochiton stelleri* (Meeuse and Fluegel, 1958), and in some other species.

Galli and Giese (1959) who had careful observations in the field and examination of extracts of the foregut and midgut of a marine snail, *Tegula funebralis*, mentioned that this species demonstrated to digest not only the reserve carbohydrates (starch and laminarin), but also some of the structural polysaccharides (cellulose, alginic acid, fucoidin, and iridophycin). In all cases measurable amount of reducing sugar appeared with incubation of gut extract and the polysaccharides, thus demonstration hydrolysis. They also suggested that intestinal flora did not appear to play a very useful part in digestion of algal cellular material since a majority of species cultured, including those present in the largest numbers, were incapable of attacking any of the structural polysaccharides studied.

On the digestive tract of a land snail, Holden and Tracey (1950) reported wide variety of substrate which are attached by *Helix* sp. digestive juice. Myera and Northcote (1958) investigated gastro-intestinal extract of *Helix pomatia* to assess the relative amounts of cellulase and other enzymes. They quantatively analysed proteinases, lipases and twenty different

carbohydrases including cellulase.

The cellulase was assayed by measuring the reducing sugar formed from Cellofas B solutions, as in the determinations of cellobiase, and also by the production of reducing sugars from phosphoric acid-swollen cotton linters(an insoluble substrate).

Recently, Maeda *et al.* (1996) purified a cellulase with activity as a cellobiohydrolase (EC3.2.1.91) from the intestinal juice of the giant snail *Achatina fulica*.

Jeong and Lee (1997b) confirmed cellulase activity in the stomach epithelium of the oriental land snail *Nesiohelix samarangae* with cytochemical and immunogold labelling methods. Jeong *et al.* (1998) investigated the epithelia of the digestive tracts such as the esophagus, gizzard, and intestine of the snail *N. samarangae*. They also confirmed cellulase activity in the epithelium only of the intestine with the same methods. Present study was emphasized in localizing the cellulase activity in the accessory glands belonging to the digestive organ with the immunogold labelling method.

Present study should be focalized on the digestive gland because this gland was the only accessory gland showing positive reaction in the screening method for carboxy methyl cellulase. This screening method has been known as one of the most effective methods for the detection of endoglucanase activity. This method is based on the specific interaction of direct dyes such as Congo Red with polysaccharides.

Based on the TEM observations, the epithelial cells of the digestive gland were classified into five types even though two of them, T4 and T5 were not identified under LM.

According to the reactions against the histochemical dyes applied, the secretory granules found in the lower part of the T1 and T3 epithelial cells seem to be neutral mucopolysaccharides, otherwise the granules observed in the upper part of the T1 cell seem to be acid mucopolysaccharides. These two types of the epithelial cells supposed to secrete the cellulase in the digestive gland. The amount of cellulase secreted by the digestive gland is probably very little because it was not detected in the cytochemical examination, but in screening method for CM-cellulase.

It was reported that the secretory cells of the digestive glands of a freshwater mesogastropod, *Pomacea canaliculata* provide strong cellulase and digestion is exclusively extracellular, in the stomach (Andrews, 1965). The results obtained from the present study, conducted in cellular level, strongly support the above report saying that digestive gland secrete cellulase. The amount of cellulase secreted by the digestive gland of *N. samarangae* seems to be relatively little in compare with that secreted by the stomach and the intestine.

When review the cellulase activities in the digestive system of the oriental snail *N. samarangae*, depend on the results obtained from a series of immuno-cytochemical studies so far reported since Jeong and Lee (1997b), the most important digestive enzyme in the herbivorous land snail, the cellulase, is secreted from the epithelial cells of the stomach, the intestine, and the digestive gland.

ACKNOWLEDGEMENT

This work was supported by grant from the basic science institute supporting program of the Ministry of Education, Korea in 1998.

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