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Properties of Tunic Acid in the Ascidian *Phallusia nigra* (Ascidiidae, Phlebobranchia)

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ABSTRACT—*Phallusia nigra*, a solitary ascidian, has no epibionts on the surface of tunic. Moreover, the tunic contains sulfuric acid. The pH inside the tunic was about 1 as measured with a needle-tip pH electrode, and the pH at the tunic surface was 4–5 as measured with a flat-surfaced pH electrode. The surface pH decreased to 2 when the tunic surface was gently wiped with tissue paper. Thus, the tunic seems to release acid in response to mechanical stimuli. The time course of the pH recording showed that the acidity is stable at pH 1–2 for more than five minutes after the needle-tip electrode is inserted, so neutralization of tunic acid with seawater would not occur rapidly. The acid is contained in highly vacuolated cells (tunic bladder cells), and most of these cells are located beneath the tunic surface (Stoecker, 1978; Hirose, 1999). The major anion in the tunic acid was sulfuric acid, and the SO_4^{2-}/Cl^- ratio was 4.63. In histological sections, the vacuolar lumen appear to occupy 25% of the total tunic and 75% of the tissue lying just beneath the surface of the tunic. The relationship between the properties of tunic acid and anti-predation, anti-fouling, and anti-infection is discussed.

INTRODUCTION

Sessile marine organisms cannot escape from such severe environmental pressures as irradiation, drying, predation, infection, and bio-fouling, and such organisms are also usually equipped with highly protective integuments. Ascidians, the only sessile chordates, have a unique integumentary tissue called the tunic, a kind of extracellular matrix that contains cellulosic materials and entirely overlays the epidermis. Within the tunic, free mesenchymal cells (tunic cells) are distributed, and some of them add protective functions to the tunic. For example, tunic phagocytes ingest foreign bodies and aged cells (De Leo *et al*., 1981; Hirose *et al*., 1994), pigmentary tunic cells shade the animal from irradiation (Hirose, 1999), and the contraction of the cellular networks promotes the tunic shrinkage after a wound has occurred (Hirose and Ishii, 1995; Hirose *et al*., 1997).

The tunic of some ascidians contains a strongly acidic fluid. The acid is located within the vacuoles of tunic bladder cells, a type of tunic cells (cf. Goodbody, 1974). Stoecker (1978, 1980a, b, c) reported acidity and heavy metals in the tunic of some ascidian species, and showed that tunic acidity is significantly associated with lack of epibionts (Stoecker, 1980c). On the other hand, Parry's (1984) research does not support a major defensive function for tunic acid, because the capacity to produce acid does not prevent predation and fouling on some ascidians in the field. Furthermore, Parry (1984) claimed that the defensive functions of tunic acid are doubtful because the undamaged tunic is neutral, and the acid released from the damaged tunic is rapidly neutralized in seawater in some ascidians. To date, information about properties of tunic acid is not enough to discuss the defensive function of the tunic acid. For instance, there are few studies about the possibility of fluctuations of tunic pH depending on season/body size, the distribution of the acid containers in the tunic, and the amount of the tunic acid.

Phallusia nigra Savigny 1816 is a solitary ascidian of tropical waters. Many bladder cells are distributed within the tunic, and the vacuolar lumen of these cells is filled with strong acid (Stoecker, 1978; Hirose, 1999). Although this species often inhabits the substratum, where it is exposed to full sunlight and to predators, the surface of its tunic is always free of epibionts. Therefore, the tunic acid may be effective against predators and/or epibionts in this species, as suggested by Stoecker, but the validity of Parry's claim should be verified. In the present study, we examined the properties of the tunic acid in *P. nigra*: the acidity, distribution, amount, and the counter anions. We also described the time course of neutralization of the tunic pH at the wounds and the pH of tunic surface of undamaged specimen in order to verify the Parry's claims.

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MATERIALS AND METHODS

Animals

Phallusia nigra, which attaches to floating piers or ropes in the Ginowan Port Marina and the Ginowan Fishery Port, Okinawa Island, Okinawa Prefecture, Japan, were collected and brought immediately to the laboratory, where they were temporarily kept in an aquarium. The wet weight of each animal was measured before the investigation.

Measurement of pH

The pH inside the tunic was measured by the insertion of a needle-tip (16 gauge) micro-pH electrode (Orion 9863BN; Beverly, MA). Ten or more specimens were examined at four times during one year (1999): January, April, July, and November. The pH was routinely measured at the central part of the right side of the body, but in November, the pH measurement was made at eight points in the tunic (see Results).

To estimate the effect of neutralization by seawater at the wound (the site insertion of the pH electrode), the time course of pH change was recorded (Fig. 1) with a SensorLink pH measurement system (Orion PCM500). The measurement was carried out in a 20-L container filled with seawater. The needle-tip electrode was inserted just below the tunic surface, and the pH was recorded at 10-s intervals, beginning 20 s before insertion. The position of the inserted electrode was maintained during the measurements, and in some experiments we continuously stirred the seawater in the container.

The pH of the tunic surface was measured by pressing a flat-surfaced electrode (Orion 8135BN) on the tunic surface of the central part of the right side of the body. After the measurement, the tunic surface was gently wiped with tissue paper (Kim wiper; Kimberiy-Clark, Tokyo) and the surface pH was then measured again.

Microscopy of tunic cells and tunic acid

The tunic over the central part of the right side of the body was cut into pieces with a razor blade. The tunic pieces were fixed in 10% formalin–seawater, dehydrated through a butanol series, and embedded in paraffin. Serial sections, 6 μm thick, were stained with Delafield's hematoxylin and eosin. Some sections were stained with periodic acid-Schiff (PAS) and alcian blue.

Tunic acid was visualized by vital staining with a pH-sensitive fluorescent dye, as follows. Fresh, unfixed pieces of tunic were cut from five parts of the tunic and were sectioned with a razor blade by hand. The sections (about 0.5 mm thick or less) were vitally stained with 10 μM LysoSensor Yellow/Blue DND60 (Molecular Probes Inc., Eugene, OR) in seawater for 30 min at room temperature and observed with violet light (380–425 nm) excitation. This dye accumulates in acidic organelles, and the color of the emitted fluorescence is pH dependent—predominantly yellow in acidic environments, blue in less acidic environments, and no fluorescence in neutral or basic environments. The intensity of the fluorescence is also pH dependent: the intensity is stronger in a more acidic environment.

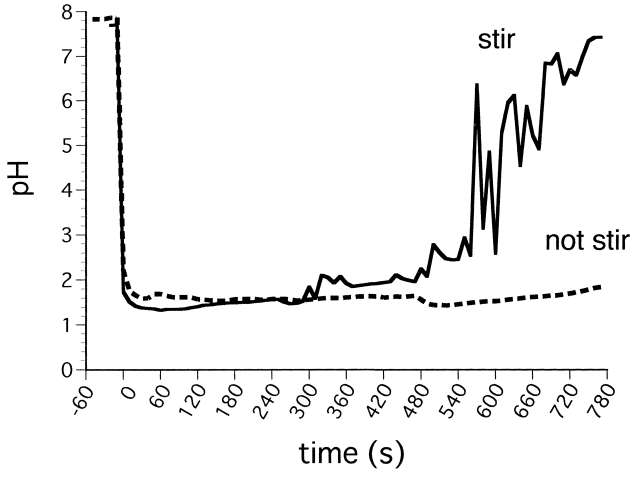

Fig. 1. Time course of the pH recording during and after insertion of the electrode into the tunic. The electrode was inserted at 0 s. The dotted line represents measurement in still seawater. The solid line represents measurement in seawater that was being stirred.

Area measurement in the sections

A rectangular area was randomly selected in each paraffin section from five animals. The selected area, 300 μm wide, extended from the surface of the tunic to its innermost end, adjacent to the epidermis. A square area (300×300 μm), at the top of this rectangle, was also selected so as to encompass the region beneath the tunic surface. An illustration of the selected areas is shown in Fig. 2. The proportion of the selected area occupied by tunic or by the vacuolar lumen of the tunic bladder cells were measured by digital images on a Macintosh computer using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/).

Measurement of tunic volume

We used 14 specimens ranging from 5.1 to 39.1 g wet weight. Each specimen was dissected and all tissues other than the tunic were removed. The tunic was submerged in water in a measuring cylinder, and the volume of displaced water was taken as a measure of tunic volume.

Determination of the counter anions

We peeled the surface layer of the tunic and cut it into pieces with a razor blade. The tunic piece (about 20×15×2 mm) was rinsed in distilled water for 30 min and then minced in 20 ml distilled water. The acidic fluid exuded from the tunic was diluted in the distilled water, and this solution was used as the sample. Twenty microliters of the sample was injected into an ion-chromatograph HIC-6A with an electric conductivity detector CDD-6A (Shimadzu, Kyoto, Japan). A Shim-Pack IC-A1 (Shimadzu) column was used, and the mobile phase was 2.5 mM phthalic acid–2.4 mM Tris (pH 4.0) at 40°C. The flow rate was 1.5 ml/min.

RESULTS

Acidity of the tunic

In 41 specimens of various wet weights (3–40 g), the pH inside the tunic at the central part of the right body ranged from 0.8 to 1.9. The correlation between wet weight and tunic pH was not significant ($p>0.05$, Spearman's correlation coefficient by rank test). The tunic pH, measured four times in a single year, was almost unchanged (Table 1).

The tunic pH was measured at eight different points for each of 10 individuals on November 4, 1999. The pH was almost the same at all points of the tunic, except that the basal part of the body, which attaches to the substratum, had a higher pH than the other points (Table 2).

Fig. 1 shows two examples of the time course of the pH recording during the insertion of the needle-tip pH electrode. When the electrode was inserted, the pH decreased rapidly from about pH 8 to below pH 2 and remained stable for more than 5 min. When the seawater was stirred, the pH initially dropped from pH 8 to below pH 2 and then began to rise after

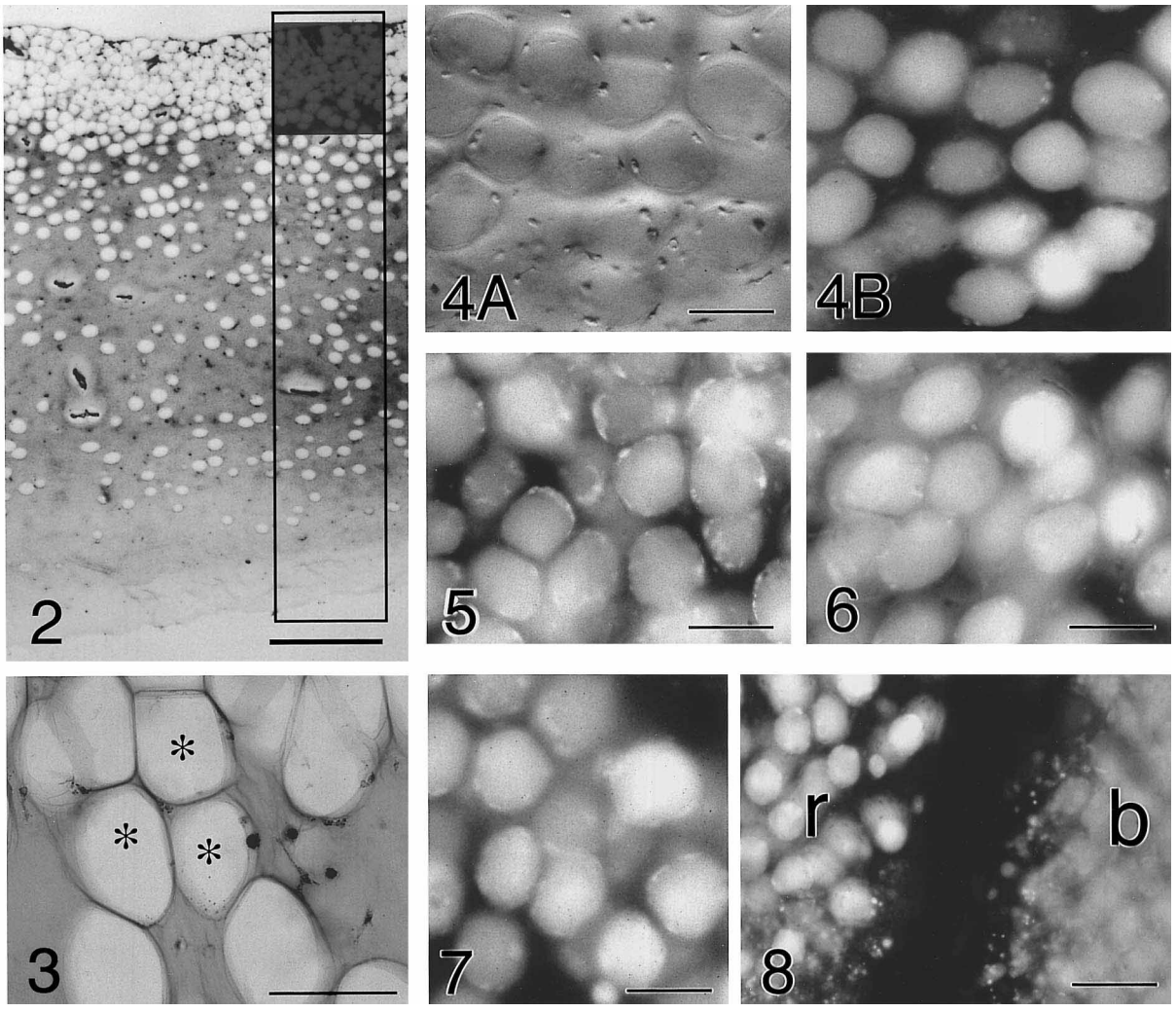

Fig. 2. Histological section of the tunic (hematoxylin–eosin staining). Many of the tunic bladder cells are distributed beneath the tunic surface. The rectangle and shaded square indicate sample areas for measurement of area occupied by vacuolar lumen of tunic bladder cells. Scale bar, 300 μm.
Fig. 3. The tunic cells in a section stained with alcian blue and PAS. The vacuolar lumen of the tunic bladder cells (asterisks) is barely stained. Scale bar, 50 μm.
Figs. 4–8. Unfixed tunic slice stained with LysoSensor: **Fig. 4.** Paired images of the tunic of the branchial siphon observed with differential interference contrast optics (A) and epifluorescence (B). **Fig. 5.** The tunic around the right side. **Fig. 6.** The tunic around the dorsal side. **Fig. 7.** The tunic around the basal part. **Fig. 8.** The tunic of the right side (r) and the basal part (b). Scale bars indicate 50 μm for Figures 4–7 and 100 μm for **Fig. 8**.

Table 1. Tunic pH measured during 1999

Date	Average	*SD*	Temperature*
Jan. 21	1.1	0.14	21.3
Apr. 15	1.0	0.11	23.6
Jul. 21	1.5	0.27	29.6
Nov. 4	1.1	0.24	24.6

* Average of the surface water temperature in each month at Sesoko Island (Okinawa Prefecture).

Table 2. pH inside the tunic at eight points

Point	pH (average)	*SD*
Branchial siphon	1.1	0.2
Atrial siphon	1.1	0.2
Midpoint between the siphons	1.1	0.2
Center of the right s ide of the body	1.1	0.2
Center of the left side of the body	1.1	0.2
Center of the dorsal side of the body	1.1	0.2
Center of the ventral side of the body	1.1	0.3
Basal part attaching to the substratum	3.3	1.3

about 5 min. About 11 min after insertion of the electrode, the pH was greater than 7.

The pH at the tunic surface averaged 4.7 (*SD*=0.77) in 23 specimens. In 15 specimens, the average surface pH was 4.8 (*SD*=0.76) decreased to 1.9 (*SD*=0.36) after gentle wiping with tissue paper (Kim wiper).

Distribution and amount of tunic acid

Tunic acid has been found in tunic bladder cells, which are highly vacuolated tunic cells of about 50 μm in diameter (cf. Goodbody, 1974). Our histological sections show that the tunic bladder cells are densely distributed beneath the tunic surface (Fig. 2). The tunic matrix was stained with alcian blue, and the cytoplasm of some tunic cells was PAS positive, whereas the vacuolar lumen of the tunic bladder cells was not stained (Fig. 3).

The distribution of tunic acid was compared among five parts of the tunic: branchial siphon, right side, ventral side, dorsal side, and basal part of the tunic. Paired images of differential interference contrast microscopy and LysoSensor staining (Fig. 4) showed that the vacuolar lumen of the tunic bladder cells was filled with tunic acid, as previously reported (Stoecker, 1978; Hirose, 1999). The distribution pattern and the number of cells per unit area were almost the same among the tunic areas of branchial siphon, right side, dorsal side, basal part (Figs. 4–7), and ventral side (data not shown). The intensity of the fluorescence in the vacuolar lumen was slightly weaker in the basal part of the tunic than in the other four parts. The intensities cannot be directly compared among the Figs. 4–7, because the exposure time was different in each micrograph. However, the fluorescence intensities in the tunic of right side and basal part can be compared in Fig. 8, where the basal vacuolar lumens are clearly darker.

Table 3 shows the relative area of the histological sections occupied by the vacuolar lumens of the tunic bladder cells. The vacuolar lumen occupies 25% of the total tunic within rectangular areas (300 μm wide) that extends from the surface of the tunic to its innermost end (rectangle in Fig. 2). Moreover, the vacuolar lumen occupies 70% of the square (300×300 μm) just beneath the tunic surface (blackened square in Fig. 2).

Table 3. The proportion of the area occupied by vacuolar lumen in the tunic

	Average (%)	*SD*	*n*
In total tunic	25	3.5	5
In 300×300 μm area beneath the tunic surface	70	3.9	5

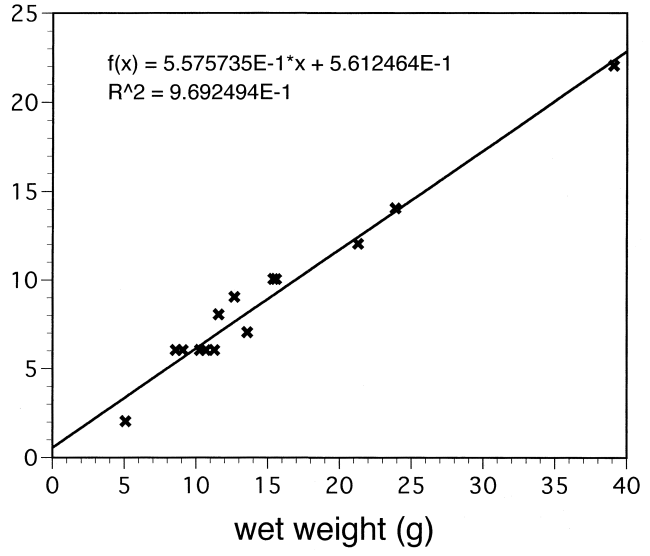

Fig. 9. Correlation between the wet weight of the ascidians and their tunic volume.

Simple regression analysis showed that there is a high correlation between the wet weight of the ascidians and their tunic volume (Fig. 9). For example, the tunic volume is about 6 ml in an animal of 10 g wet weight according to this regression line.

Major anion in tunic acid

The anion composition in the exudates from samples of minced tunic was determined by anion chromatography.

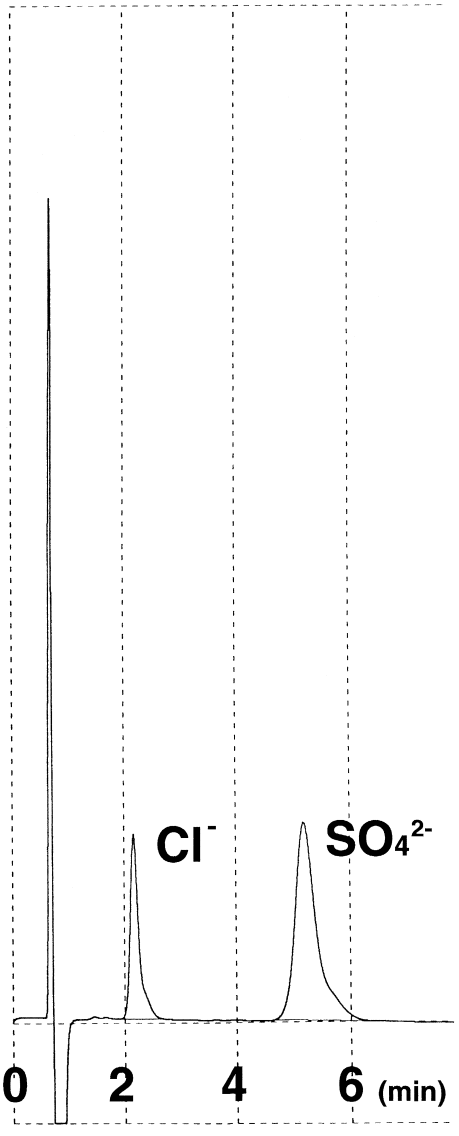

Fig. 10. Anion chromatograph showing that the major anion in the acidic fluid exuding from the tunic is SO_4^{2-}.

Because the specimens were cut from the surface area of the tunic, the exudate was mostly tunic acid. In these preparations, sulfuric ion was the major anion, and the SO_4^{2-}/Cl^- ratio was 4.63 (Fig. 10).

DISCUSSION

The tunic of *Phallusia nigra* contains acidic fluid of about pH 1 as measured with the needle-tip pH electrode. Because the acidic fluid is "mixed" with seawater and blood plasma that is not acidic (cf. Hirose, 1999) in this method, the real pH of the acidic fluid may be even lower. The pH of the tunic is invariable throughout the year and among individuals of various sizes. The tunic pH is also invariable among the seven points tested in the body (branchial siphon, atrial siphon, midpoint between the siphons, right side, left side, dorsal side, and ventral side), whereas the pH is higher at the basal part of the body where the ascidian attaches to the substratum. The acidic fluid is contained exclusively in the vacuolar lumen of tunic bladder cells (Stoecker, 1978; Hirose, 1999), and the density of these cells in the basal part of the tunic is almost the same as that in other parts of the tunic (Figs. 4–7). Therefore, the higher tunic pH in the basal part might not be caused by a difference in the amount of tunic acid. The staining with LysoSensor, a pH-sensitive fluorescent dye, showed that the intensity of the fluorescence was weaker in the basal part of the tunic than in the other parts (Fig. 8). Because the intensity of the fluorescence is stronger in a more acidic environment, the bladder cell content in the basal part of the tunic is probably less acidic than that in other parts. In *Rhopalea birkelandi*, Stoecker (1980b) reported that the tunic around the siphons is acidic and is not fouled, whereas the tunic of the basal half of the body is non acidic and fouled. In *P. nigra*, the entire tunic is acidic and free of epibionts. Tunic acid appears to be involved in anti-fouling as well as anti-predation (Stoecker, 1978, 1980 a, b, c).

Parry (1984) claimed that the defensive functions of tunic acid are doubtful because the undamaged tunic is neutral, and the acid released from the damaged tunic is rapidly neutralized in seawater in some aplousobranchian ascidians. In contrast, our report shows that the undamaged tunic surface is slightly acidic in *P. nigra* (about pH 4.5), and this condition would be effective in preventing some epibionts from settlement and growth. The surface pH decreases after stimulation by gentle wiping. Therefore, mild stimuli may easily lead to the release of tunic acid that may kill or clear epibionts from the tunic surface and irritate predators. It is also possible that the tunic surface is neutral in intact animals, and that handling the animals or pressing on the surface with a flat-surface pH electrode induces some acid release from the tunic. When the needle-tip pH electrode was inserted slightly into a tunic immersed in seawater, the low pH (about 2) was maintained for 5 min. This finding indicates that neutralization of tunic acid is not very rapid at the wound site, and that tunic acid is probably effective in anti-predation and anti-infection.

In histological observations, alcian blue stains nothing in the vacuolar lumen of the tunic bladder cells where tunic acid is contained, and thus, acidic polysaccharides are probably not involved in the acid storage. Many tunic bladder cells are concentrated beneath the tunic surface, and this type of distribution is consistent with the defensive function of tunic acid. Measurements indicate that the vacuolar lumen occupies 25% of the total tunic at the central part of the body. If this value of vacuolar lumen occupation is consistent in all parts of the tunic, an individual of 10 g wet weight can be estimated to contain tunic acid of 1.5 ml (Table 3 and Fig. 9).

The major anion is SO_4^{2-} in the tunic exudate. Webb (1939) reported that the SO_4^{2-}/Cl^- ratios of seawater and ascidian blood plasma are 0.646 and 0.640, respectively. The ratio (4.63) in the tunic exudate in *P. nigra* indicates that this fluid contains much more SO_4^{2-} than Cl^-, especially when compared to the ratios in seawater and ascidian blood plasma. We estimate that most of the tunic acid is sulfuric acid. The presence of free sulfuric acid in vacuolated blood cells has been reported in some ascidian genera (reviewed in Wright, 1981). These vacuolated blood cells may share a common cell lineage with tunic bladder cells.

The present study revealed several properties of the tunic acid in *P. nigra*; 1) the acidity of the tunic is about pH 1 and almost stable regardless of season, body size, or location of the tunic (except for the basal part), 2) the tunic contains large amount of sulfuric acid just beneath the tunic surface, 3) the pH of tunic surface is slightly acidic and the acidity increase responding to physical stimuli, and 4) the acidity at the wound is stable at pH 1–2 for more than five minutes. These results support the possibility that tunic acid could be an effective agent in *P. nigra* to protect the organism from predation, fouling, and infections. This species is usually free of epibionts, and it is exposed to predators. Acidic fluid of about pH 1 could be released from the tunic in response to an attack by predators or the settlement of epibionts. When the tunic is wounded, tunic acid could disinfect the wound surface and prevent microorganisms from invading the tunic. The mechanism of acid production and release should be investigated in future studies.

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