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Occurrence of Cellulose Activities in Planktonic Crustaceans Inhabiting Mangrove Areas in Malaysia

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Abstract

We assessed cellulase activity in several crustaceans of different taxonomic groups inhabiting the mangrove areas of Malaysia to clarify whether these animals could digest cellulose derived from mangrove trees. We investigated four Copepod species, two Mysida species, and ten Decapoda species. Three copepod species demonstrated multiple cellulase active bands that differed among species in a zymographic assay, suggesting that these animals were equipped with distinct cellulases. Interestingly, the way the cellulase were expressed in the zymographic assay differed, even among members of the same species collected at different locations, suggesting that cellulase expression patterns of copepod species are regulated by environmental factors. Although no significant cellulase activity was detected in two of the Mysida species, widespread distribution of cellulases was also detected in decapod species. Multiple common active bands in the various organs of decapod species were detected by the zymographic assay, while remarkable activity was detected in the hepatopancreas in the reducing sugar assay. The above findings suggested that cellulases are synthesized in the hepatopancreas and then secreted into digestive tracts such as the stomach and intestine. The present study shows that various crustaceans comprising most of the biotic resources in mangrove areas may be there because of their ability to digest cellulose.

Discipline: Aquaculture
Additional key words: Cellulase, Copepod, Decapoda, Digestion, Mysida

Introduction

All zooplanktons are heterotrophic, although the individual species differ in how they obtain organic energy; classified as herbivores, carnivores, detritivores and omnivores (Lalli & Parson 1997a). In temperate areas, zooplankton mainly feed on phytoplankton and are classified as carnivores (Bouillon et al. 2000). In tropical mangrove areas, however, the amount of phytoplankton is assumed to decrease due to the low transparency of water caused by the suspended clay particles (Okamura et al. 2010, Robertson et al. 1992). Conversely, a remarkable number of zooplankton inhabit mangrove areas, including small crustaceans such as mysid crustaceans, despite the turbid water (Hanamura et al. 2008). Therefore, it seems likely that small crustaceans in the mangrove areas feed on alternative carbon sources and not solely on phytoplankton.

Recently, our studies showed that various aquatic invertebrates in temperate zones could break down cellulose using cellulases (Niiyama & Toyohara 2011). In addition,

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tropical bivalves and shrimp in mangrove areas have cellulolytic activities in their digestive organs (Niiyama et al. 2012a, 2012b). These findings led us to hypothesize that small crustaceans inhabiting mangrove areas are equipped with cellulolytic activities, which enable them to digest cellulose derived from mangrove trees. To confirm this hypothesis, we attempted to detect cellulolytic activities in different taxonomic groups of crustaceans collected in the mangrove areas of Malaysia.

Materials and Methods

1. Materials

Species of Copepod, Mysida, and Decapoda were collected at the Matang Mangrove Forest Reserve (MMFR) and Selangor Estuary. Both sampling sites were located on the western coast of Peninsular Malaysia (Fig. 1). The water in the MMFR and Selangor Estuary is relatively shallow; averaging 5 m. Samples of planktonic crustaceans were collected using a 2 m-wide otter trawl net with 10 mm cod-end mesh openings. Fig. 1 and Table 1 show details of the collecting sites and dates. Samples were transferred on ice to the Fisheries Research Institute on Penang Island. Each species was identified and separated using a binocular microscope and then stored as whole bodies in Eppendorf tubes with local water at -80˚C until use. All reagents not specifically mentioned were purchased at G.R. grade from nacalai tesque (Kyoto, Japan).

2. Methods

To determine cellulase activity, we applied three enzyme assays: agar-plate, SDS-PAGE zymographic and reducing-sugar assays. Initially, we performed an agar-plate assay because it was the most adequate way to determine the occurrence of cellulase activity. When the activity was detected in the agar-plate assay, we performed a SDS-PAGE zymographic assay to determine the molecular sizes of cellulase as a second step. To compare the level of cellulose activity among digestive organs, we performed reducing sugar assay as the third step.

(1) Agar-plate assay

Eighty of each copepod species were counted on a slide glass using a binocular microscope and collected. After removing the water using a paper towel, each of the 80 samples was homogenized with 20 µL of phosphate buffered saline (PBS) containing 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 and 1.5 mM KH_2PO_4 (pH 7.4). The homogenate was applied on carboxymethyl-cellulose (CMC) (Sigma, St. Louis, MO, USA) agar plates. The plates were then incubated for 48 h at 37˚C, stained with 0.1% Congo Red and distained with 1 M NaCl. Cellulase activity was detected as non-stained halos. Purchased *Aspergillus niger* cellulase (MP Biomedicals, Santa Ana, CA, USA) or *A. japonicus* samples were used as positive controls.

(2) SDS-PAGE zymographic assay

One hundred copepod and 100 mysid species were homogenized as described above. The hepatopancreas, stomach and intestine were separated from the body of large decapods, if possible, and then each organ and the rest of the body were separately homogenized with aliquots of PBS. The obtained homogenates were centrifuged for 10 min at 8,000 *g*. The protein concentration of the supernatant was measured by the Bradford Method (Bradford 1976), adjusted to 0.1 µg/µL with PBS and used as the enzyme solution. Ten microliters of the enzyme solution was applied on a 10% SDS-PAGE gel containing 0.1% CMC. After electrophoresis, the gels were immersed in 10 mM acetate buffer (pH 5.5) containing 0.1% TritonX-100 for 30 min to remove SDS and the gels were transferred into a new 10 mM acetate buffer and incubated for 48 h at 37˚C. The

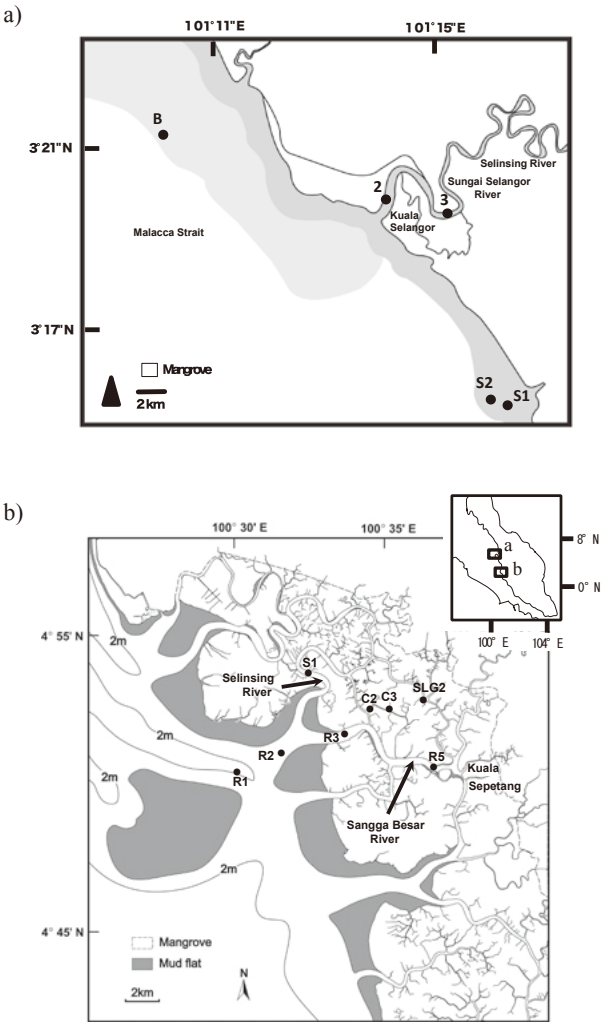

Fig. 1. Sampling sites in Selangor Estuary (a) and Matang Mangrove Forest Reserve (b), Malaysia.

Table 1. Copepod, mysid and decapod species investigated in the present study

Area	Order/Subclass	Species	Date	Site
Selangor Estuary	Copepod	*Acartia spinicauda*	Sep. 18, 2012	B, 2, 3
		Pseudodiaptomus trihamatus	Sep. 18, 2012	B
	Mysid	*Notacanthomysis hodgarti*	Sep. 23, 2012	S1, S2
	Decapoda	*Acetes japonicus*	Sep. 23, 2012	S1
		Penaeus monodon	Sep. 23, 2012	Purchased
Matang Mangrove Forest Reserve	Copepod	*Acartia spinicauda*	Nov. 7, 2012	R1. R3. S1
		Acarita sp	Nov. 7, 2012	R5, SLG2
		Pseudodiaptomus annnandalei	Nov. 7, 2012	C3
	Mysid	*Mesopodopsis tenuipes*	Sep. 28, 2012	C2
	Decapoda	*Metapenaeus ensis*	Sep. 28, 2012	C2
		Acetes sibogae	Sep. 28, 2012	C3
		Palaemon semmelinkii	Sep. 28, 2012	C3
		Metapenaeus lysianassa	Sep. 28, 2012	R2
		Mierspenaeopsis sculptilis	Sep. 28, 2012	R2
		Acetes indicus	Sep. 28, 2012	R2
		Exopalaemon styliferus	Sep. 28, 2012	R2
		Fenneropenaeus merguiensis	Sep. 28, 2012	R2, R5, S1
		Penaeus monodon	Sep. 28, 2012	S1

active bands were detected as non-stained bands after being stained by 0.1% Congo Red and distained by 1 M NaCl.

(3) Reducing sugar assay

The hepatopancreas, stomach and intestine of the decapods were separated and homogenized with aliquots of PBS, as described in the SDS-PAGE zymographic assay protocol. For small mysid of planktonic size, whose organs could not be separated, the whole body was homogenized instead. After centrifugation for 10 min at 8,000 *g*, the supernatant was collected and its protein concentration adjusted to 1 μg/μL as described above. The obtained solution was then used as the enzyme solution. Five micro liters of the enzyme solution, 5 μL of acetate buffer (pH 5.5) and 40 μL of CMC (1%) were mixed and incubated at 37˚C for 8 h, with distilled water used in a control instead of the enzyme solution. After incubation, the reaction was terminated by heating at 100˚C for 3 min. The amount of reduced sugar released from CMC was measured using tetrazolium as a coloring agent according to our previous work (Niiyama et al. 2012a). Absorbance at 660 nm was measured using a spectrophotometer (Lambda 20, Perkin Elmer Co. Ltd) and converted into the glucose concentration using a standard curve. The significance was analyzed by ANOVA followed by post-hoc analysis (Tukey-HSD) using SPSS (IBM, ver. 22).

Results

1. Agar-plate assay and zymographic assay of Copepods

The cellulase activities of four Copepod species were measured by a semi-quantitative plate assay, using CM-Cellulose as a substrate. All copepods showed cellulase activities. In the Selangor Estuary, *Acartia spinicauda* showed the strongest activities, regardless of the collecting sites. *Pseudodiaptomus trihamatus* also showed cellulase activity but at a lower level than *A. spinicauda*. (Fig. 2). In the Matang Mangrove Forest Reserve meanwhile, *Pseudodiaptomus annandalei* showed the strongest cellulose activity. *Acartia spinicauda* also showed high level of cellulase activity, regardless of the collecting sites, while *Acartia sp* showed a very low level of activity at both its two sites (Fig 3).

The extract of copepods was separated using SDS-PAGE by a CMC-containing gel to detect the molecular sizes of the cellulases in copepods sampled in the Selangor Estuary. As shown in Fig. 4, *Pseudodiaptomus trihamatus* showed active bands at 22 and 42 kDa respectively. On the other hand, *Acartia spinicauda* showed multiple active bands from 18 to 130 kDa, while the distribution of active bands varied according to the sampling sites.

At the Matang Mangrove Forest Reserve, cellulase ac-

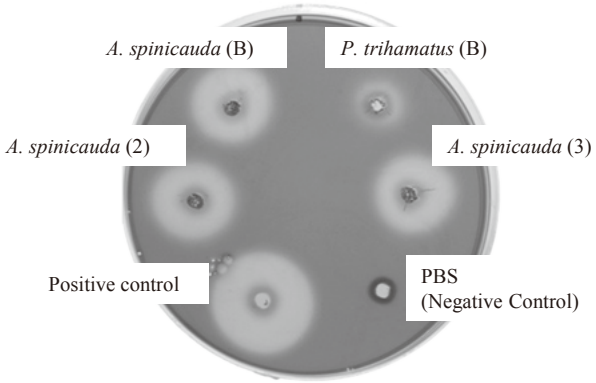

Fig. 2. Plate assays of copepod species in Selangor Estuary, Malaysia. Sampling sites are shown within parentheses. A positive control was prepared with commercially purchased cellulase.

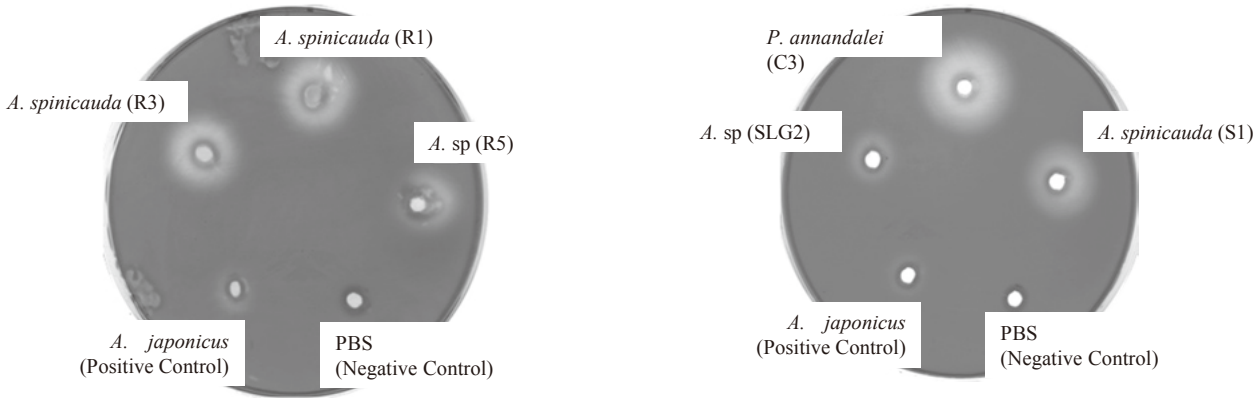

Fig. 3. Plate assay of copepod species in the Matang Mangrove Forest Reserve, Malaysia. Inside parentheses show the sampling sites. A positive control was prepared with an *Acetes japonicus* whole-body sample collected from the same area.

tivities were only detected in *Pseudodiaptomus annandalei* at site C3 and *Acartia* sp. at site SLG2 (Fig. 5), A 24 kDa cellulase was detected in *Acartia spinicauda* at site C3. A 28 kDa and a 34 kDa cellulase were detected in *Pseudodiaptomus annandalei* at site SLG2. No cellulose activities were detected in *Acartia spinicauda* at sites S1, R1 and R3, or in *Acartia* sp. at site R5, even though all these samples had their activity confirmed by a plate assay, as shown in Fig. 3.

There was no result of reducing the Copepods sugar assay because the samples collected were insufficient for the assay in question.

2. Zymographic assay and reducing sugar assay of mysid and decapods

Extracts of the whole body or of certain digestive organs separated from mysid and decapod species were submitted for zymographic assay. Indeed, the agar-plate assay of mysid and decapods was also performed, but the results were not shown because they were redundant compared to those of zymographic assays. As a result of zymographic assay, multiple active bands were detected in most kinds of decapods (Fig. 6: a-e; g, h) except mysid *Mesopodopsis tenuipes* (Fig. 6: f), suggesting that cellulases are widely distributed in various taxa across Decapoda. Interestingly, the multiple active bands in the hepatopancreas were commonly detected in other digestive organs (e.g. the 34-kDa active band of *Palaemon semmelinkii* (Fig. 6: a)), indicating that cellulases may possibly be expressed in the hepatopancreas and subsequently transported to other digestive organs.

To compare the level of cellulase activity among the digestive organs of mysid and decapod species, the total reducing sugar production was measured by incubating

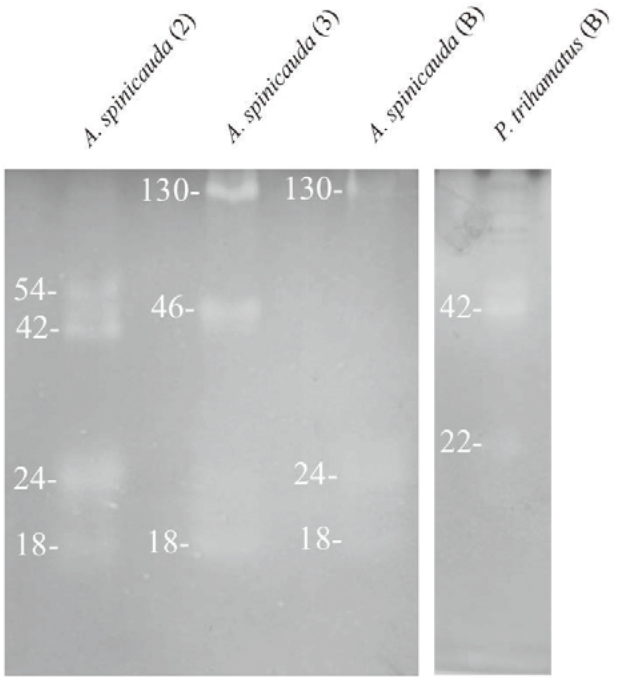

Fig. 4. Zymographic assay of copepod species in Selangor Estuary, Malaysia. Sampling sites are shown within parentheses, according to Table 1. Numbers show the molecular sizes of each active band (kDa).

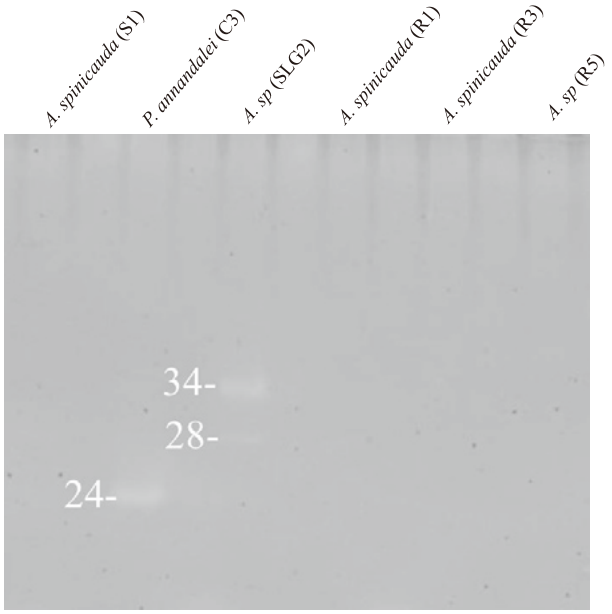

Fig. 5. Zymographic assay of copepod species in Matang Mangrove Forest Reserve, Malaysia. Inside the parentheses show the sampling sites, according to Table 1. Numbers show the molecular sizes of each active band (kDa).

protein concentration-adjusted organ extracts with CMC. As shown in Table 2, the cellulase activities in the hepatopancreases in decapods *Acetes indicus*, *Acetes japonicus*, *Acetes sibogae*, *Fenneropenaeus merguiensis*, *Metapenaeus ensis*, *Metapenaeus lysianassa* and *Penaeus monodon* significantly exceeded the levels in any other digestive organs or whole bodies. However, no cellulase activity was detected in the whole bodies of two of the mysid species.

Discussion

Interestingly, the level of cellulase activity differed, even among the same copepod species, at different sampling sites (Figs. 4 and 5). It goes without saying that the aquatic environment changes violently and invariably (Lalli et al. 1997b), which forces aquatic inhabitants to adapt by changing their status. Considering the fact that these copepods were sampled in the same week, the different patterns of their active bands could be due to their differences in the environment of their habitats or possibly their feeding status. On the other hand, *Acartia spinicauda* in sites S1, R1, and R3 and *Acartia* sp. in site R5 had cellulase activities detected in the plate assay (Fig. 3), but showed no activities in the zymographic assay (Fig. 5). We tried to concentrate the samples or extend the reaction time to enhance the signal, but no activity bands were detected. We attribute this to the smaller application volume in the zymographic assay and the sample dilution while adjusting the protein concentration prior to zymographic assay, both of which making the quantity of cellulase insufficient for detection.

No cellulase activities were detected in the two species of Mysida investigated in the present study, which contradicts our previous work that one of the two mysid species (*N. hodgarti*) had cellulase activity in a zymographic assay, suggesting that the expression of their cellulase is also regulated by certain environment factors. Recent stable isotope analyses suggested that higher consumers such as fish might indirectly utilize original mangrove organic matters as a carbon source *via* mangrove small crustaceans (Chong 2007, Hayase et al. 1999, Tanaka 2011). Our data support this theory, at least partly, by demonstrating the occurrence of cellulase activity in the mangrove small crustaceans, prey of these higher consumers.

The results of the zymographic assay showed that most decapod species had active bands of equivalent molecular size among the hepatopancreas and other digestive organs. On the other hand, the hepatopancreas of decapods showed significantly higher levels of cellulase activities compared to other digestive organs (Table 2), indicating that the hepatopancreas is the cellulase-producing organ in decapod species. The hepatopancreas of crustaceans is reportedly a digestive gland mainly synthesizing and secreting digestive

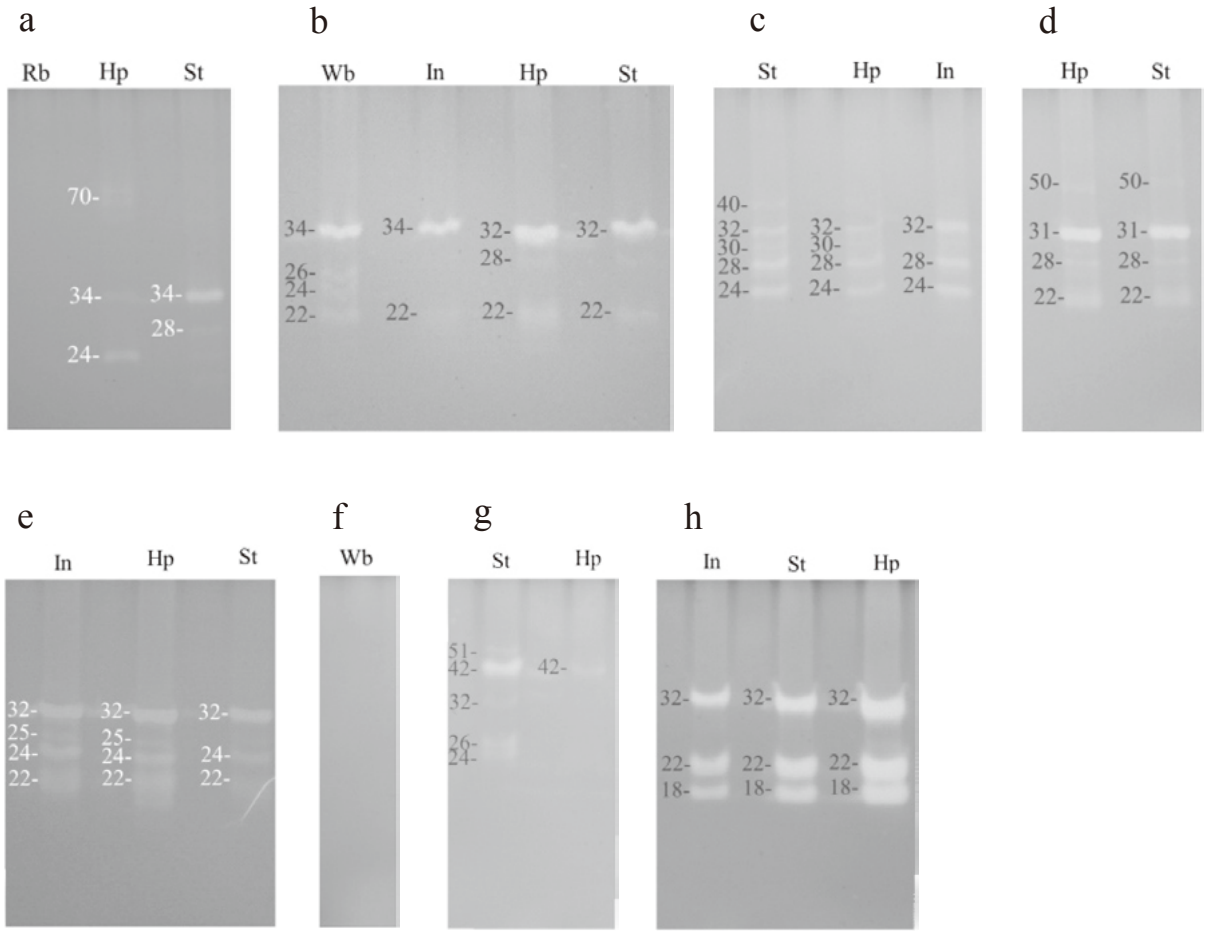

Fig. 6. Digestive organ distribution of mysid and decapod species by Zymographic assay. (a):*Palaemon semmelinkii*; (b): *Fenneropenaeus merguiensis*; (c): *Mierspenaeopsis sculptilis*; (d): *Metapenaeus lysianssa*; (e): *Metapenaeus ensis*; (f): *Mesopodopsis tenuipes*; (g): *Exopalaemon styliferus*; (h) *Penaeus monodon*. Abbreviated names of organs are shown on each sub-figure. Hp: hepatopancreas; St: Stomach; In: Intestine; Rb: rest of the body; Wb: whole body. Numbers the molecular sizes of each active band (kDa).

enzymes (Al-Mohanna 1987, Lovett et al. 1990). Based on these facts, the cellulase activities detected in the present study in decapod species would have been derived from enzymes whose genes are encoded on the chromosomal DNA. Further studies, however, are required to identify the genomic structure of cellulase in decapods.

Planktonic crustaceans are the dominant animals in the river water of mangrove areas (Chong 2007). It is thought that they normally feed on phytoplankton, as was previously reported (Mauchline 1980). However, the number of phyotoplankton is assumed to decline in our sampling sites, due to the low transparency of water limiting photosynthesis (Okamura et al. 2010, Robertson et al. 1992), meaning their feeding habitats remain unclear. To date, the widespread distribution of cellulase among aquatic animals has only been reported in Japanese wetlands (Niiyama & Toyohara 2011), The occurrence of cellulase activities in mysid species and *Acetes* shrimps in the Matang Mangrove Forest Reserve was also reported in our previous study (Niiyama et al. 2012b). Together with these findings, we conclude that the organic matter accumulated in the mangrove areas is used as an alternative carbon source for various crustaceans, including planktonic crustaceans.

In the present study, cellulase activities were successfully detected in the major taxonomic groups such as copepods and decapods (Figs. 2, 3, 4, 5 and 6), which effectively correlates with our hypothesis, whereby that small crustaceans inhabiting mangrove areas are capable of cellulolytic activities. Moreover, it has also been reported that *Acetes* shrimps dominate brackish waters worldwide (Hanamura et al. 2008) as well as being the key food source for many fish (Chong 2007). Accordingly, the copepod and decapod species may be a bridge between higher consumers, as their prey, and original mangrove carbon sources, as

Table 2. Reducing the oligosaccharide production of digestive organs in mysid and decapod species

Order	Species	Hepatopancreas	stomach	Intestine	Rest of the Body	Whole Body
Mysid	*Mesopodopsis teruipes*	ND	ND	ND	ND	0.03 ± 0.01
	Natacanthomysis hodgar	ND	ND	ND	ND	0.02 ± 0.01
Decapos	*Acetes indicus*	0.93 ± 0.47*	ND	ND	ND	0.03 ± 0.00
	Acetes japonicus	0.19 ± 0.02*	ND	ND	ND	0.02 ± 0.01
	Acetes sibogae	0.53 ± 0.12*	ND	ND	ND	0.01 ± 0.00
	Exoplaemon styleus	0.05 ± 0.00	0.05 ± 0.00	ND	ND	ND
	Fenneropenaeus merguiensis	0.26 ± 0.03*	0.06 ± 0.00	0.04 ± 0.01	ND	0.06 ± 0.02
	Metapenaeus ensis	0.31 ± 0.05*	0.12 ± 0.01	0.11 ± 0.02	ND	ND
	Metapenaus lysianassa	0.33 ± 0.02*	0.10 ± 0.01		ND	ND
	Mierspenaeopsis sculptilis	0.09 ± 0.01	0.07 ± 0.01	0.02 ± 0.00	ND	ND
	Pemaeus mondon	0.41 ± 0.01*	0.22 ± 0.02	0.05 ± 0.01	ND	ND
	Plaemon semmklinkii	0.04 ± 0.00	0.03 ± 0.00	ND	0.01 ± 0.00	ND

Date are mean (μmol/min mgprotein) ± standard error.
Asterisks indicate that the value is significantly high compared with others in the same species.
ND: Not determined.

their decomposers.

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