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Biosynthesis of *Astacus* protease, a digestive enzyme from crayfish*

G. Vogt[1, **], W. Stöcker[2], V. Storch[1], and R. Zwilling[2]

[1] Zoologisches Institut I (Morphologie/Ökologie), Universität Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg, Federal Republic of Germany

[2] Zoologisches Institut II (Physiologie), Universität Heidelberg, Im Neuenheimer Feld 230, D-6900 Heidelberg, Federal Republic of Germany

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Summary. For the first time, the site of biosynthesis of a well characterized invertebrate digestive enzyme is localized. The enzyme chosen, *Astacus* protease, is a zinc-metalloenzyme occuring in high concentration in the gastric fluid of the freshwater crayfish *Astacus astacus*. Enzyme production was stimulated in adult crayfish either by feeding or by removal of the gastric fluid. Immunohistochemistry, cytology and investigation with radioactive tracers demonstrate that in the hours following stimulation, new enzyme was produced in the F-cells of the midgut gland and subsequently discharged into the midgut gland lumen. The enzyme was then accumulated and stored extracellularly in the cardiac stomach in active form. The mechanism of enzyme production observed in *Astacus* differs considerably from vertebrates suggesting an alternative model for synthesis and storage of digestive enzymes.

Introduction

The midgut gland (hepatopancreas) of decapod crustaceans is generally considered to play a central role in digestion (review articles: Gibson and Barker 1979; Dall and Moriarty 1983). Its involvement in absorption and further intracellular catabolism of digestion products and in storage of lipid and glycogen has been deduced from a considerable number of morphological (e.g.: Stanier et al. 1968; Storch et al. 1982; Vogt et al. 1986) and physiological (e.g.: Speck and Urich 1972; Dall 1981) studies. The organ was first interpreted as liver (for literature see Weber 1880). Since Krukenberg (1878) demonstrated enzyme activities in the homogenate of the midgut gland, the pancreatic function of the organ was apparently established. It must be noted, however, that all liquid material which can be isolated from the cardiac stomach is eventually transported into the midgut gland tubules. Therefore, enzymes produced elsewere, but secreted into the cardia would ultimately also appear in the hepatopancreas homogenate. Thus, the basis for the hypothesis that the midgut gland is the site of biosynthesis of the digestive enzymes is potentially in error.

Hirsch and co-authors (Hirsch and Jacobs 1928, 1930; Hirsch and Buchmann 1930) tried to identify the enzyme producing cells by correlating the enzyme levels in the hepatopancreas homogenate with the quantity of the four cell types, i.e. E-cells, R-cells, F-cells and B-cells. Since then, it has been generally accepted that the digestive enzymes are discharged by holocrine secretion of the B-cells (Dall and Moriarty 1983; Caceci et al. 1988). Electron microscopy, however, demonstrated that cytological features of F-cells resemble those of the acinus cells of the mammalian pancreas (Loizzi 1971; Jamieson and Palade 1971) and, therefore, they appear as primary candidates for sites of enzyme production.

In the present study, the intracellular site of biosynthesis is localized directly and unambiguously through the use of specific antibodies against a well characterized digestive enzyme. For this purpose, *Astacus* protease, a digestive enzyme of the freshwater crayfish *Astacus astacus* (EC 3.4.99.6), was chosen. The enzyme is one of only four proteases in decapods for which the complete amino acid sequence is known and thus one of the best characterized invertebrate digestive enzymes. It is a zinc-metalloproteinase (Stöcker et al. 1988) consisting of a single 200 amino acid polypeptide chain and has a molecular weight of 22.614 (Titani et al. 1987).

Materials and methods

Animals. Adult males of *Astacus astacus* (L.) 1758 (Crustacea, Decapoda) were obtained from a commercial crayfish farm (Keller, Augsburg, FRG) and adapted to laboratory conditions for 4 weeks. The size range at the beginning of the experiments was 19.2–36.4 g with a mean of 29.2 g. The hepatopancreatic-somatic index, a parameter for the health of the animals, was 5.7 ± 0.8. All experiments were conducted in $120 \times 80 \times 20$ cm plastic tanks which could be divided into individual boxes. The tanks were filled with aerated fresh water of approximately 18° C. Water was changed every second day. Lighting was natural. For all studies only animals in intermolt stages were used.

The crayfish were fed every second day with minced pork enriched with vitamins and minerals. This food was chosen due to its high content of lipids. In our earlier experiments, feeding of lipid diets led to accumulations of lipid deposits in the R-cells of *Penaeus monodon* (Vogt et al. 1985). Therefore, since R-cells and F-cells are difficult to distinguish, lipid droplets can act as a "marker" for the R-cells. Application of this pre-experimental measure in *Astacus* should make it possible to differentiate R- and F-cells in light microscopy even without staining.

Experiment 1

Food digestion and refilling of the cardiac stomach in a normal digestive cycle and after artificial emptying of the stomach. Crayfish were starved for 1 week to exclude any overlapping of pre-experi-

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** To whom offprint requests should be sent

mental and experimental digestion processes. Thereafter, 21 crayfish were fed individually with 0.2 g minced meat. For feeding, the animals were removed from the water and the food was placed with a forceps directly onto the mouth to control the exact time of ingestion. At hourly intervals for 7 h, groups of 3 crayfish were removed and the content of the cardia was collected by inserting a curved glass capillary through the oesophagus. The amount and consistency of both the food and the gastric fluid were chosen as parameters to evaluate the status of food processing.

In addition, the gastric fluid from three unfed crayfish was drained to stimulate the refilling of the cardia under artificial conditions. The fluid of these animals was collected again after 6 h.

Experiment 2

Radioactive labeling of newly synthesized gastric fluid. Three crayfish were starved for 1 week. Then L-(4,5-^{3}H)-leucine (10 µCi = 0.37 MBq per specimen, specific activity 5.66 TBq/mmol, supplied as aqueous solution, Amersham Buchler, Braunschweig, FRG) was injected into the hemolymph. Ten minutes after injection, de novo production of digestive enzymes was stimulated by the complete removal of the gastric fluid. Newly synthesized fluid was collected from all animals 1, 2, 3, 4, 5, 6, 8, 10, 24, 32 and 48 h after stimulation. For determination of radioactivity in the gastric fluid, 0.1 ml of the fluid was mixed with 3.6 ml PCS™-liquid scintillant (Amersham/Searle, Arlington Heights, IL, USA). Incorporation of the label into protein was measured in aliquots of some samples by precipitation of the proteins with trichloro acetic acid (TCA). For this purpose 0.1 ml gastric fluid was mixed with 0.1 ml 10% TCA. After centrifugation in an Eppendorf centrifuge (Eppendorf, Hamburg, FRG) for 5 min at 12000 × *g*, 3.6 ml PCS™-scintillant was added to 0.1 ml of the supernatant. Radioactivity of the TCA-pellet was calculated by difference from the values of the supernatant. Samples were counted in a Beckman liquid scintillation counter LS 8000 (Beckman, München, FRG).

Experiment 3

Immunohistochemistry. For immunohistochemistry, antiserum against *Astacus* protease was prepared. The enzyme was isolated and purified from the gastric fluid by a combination of anion-exchange chromatography and gel filtration according to Zwilling and Neurath (1981). The purity of the antigen was tested by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) in a custom-built vertical slab gel apparatus.

Antiserum was prepared in rabbits by subcutaneous injection of a solution of 1 mg *Astacus* protease dissolved in 0.5 ml *Tris*/HCl buffer (0.1 *M*, pH 8.0) and emulsified with 0.5 ml complete Freund's adjuvant (Difco, Detroit, USA). Booster injections were given at 2-week intervals. Small aliquots of blood were withdrawn and the titer of the antisera was estimated by Ouchterlony immunodiffusion (Ouchterlony 1958). Therefore, 20 µg *Astacus* protease dissolved in 0.1 *M Tris*/HCl buffer, pH 8.0, was tested against 20, 10, 5 and 2.5 µl pure serum in a 1% agarose gel (Serva, Heidelberg, FRG) in 0.02 *M* sodiumbarbiturate buffer, pH 8.6. Rabbits were bled when the enzyme was precipitated by 2.5 µl serum. The blood was allowed to clot overnight and the clot was removed by centrifugation. Sera were stored at −80° C.

Specificity tests with 20 µl anti-*Astacus* protease antiserum were performed by Ouchterlony immunodiffusion against *Astacus* protease (20 µg), trypsin (20 µg), carboxypeptidase (20 µg), crude gastric fluid (40 µl) and hemolymph (40 µl) obtained from *Astacus astacus*. Trypsin and carboxypeptidase were isolated from the gastric fluid of *Astacus* by affinity chromatography with immobilized soybean trypsin inhibitor and potato carboxypeptidase inhibitor, respectively (Zwilling and Neurath 1981). For immunodiffusion, the three enzymes were dissolved in 0.1 *M Tris*/HCl buffer, pH 8.0. Hemolymph was withdrawn from the abdomen of crayfish and mixed 1:1 with 0.1 *M* sodium citrate buffer, pH 6.9, to prevent coagulation.

The purity of the anti-*Astacus* protease antisera was tested against crude gastric fluid by two-dimensional Laurell immunoelectrophoresis (Laurell 1965) in a LKB 2117 Multiphor apparatus (LKB, Gräfelfing, FRG).

To locate the site of biosynthesis, 21 crayfish were starved for 1 week. Then production of digestive enzymes was stimulated by draining the gastric fluid. Groups of three specimen were sacrificed immediately and at 1 h intervals for the next 6 h. The midgut glands were processed for light and electron microscopy.

For light microscopy, the tissues were fixed either in a modified Bouin's solution (15 parts picric acid and 5 parts formaldehyde, pH 3.7) or in Susa-fixative (pH 2.5), then dehydrated in 70%, 80%, 90%, 95% and 100% ethanol and embedded in paraffin using methylbenzoate as intermedium. Sections for routine light microscopical investigations of the midgut glands were deparaffinized in xylene and graded ethanol and then stained with hematoxylin (6 min) and erythrosin (3 min).

Astacus protease was localized by indirect immunofluorescence. Sections (6 µm thick) were deparaffinized and incubated at room temperature for 2 h in anti-*Astacus* protease antiserum diluted 1:10 with 0.15 *M* PBS (phosphate buffered saline), pH 7.2. After three 15-min washings in PBS, the sections were overlaid for 1 h with FITC-conjugated anti-rabbit-IgG from goat (Sigma, Deisenhofen, FRG) diluted 1:40 in PBS. Then the sections were rinsed with PBS, stained with 1% Evans blue and mounted either with PBS or glycerol/PBS 9:1, both containing 25 mg DABCO (Sigma, Deisenhofen, FRG) per ml to stabilize fluorescence. For examination of the slides Leitz Aristoplan (filters BP 450-490, RKP 520, LP 515; Leitz, Wetzlar, FRG) and Zeiss IM 35 (filters BP 450–490, FT 510, BP 520–560; Zeiss, Oberkochen, FRG) microscopes were used. The results were documented with Ilford Pan F films (50 ASA).

Control screenings were performed by treating with only one of either *Astacus* protease antiserum, FITC-conjugated anti-rabbit IgG, Evan's blue or PBS. Additionally, the entire immunohistochemical procedure was carried out with normal rabbit serum.

For electron microscopy the midgut glands were fixed in cold 2.5% glutaraldehyde-0.1 *M* Sörensen's buffer solution (4° C, pH 7.2) for 2 h. The tissues were then rinsed for 30 min in Sörensen's buffer and postfixed in a 1% osmium tetroxide-Sörensen's buffer solution for another 2 h. After dehydration through 70%, 80%, 90%, 95% and 100% ethanol, the tissues were transferred to propylene oxide for 20 min and embedded in araldite (Serva, Heidelberg, FRG). Sections were cut with glass knives on a Reichert OM U-2 ultramicrotome (Reichert-Jung, Nußloch, FRG). Semithin sections were stained with Richardson solution (methylene blue – azur II) for 1 min. Ultrathin sections were mounted on copper grids and contrasted for 7 min with uranyl acetate (saturated solution in 70% methanol) and for 5 min in 10% (w/v) lead citrate. Examination was done with a Zeiss EM 9-S2 electron microscope (Zeiss, Oberkochen, FRG).

Results

Gross morphology of the digestive tract, histology of the midgut gland and "conditioning" of the tissue

The digestive tract of crayfish is composed of two duct systems: (*1*) the continuous oesophagus-stomach-gut tract and (*2*) the two blind ending lateral duct systems of the midgut gland (Fig. 1). In *Astacus*, the oesophagus-stomach-gut tract is completely coated with a cuticle. The anterior part of the stomach, called the cardia, bears cuticle teeth and serves as mill for mastication of food. Enzyme containing gastric fluid is present in the cardia at almost all times. The posterior part of the stomach, the pylorus, has a complicated filtering system which separates the fluid mixture of nutrients and digestive enzymes from the solids. This fluid is then pressed through ventrolaterally located open-

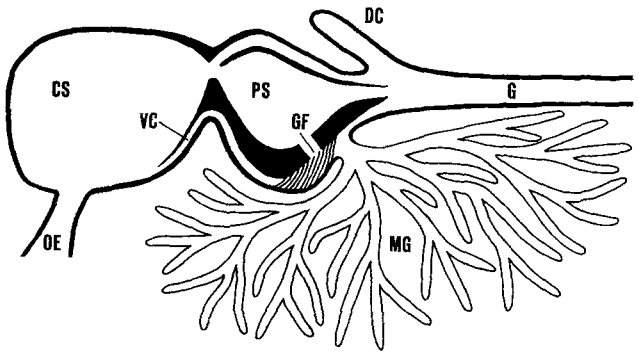


Fig. 1. Schematic diagram to show the anatomy of the digestive tract in *Astacus*. *Thick lines* indicate cuticular coated parts of the digestive tract. *CS*=cardiac stomach, *DC*=dorsal coecum, *G*=gut, *GF*=gland filter, *MG*=midgut gland, *OE*=oesophagus, *PS*=pyloric stomach, *VC*=ventrolateral channel

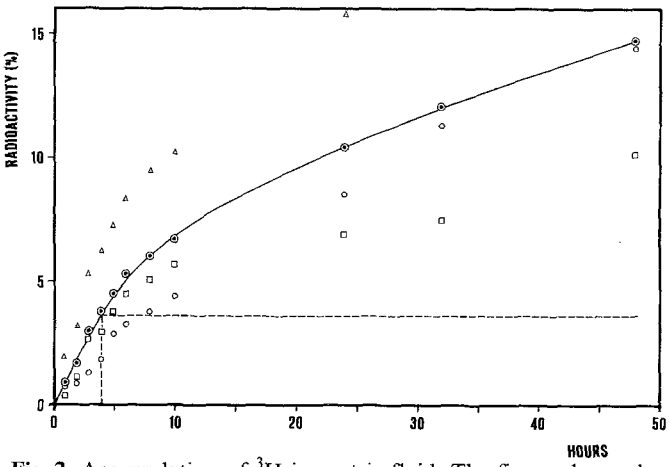


Fig. 2. Accumulation of ^3H in gastric fluid. The figure shows the cumulative radioactivity (i.e. each value was added to previous values) in the gastric fluid in the hours following the emptying of the cardia. Filled dots represent the average from three animals. The values of the three individuals are marked by the signs *square*, *triangle* and *circle*. Radioactivity is expressed as percent of the per gram dose measured in 1 ml of gastric fluid. The amount of radioactivity accumulated during the time from 10–24 h is representative of a complete, undisturbed filling of the cardiac stomach. This amount is shown with the broken horizontal line and is projected onto the cumulative curve to reveal a filling time of about 4 h

ings into the duct systems of the midgut gland, where resorption of nutrients takes place.

The hepatopancreas of *Astacus* is not a compact organ as in some other decapods such as the penaeids. It is rather divided into two halves which are not connected with each other. Each part of the organ is composed of several hundred blind-ending tubules (Fig. 1) which consist of a single cell layer. The histology of the midgut gland tubules, however, does follow the same scheme published for other decapods (e.g. Loizzi 1971; Storch and Welsch 1977; Vogt 1985; Al-Mohanna and Nott 1987). In addition to the E-cells (embryonic cells) at the tips of the tubules, three morphologically distinct cell types, R-cells (resorptive cells), F-cells (fibrillar cells) and B-cells (blister-like cells) occur in the epithelium (Fig. 5).

In light microscopy, the B-cells are easily recognizable by their large central vacuole regardless of the processing of the tissue. R-cells and F-cells can be clearly distinguished after staining with hematoxylin-erythrosin (two-component staining) since F-cells stain blue whereas R-cells show a slight red colour. In contrast, after staining with Evan's Blue (one-component staining) or in unstained sections, R- and F-cells are difficult to distinguish. Thus, for immunohistochemical investigations, a "lipid-marker" was introduced into the R-cells. As expected, the R-cells accumulated large amounts of reserve lipids after feeding of a lipid-rich diet (Fig. 12). Lipid droplets were found only in R-cells. The starvation period between this pre-experimental procedure and the experiments did not significantly reduce that marker. Thus the midgut gland tissue could be optimally "conditioned" for immunohistochemistry.

Food digestion and refilling of the cardiac stomach

During the 4 h following feeding, the food particles obtained from the cardiac stomach became gradually smaller. Their consistency changed from solid to soft and slimy. The gastric fluid, which was oily and dark brownish before feeding, was altered to milky and whitish. After 5 h, the food particles had disappeared and the gastric fluid again showed its original characteristics. Apparently, the cardiac stomach was refilled with new gastric fluid between 4 and 5 h after feeding, whereas the chymus, a mixture of food and gastric fluid, was transported to the pyloric stomach to be filtered for the eventual resorption of the nutrients in the hepatopancreas.

The cardiac stomach of unfed animals whose gastric juice had been removed was also found to be refilled after 6 h. The new gastric fluid was brown and oily, as described for the animals before induction of digestive enzyme synthesis. This demonstrates that the refilling of the cardiac stomach under artificial conditions requires approximately the same time as in a normal feeding cycle.

Radioactive labeling of newly synthesized gastric fluid

During the first 6 h after stimulation of digestive enzyme production, radioactive material was continuously accumulated in the cardiac stomach as was monitored by hourly withdrawals of gastric fluid (Fig. 2). Up to 88% of the radioactivity could be precipitated with TCA, demonstrating that it was incorporated into newly synthesized proteins. It must be noted that enzyme synthesis might have been stimulated again and again by the hourly removal of the gastric fluid. Therefore, the time span necessary for a complete refilling of the cardiac stomach was deduced from the radioactivity which was accumulated between 10 and 24 h. During this time, no gastric fluid was collected and thus the content of the stomach should represent a complete filling. From the projection of this amount of radioactivity onto the time scale, a refilling time of around 4 h is calculated (Fig. 2).

Antigen and antisera

The purity of the preparation of *Astacus* protease used for antiserum production is documented by a single band in the SDS-PAGE (Fig. 3). The polyclonal anti-*Astacus* protease antiserum was free of impurities as shown by the single precipitation peak in Laurell immunoelectrophoresis when tested against crude gastric fluid (Fig. 4). In Ouchterlony immunodiffusion tests, anti-*Astacus* protease antiserum was

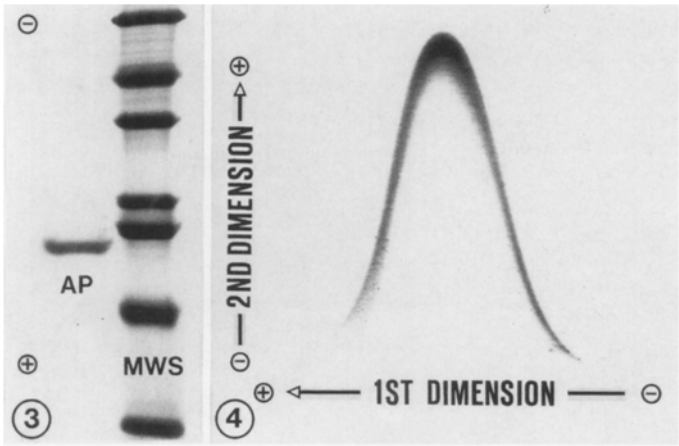


Fig. 3. Purity of *Astacus* protease used for production of antisera. SDS-polyacrylamide-gel of purified *Astacus* protease according to Laemmli (1970). Separation gel 12.5%; stacking gel 3%; 0.025 *M Tris*-buffer/0.6 *M* glycine pH 8.4; 120 µg *Astacus* protease (*AP*); 60 µg molecular weight standard (*MWS*), Dalton Mark VII-L (Sigma, Deisenhofen, FRG). MWS from top to bottom: albumin (bovine) 66000; albumin (egg) 45000; glyceraldehyde-3-phosphate dehydrogenase (rabbit) 36000; carbonic anhydrase (bovine) 29000; trypsinogen (bovine) 24000; trypsin inhibitor (soybean) 20100; α-lactalbumin (bovine) 14200. 5 h at 90 V. Staining with Coomassie Brillant Blue R 250 (Serva, Heidelberg, FRG)

Fig. 4. Purity of anti-*Astacus* protease antiserum. Two dimensional immunoelectrophoresis according to Laurell (1965). *1st dimension:* electrophoretic separation of gastric fluid, 2 µl crude fluid diluted 1:10 in sodium barbiturate/barbituric acid buffer; 1% agarose *M* gel (Pharmacia-LKB, Freiburg FRG) in 0.12 *M* sodium barbiturate/barbituric acid buffer pH 8.6; 30 min at 500 V. *2nd dimension:* electrophoresis into a gel containing antiserum against purified *Astacus* protease; 100 µl anti-*Astacus* protease antiserum per 2 ml 1% agarose *M* in 0.12 *M* sodium barbiturate/barbituric acid buffer, pH 8.6; 12 h at 300 V

precipitated by *Astacus* protease and crude gastric fluid. No crossreactions were observed with *Astacus* trypsin, *Astacus* carboxypeptidase or hemolymph proteins.

Immunohistochemistry

The immunohistochemical procedure itself produced no ambiguities, since all immunohistochemical control tests for non-specific side reactions were negative.

In the hours after induction of enzyme production, extracellular immunofluorescence was scattered throughout the lumen of the midgut gland tubules (Fig. 6) and associated with the microvillous border, particularly of the F-cells (Figs. 7 and 9). Intracellular labeling of *Astacus* protease was found only in F-cells (Figs. 7–10). Small roundish or crescent shaped areas approximately 2 to 4 µm in diameter showed strong immunofluorescence (Fig. 11). These positively reacting areas were predominantly observed in the apical part of the F-cells and around the cell nucleus. By light microscopy, no accumulation of the fluorescent areas into larger complexes was observed.

Cytology

It can be concluded from electron micrographs of the midgut gland cells that the large fluorescent cell compartments are Golgi stacks (compare Fig. 11 with Figs. 12 and 13). In synthesizing F-cells, electron dense transport vesicles are budding from the transitional elements of the rough endoplasmic reticulum (Figs. 16 and 17). They are clustered in high numbers between the rough endoplasmic reticulum and the cis face of the dictyosomes (Figs. 15 and 16). On the trans face, the secretory products of the dictyosome are packaged into vacuoles around 0.5 µm in diameter (Figs. 15 and 18). Immunohistochemistry indicates that one of these secretory products is *Astacus* protease. Since it is not clear whether the *Astacus* protease is packaged as active enzyme or as proenzyme (zymogen) these compartments will be referred to as enzyme vacuoles. After formation at the dictyosomes these enzyme vacuoles are apparently transported directly to the apical microvillous border without change in either size or histological appearance (compare Figs. 15 and 19). At the cell apex, their contents are released into the lumen of the midgut gland tubules by exocytosis (Fig. 20).

Discussion

Many reports have been published on the localization of digestive enzymes in various invertebrate taxa such as trematoda (Bogitsh and Dresden 1983), nemertini (Gibson and Egan 1976), sipunculida (Michel and DeVillez 1984), gastropoda (Onishi et al. 1985), bivalvia (Palmer 1979), oligochaeta (Prentø 1987), polychaeta (Michel and DeVillez 1979), insecta (Lehane 1976), arachnida (Ludwig and Alberti 1988) or echinodermata (Tokin and Filimonova 1977). As in crustaceans, the site of biosynthesis of digestive enzymes often was deduced from enzyme activities in tissue homogenates or evaluated by histochemistry using synthetic substrates. The first method, however, is subject to potential errors as discussed above for crustaceans. A problem in interpretation of results obtained by enzyme-histochemistry is the unsatisfactory specificity of the chromogenic or fluorogenic substrates used. Further complication arise from lack of information about the tested enzymes since only few invertebrate digestive enzymes are well characterized. The more specific immunohistochemistry with antibodies raised against purified digestive enzymes has, to our knowledge, not yet been utilized to investigate the site of biosynthesis of digestive enzymes in invertebrates.

Fig. 5. Cell types in the epithelium of the hepatopancreas. The epithelium is composed of three cell types: resorptive cells (*R*), fibrillar cells (*F*) and blister-like cells (*B*). *H* = hemolymph space, *L* = lumen of hepatopancreas tubule, *N* = cell nucleus. Bright field light microscopy; Susa's fixed; × 560

Fig. 6. Immunohistochemistry; extracellular fluorescence. Strong fluorescence after incubation with anti-*Astacus* protease antiserum is localized in the lumen of a midgut gland tubule. As a result of adequate preservation and representation of extracellular *Astacus* protease, in this photographic reproduction, intracellular fluorescence is not recognizable. Susa's fixed; × 240

Fig. 7. Extracellular and intracellular fluorescence. In addition to the lumen small cell compartments and certain cell apices show immunofluorescence. Susa's fixed; × 460

Fig. 8. Intracellular fluorescence. The positive reacting cells are F-cells. Bouin's fixed; × 180

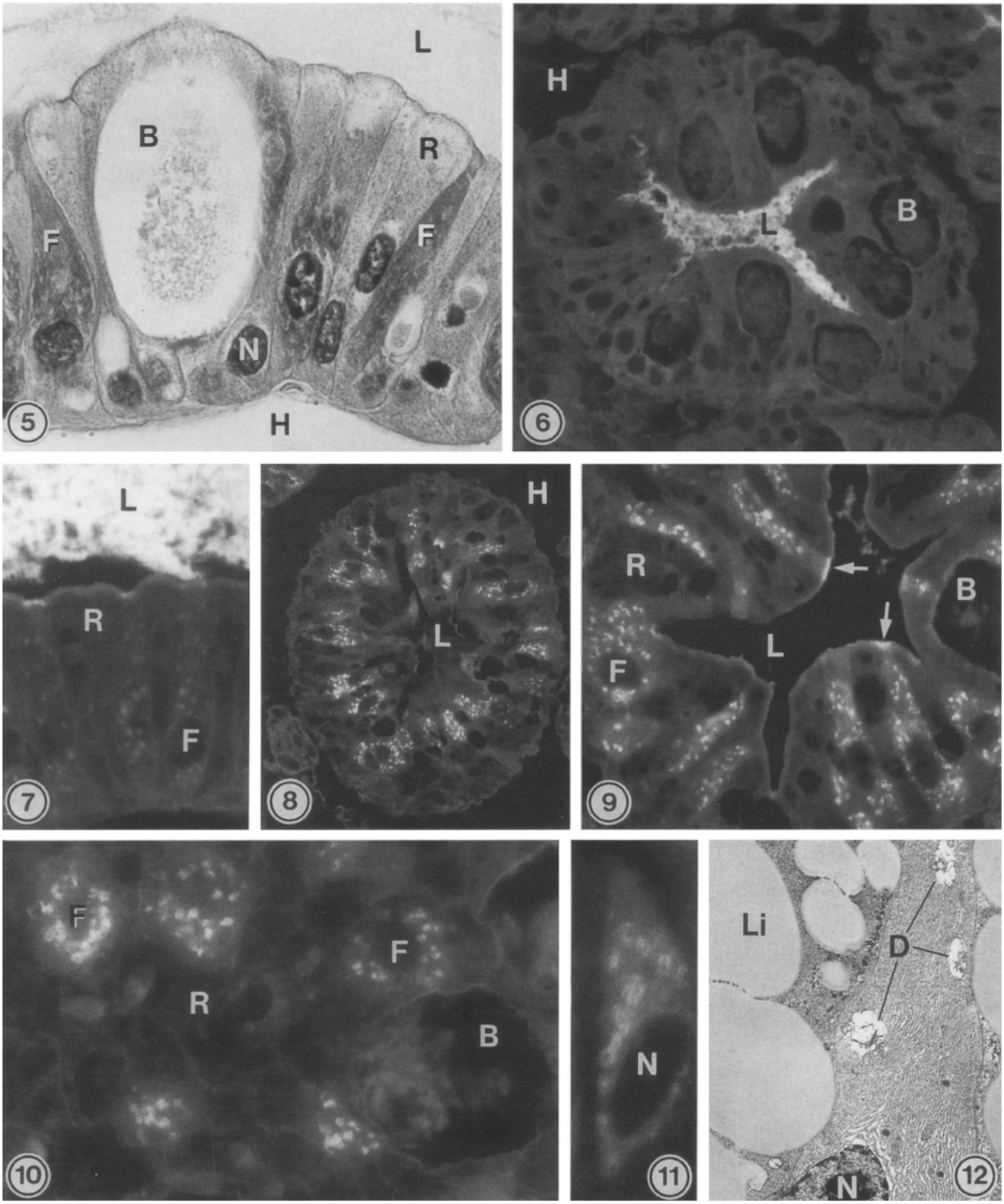
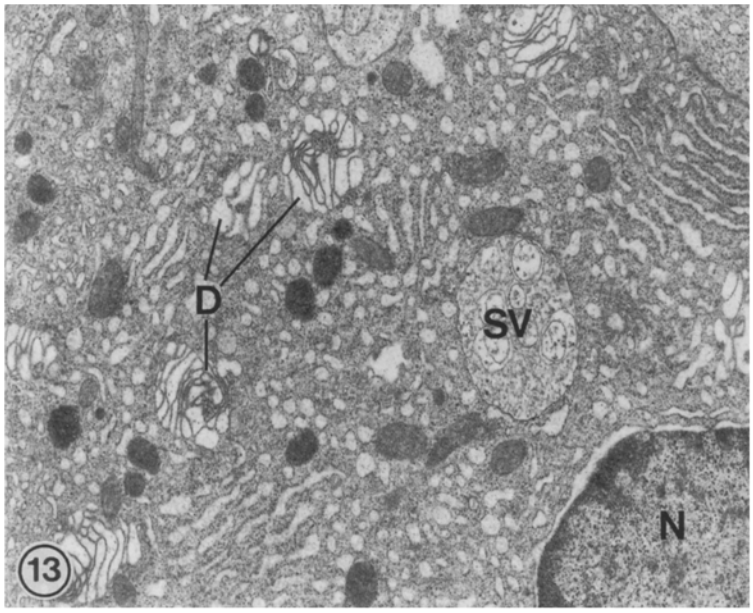


Fig. 9. Longitudinal sections through midgut gland cells. The *arrows* indicate strong fluorescence in cell apices of F-cells. Bouin's fixed; ×400

Fig. 10. Cross sections through midgut gland cells. The micrograph confirms that *Astacus* protease is localized only in F-cells. Bouin's fixed; ×840

Fig. 11. Higher magnification of a single F-cell. The immunofluorescence is mainly localized in large compartments in the apical part of the cell. Bouin's fixed; ×1070

Fig. 12. Electron micrograph of an F-cell. The positively reacting areas in Fig. 11 can be identified as dictyosomes (*D*). The cells with large lipid droplets (*Li*) are R-cells; ×2200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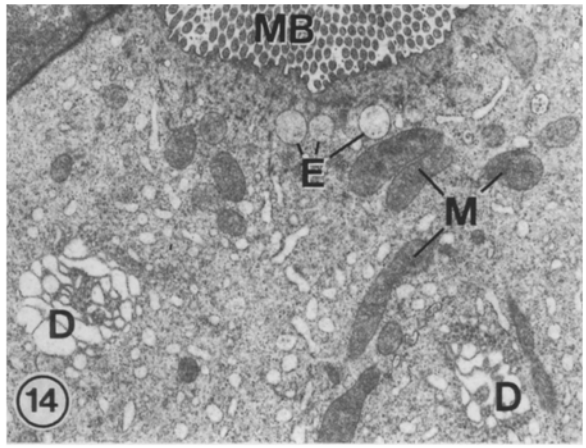
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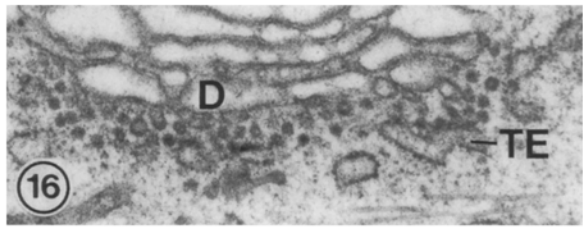
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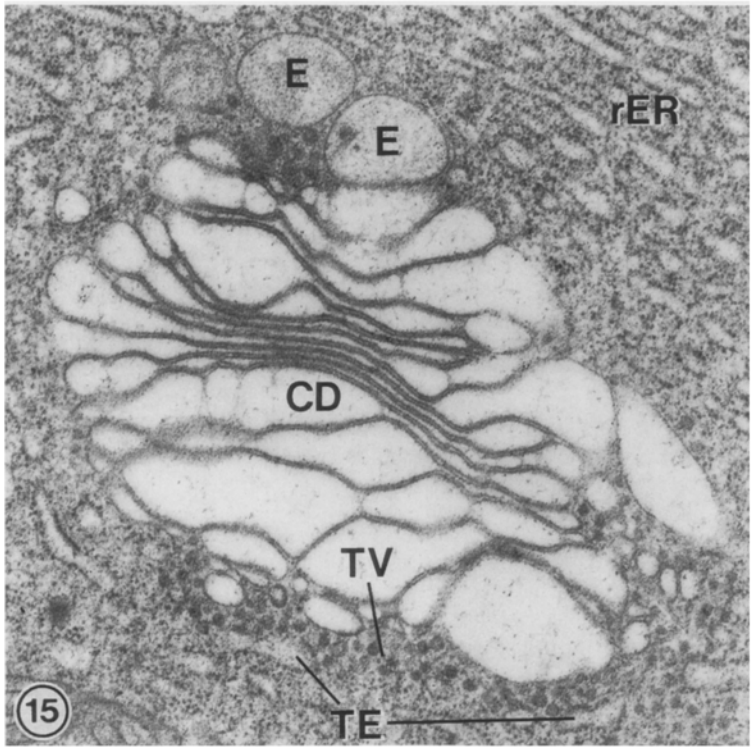
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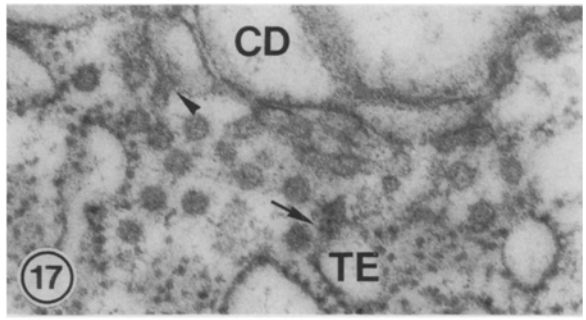
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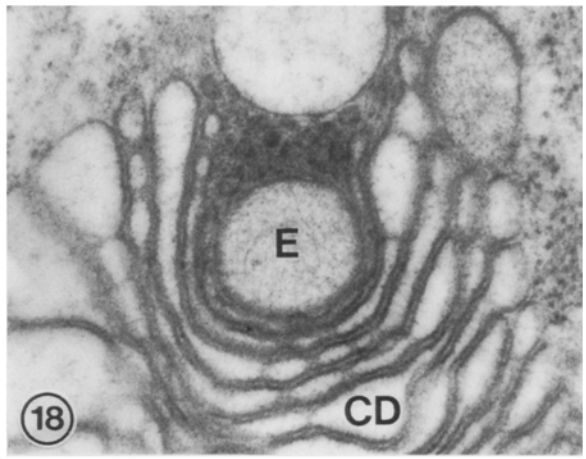
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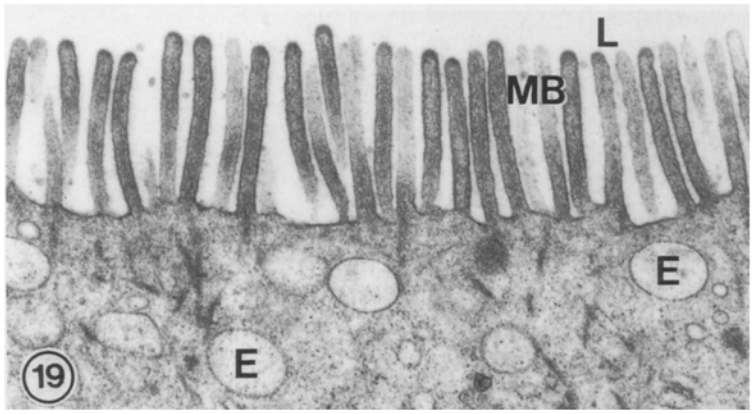
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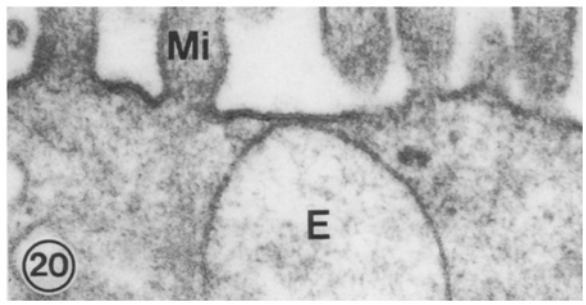
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In vertebrates, the production of digestive enzymes has been evaluated in detail during the recent decades. The intracellular occurrence of digestive enzymes was demonstrated for the first time by Marshall (1954) in the acinus cells of the vertebrate exocrine pancreas using immunofluorescence. The exact localization of the site of biosynthesis was achieved by Kraehenbuhl et al. (1977) by immunocytochemical techniques with ferritin conjugated antibodies. Bendayan et al. (1980) reevaluated the previous results with the more sophisticated immunocytochemical protein A-gold method and found that the nine digestive enzymes tested were synthesized synchronously in all acinar cells of the exocrine pancreas. Whereas the acinus of the vertebrate exocrine pancreas consists of a single cell type, the existence of three morphologically different cell types in the decapod hepatopancreas complicates the problem.

By using immunohistochemistry under experimental conditions that exclude overlapping of secretion and resorption processes, we have now clearly identified the F-cells as the site of biosynthesis of *Astacus* protease in the crayfish *Astacus astacus*. The fact that enzyme was newly synthesized was demonstrated by incorporation of ^{3}H-leucine.

From our results, a new model of production and storage of digestive enzymes can be derived which is different from the mechanisms in vertebrates. In the exocrine pancreas of mammals, newly synthesized enzymes are stored in intracellular compartments (zymogen granules) of the acinus cells as inactive proenzymes (zymogens). The formation of these compartments is very time consuming. The period from the synthesis of the pre-proenzymes at the ribosomes to the inclusion of the proenzymes in the condensing vacuoles lasts only around 30 min, whereas further 3–4 h are needed for the transformation of the condensing vacuoles to mature zymogen granules (Ermak and Rothman 1978). During this time, additional substances are incorporated into the condensing vacuoles by small vesicles or by the rigid lamella of the GERL (Slot et al. 1976; Novikoff et al. 1978). The entire process leads first to the enlargement of the vacuoles and then to the condensation of the incorporated material (Bendayan 1984). In mature zymogen granules, the enzymes exist as an ordered solid-state array (Ermak and Rothman 1978). The zymogens are discharged upon hormonal and nervous signals in response to the uptake of food (Palade 1975). After activation in the intestine, these enzymes are stable only for a relatively short period of time (Walsh 1970).

In *Astacus*, the intracellular transport of the newly synthesized enzymes from the endoplasmic reticulum to the dictyosomes via transport vesicles seems to be similar to the vertebrate system. The following cytologically visible steps of enzyme processing, however, are in contrast to the vertebrate model. From the combined application of immunohistochemistry and cytology and from the time schedule of the production of gastric fluid, it can be concluded that the enzyme vacuoles in *Astacus* are transported directly from the dictyosomes to the cell apex without any change in size or appearance. There, they are discharged into the midgut gland lumen by exocytosis. Vacuoles resembling the zymogen granules of vertebrates can be found neither in the hours following the artificial emptying of the cardiac stomach nor after feeding of the crayfish. Moreover, cytological structures which are involved in the formation of zymogen granules are missing. The continuous refilling of the cardia with gastric fluid after artificial stimulation of enzyme production and the observation that no significant accumulation of enzyme vacuoles occurred in the F-cells of the hepatopancreas are consistent with this interpretation.

From the midgut gland tubules, the digestive enzymes are transported to the cardiac stomach where they are accumulated and stored extracellularly. As reported by Zwilling and Neurath (1981) for *Astacus* trypsin, *Astacus* carboxypeptidase and *Astacus* protease the enzymes in the cardia are fully active. They remain stored in the cardia in active form until the next food uptake which often takes place several days after enzyme synthesis. Thus, the function of zymogen granules in the exocrine pancreas cells of vertebrates as intracellular storage compartment of digestive enzymes is replaced in *Astacus* by the extracellular storage of digestive enzymes in the cardiac stomach.

Ultrastructural features similar to the F-cells of *Astacus* have been observed in *Penaeus monodon* (Vogt 1985) and *Penaeus semisulcatus* (Al-Mohanna et al. 1985). Vacuoles resembling the zymogen granules of vertebrates have never been reported, neither for fed decapods (Gibson and Barker 1979; Dall and Moriarty 1983; Al-Mohanna et al. 1985; Merrill et al. 1985; Vogt 1985; Caceci et al. 1988), nor in the starved species *Hyas araneus* (Storch and Anger 1983) and *Penaeus monodon* (Vogt et al. 1985) nor in the present study of *Astacus astacus*. In vertebrates, high amounts of

Fig. 13. Middle region of an F-cell. Large dictyosomes (*D*) are characteristic features of F-cells. *SV*=supranuclear vacuole, *N*=cell nucleus; ×9500

Fig. 14. Apex of an F-cell. During the period of digestive enzyme synthesis, enzyme vacuoles (*E*) can be found in F-cells along the microvillous border (*MB*). *M*=mitochondria; ×9600

Fig. 15. Dictyosome. The cis face of a dictyosome in *Astacus* protease synthesizing F-cells is characterized by transport vesicles (*TV*) and the trans face by enzyme vacuoles (*E*). *CD*=cisterna of the dictyosome, *rER*=rough endoplasmic reticulum, *TE*=transitional elements of the rER; ×23900

Fig. 16. Transport vesicles. Transport vesicles are clustered between the transitional elements (*TE*) of the rough endoplasmic reticulum and the trans-most cisterna of the dictyosome (*D*); ×24300

Fig. 17. Vesicle shuttle system between rER and dictyosome. Vesicles are transported in both directions. Those vesicles budding from the transitional elements (*TE, arrow*) most probably carry digestive enzymes or precursors to the cisternae of the dictyosome (*CD*). Vesicles migrating from the dictyosome to the rER may recycle membrane material. *Arrowhead:* vesicle budding from the trans-most cisterna of the dictyosome; ×60100

Fig. 18. Formation of enzyme vacuole. The enzyme vacuoles (*E*) originate at the trans face of the dictyosome. The exact mechanism of formation is not yet clear; ×45150

Fig. 19. Enzyme vacuoles at the microvillous border. The vacuoles do not change in size or appearance during the time from their formation at the dictyosomes until exocytosis at the microvillous border (*MB*); *L*=lumen of midgut gland tubule; ×19300

Fig. 20. Exocytosis of enzymes. The membrane of an enzyme vacuole is fusing with the plasmamembrane. *Mi*=microvillus; ×73650

zymogen granules are found particularly in starved specimens (Hirsch 1932).

In both normal food processing and after stimulation by artificial removal of the gastric fluid, the cardiac stomach was refilled within 4–6 h. The results of the radioactive tracer experiment confirm this filling time. An artificial emptying of the stomach is followed by continuous refilling. In contrast, during a normal digestive cycle two processes might take place in parallel. While the food is triturated in the cardiac stomach, the F-cells of the midgut gland may synthesize digestive enzymes and continuously secrete them into the tubules of the hepatopancreas. Then, 4–5 h after food ingestion, the triturated food (chymus) may be transported into the pyloric stomach and, at the same time, the digestive enzymes might move from the tubules through the gland filter and the ventrolateral channels into the emptied cardiac stomach to be stored for the next food uptake. Powell (1974) presented evidence that this passage is used for the transport of liquids. Thereafter, the chymus from the pyloric stomach can be pressed through the gland filter and the filtrate can be transferred into the midgut gland tubules where the R-cells start to resorb the nutrients.

The biochemical properties of the digestive enzymes of decapods are obviously well adapted to the unique mode of storage as active enzymes in an extracellular compartment. As demonstrated for *Astacus* trypsin, *Astacus* carboxypeptidase and *Astacus* protease (Zwilling and Neurath 1981), they are resistant to self-digestion and therefore stable for many days. This resistance can be directly related to the primary structure of the enzymes. *Astacus* trypsin, for example, contains only seven potential sites for autocatalytic cleavage whereas the relatively unstable bovine trypsin has 16 (Titani et al. 1983). Moreover, the dissociation-halflife of the active site zinc-atom in *Astacus* protease is higher than in other metalloenzymes. This indicates that the zinc is bound very tightly to the protein thus underlining the stability of the enzyme (Stöcker et al. 1988).

In conclusion, differences between the vertebrate and decapod models of production of digestive enzymes are found in parts of the intracellular processing, in enzyme storage and in related biochemical characteristics of the enzymes concerned.

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