Excellent methods for processing crustacean larvae for scanning electron microscopy

Masaki Ueno

Abstract.—In this study, a novel processing technique was developed for observing artemia nauplii by scanning electron microscopy (SEM). The specimens obtained by this method could be observed under very low to high magnification, and minimal morphological transformation was noted in the larval body or in the setae at the tips of the antennae. The technique involves a combination of chemical fixation and microwave irradiation, ultra-high-vacuum freeze-drying, and osmium coating. It could prevent the formation of a charge at the tips of the setae a phenomenon that often occurs during sample processing for SEM. This method may facilitate research on the morphology of crustacean larvae.

Introduction

Crustaceans, which are arthropods, possess a cuticle within the digestive duct and also serves as the exoskeleton. Their hard exoskeleton facilitates experimental observation of the adult body surface: even with inadequate fixation of the epidermis and internal organs, the morphology of the exoskeleton restricts the transformation of these internal structures. Further, crustaceans can be stably observed by scanning electron microscopy (SEM) if the conductivity of the target surface is secured. For the observation of an internal organ, inadequate fixation can usually be prevented by dissecting the target organ. However, in the case of a minute larva or an imago, the situation is entirely different: the exoskeleton, although it may be thin, remarkably hampers the penetration of the fixative. Owing to the small size of the larval body, dissection of the internal organs is difficult, and even if the organism is entirely immersed in the fixative, the fixative rarely penetrates the tissue within a short duration. Moreover, the fixative cannot easily penetrate the intestinal lumen because this region is largely covered by a thin cuticle. In fact, the organisms are often found to be swimming around vigorously in the fixative even at 4 h after immersion. The exoskeletal cuticle is reported to be 0.3–1.0 μm thick throughout the integument (Freeman, 1989). This thin cuticle does not prevent transformation of the internal structures during dehydration and drying, and many wrinkles are formed on the surface of the organism; thus, the organism cannot be observed in a true-to-life manner. It is extremely difficult to prepare a crustacean larval sample that can withstand the treatment required for observation under low magnification. Microwave irradiation (MWI) has been shown to improve the results of immunohistochemical staining and fixation for subsequent observation by light and transmission electron microscopy (Login & Dvorak, 1985; Leong et al., 1985; Login et al., 1986; Login & Dvorak, 1988; Login et al., 1990; Smid et al., 1990; Login et al., 1995). Koga et al. (2003) described the advantages of MWI in fixing insect tissue for SEM; these researchers obtained high-quality scanning electron micrographs of the pitinum of the fly and the compound eye of the mosquito, which are very fragile
tissues. For drying samples to be observed by SEM, 2 methods—\textit{t}-butyl alcohol freeze-drying and critical-point drying—are conventionally adopted. Further, the use of ultra-high-vacuum freeze-drying for this purpose has been investigated, although this technique was originally developed for ultrastructural analysis and immunoelectron microscopy (Linner et al., 1986). Since ultra-high-vacuum freeze-drying removes water from the tissues without causing ice crystal damage, it may also be useful for preserving the external morphology of organisms. In this study, I aimed to design a method that overcomes the limitations posed by the cuticle of crustaceans and yields images of larvae in the most true-to-life manner possible. I used the MWI technology for fixation and the ultra-high-vacuum freeze-drying technology for drying.

Materials and Methods

Nauplii of artemia (\textit{Artemia franciscana}) were used for the experiment. Diapaused eggs of artemia, which are used to feed tropical fish, were purchased and allowed to hatch in 2.0% salt solution at 28°C.

\textit{Larval collection}

A bottle-top filter (Becton Dickinson Labware Co., NJ, USA), in which the sterilization filter was replaced with a nylon mesh, was used for efficient larval collection; this filtration method ensured minimal damage to the larvae.

\textit{Fixation}

The larvae were fixed in 7\% glutaraldehyde diluted with 0.1 M phosphate buffer (pH 7.3), for 6 h at room temperature. Thereafter, they were washed with phosphate buffer and immersed in 2\% osmium tetroxide for 2 h at room temperature.

\textit{MWI}

During glutaraldehyde fixation, the larval samples were irradiated in a microwave oven (MI-77; Azumaya Co., Tokyo, Japan). The bottle containing the larval samples was submerged in 400 ml of cold water (1\(^\circ\)C) in a heat-resistant glass dish (diameter, 13 cm; height, 6 cm) in order to ensure uniform irradiation and to prevent a rapid increase in the temperature of the fixative. The glass bottle (capacity, 20 ml) containing the larvae and 10 ml of glutaraldehyde was lightly closed, and the level of the fixative was maintained slightly lower than that of the water outside the bottle. The turntable of the oven rotated during the irradiation.

\textit{Irradiation conditions}

The larval samples were subjected to continuous irradiation for 3–4 min in the microwave oven (output, 350 W). After irradiation, the temperature of the fixative increased to approximately 80\(^\circ\)C. Subsequently, the samples were left undisturbed in the bottle containing glutaraldehyde for 6 h at room temperature.

\textit{Dehydration}

After fixation, the larval specimens were washed with 0.1 M phosphate buffer and dehydrated by treatment with a graded series of ethanol (70\%, 80\%, 90\%, and 95\% for 30 min each, followed by 100\% for 3 cycles of 30 min each).

\textit{Drying}

(1) Critical-point drying

Samples that have been dehydrated through a graded ethanol series are usually transferred from the ethanol solution into isoamylacetate for drying. However, in my experiment, I omitted this step because it can alter the morphology of the specimens. Instead, I directly transferred the specimens from absolute ethanol into liquefied carbon dioxide and dried them in a critical-point dryer (CP-5; Topcon Co., Tokyo, Japan).

(2) \textit{t}-Butyl-alcohol freeze-drying

As per the conventional method for the sample drying, absolute ethanol is used for the final dehydration step, after which the
specimens are transferred to 100% t-butyl alcohol. However, since this transfer can alter the morphology of the specimens, I omitted the step of dehydration in absolute ethanol in my experiment. Instead, I used 95% ethanol in the final step of dehydration and subsequently transferred the specimens into 99% t-butyl alcohol and freeze-dried them at −20°C for 2h in a VFD-21S freeze-dryer (Vacuum Device Inc., Ibaraki, Japan).

(3) Ultra-high-vacuum freeze-drying

The fixed larvae were washed, made to float on a small amount of water in a brass saucer (diameter, 4 mm), and immediately frozen in liquefied propane. The saucer was then placed in an ultra-high-vacuum freeze-dryer (VFD-300; Vacuum Device Inc., Ibaraki, Japan). The freeze-drying process ensued with a time-controlled linear increase in the temperature, from −120°C to 25°C, as follows: the temperature increased from −120°C to −110°C over 1 h, remained constant at −110°C for 1 h, increased from −110°C to −80°C over 24 h, remained constant at −80°C for 1 h, and finally increased from −80°C to 25°C over 6 h. This process was performed under ultra-high vacuum (1 × 10⁻⁸ Torr), and the duration of the step in which the temperature increased from −110°C to −80°C was either 24 h or 36 h.

Sample coating

The dried samples were mounted on an aluminum base with the help of carbon tape and were coated with a 20-nm-thick osmium film by using an osmium plasma coater (Neo osmium coater Neoc-ST; Meiwafosis Co., Ltd. Osaka, Japan). For comparison, control specimens were subjected to conventional sputter coating with platinum-palladium in a magnetron sputtering unit (MSP-10; Vacuum Device Inc., Ibaraki, Japan).

Results

Compared to the fixation, dehydration, and sample-coating processes, the drying process yielded remarkably different results. The results obtained with the 3 drying processes were as follows.

Critical-point drying

Figure 1 shows images obtained using the conventional method of critical-point drying: a large wrinkle was formed on the surface of the integument. The modified method, wherein the step of isoamylacetate treatment was omitted, yielded slightly better results. The setae growing at the tips of the upright antennae were prominent and showed only slight morphological transformation. However, the larval body surface had many large and small wrinkles, most of which seemed to have developed during sample processing, i.e., through artificial induction (Fig. 2).

t-Butyl-alcohol freeze-drying

The conventional method of t-butyl-alcohol freeze-drying resulted in drastic morphological transformations over the entire larval body surface (Fig. 3). On the other hand, better results were obtained when the samples were transferred from 95% ethanol to 99% t-butyl alcohol and freeze-dried (Fig. 4). The setae on the upright antennae appeared wavy and were morphologically transformed, but only slight body-surface transformation was observed in these larvae when compared with the larvae that were subjected to critical-point drying. However, in a magnified image (Fig. 5), the wrinkles on the cuticle covering the epidermis appeared large and seemed to have developed through artificial induction. Moreover, most of the setae appeared flattened.

Ultra-high-vacuum freeze-drying

Figure 6 shows the results obtained by freeze-drying the larval specimens, using the temperature/time-controlled program detailed in the methods section. The setae at the tips of the antennae had grown normally, and minimal transformation was noted in the larval head. Although few wrinkles were present on the cuticle covering
SEM ANALYSIS OF NAUPLII

Fig. 1. Specimen subjected to critical-point drying by the conventional method (without omitting the step of immersion in isoamylacetate): a large wrinkle is observed on the surface of the first and second antennae and the labrum. Bar: 30 μm.

Fig. 2. Specimen subjected to critical-point drying: the setae on the antennae have grown normally and do not show transformation. However, the whole body surface is covered with small and large wrinkles. The final specimen does not resemble living larvae. Bar: 50 μm.

Fig. 3. Specimen subjected to t-butyl alcohol freeze-drying by the conventional method (without omitting the step of dehydration in absolute ethanol): the whole body surface shows drastic transformations. The labrum and antennae are largely dented. Bar: 30 μm.

Fig. 4. Specimen subjected to freeze-drying with 99% t-butyl alcohol: fewer wrinkles are formed than in the case of the critical-point-drying method; however, the setae appear wavy and shrunken. Bar: 50 μm.

the epidermis, the whole larval body appeared rugged and was morphologically transformed. Figures 7–12 show the results obtained when the duration of the step in which the temperature increased from −110°C to −80°C was extended to 36 h. Figure 7 shows a frontal image of the whole larval body. The setae at the tip of each antenna had grown upright, and there was minimal transformation in and few wrinkles on the body surface. Moreover, the specimens lost the rugged appearance they acquired when the temperature increased from −110°C to −80°C over 24 h (Fig. 6); in fact, the body of the larvae that were subjected to the temperature increase for 36 h appeared smooth and untransformed (Fig. 7). Figure 8 shows the whole larval body in the right lateral view. The first and second antennae that developed into the
Fig. 5. Magnified image of the second antenna in a specimen subjected to freeze-drying with 99% t-butyl alcohol: although only few small wrinkles are formed on the antenna surface, a large wrinkle is observed on the cuticle. The setae appear slightly wavy. Bar: 10 μm.

Fig. 6. Specimen subjected to ultra-high-vacuum freeze-drying with the temperature increasing over 24 h: an artificially induced large wrinkle is observed on the body surface, and the whole body surface appears rugged. However, the setae show minimal morphological transformation as compared to after drying with t-butyl alcohol. Bar: 30 μm.

Fig. 7. Frontal image of the specimen subjected to ultra-high-vacuum freeze-drying with the temperature increasing over 36 h. The body surface appears smooth with no conspicuous transformation. Moreover, the setae have grown upright and are not transformed. Bar: 50 μm.

Fig. 8. Right lateral image of the specimen subjected to ultra-high-vacuum freeze-drying with the temperature increasing over 36 h: no morphological transformation is noted in the first and second antennae or the abdomen. A few wrinkles and separation of the cuticle from the epidermis are observed toward the base of the salt gland. Bar: 50 μm.

Thorax used for swimming were observed, and no noteworthy transformation was noted. The nauplii grew rapidly, and their external morphology was altered within a short duration. Figure 9 shows extension of the labrum as well as the abdomen. The whole body surface appeared smooth and lacked conspicuous wrinkles that seemed to have developed through artificial induction. Figures 10 and 11 present a magnified image of the larval head and a frontal image of the abdomen respectively. Minute wrinkles that seemed to have developed naturally were noted on the upper and lower abdomen.
Fig. 9. Whole body of the specimen subjected to ultra-high-vacuum freeze-drying: the nauplius has grown rapidly. The labrum has become narrow, and the abdomen has stretched. A relatively large wrinkle is noted on the head, but the body surface appears smooth with few wrinkles. Bar: 50 μm.

Fig. 10. Magnified image of the head of the specimen subjected to ultra-high-vacuum freeze-drying: the surface appears smooth, both at the end of the labrum and on the setae at the tips of the antennae; the setae have elongated normally. Bar: 30 μm.

Fig. 11. Magnified abdominal image of the specimen subjected to ultra-high-vacuum freeze-drying. The bulges along the abdomen are attributable to the epithelial cells beneath the cuticle. No wrinkles are observed on the cuticle. Bar: 30 μm.

Fig. 12. Magnified dorsal image of the specimen subjected to ultra-high-vacuum freeze-drying: a characteristic pattern is noted over the salt gland. Very few artificially induced wrinkles are noted. Bar: 30 μm.

Figure 12 presents a dorsal image of the whole larval body, showing a characteristic pattern over the salt gland. Many small circular bulges were observed along the lower back; these bulges were attributable to the underlying epidermal cells that formed a simple squamous epithelium. Figure 13 shows an enlarged larval head: the end of the labrum is invaginated and forms a deep wrinkle. Figure 14 presents a magnified image of the first antenna, with 3 setae at the tip and minute setae arising from each segment. Figure 15 shows the second antenna; an image of the setae shows that they are probably tubular, but their surface is not smooth. The end of the labrum moves
Fig. 13. Magnified frontal image of the head of the specimen subjected to ultra-high-vacuum freeze-drying: although a deep wrinkle, which seems to have developed naturally, is noted toward the end of the labrum, each region seems to accurately reflect the living condition. Bar: 30 μm.

Fig. 14. Magnified image of the first antenna of the specimen subjected to ultra-high-vacuum freeze-drying: 3 setae differing in thickness have originated from the tip of the antenna, and minute setae are observed in each segment. Bar: 10 μm.

Fig. 15. Second antenna of the specimen subjected to ultra-high-vacuum freeze-drying: minute bulges are observed on both the surface of the antennae and the setae. The surface of the setae is not smooth. Bar: 10 μm.

Fig. 16. Specimen fixed with glutaraldehyde and osmium tetroxide by the conventional method (without MWI): many wrinkles are formed on the surface of the antennae, labrum, and thorax. The setae of the second antenna are wavy. Bar: 50 μm.

preserving the external morphology of fragile insect tissue (Koga et al., 2003). However, this is the first report on its application for the preservation of nauplii. Structural preservation is more difficult to achieve in the case of artemia nauplii than in that of insects, because the body length of the former is approximately 0.4 mm (one-tenth that of the fly) and the cuticle covering the body is only 1 μm thick (Freeman,

rhythmically when the full-grown organism swims; this results in the formation of a deep wrinkle in this area.

Discussion

Effects of MWI

MWI is reported to be effective in
1989). Moreover, most of the nauplius body surface comprises a simple epithelium covered by a cuticle, and the epithelium can easily detach from the cuticle; this leads to morphological transformation and the appearance of wrinkles during fixation, owing to shrinkage. In addition, since the cuticle restricts the penetration of fixatives, the underlying epithelium cannot easily be fixed. Figure 16 shows the result of fixation without MWI: many wrinkles were formed on the integument surface. In the present study, MWI probably facilitated penetration of the fixative through the cuticle and epithelium. With MWI, the temperature of the fixative increases and the vital processes of the larvae are rapidly arrested (Hopwood et al., 1990); thus, the cuticle is no longer functional in restricting the penetration of the fixative, and this leads to enhanced fixation. The effects of applying a high temperature to achieve the fixation of human, mouse, and insect tissues have been discussed (Mayers, 1970; Hopwood et al., 1988; Hernandez & Guillen, 2000). Caution should be exercised when increasing the temperature of the fixative because a fixative at 80°C or more can drastically transform the concerned tissue specimen.

**Optimum duration of ultra-high-vacuum freeze-drying**

The results of the present study reveal the importance of optimizing the duration of each step in the sample-drying procedure and the difficulties associated with selecting an appropriate program. A program in which the temperature increased from −110°C to −80°C over 24 h yielded unsatisfactory results, while one in which this step lasted for 36 h yielded satisfactory results. Thus, it is possible that the optimal temperature-controlled program for fixation varies depending on conditions such as the amount of sample used and the volume of water on which it is made to float. Further studies are required to examine this possibility. In addition, the optimal conditions for drying specimens are affected by the rate of larval growth and the thickness of the cuticle: rapid larval growth and a thick cuticle hinder efficient drying. Nevertheless, I consider ultra-high-vacuum freeze-drying to be a useful method for research on the morphology of crustacean larvae, because it yields satisfactory results without transformation of the larval cuticle and body surface.

**Advantages of osmium coating**

Evaporation coating with a metal or sputter coating with gold or platinum-palladium is often performed during sampling processing for conventional SEM. These methods provide excellent contrast for sample visualization, and the processed samples do not appear grainy on observation at the normal magnification. However, an inherent morphological limitation of crustacean larvae is that the body surface may be considerably uneven and the setae may vary in size; this leads to friction between the sample and the aluminum base and can result in the development of a charge on the larval body surface or at the tips of the setae. Figure 17 shows the result of sputter coating with platinum-palladium: some parts of the body surface

![Specimen subjected to sputter coating with platinum-palladium: the end of the labrum, the surface of the second antennae, and the setae on the second antenna appear shiny because of the development of a charge. Bar: 50 μm.](image-url)
appear shiny because of the development of a charge. Osmium can be used to lend samples a smooth coating without a grainy appearance. Evaporation coating with osmium gas is superior to sputter coating, and with the former technique, minimal charge develops at the tips of the setae and on the sample surface that is in contact with the aluminum base. Moreover, after osmium coating, the original shape of the specimen is efficiently retained; therefore, this technique is also useful for observation under high magnification. In conclusion, osmium coating is an effective technique for studies on the morphology of crustacean larvae.

Acknowledgements

I would like to express my gratitude to Dr. Jiro Nambu (University Of Occupational and Environmental Health, Japan) for providing valuable suggestions with regard to the hatching and breeding of artemia larvae.

Literature Cited


Address: Department of Histology, Kitasato University, School of Allied Health Sciences, 1-15-1 Kitasato, Sagamihara, Kanagawa 228-8555, Japan

E-mail: matt@ahs.kitasato-u.ac.jp