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Authors: Yoshikawa, Akihiro, Nakazawa, Shiori, and Asakura, Akira

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# A Brief Description of Surface Structure and Composition of the Pseudo-Snail Shell Formed by a Sea Anemone *Stylobates* sp. Symbiotic with Hermit Crabs from the Deep-Sea Floor

Akihiro Yoshikawa<sup>1\*</sup>, Shiori Nakazawa<sup>2†</sup>, and Akira Asakura<sup>1</sup>

<sup>1</sup>*Seto Marine Biological Laboratory, Field Science Education and Research Center, Kyoto University, 459 Shirahama, Nishimuro, Wakayama 649-2211, Japan*

<sup>2</sup>*Sugashima Marine Biological Laboratory, Graduate School of Science, Nagoya University, 429-63 Sugashima, Toba, Mie 517-004, Japan*

Sea anemones belonging to the genera *Adamsia* and *Stylobates* have a remarkable symbiotic relationship with hermit crabs. These symbiotic sea anemones produce a shell-like structure, called a “carcinoecium,” that covers and extends over the gastropod shell of the host hermit crab as hermit crabs grow. This structure has been described as “chitinous carcinoecium” or “chitinous coating.” A previous study investigated carcinoecia of *Stylobates aeneus*, the results of which indicated that it contained at least 1.7% chitin, while the remaining components were unidentified. Moreover, the microscopic structure of a carcinoecium still remains to be detailed. We, therefore, conducted detailed observations using a stereoscopic microscope and scanning electron microscope (SEM) and the analyses of the chemical composition of carcinoecia produced by *Stylobates* sp. (apparently conspecific with *Isadamsia* sp. “J” reported in Uchida and Soyama, 2001) associated with a pagurid hermit crab *Pagurodoyleinia doederleini* collected in the south of the Shima Peninsula, Mie, Honshu Island, Japan at a depth of 294–306 m. Our results indicate that carcinoecia of *Stylobates* sp. contain HCl-soluble components (13%), NaOH-soluble components (38%), chitin (11%) and unidentified remnants (39%). Additionally, our observations show that *Stylobates* sp. incorporates dark- and white-colored particles that could be sand and/or mud into the carcinoecium.

**Key words:** carcinoecium, chitin, protein, symbiosis, shell-forming, SEM

## INTRODUCTION

Symbiosis is a widespread phenomenon in the sea, and ecological relationship in the symbiosis is one of major forces of evolution of interspecific interaction and shaping marine communities. Decapod crustaceans are a major component in marine community, and, in particular, hermit crabs are known to have a wide variety of symbiotic relationships with other invertebrates (Williams and McDermott, 2004; Goto et al., 2006; Yoshikawa et al., 2018a).

The symbiotic relationship between hermit crabs and sea anemones is a well-known example of mutualism in the sea. Many species of hermit crabs carry symbiotic sea anemones on their chelipeds or the external surfaces of the shells they inhabit (Williams and MacDermott, 2004; Antoniadou et al., 2013). The benefits for host hermit crabs are mainly protection from predators, such as cephalopods and fish, by the nematocysts of sea anemones (Ross, 1971; Balasch and Mengual, 1973; Ross and Boletzky, 1979; McLean, 1983) and use of the sea anemones as stock food (Imafuku et al., 2000). The symbiotic sea anemones also

benefit from their association with hermit crabs, as the sea anemones gain access to food resources (Stachowitsch, 1979, 1980), substratum availability (Conover, 1979; Brooks, 1989), protection from predators, and increased dispersal (Balss, 1924; Jonsson et al., 2001; McLean and Mariscal, 1973). Since both hermit crabs and sea anemones gain benefits from their symbiotic associations, those relationships have been recognized as “mutualism” (Antoniadou et al., 2013). Some species of hermit crabs have the ability to transfer the symbiotic anemones onto their new shells when they change shells (Ross, 1974, 1975; Yoshikawa et al., 2018b).

A remarkable mutualistic relationship is seen between sea anemones of the genera *Adamsia* Forbes, 1840, and *Stylobates* Dall, 1903 and hermit crabs. These anemones settle on gastropod shells inhabited by hermit crabs and produce a bronze-colored shell-like structure that first covers, and then extends over the host hermit crab shell as the host hermit crab grows (Dunn and Liberman, 1983; Antoniadou et al., 2013). Both parties benefit from this symbiotic relationship; the hermit crab might not have to seek new, larger shells as it grows, and the anemone does not have to change the substrate. The protective “shell” produced by the anemone, the carcinoecium, has been described previously as “chitinous carcinoecium” or “chitinous coating” (Antoniadou et al., 2013; Crowther et al., 2011; Dunn et al., 1980; Fautin,

\* Corresponding author. E-mail: akj.kt.ex80@gmail.com

† Current affiliation: Hitachi, Ltd. Research and Development Group, 2520 Akanuma, Hatoyama, Hiki, Saitama 350-0395, Japan  
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1987). The carcinoecium of *Stylobates aeneus* Dall, 1903 has been reported to contain chitin, but this represents only 1.7% of the total components (Dunn and Liberman, 1983). To date, the remaining components of carcinoecia have remained unidentified, meriting further detailed investigation.

During a survey that was conducted on benthic organisms around Kumano-Nada, south of the Shima Peninsula, Mie, Honshu Island, Japan (at a depth of 294–306 m), we collected specimens of a species of *Stylobates* sp. found on shells used by a pagurid hermit crab *Pagurodoyleinia doederleini* (Doflein, 1902) (Fig. 1). This sea anemone is conspecific with *Isadamsia* sp. J reported in Uchida and Soyama (2001) and Yanagi (2006) judged from morphological characters including body shape and coloration as well as direction of the mouth (Fig. 1A). It is unique, unlike other *Stylobates* species, in producing only an extension portion of the lip of an aperture of a gastropod shell inhabited by a hermit crab (Fig. 1B, C). Since *Isadamsia* is now a junior synonym of *Stylobates* (Dunn et al., 1980), we hereinafter refer to this sea anemone as *Stylobates* sp.

Our research focused on describing the surface structure and the chemical components of carcinoecia produced by *Stylobates* sp. We observed the surface structure of carcinoecia using stereoscopic microscope and scanning electron microscope (SEM). This is the first description of the surface structure of the carcinoecia part with high magnification images from SEM. In addition, we also investigated the components by extracting minerals and proteins from shells based on the methods for chitin isolation from crustacean shells (Younes and Rinaudo, 2015). We then confirmed the presence of proteins and chitin in carcinoecia by protein analysis and chitinase treatment.

## MATERIALS AND METHODS

### Field sampling and preparation of material for analysis

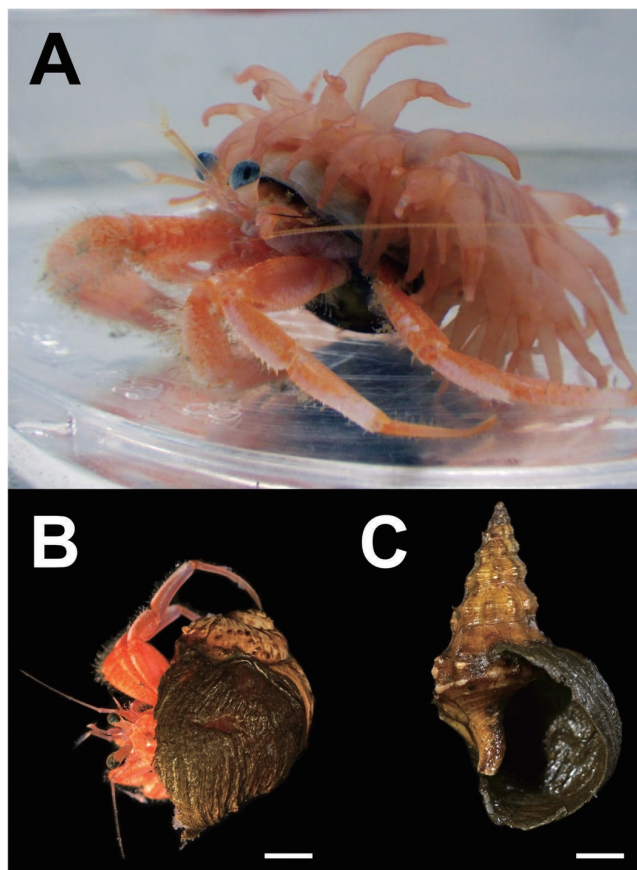
Specimens of the hermit crab *P. doederleini* were collected by beam trawl from the south of the Shima Peninsula, Mie, Honshu Island, Japan, on 7 November 2017 (depth 294–306 m; 34°07.56N, 136°40.47E to 34°07.91N, 136°39.44E), and those bearing *Stylobates* sp. sea anemones were used for chemical analysis.

In the laboratory, specimens of *Stylobates* sp. were removed from the gastropod shells. Three gastropod shells, each bearing a carcinoecium, were dried at room temperature (25°C) for 12 h.

Once dry, the bronze-colored carcinoecia, which exclusively contain the extended parts of carcinoecia, were carefully removed from the gastropod shells and powdered using a pestle and mortar. Because *Stylobates* sp. extends the portion of the lip from the aperture of a snail shell (Fig. 1B, C), we used the extended parts for

experiments to avoid contamination by the snail shell part. Approximately 100 mg of carcinoecium powder was divided into four 1.5-ml tubes.

The surface structure of both inner and outer sides and partial cross-section were observed with the stereoscopic microscope. Additionally, the high-magnification images of surface structure of carcinoecium were obtained by using a scanning electron microscope (SEM). For SEM imaging, we fixed the dry carcinoecia to a



**Fig. 1.** *Stylobates* sp. and its carcinoecium associated with *Pagurodoyleinia doederleini* collected by beam trawl from the south of the Shima Peninsula, Mie, Honshu Island, Japan, on 7 November 2017 (depth 294–306 m; 34°07.56N, 136°40.47E to 34°07.91N, 136°39.44E). (A) the gastropod snail shell covered by *Stylobates* sp.; (B) and (C) the gastropod shell (pale colored portion) and the carcinoecium portion as an extension of a lip of the aperture of the gastropod shell (black portion) (B, Voucher ID, SMBL-V00002; C, Voucher ID, SMBL-V00003). Scale bar = 5 mm.

**Table 1.** Specimens of *Stylobates* sp. used for structural observation and analysis of the chemical composition of carcinoecia, and their host hermit crabs and gastropod shells. All specimens were collected from Kumano-Nada, South of the Shima Peninsula, Mie, Honshu Island, Japan (294–306 m deep) on 7 Nov 2017 by beam trawl. \* indicates the specimens used for chemical analysis. \*\* indicates the specimen used for structural observation.

Species			Voucher ID		
Sea anemone	Hermit crab	Gastropod shell	Sea anemone	Hermit crab	Gastropod shell
<i>Stylobates</i> sp.	<i>Pagurodoyleinia doederleini</i>	Naticidae gen. et sp. indet.	SMBL-V00001*	SMBL-V00009	SMBL-V00013
<i>Stylobates</i> sp.	<i>Pagurodoyleinia doederleini</i>	<i>Bulbus tenuiculus</i>	SMBL-V00002**	SMBL-V00010	SMBL-V00014
<i>Stylobates</i> sp.	<i>Pagurodoyleinia doederleini</i>	<i>Fusinus</i> sp.	SMBL-V00003*	SMBL-V00011	SMBL-V00015
<i>Stylobates</i> sp.	<i>Pagurodoyleinia doederleini</i>	Buccinidae gen. et sp. indet.	SMBL-V00004*	SMBL-V00012	SMBL-V00016

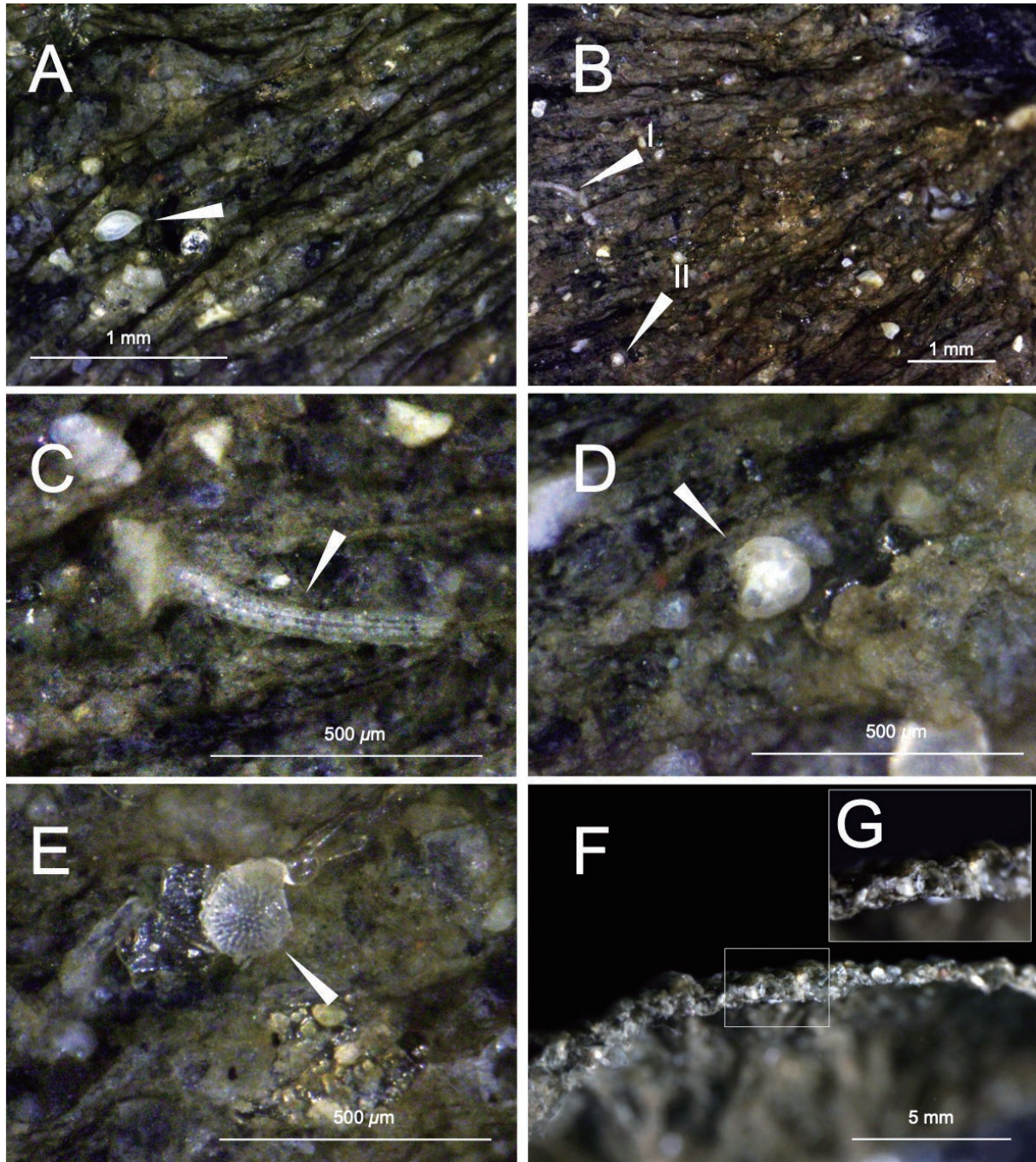


stage with adhesive tape and coated them for 1 min with platinum using an auto-fine ion coater (QUICK AUTO COATER SC-701AT, Sanyu Electron Co., Ltd., Tokyo, Japan). SEM images were obtained by an SS-550 scanning electron microscope (Shimadzu) at an accelerated voltage of 15 kV under high vacuum.

#### Investigation of carcinoecium components

For demineralization (Younes and Rinaudo, 2015), 1000  $\mu$ l of

1 M HCl was added to the powdered carcinoecium, the samples were centrifuged at 3000  $\times$ g for 1 min, and the supernatant was discarded. This process was repeated until the supernatant became strongly acidic. After confirming that the pH of the supernatant had dropped to pH 1 or 2 using indicator paper (UNIV, 07011030, Toyo Roshi Kaisha, Ltd., Tokyo, Japan), the powdered samples were resuspended in acetone and dried in a centrifugal concentrator for 10 min. Finally, the weight of the sample was measured.



**Fig. 2.** Surface and partial cross-section of the carcinoecium produced by *Stylobates* sp. (part of SMBL-V00002) observed by the stereoscopic microscope. (A) image of the outer surface; (B) images of the inner surface; (C) unidentified biological-organisms indicated by "I" in (B); (D) included foraminiferans, Elphidiidae gen. et sp. indet., indicated by "II" in (B); (E) fragment of diatoms, Coscinodiscaceae gen. et sp. indet., in the carcinoecium; (F) the partial cross-section of the carcinoecium; (G) the enlarged view of the partial cross-section indicated by white square in (F).



For protein content analysis, 1000  $\mu\text{l}$  1 M NaOH was added to the HCl-extraction residue to extract crude proteins (Younes and Rinaudo, 2015). The samples were centrifuged at 3000  $\times g$  for 1 min and the supernatant was discarded; 1000  $\mu\text{l}$  NaOH was added and the sample was agitated at 60°C for 60 min with moderate mixing. After 60 min, the samples were centrifuged at 3000  $\times g$  for 1 min and the supernatant was discarded. This process was repeated five times. The samples were then resuspended in 1000  $\mu\text{l}$  deionized water (DW) to wash them, centrifuged at 3000  $\times g$  for 1 min, and the supernatant was discarded. After washing with DW, the samples were resuspended in acetone and dried in a centrifugal concentrator for 10 min. Finally, the decrease in weight was measured.

#### Biuret test and xanthoproteic reaction

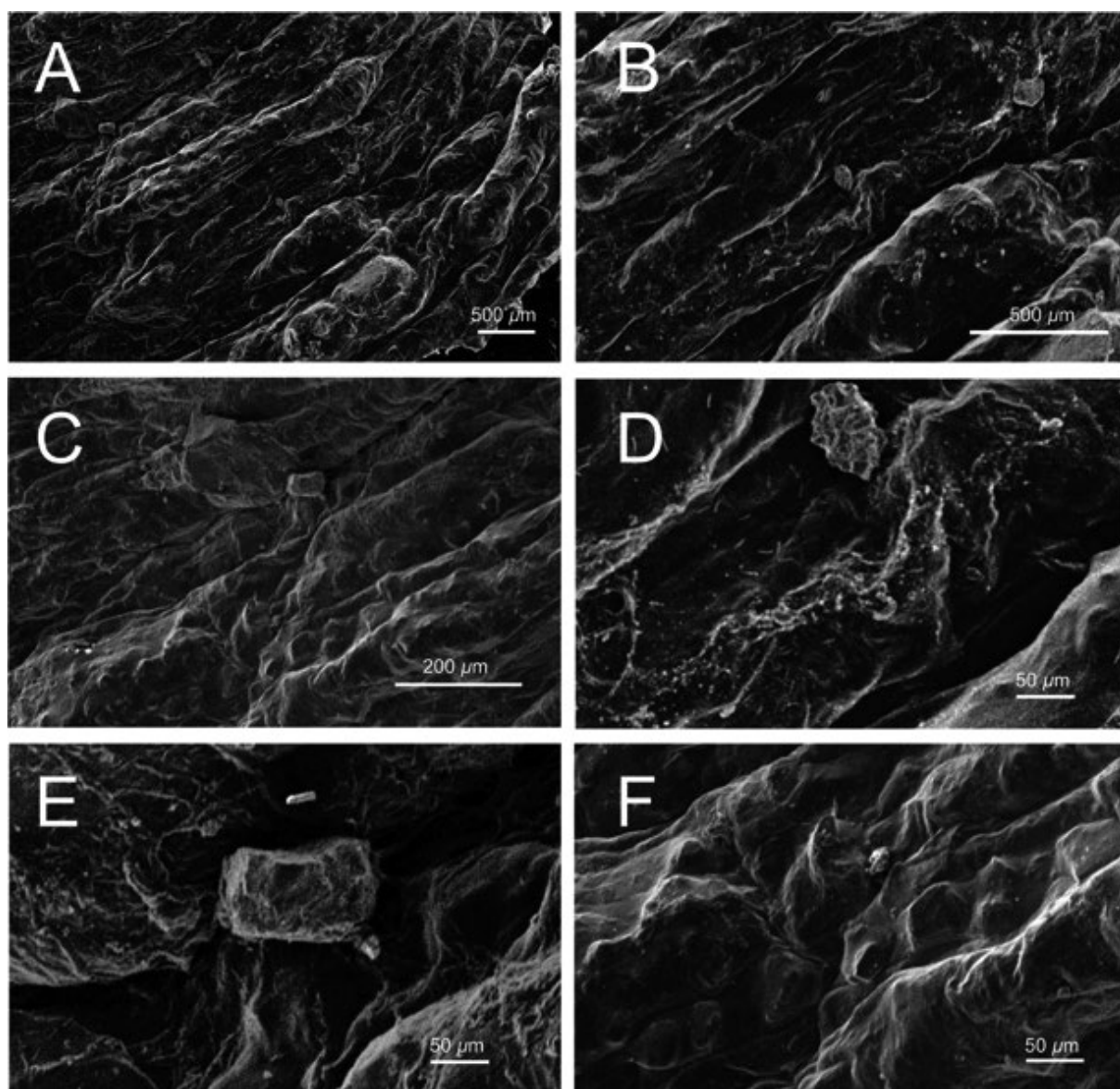
The NaOH extract was neutralized with 35% hydrochloric acid and desalted using a gel filtration column (PD-10, 17085101; GE Healthcare, Chicago, IL, USA), according to the manufacturer's gravity flow protocol. It was eluted with 3.5 ml of ultrapure water,

every 1 ml and the last 0.5 ml of which were pooled as fractions 1, 2, 3, and 4. Ultrapure water and 0.1% skimmed milk solution (Morinaga Milk Industry Co., LTD., Tokyo, Japan) fractions were subjected to a biuret test by mixing 100  $\mu\text{l}$  sample solution with 5  $\mu\text{l}$  1 M sodium hydroxide and 2  $\mu\text{l}$  1% copper (II) sulfate. To intensify the color, the mixtures were condensed with a centrifugal concentrator.

For the xanthoproteic reaction, 50  $\mu\text{l}$  of the eluates from the gel filtration were mixed with one drop of nitric acid (approximately 60%), heated at 95°C for 15 min, then neutralized with 50  $\mu\text{l}$  10 M sodium hydroxide.

#### SDS-PAGE and Coomassie Brilliant Blue (CBB) staining

The NaOH extracts were neutralized with 35% hydrochloric acid, desalted by adding an equal volume of water and a 6-fold volume of ice-cold acetone, incubated at  $-20^{\circ}\text{C}$  for 2 h, and centrifuged at 15,000 rpm for 10 min for acetone precipitation. Desalted NaOH extracts were dissolved in 6 M urea, mixed with sodium



**Fig. 3.** Scanning electron microscope (SEM) images of the outer surface of the carcinoecium produced by *Stylobates* sp. (part of SMBL-V00002). (A) surface structure ( $\times 20$ ); (B) and (C) surface structure ( $\times 50$ ); (D) and (E) included-sand on the carcinoecium ( $\times 200$ ); (F) included-materials covered by the substance produced by *Stylobates* sp. ( $\times 100$ ).

dodecyl sulfate (SDS) sample buffer (final concentration: 10% glycerol, 2% SDS, 0.1% bromophenol blue, and 50 mM Tris-HCl at pH 6.8) with or without 1% by volume 2-mercaptoethanol, heated at 95°C, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 5%–20% gradient polyacrylamide gel (E-T520L; Atto Corporation, Tokyo, Japan) with a protein molecular weight marker (26614; Thermo Fisher Scientific, Waltham, MA, USA). The gel was then equilibrated with 10% acetic acid and 50% methanol, stained in 0.25% CBB-R250, 5% acetic acid, and 50% methanol for 2 h, and finally destained in 7% acetic acid and 25% methanol.

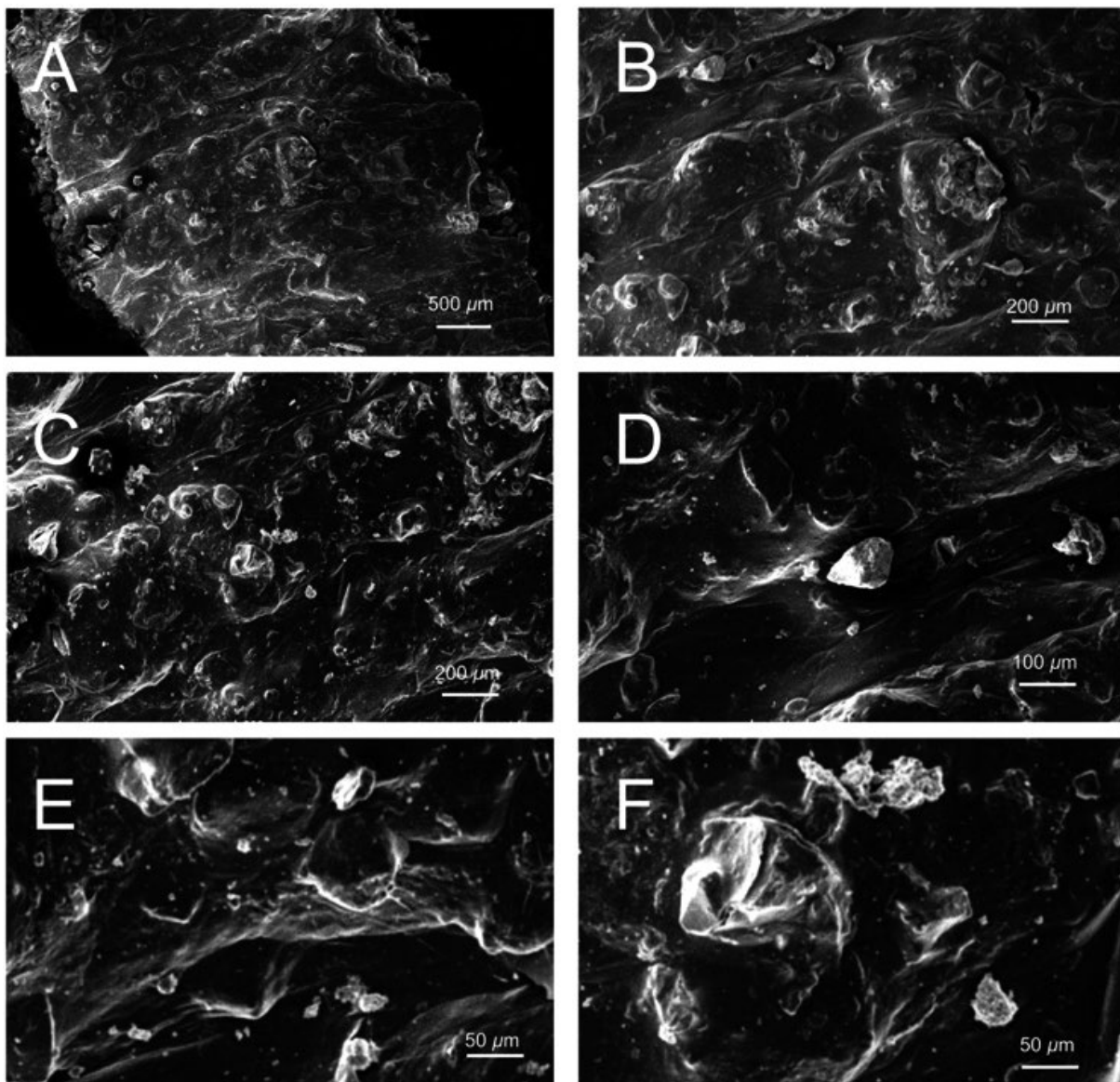
#### Liquid chromatography/mass spectrometry (LC/MS)

CBB-stained protein bands were excised from the polyacrylamide gel and subjected to in-gel digestion, LC/MS, and a database search, as described previously (Nakazawa et al., 2015). Briefly, samples were reduced with dithiothreitol and carbamidometh-

ylated and carbamidomethylated, digested with modified trypsin (V511A; Promega, Madison, WI, USA), separated in an octadecyl silica gel column over a gradient of acetonitrile containing 0.1% formic acid, and analyzed with a mass spectrometer (LTQ-XL; Thermo Fisher Scientific) equipped with an electrospray ionization unit. The spectra were compared against a database of 105,046 Actiniaria protein sequences retrieved from the NCBI Protein database on January 16, 2018, and a database of 556,388 SwissProt database entries retrieved on December 26, 2017, using Mascot ver 2.4.1 software. Hits with protein scores of 40 or more were regarded as “detected.” Tryptic digests of 100 fmol bovine serum albumin (BSA) gave protein scores of 522 and 683 in two repeated scans conducted just before the analysis of the NaOH extracts.

#### Chitinase treatment

For the chitinase treatment, we used part of the residue after NaOH treatment and divided the samples into four 1.5-ml tubes to



**Fig. 4.** Scanning electron microscope (SEM) images of the inner surface of the carcinoecium produced by *Stylobates* sp. (part of SMBL-V00002). (A) surface structure ( $\times 20$ ); (B) and (C) surface structure with included sand on the carcinoecium ( $\times 50$ ); (D) included-sand on the carcinoecium; (E) included-materials covered by the substance produced by *Stylobates* sp. ( $\times 100$ ); (F) semi exposed-materials from substance produced by *Stylobates* sp. ( $\times 200$ ).



increase reaction efficiency. Two of the aliquots were treated with chitinase and two were chitinase-free blanks (Supplementary Table S1). First, the samples were made colloidal with 35% hydrochloric acid and neutralized with 10 M NaOH. Then, 1000  $\mu$ l DW was added, the samples were centrifuged at 3000  $\times$ g for 1 min, and the supernatant was discarded. We replaced the supernatant with 1000  $\mu$ l chitinase buffer A (25 mM Tris-HCl pH 7.5, 1 mM Ethylenediaminetetraacetic acid (EDTA), 25 mM sodium chloride) (Oku and Ishikawa, 2014). Then, 150  $\mu$ l chitinase (Thermostable, Recombinant, Solution. 034-19891 Wako, Lot WDP4902 [EC 3.2.1.14], Activity 140 units/ml) was added to one of the divided samples, and nothing to the other. We maintained both samples at 85°C for 8 d. After 8 d, we resuspended the samples in acetone and dried them using a centrifugal concentrator for 10 min. Finally, the weight was measured.

### Sample deposition

All *Stylobates* sp. specimens, along with their host hermit crabs and shells as well as the residues from all experiments, were deposited in the Seto Marine Biological Laboratory, Kyoto University, with the catalog numbers (Table 1 and Supplementary Table S1).

## RESULTS

### Microscopic structure of a carcinoecium

The carcinoecium was thinner than the snail shell from which it extended (approx. 0.5 to 1.0 mm) (Fig. 2F, G), and the growth rings were recognized on the surface. The carcinoecium included many dark- and white-colored particles including sand and foraminiferans (Elphidiidae Galloway, 1933 gen. et sp. indet.) (Fig. 2A, B, D), fragment of unidentified organism (Fig. 2C) and diatoms (Coscinodiscaceae Kützing, 1844 gen. et sp. indet.) (Fig. 2E). These materials were uniformly scattered rather than being patchy on the carcinoecium. Layered structures as observed in gastropod snail shells were absent in the carcinoecium (Fig. 2F, G). In the SEM images, the outer surface of carcinoecium was smoother than the inner surface was. SEM images showed that the included materials were covered by the substances produced by *Stylobates* sp. (Figs. 3, 4). In the inner surface, more exposed particles were observed than in the outer surface of the carcinoecium (Fig. 4).

### Carcinoecium components

We obtained a total of 385.36 mg of carcinoecium powder. The amount of carcinoecium powder and the decreases in weight after each treatment are shown in Supplementary Table S2. The total decreases in weight after HCl treatment for demineralization and NaOH treatment for crude protein extraction were 49.27 mg and 144.79 mg, respectively, leaving a residue of 191.30 mg. Thus, the carcinoecium comprised 13% HCl-soluble component and 38% NaOH-soluble component (crude protein). The carcinoecium vigorously released bubbles when exposed to HCl. The residual 49.64% was examined next.

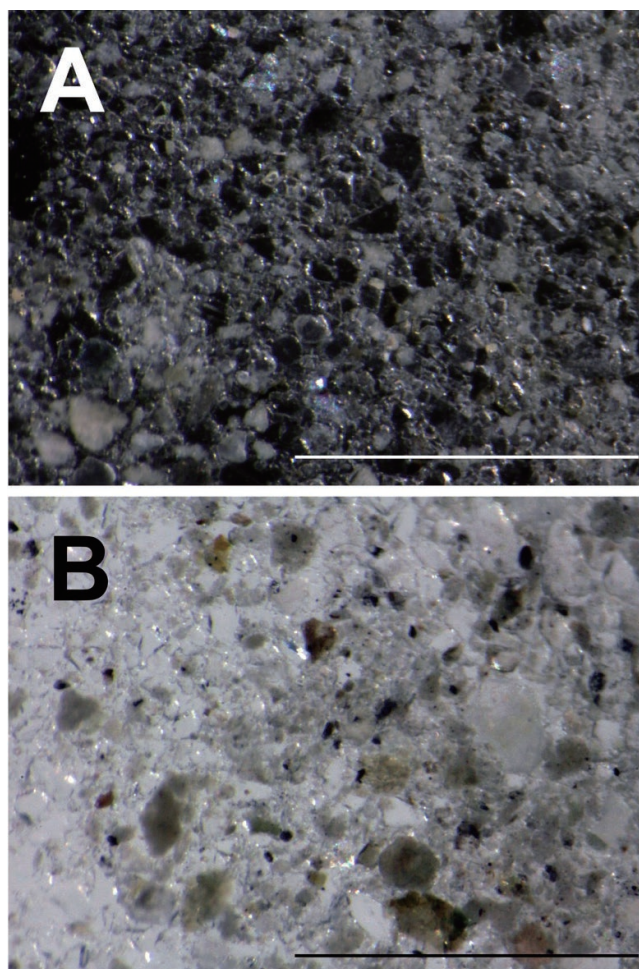
Of the 191.30 mg residue obtained after NaOH treatment, 55.01 mg was used to test for the presence of chitin (28.69 mg for chitinase treatment and 26.32 mg for chitinase-free control). The weights of the samples before and after chitinase treatment are shown in Supplementary Table S1. The decreases in sample weights after chitinase treatment were 7.12 mg (chitinase 1), 0.94 mg (chitinase-free 1, blank), 0.86 mg (chitinase 2, 10-fold dilution), and 0.74 mg

(chitinase-free 2, blank) (Supplementary Table S1). Therefore, the 28.69 mg of residue after NaOH treatment contained at least 6.30 mg (22%) chitin. Thus, the carcinoecium contained at least 11% of chitin and 39% of other unidentified components. The unidentified components contained insoluble dark- and white-colored particles (Fig. 5A, B).

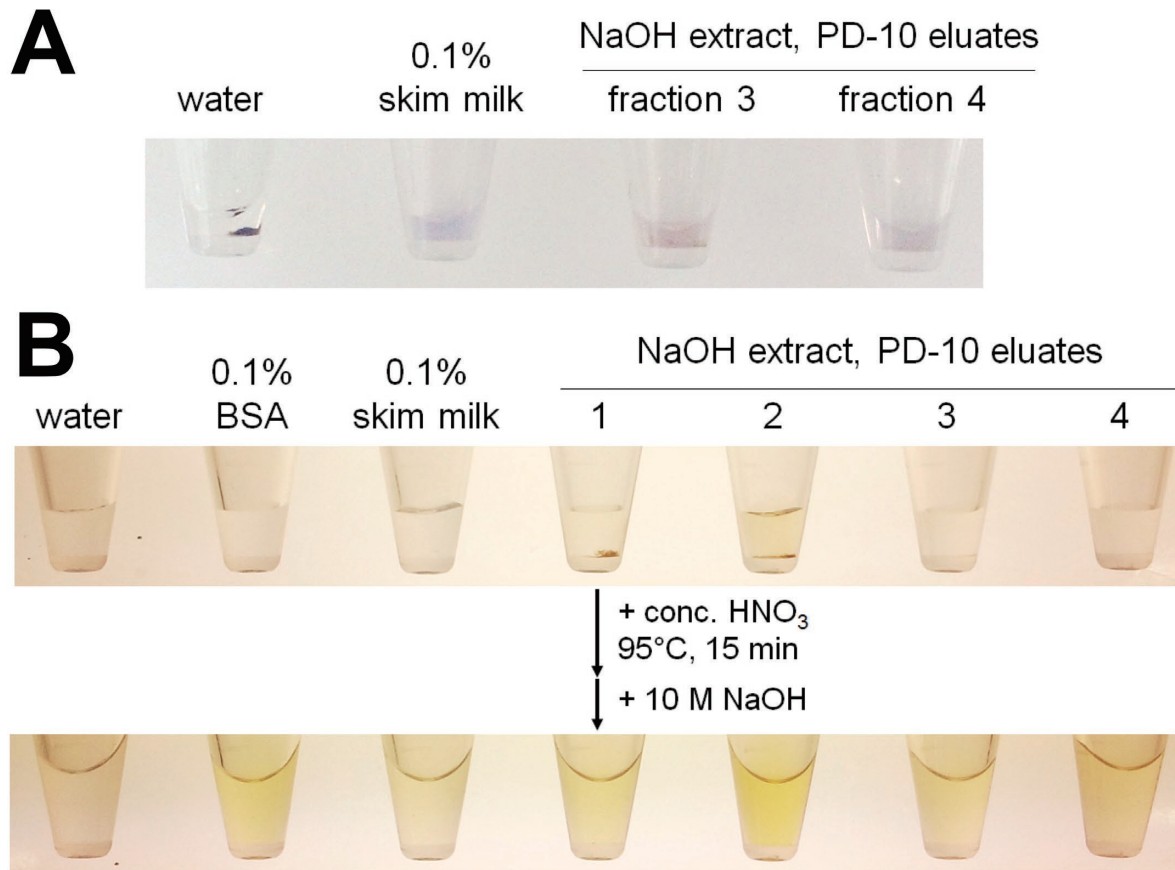
To summarize, our preliminary investigation indicated that the carcinoecium comprised 13% HCl-soluble component, 38% NaOH-soluble component (crude protein), 11% chitin, and 39% other unidentified components.

### Protein analyses

The biuret test and the xanthoproteic reaction resulted in reddish-purple and yellow coloration, respectively (Fig. 6A, B), indicating the presence of proteins in the NaOH extracts. The extracts were desalted and subjected to SDS-PAGE to assess the component proteins. Most prominent were two bands at approximately  $10 \times 10^3$  Da and  $15 \times 10^3$  Da, accompanied by a long smear (Fig. 7). The two bands were subjected to LC/MS, although no proteins other than the trypsin used in the in-gel digestion procedure were detected.



**Fig. 5.** Photographs of the unidentified remnants after HCl treatment, NaOH treatment, and chitinase treatment. (A) remnants on a black background; (B) remnants on a white background (white and black bars indicate 500  $\mu$ m).



**Fig. 6.** Results of protein analyses. **(A)** Biuret test of alkaline extracts gave reddish-purple colors, indicating the presence of proteins. Ultrapure water and 0.1% skimmed milk solution were used as negative and positive controls, respectively; **(B)** xanthoproteic reaction of the alkaline extracts gave yellow colors, indicating the presence of proteins. Ultrapure water was used as a negative control and 0.1% BSA solution and 0.1% skimmed milk solution were used as positive controls.

## DISCUSSION

### Presence of protein

The NaOH carcinoecium extract registered positive for proteins/peptides to the same extent as 0.1% skimmed milk solution and 0.1% BSA solution in biuret test, xanthoproteic reaction, and CBB staining. This clearly indicates that the carcinoecium formed by *Stylobates* sp. contains a substantial amount of protein (maximum 38%) and is the first evidence of the presence of proteins in the carcinoecium, supporting the prediction of Dunn and Liberman (1983) based on research on *S. aeneus*.

However, our data reveal that the proportion of protein is far less than the 98% predicted by Dunn and Liberman (1983) and that the carcinoecium contains at least 23% non-protein components (HCl-extractable components and chitin). One possible reason for the discrepancy in the contents of chitin and proteins in this study and in that conducted by Dunn and Liberman (1983) may be due to the newly cloned chitinase, which was unavailable at that time, and/or the radical conditions for HCl treatment and NaOH treatment. These different approaches would have enabled more detailed elucidation of the composition of the carcinoecium. However, because our qualitative analysis could only indicate that NaOH-extractable components contain some

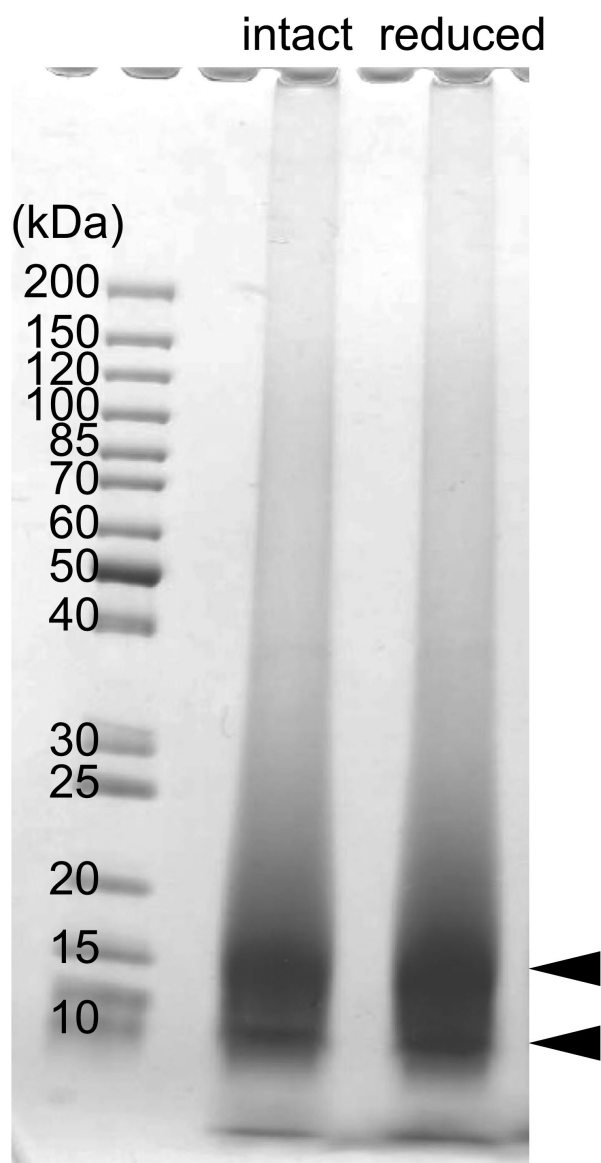
amount of proteins, future investigation of the comprehensive profiling of the small compounds of carcinoecia including alkaline-soluble compounds is also needed.

### Protein identification

Likely due to the poor solubility of NaOH-extracted proteins under neutral and acidic conditions, almost no peptides were detected by LC/MS; the proteins and peptides would have aggregated during desalting, in-gel digestion, or LC and thus, would have been reduced to very low levels for mass spectrometry. The smear signal at low molecular weight might have been the proteins degraded during HCl- and NaOH-extraction. The solubility of the peptides in the SDS-PAGE sample buffer, that in the in-gel digest extraction solution (3% formic acid/50% acetonitrile), and that in the resolubilizing solution for LC (0.1% formic acid/water) totally differ from each other. Therefore, it is possible that the proteins were soluble during electrophoresis but insoluble at the stage of LC/MS.

Otherwise, there was no corresponded protein with the reference protein databases, except trypsin; it might also be because one or the two low molecular mass bands are the unidentified proteins. Therefore, top-down proteomics or protein sequencing if the amino terminus of the protein is exposed at pH 8 may be solutions for identifying the compo-





**Fig. 7.** Gel image of carcinoecium separated by SDS-PAGE and stained with CBB-R250. Arrowheads indicate bands.

nent proteins.

#### Presence of chitin

The present study indicates that the carcinoecium of *Stylobates* sp. contains 11% chitin, though Dunn and Liberman (1983) found that the carcinoecium of *S. aeneus* contained at least 1.7% chitin. Dunn and Liberman (1983) showed the direct evidence of the existence of chitin in the carcinoecium with infrared absorption spectra, but the portion of chitin remains as a matter to be discussed. They conducted ultrasonic washing in sodium dodecyl sulfate (SDS) solution to remove proteins, washed the residues with deionized water and dried them on a filter paper at 100°C to obtain chitin which yielded 1.7% by weight. Deacetylation of chitin would not explain this discrepancy because they clearly detected the characteristic infrared absorption of the amide groups at around 1640  $\text{cm}^{-1}$  and near 1540  $\text{cm}^{-1}$  as

observed in standard chitin, indicating the acetyl group, which accounts for 19% of the molecular weight of chitin, was mostly present on their sample. Notably, materials like the unidentified remnants in our study were not mentioned to be present in their samples, although muds and diatoms shells should be resistant to SDS extraction. The absence of these materials suggests either that they may have lost a substantial amount of water-insoluble components through the attempt to gain chitin at an infrared-grade purity, or that the carcinoecium of *S. aeneus* consists of different proportions of chitin, muds and other insoluble particles. Thus, it is possible that the discrepancy is explained by species differences, and further research on carcinoecia of other *Stylobates* species is needed to compare the contents of the chitin. However, our study has confirmed that, at least to some extent, the term “chitinous” is correct when used to describe the carcinoecium.

#### Presence of HCl-soluble component

The carcinoecium formed by *Stylobates* sp. contains 13% of HCl-extractable components. Although the exact composition of the HCl-soluble components remains undefined, it would contain a substantial amount of carbonate(s), given that the carcinoecium vigorously released bubbles when exposed to HCl and that calcium carbonate is a common seawater-insoluble compound produced by animals. The previous study also detected the existence of mineral (Ca, Si, Mg, Al Cu, and several other metals) (Dunn and Liberman, 1983). In addition to that, our observation shows that dark/white-colored particles, foraminiferans, diatoms, and fragment of unidentified biological-organisms were included on the surface of carcinoecium (Fig. 2); *Stylobates* sp. may construct the carcinoecium in part by incorporating materials from outside environments.

There are no records of mineralized hard skeletal axes and/or hard materials from Actiniaria species, although some members of the phylum Cnidaria are able to produce hard, mineralized skeletal axes (e.g., some species of Octocorallia and Scleractinia) (Cairns and Macintyre, 1992; Dauphin, 2006; Martin and Le Tissier, 1991; Tor, 1976). Further investigation will be needed to understand the origin of HCl-soluble components of the carcinoecium part.

#### Possible components of unidentified remnants

After conducting the methods of preparing the pure chitin from crustaceans (Younes and Rinaudo, 2015), 39% of the shell remained unidentified in this study. The insoluble dark- and white-colored particles (Fig. 5) and the surface structure (Fig. 2) also indicated that *Stylobates* sp. incorporate sand, mud and the remains of other organisms (foraminiferans, diatoms and fragment of unidentified organisms) into the carcinoecium during formation. These silicate materials would account for part of the unidentified remnants that were resistant to HCl, NaOH and chitinase.

Additionally, the unidentified remnants after these treatments might be also explained by incomplete extraction of supposed components and/or presence of components we did not test for. One possibility is that one or more treatments were incomplete, leaving minerals, proteins, or chitin in the sample. Another possibility is that the N-acetylglucosamine residues in chitin were partially deacetylated in the alkaline

treatment (Younes and Rinaudo, 2015), resulting in the loss of interaction with chitinase. This would result in some of the chitin remaining undigested, and although we demonstrated that the carcinoecium contained at least 11% chitin, the amount of chitin in the unidentified fraction should be investigated further.

### Surface structure of carcinoecium

The outer surface of the carcinoecium was smoother than the inner surface, and many of the included-particles were covered by brown materials produced by the sea anemone. Thus, *Stylobates* sp. may produce the carcinoecium based on chitin and proteins, actively or passively incorporating other elements from the sea bed.

In other *Stylobates* species, most individuals fully cover the gastropod shells and extends the lip of it by a thin or thick carcinoecia (Crowther et al., 2011; Dunn et al., 1980; Fautin, 1987), while *Stylobates* sp. only extends the portion of the lip of an aperture of a gastropod shell by the relatively thin carcinoecia (Uchida and Soyama, 2001; Yanagi, 2006) with many particles (Figs. 2–4). Perhaps, as the results of incorporated particles, this species could strengthen own carcinoecium.

### The future of research on interspecific variation in the carcinoecium

*Stylobates* species live in a wide range of habitats, from the continental shelf to deep sea environments (100 m to approximately 1000 m) in the Indo-Pacific Ocean, and each species exhibits species specificity or preference for host hermit crabs (Fautin, 1987; Crowther et al., 2011; Uchida and Soyama, 2001; Yanagi, 2006). The shell utilization pattern of hermit crabs varies among species (Reese, 1969; Fotheringham, 1976; Imazu and Asakura, 1994). The rigidity of the carcinoecium, which would be affected by the composition, thus may vary among *Stylobates* species. In addition, our structural observation showed that carcinoecium includes many particles probably from the sea floor. It might work as strengthening the extension part and/or accelerating the constructing speed, otherwise just reflecting the environmental difference by passively incorporating the substrates on the sea floor. Therefore, the intraspecific differences of carcinoecium depending on the benthic substrate may also exist. Future research in this field is needed to strengthen and/or verify (or to refute and extend) our observation of the chemical composition and structural differences of carcinoecia produced by different *Stylobates* species in various regions and at various depths.

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### COMPETING INTERESTS

The authors declare that they have no conflict of interest.

### AUTHOR CONTRIBUTIONS

AY, SN, and AA designed and performing research. AY collected animals. AY and SN conducted experiments and analyzed data. AY, SN, and AA wrote the manuscript. All authors read and approved the final manuscript.

### SUPPLEMENTARY MATERIALS

Supplementary materials for this article are available online. (URL: <https://bioone.org/journals/supplementalcontent/10.2108/zs180167/10.2108.zsj.36.284.s1.pdf>).

**Supplementary Table S1.** Results of chitinase treatment.

**Supplementary Table S2.** Results of HCl treatment and NaOH treatment.

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