

# A simple enzymatic method for examining calcite ossicles of Echinodermata

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## Summary

The various shapes, size and fine details of ossicles are of paramount importance as systematic characters in the Echinodermata. The ossicles in this group are endodermal and a method to free them from the dermis is essential for their study. To date, a small quantity of hypochlorite solution (usually household liquid bleach) has been used to dissolve a small piece of tissue and, consequently, free these ossicles. A new method, using a proteolytic enzyme instead of hypochlorite solution to dissolve holothurian tissues, is proposed. This method was tested on fragments of body tissues from three species in three different orders of holothurians: *Holothuria grisea* Selenka, 1867 (Aspidochirotida), *Duasmodyctyla seguroensis* (Deichmann, 1930) (Dendrochirotida) and *Synaptula secreta* Ancona Lopez (1957) (Apodida). Observations of material prepared using the hypochlorite and the enzyme methods were made using scanning electron microscopy (SEM). The SEM images show the striking differences in the surface of the ossicles obtained by each method, and reveal the advantages of the enzyme method.

## Introduction

Echinoderm identification is based mainly on the morphology of the calcite skeleton. This skeleton is composed of numerous ossicles, united by muscles and other tissues (Ophiuroidea, Asteroidea, Echinoidea and Crinoidea) or scattered in the dermal tissues of the animal (Holothuroidea). The classification of the echinoderms is based on the type, shape and fine details of the calcareous skeleton. The identification of these animals depends on the use of suitable techniques to clean the skeleton, or to isolate the ossicles.

The idea of developing a new method to isolate echinoderm ossicles arose because we had started to work with holothurians with minute ossicles, that usually are damaged with the

traditional method which uses hypochlorite solution (household bleach) to dissolve the tissues (Deichmann, 1954; Pawson, 1986; Ditadi, 1987; Hendler *et al.*, 1995; Cutress, 1996). However, this hypochlorite solution, when not properly buffered, can corrode the calcareous ossicles, resulting in modification of their shape, dimensions and fine details sometimes are completely lost (A. S. F. Ditadi, personal communication). Alterations in the characteristics of the ossicles can even compromise the species identification, especially when identification is based on slight differences in dimensions and surface details of ossicles or when the ossicles in young individuals are very different from those in adults (Cutress, 1996).

Proteolytic enzymes are used in several cleaning processes, such as in domestic protein-cleaning process of contact lenses, in clarification of halacarid mites (Newell, 1947) or in the procedure of clearing and counterstaining cartilages and bones of fishes (Taylor & Van Dyke, 1985).

The new method, developed to isolate the ossicles of echinoderm tissues, is based on the step of clearing the muscle tissue with proteolytic enzyme in a borax buffer in the Taylor & Van Dyke (1985) procedure.

## Materials and method

The following materials were used for the method (see Fig. 1): pieces of body tissues of echinoderms (fresh or preserved); distilled water; sodium tetraborate (borax:  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ); proteolytic enzyme; glycerine jelly (Green & Macquitty, 1987), Permout® or Entellan®.

The enzymatic method for isolating ossicles was tested with pieces of body tissues of three species of three different orders of holothurians: *Holothuria grisea* Selenka, 1867 (Aspidochirotida), *Duasmodyctyla seguroensis* (Deichmann, 1930) (Dendrochirotida) and *Synaptula secreta* Ancona Lopez (1957) (Apodida). It is possible to use either fresh or preserved pieces of body tissues, unless the animal or the tissues were preserved in non-buffered formalin, which corrodes the ossicles and precludes the process of identification in echinoderms.

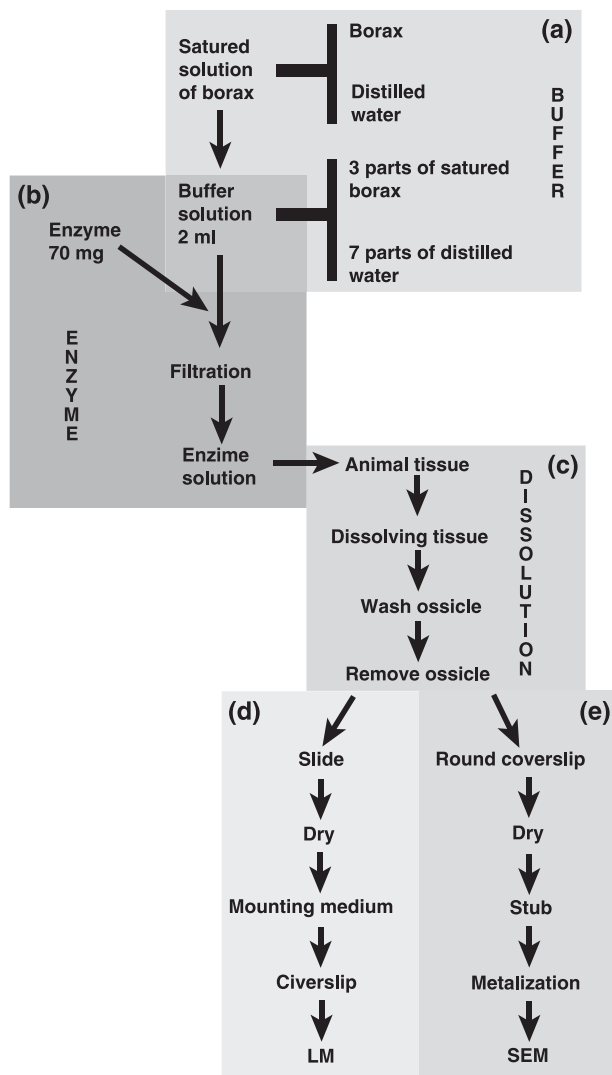


Fig. 1. Procedure step by step.

To use the proteolytic enzyme it is necessary to prepare a buffer solution made of 30% borax (sodium tetraborate;  $\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$ ). This solution consists of three parts of saturated borax solution and seven parts of distilled water. The solution of saturated borax is prepared by adding an excessive amount of borax in a flask with distilled water over a shaker. The solution is ready when undissolved borate settles on the bottom of the flask (Fig. 1a).

For small pieces of tissue, up to  $0.5 \text{ cm}^2$ , approximately 70 mg of pancreatin or trypsin is mixed with 2 mL of the buffer solution. The enzyme solution must be prepared at temperatures below  $25^\circ \text{C}$  and used immediately after its preparation. The mixture is then filtered to remove undissolved solids. For animals with minute ossicles, filtration in a  $0.22 \mu\text{m}$  Millipore® filter is recommended (Fig. 1b).

Tissues were placed in watch glasses in an oven ( $30\text{--}40^\circ \text{C}$ ). Lower temperatures will make the reaction extremely slow, whereas higher temperatures will cause denaturation of the enzyme. Complete dissolution usually takes from 10 to 30 min, depending on the size of the piece of tissue. For larger pieces the enzyme and buffer solution quantities must be proportionally increased up to a considerable amount of solution until the tissue is fully covered with solution (Fig. 1c). At the end of the process, after the complete dissolution of tissue, ossicles will settle on the bottom of the watch glass. Using a Pasteur pipette, the preparation is washed with distilled water to remove the enzyme remnants. When the preparation has been cleaned, the ossicles can be removed with a Pasteur pipette, under a stereomicroscope (Fig. 1c). In order to verify the conditions of the obtained material, a preliminary analysis in the light microscope (LM) is necessary. Thus, a slide containing the ossicles should be left aside to dry and then covered with mounting medium (glycerine jelly, Permunt® or Entellan®) and a coverslip. If using a glycerine jelly (Green & Macquitty, 1987), the coverslip should be sealed with a standard microscopical sealing agent or with a good quality nail varnish (Fig. 1d). A JEOL (JSM – 840A) scanning microscope was used for scanning electronic microscope (SEM) study. The material was placed on a circular glass coverslip and set aside to dry. Once dried, it was examined in the LM to verify the presence of suitable material for the SEM analysis. Due to the calcareous nature of the material, there was no need to use the critical point dryer. Only metal coating of the material was necessary (Fig. 1e).

In order to compare the proposed method with the traditional method, ossicles from the three species of holothurians were obtained with hypochlorite solution (traditional method). Small pieces of tissue were placed on separate watch glasses and covered with several drops of hypochlorite solution. When the tissue was completely dissolved the hypochlorite was washed off with distilled water and the ossicles were removed with a Pasteur pipette, under a stereomicroscope. The procedure for examining the conditions of the ossicles under light microscope and for SEM study was identical to the procedure described for the material obtained with the proteolytic enzyme.

## Results

The results of SEM analysis show that the hypochlorite solution, when not properly buffered, can corrode the calcite pieces and modify the original shape, dimensions and fine details of the ossicles, whereas the enzymatic method discloses details in a smooth surface that cannot be seen in material obtained with hypochlorite.

SEM images of the material prepared using the hypochlorite and the enzymatic methods are shown in Figs 2–6. The striking differences in the surface of the ossicles obtained by each method can be observed. The teeth in the apical portion of the

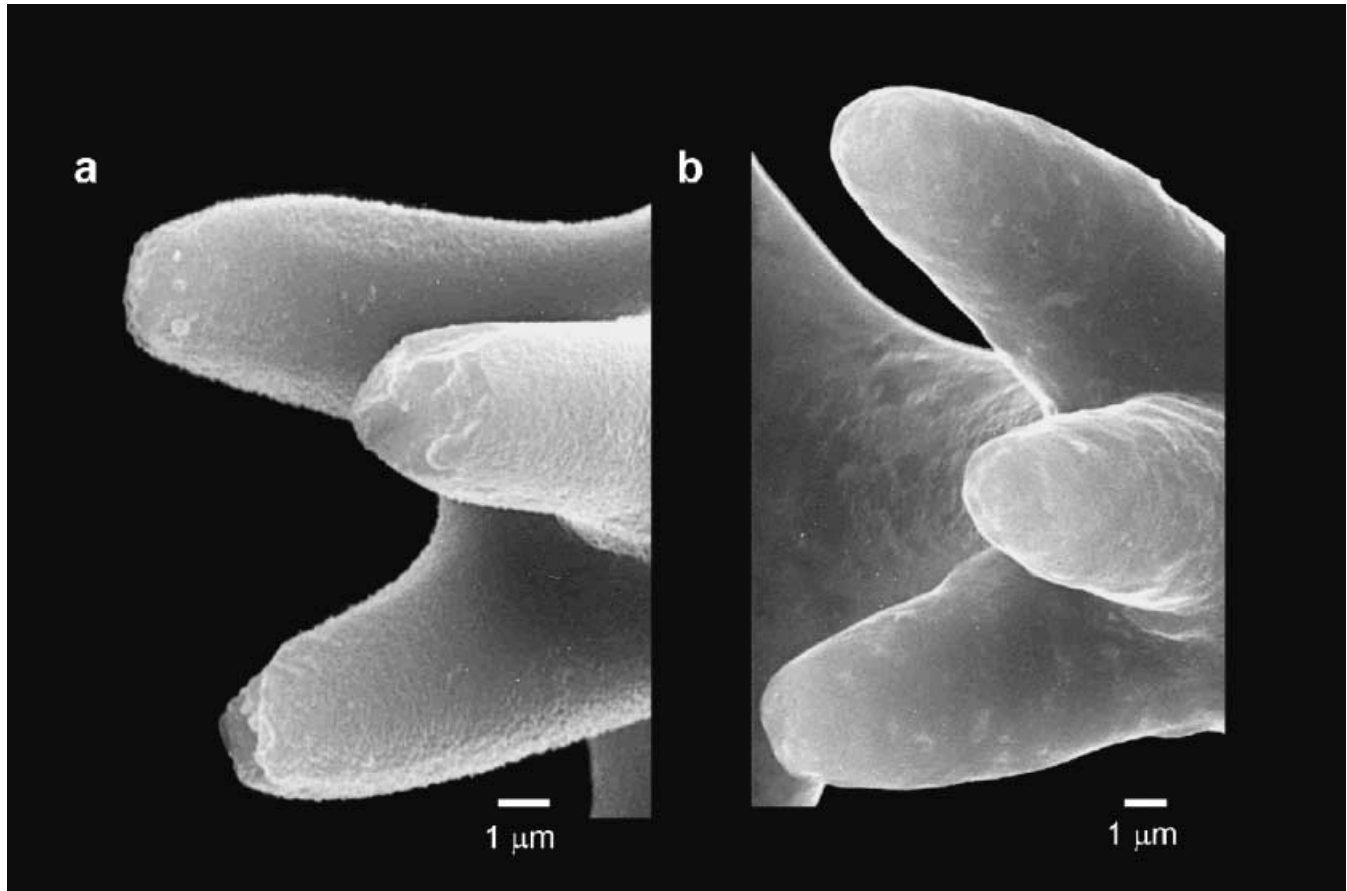


Fig. 2. Teeth in the apical portion of a tower from *Duasmodactyla seguroensis*: (a) tower isolated with hypochlorite; (b) tower isolated with pancreatin.

tower isolated by the hypochlorite method from *D. seguroensis* tube feet (Fig. 2a) show an irregular surface and a porous appearance in contrast to Fig. 2(b), where the entire apical surface appears smooth, as a result of the enzymatic method. In this figure it is possible to see how the hypochlorite can corrode the surface of the ossicles and make it frail.

The difference between a tower isolated with the enzymatic method (Fig. 3a) and a tower obtained with hypochlorite (Fig. 3b) is easily recognized. Figure 3(c) shows a detail of the tower base surface corroded by hypochlorite.

In Fig. 4(a) it is not possible to see the teeth in the arms and the crenulations of the stock of the anchor from *S. secreta* body wall due to the hypochlorite corrosion, details that can be seen in Fig. 4(b), obtained with the enzymatic method.

In *S. secreta* the anchor plates obtained using the hypochlorite method (Fig. 5a) were corroded and did not show the crenulations on the inner edge of the large holes revealed via the enzymatic method (Fig. 5b). A perforated plate from *H. grisea* tube feet obtained using the hypochlorite method was severely damaged (Fig. 6a) compared to one isolated by enzymes (Fig. 6b).

Applying this new method in holothurians with minute ossicles we found a new shape of miliary granule in *S. secreta*

(Fig. 7). This new type has a round shape about 10 µm in diameter, with irregular holes in the surface.

## Discussion

The idea of developing a new methodology for ossicle isolation arose from taxonomical studies of holothurians with minute ossicles that usually are damaged by the use of hypochlorite solution.

Although with the new method a longer time is needed to dissolve the tissues, it does not cause any alteration of the dermal ossicles. Instead, it guarantees the preservation of the original shapes, dimensions and fine details of the ossicles, thereby facilitating correct identification of the species. Furthermore, the enzymatic method allowed us to discover a new miliary granule in *S. secreta* that was not cited in the original description (Ancona Lopez, 1957), probably because the hypochlorite method used caused complete corrosion of these minute ossicles. Therefore, we could infer that the enzymatic method ensures that all ossicles, even the smallest ones, are not lost.

The first analysis of the ossicles, obtained with hypochlorite, in Figs 4 and 5 could give the impression that these ossicles could

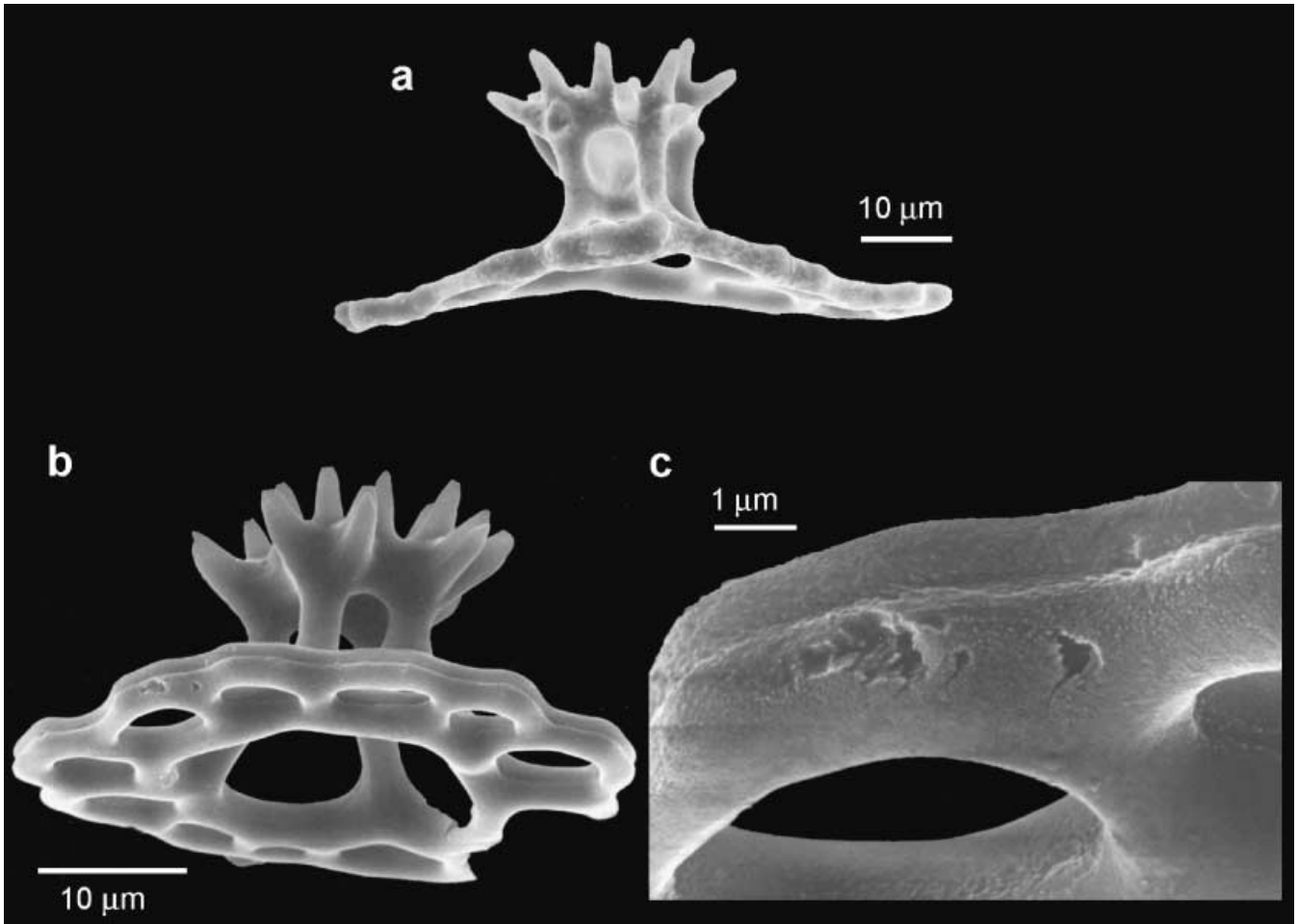


Fig. 3. Towers from *Duasmodactyla seguroensis* tube feet: (a) tower isolated with pancreatin; (b) tower isolated with hypochlorite; (c) detail of corrosion on the tower base isolated with hypochlorite.

be in different phases of development. However, the ossicles obtained with hypochlorite solution are larger than the ossicles isolated with enzyme, which indicates that the structures are corroded and are not an earlier phase of development.

The results show clearly that the enzymatic method keeps the ossicles' surface in perfect condition. Besides that, this method has the advantage of not requiring a careful monitoring of time and pH.

Preliminary experiments with other members of this phylum, such as ophiuroids and sea urchins, produced satisfactory results. Therefore, we conclude that this new procedure could be used without significant modifications for all the echinoderm classes.

Other potential application of the technique tested by one of the authors (G. Y. Kawauchi) is to isolate the protein-based (not chitin) hooks from the distal portion of the introvert of sipunculans, especially in minute animals. These structures are used in the identification of some species (Cutler, 1994). As the body wall of the sipunculans is thick and presents a

cuticle composed of collagen fibres (Edmonds, 2000), dissolution is slower than in the echinoderms. In these animals, the enzymatic method allows the extraction of the hooks when all the tissues are dissolved and only the cuticle remains.

As well as in echinoderms and sipunculans, soft tissue of hydroids could be cleaned using the method. A. E. Migotto and J. E. Borges (personal communication) tested our technique instead of hypochlorite solution in order to avoid the bubbles formed by the hypochlorite inside the hydroid structures that impair the process of illustrating the animal.

All the examples listed indicate that this method can be used with other invertebrates when it is necessary to dissociate calcareous or corneous structures from body tissues in order to identify the organism or to take an image of one structure.

Isolating the calcareous ossicles and other structures in perfect condition, including the minute ones, the enzymatic method could increase the knowledge about the characters of the species and provide data that would be useful to improve taxonomic studies of the echinoderms and other groups.

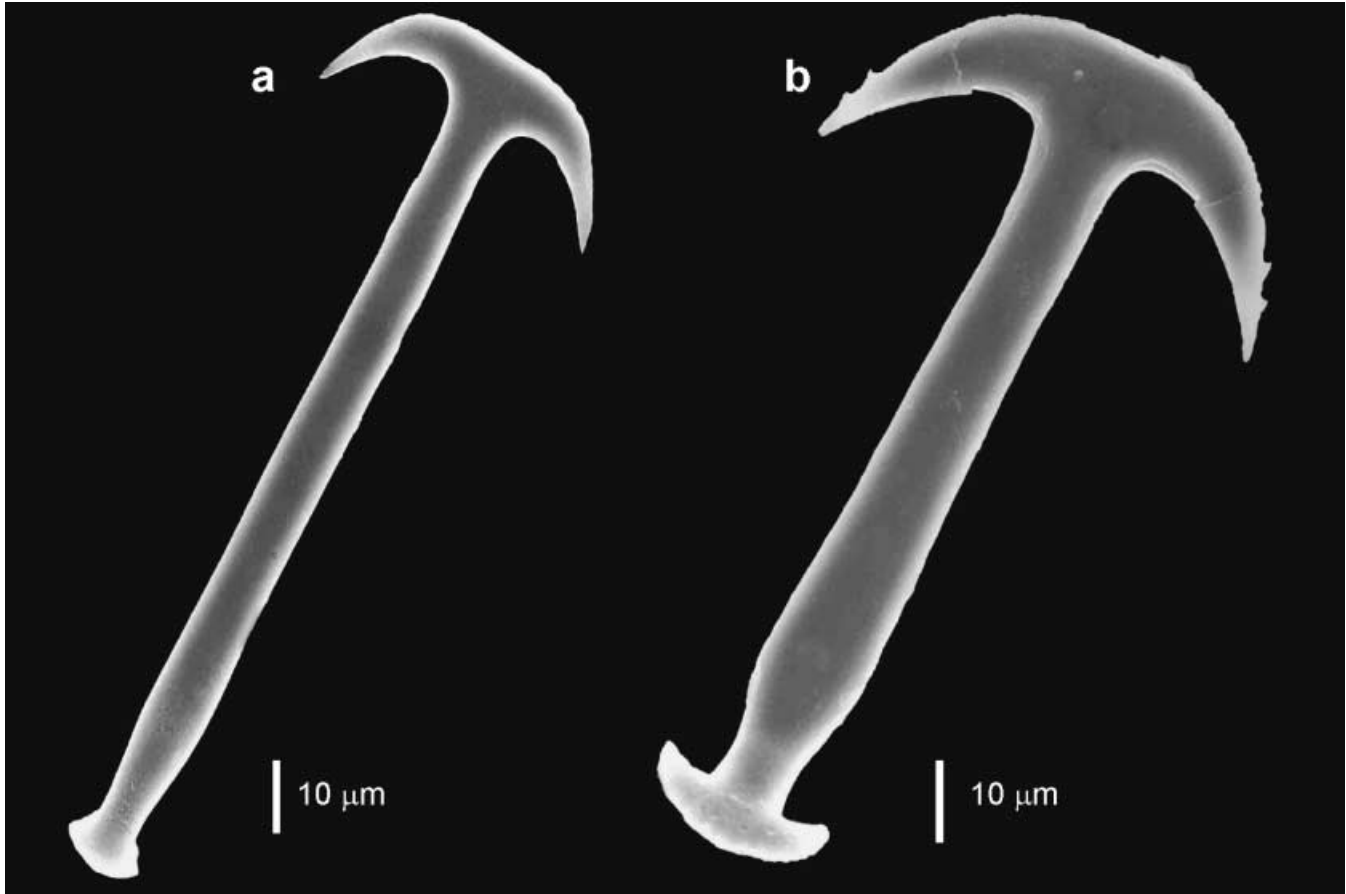


Fig. 4. Anchor of *Synaptula secreta*: (a) anchor isolated with hypochlorite; (b) anchor isolated with pancreatin.

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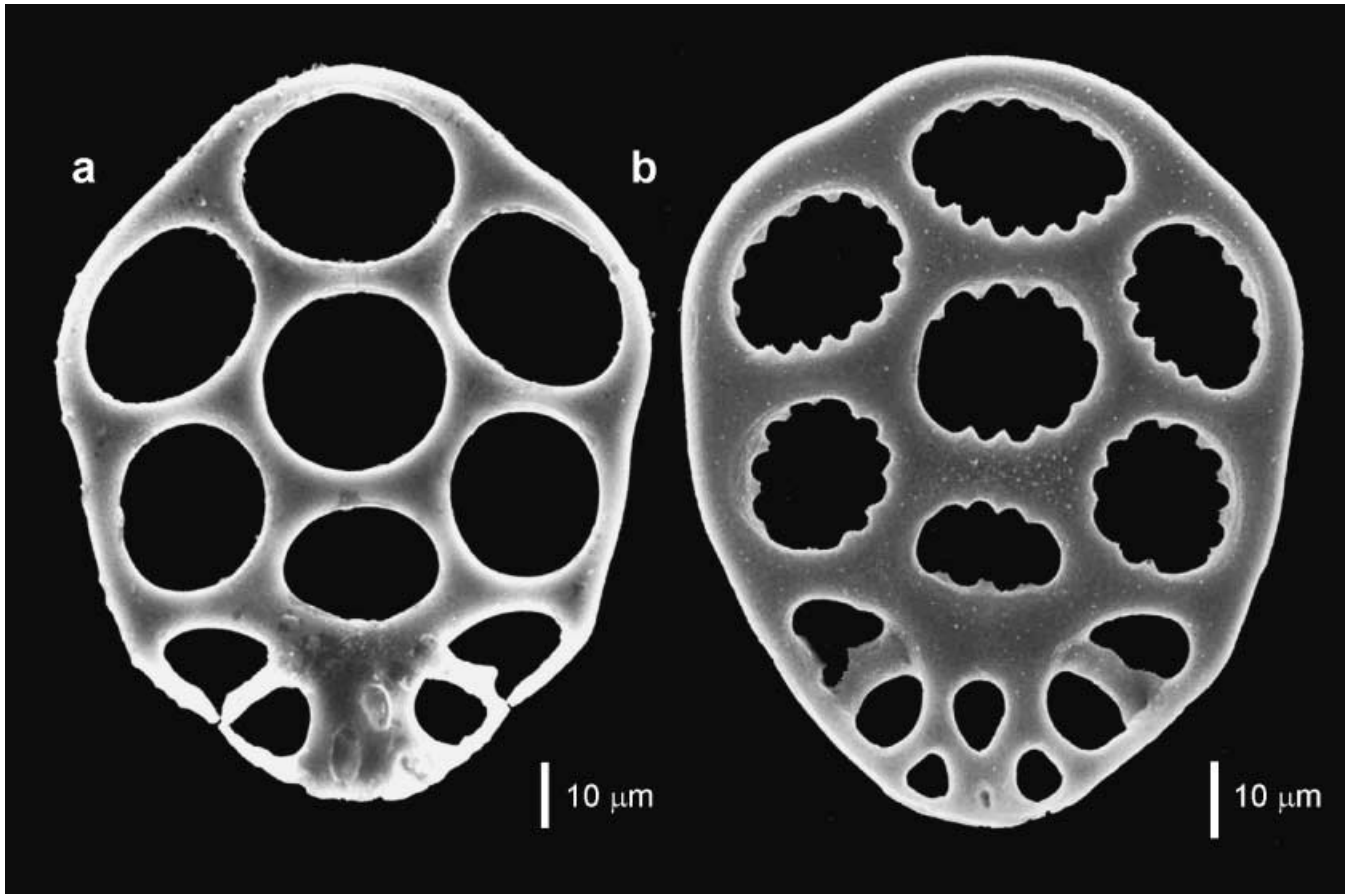


Fig. 5. Anchor plate from *Synaptula secreta*: (a) anchor isolated with hypochlorite; (b) anchor plate isolated with pancreatin.

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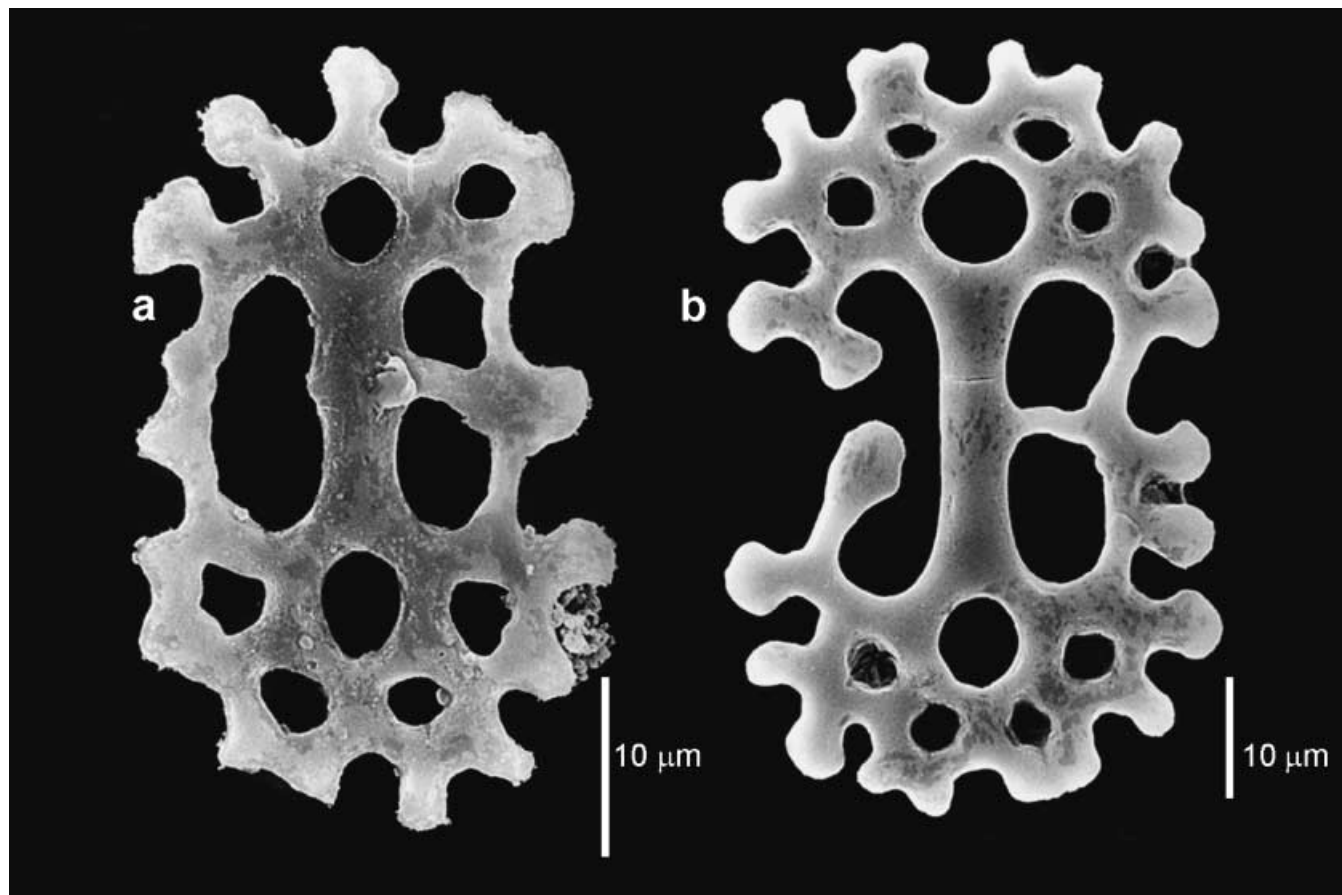


Fig. 6. Perforated plate from *Holothuria grisea*: (a) plate isolated with hypochlorite; (b) plate isolated with pancreatin.

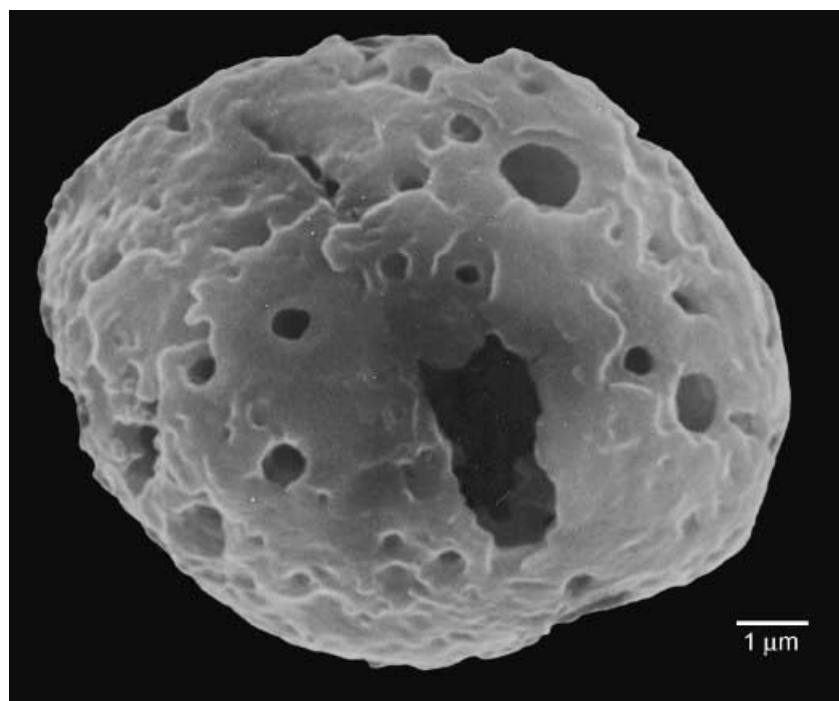


Fig. 7. Round shaped miliary granule from *Synaptula secreta* isolated with pancreatin.