BREAKDOWN OF HARD-DEGRADABLE POLYSACCHARIDES IN WETLANDS

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CONTENTS

INTRODUCTION

Vegetation on the Earth immobilizes atmospheric carbons and stores them in the form of polysaccharides including celluloses and hemi-celluloses via photosynthesis. Cellulose is the most abundant organic substance on the Earth. Since cellulose is chemically stable, they play an important role to provide physical strength as a major component of the plant cell walls[1]. This physical strength is attributed to the primary structure of cellulose. Monomeric chains of cellulose are consisted of D–glucopyranose. D–glucopyranoses are bound by β-1, 4-glycoside linkages to form cellulose microfibrils. Cellulose microfibrils are interconnected by hydrogen bonds (**Fig. 0-1**)[2]. In addition to cellulose, the cell wall contains lignin and hemicelluloses, including mannan, xylan, and laminarin. The contents of the hemicelluloses differ across plant species[3]. Enzymes that are able to decompose cellulose are collectively called cellulases. Cellulase are classified according to several characteristics.

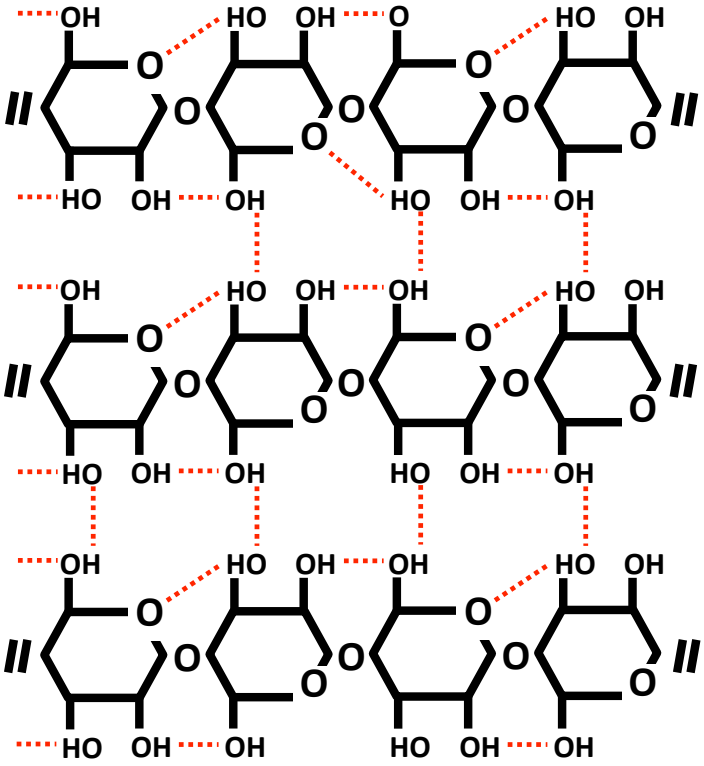

Fig. 0-1 Scheme of the crystalline structure of cellulose. D–glucopyranose units are bound by β-1,4 linkages. Mutiple cellulose monomeric chains are linked by hydrogen bonds (marked by dotted lines) to form cellulose crystals.

First, cellulases are classified according to the cleavage site on cellulose: (1) The cellulase that cleaves cellulose at random sites is called β -1, 4-endoglucanase, and (2) The cellulase that cleaves off glucose dimers from the terminal end of cellulose is

called β-1, 4- exocellobiohydrolase. Subsequently, the cellulase that cleaves glucose from the breakdown products formed by β-1, 4-endoglucanase and β-1, 4-exocellobiohydrolase is called β-glucosidase (**Fig. 0-2**).

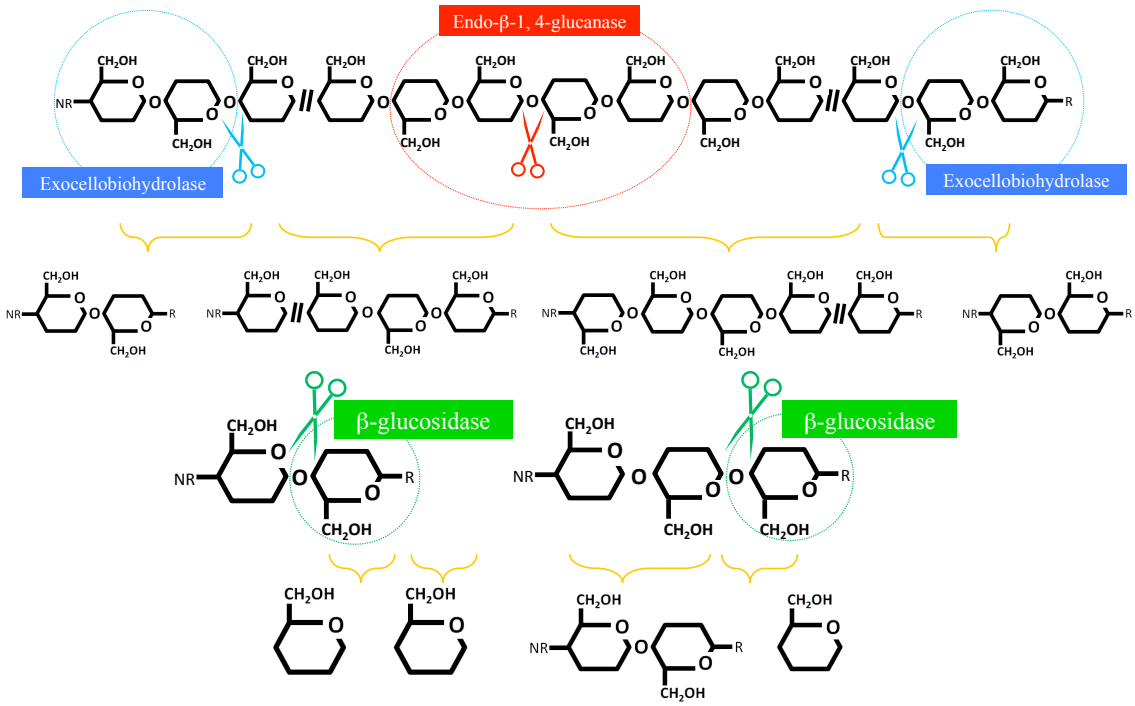

Fig. 0-2 Three types of cellulose hydrolysis enzymes that are collectively called cellulases, namely exocellobiohydrolase, endo-β-1, 4-glucanase, and β-glucosidase. Exocellobiohydrolases (exocellulase) cleave cellulose to release cellobiose from reducing (R) or nonreducing (NR) termini (dotted circle). Endo-β-1, 4-glucanases cleave the cellulose randomly at internal β-1, 4 linkages (broken circle). β-Glucosidases hydrolyze cellobiose or cello-oligomersto to release glucose from the reducing temini (double circle). Scissors represent the cutting site of each enzyme.

Second, cellulases are classified according to the presence or absence of a sub-domain called carbohydrate-binding module (CBM). Part of the carbohydrate-hydrolyzing enzymes has a CBM, which is independent to the catalytic site, binding to substrates and stabilizing the enzymatic reaction. For example, cellulases that have a cellulose-binding domain (CBD) could constantly bind to

cellulose. The enzyme molecules continuously move to the next cleavage site after each hydrolytic reaction. To the contrary, cellulases that do not have a CBD detach from the cellulose after every cleaving reaction. The enzymes have to newly search for the cleavage site of the next hydrolytic reaction[2]. Cellulases that have a CBD are assumed to have more hydrolyzing efficiency than those do not (**Fig. 0-3**).

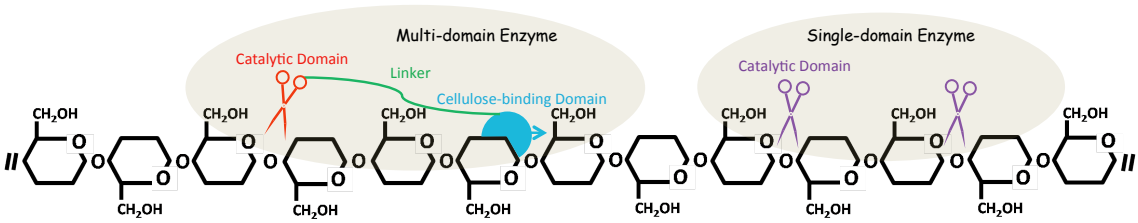

Fig. 0-3 Two different types of cellulase according to the presence of cellulose-binding domain (CBD): multidomain cellulases and single-domain cellulases. Multidomain cellulases are composed of a catalytic domain and a CBD linked by a peptide strand, termed a linker. In this instance, CBDs are assumed to bind to cellulose, which improves the efficiency of the hydrolytic reaction. In contrast, single-domain cellulases leave the cellulose surface after each catalytic action is completed, and subsequently approach a new linkage to perform another hydrolytic reaction.

Third, cellulases are classified according to their amino acid sequence similarities. For instance, glycoside hydrolases are classified into families (each of which is assigned a number, i.e., GHF1, GHF2, GHF3, ...) (see also Cazy-Web: http://www.cazy.org/)[4]. To date, 130 GH families are registered. Cellulases can be found in families of GHF1, 3, 5, 6, 7, 8, 9, 10, 12, 19, 26, 30, 44, 45, 48, 51, 61, 74, 116, and 124 (**Table 0-1**), most of which are associated with endo-β-1, 4-glucanase. In addition, only GH5, 9, 10, and 45 are found in metazoans, while GHF9 and 45 are found exclusively in aquatic invertebrates. β-Glycosidases have been found in most animal phyla of animals, hydrolyzing various dimers of sugars in addition to cellobiose.

Table 0-1 Cellulase members of various glycoside hydrolase (GH) families, and the taxa in which the cellulases have been found

GHF	Cellulase Type	Archaea	Eukaryote			
			Eubacteria	Fungus	Plant	Animal (Invertebrate)
1	β-glucosidase	+	+	+	+	+
3	β-glucosidase	+	+	+	+	+
5	Endoglucanase, β-glucosidase	+	+	+	+	+
6	Endoglucanase, Cellobiohydrolase	-	+	+	-	-
7	Endoglucanase, Cellobiohydrolase	-	-	+	+	+
8	Endoglucanase	+	+	-	-	-
9	Endoglucanase, Cellobiohydrolase, β-glucosidase	+	+	+	+	+
10	Endoglucanase	-	+	-	-	-
12	Endoglucanase	+	+	+	+	-
19	Endoglucanase	+	+	-	-	-
26	Endoglucanase	-	+	-	-	-
30	β-glucosidase	+	+	+	+	+
44	Endoglucanase	-	+	-	-	-
45	Endoglucanase	-	+	+	-	+
48	Endoglucanase	-	+	+	-	-
51	Endoglucanase	+	+	+	+	-
61	Endoglucanase	-	-	+	+	-
74	Endoglucanase	-	+	+	-	-
116	β-glucosidase	+	+	+	+	+
124	Endoglucanase	-	+	-	-	-

It has been believed for a long time that only protozoa, bacteria, and fungi, plants and photosynthetic bacteria are able to synthesize endogenous cellulases[1, 5-7]. For example, it was assumed that metazoans degrade cellulose by using cellulases derived from the symbiotic protozoa and bacteria living in their digestive organs[1]. However, a gene encoding a cellulase (GHF9; β-1, 4-endoglucanases) was identified in *Reticulitermes speratus* (Arthropoda, Insecta) in 1998[7]. After this stunning discovery,

the presence of endogenous β-1, 4-endoglucanases across various GHF families has been reported in insects, crustaceans, mollusks, echinoderms, and nematodes[8-16].

Wetlands are inhabited with various cellulase-processing invertebrate species. Besides, wetlands are well known as the purifier of the terrestrial origin polysaccharides. These invertebrates are considered to contribute to the purification (polysaccharides decomposition) ability of the wetlands, but the mechanisms and the proportion of their contribution remains unclear. This study focuses on typical wetlands such as mangroves and tidal flats in order to reveal the roles played by these invertebrates.

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CHAPTER 1

Breakdown of Hard-degradable Polysaccharides in Mangrove Area

1.1. Introduction

All zooplanktons are heterotrophic, although the individual species differ in how they obtain organic energy; classified as herbivores, carnivores, detritivores and omnivores[1]. In temperate areas, zooplankton mainly feed on phytoplankton and are classified as carnivores[2]. 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[3, 4]. Conversely, a remarkable number of zooplankton inhabit mangrove areas, including small crustaceans such as mysid crustaceans, despite the turbid water[5]. 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[6]. In addition, tropical bivalves and shrimp in mangrove areas have cellulolytic activities in their digestive organs[7, 8]. 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.

1.2. Materials and Methods

1.2.1. 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. 1-1**). 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. **Figure 1-1** and **Table 1-1** 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

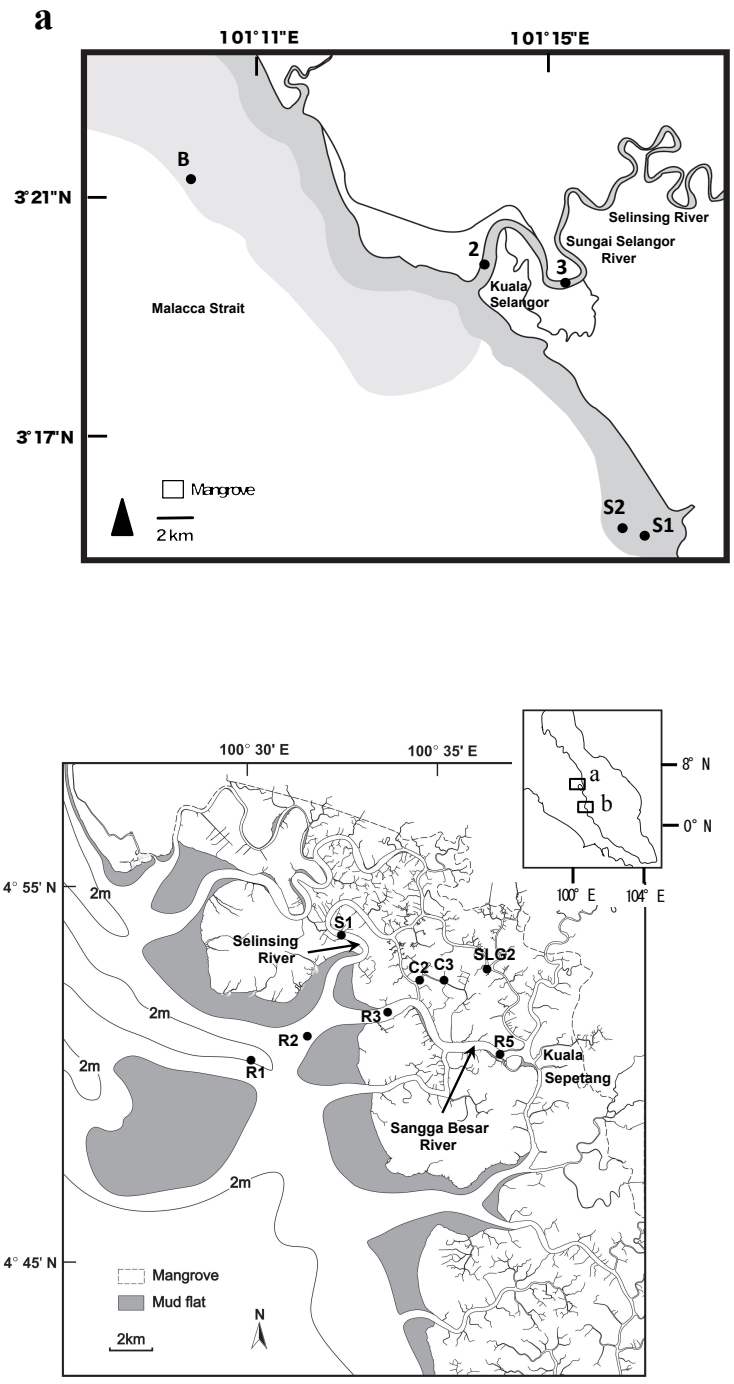

Fig. 1-1 Sampling sites in Selangor Estuary (**a**) and Matang Mangrove Forest Reserve (**b**), Malaysia.

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).

Table 1-1 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
		Acartia sp	Nov. 7, 2012	R5, SLG2
		Pseudodiaptomus annandalei	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

1.2.2. 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.

1.2.2.1.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.

1.2.2.2.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[9], 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 active bands were detected as non-stained bands after being stained by 0.1% Congo Red and distained by 1 M NaCl.

1.2.2.3.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[7]. 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).

1.3. Results

1.3.1. 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. 1-2**). 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. 1-3**).

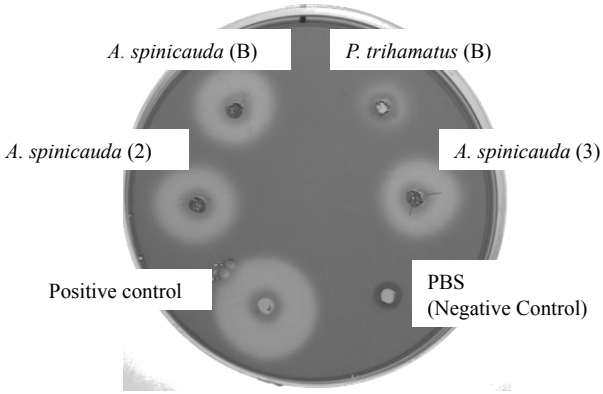

Fig. 1-2 Plate assays of copepod species in Selangor Estuary, Malaysia. Sampling sites are shown within parentheses. A positive control was prepared with commercially purchased cellulase.

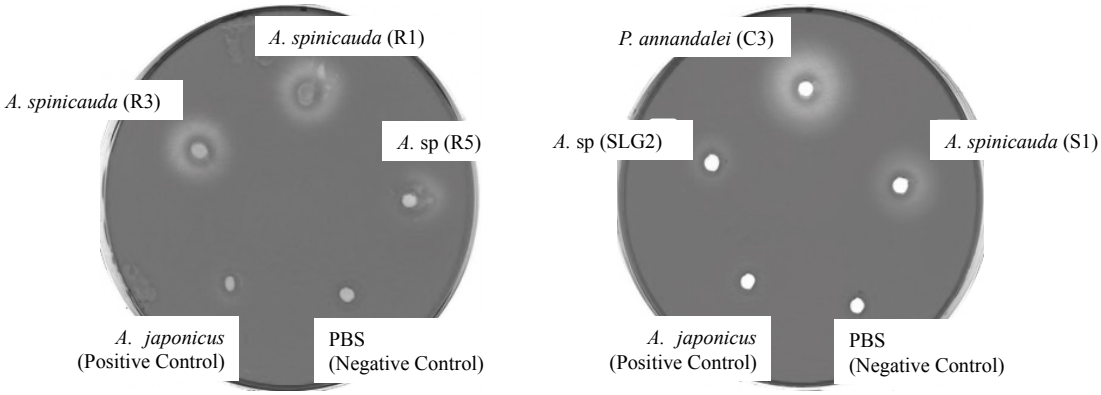

Fig. 1-3 Plate assay of copepod species in the Matang Mangrove Forest Reserve, Malaysia. Inside parentheses show the sampling sites. A positive control was prepared with an *Acetes japonicus* whole-body sample collected from the same area.

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. 1-4**, *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.

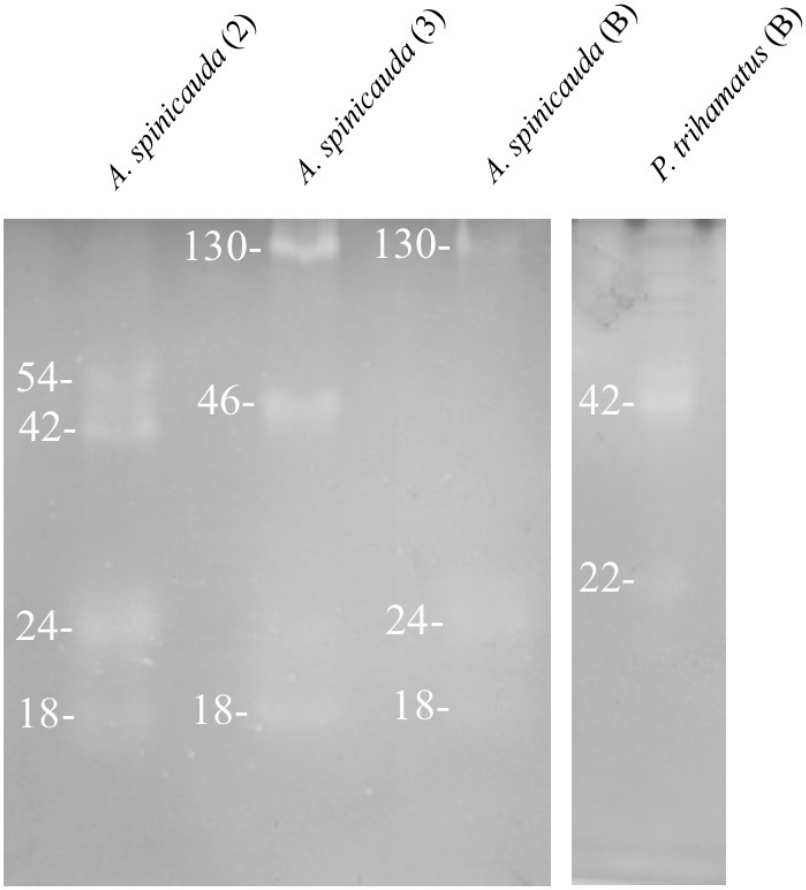

Fig. 1-4 Zymographic assay of copepod species in Selangor Estuary, Malaysia. Sampling sites are shown within parentheses, according to Table 1. Numbers show the molecular sizes of each active band (kDa).

At the Matang Mangrove Forest Reserve, cellulase activities were only detected in *Pseudodiaptomus annandalei* at site C3 and *Acartia* sp. at site SLG2 (**Fig. 1-5**), 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. 1-3**. There was no result of reducing the Copepods sugar assay because the samples collected were insufficient for the assay in question.

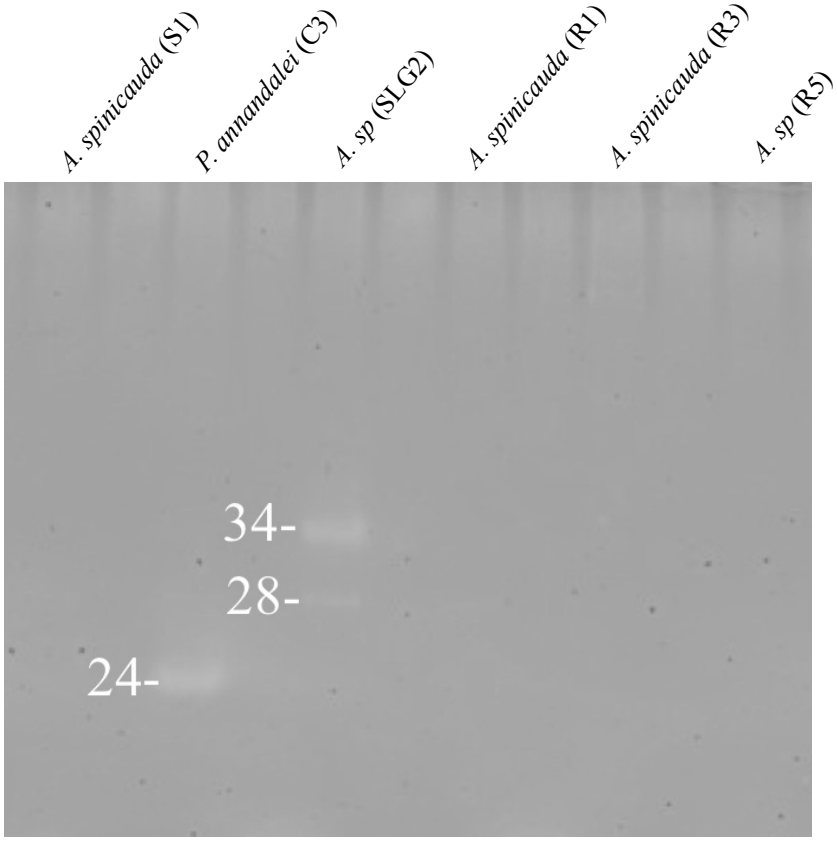

Fig. 1-5 Zymographic assay of copepod species in Matang Mangrove Forest Reserve, Malaysia. Inside the parentheses show the sampling sites, according to Table 1. Numbers show the molecular sizes of each active band (kDa).

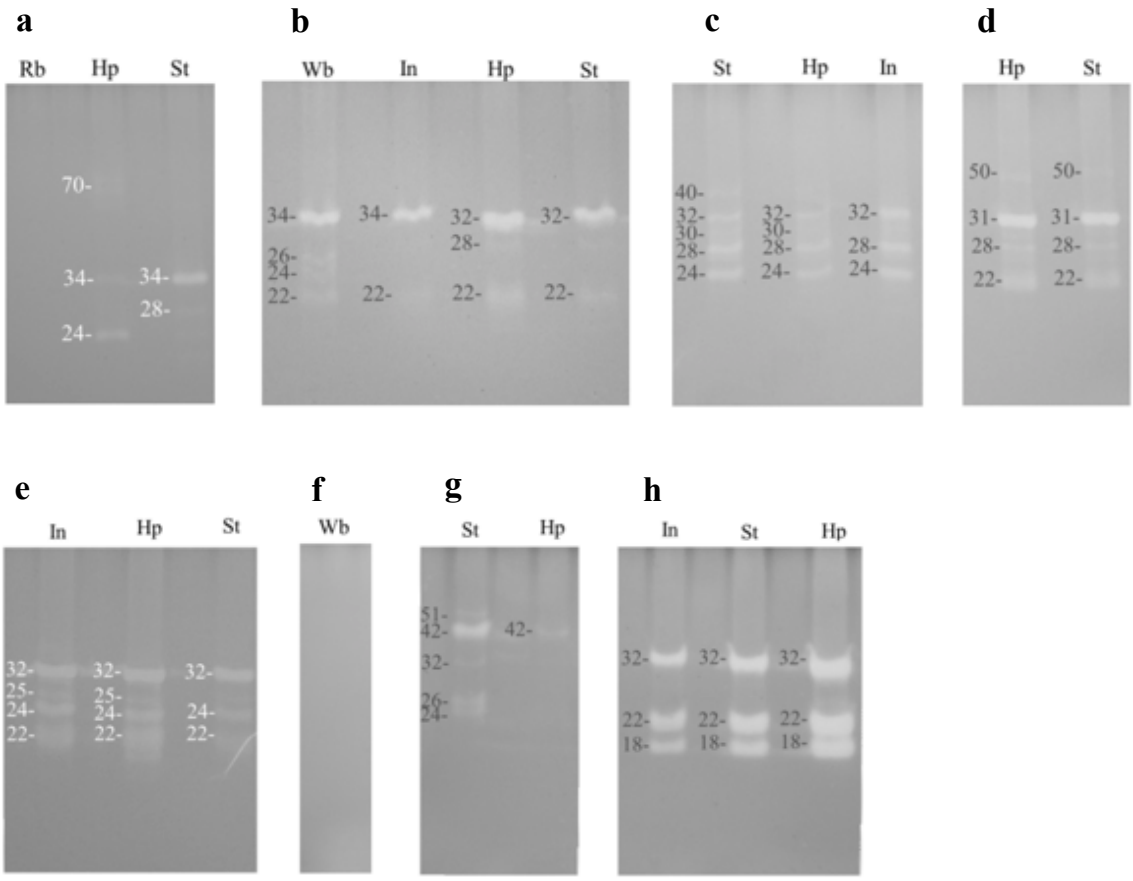

Fig. 1-6 Digestive organ distribution of mysid and decapod species by Zymographic assay. (a):*Palaemon semmelinkii*; (b): *Fenneropenaeus merguiensis*; (c): *Mierspenaeopsis sculptilis*; (d): *Metapenaeus lysianssa*; (e): *Metapenaeus ensis*; (f): *Mesopodopsis tenuipes*; (g): *Exopalaemon styliferus;* (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).

1.3.2. 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. 1-6: a-e; g, h**) except mysid *Mesopodopsis tenuipes* (**Fig. 1-6: 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. 1-6: 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 protein concentration-adjusted organ extracts with CMC. As shown in **Table 1-2**, 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.

Table 1-2 Reducing 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
	Notacanthomysis hodgarti	ND	ND	ND	ND	0.02 ± 0.01
Decapoa	*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
	Exopalaemon 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
	Palaemon semmelinkii	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.

1.4.Discussion

Interestingly, the level of cellulase activity differed, even among the same copepod species, at different sampling sites (**Figs. 1-4** and **5**). It goes without saying that the aquatic environment changes violently and invariably[10], 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. 1-3**), but showed no activities in the zymographic assay (**Fig. 1-5**). 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[11, 12]. 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 1-2**), 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 enzymes[13, 14]. 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[11]. It is thought that they normally feed on phytoplankton, as was previously reported[15]. However, the number of phyotoplankton is assumed to decline in our sampling sites, due to the low transparency of water limiting photosynthesis[3, 4], meaning their feeding habitats remain unclear. To date, the widespread distribution of cellulase among aquatic animals has only been reported in Japanese wetlands[6], The occurrence of cellulase activities in mysid species and *Acetes* shrimps in the Matang Mangrove Forest Reserve was also reported in our previous study[8]. 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. 1-2, 1-3, 1-4, 1-5** and **1-6**), 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[5] as well as being the key food source for many fish[11]. Accordingly, the copepod and decapod species may be a bridge between higher consumers, as their prey, and original mangrove carbon sources, as their decomposers.

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Chapter 2

Breakdown of Hard-degradable Polysaccharides in Subtidal Zone

2.1. Introduction

Detritus, including organic matter derived from seaweed and terrestrial plants, accumulates in estuaries via tidal flow from the sea and river flow from the land[1, 2]. Within the estuaries, specific ecological systems are formed with organisms that feed on the accumulated detritus[3, 4]. Benthic animals are the major organisms contributing to the degradation of organic matter in estuaries[5].

Estuaries are ecologically important areas, because they are the spawning sites of fish and stopover points for migrating birds[5, 6]. However, since the Second World War, land reclamation has greatly reduced the size of estuarine areas in Japan[7]. This reduction in habitat has led to a marked decrease in the numbers of estuarine species, such as the crustacean *Uca lactea lactea* and the mollusk *Ellobium chinense*, thereby resulting in the designation of these species as vulnerable[8]. In addition, populations of *Cerithidea rhizophorarum*, *Cerithidea cingulata*, and *Batillaria multiformis* have decreased, and these species have been designated as near threatened species. To prevent the populations from decreasing further, it is important to understand the feeding system and distribution of these animals.

The results of recent stable isotopic studies suggest that benthic animals in estuaries preferentially feed on benthic diatoms or microphytobenthos (MPB)[9-11]. Most benthic animals are unable directly to assimilate organic matter derived from land plants, because they lack the enzymes required to digest cellulose, which is one of the primary components of land plants[12]. On the other hand, benthic animals inhabiting estuaries and reed beds are assumed to feed on detritus and the remains of land plants that are primarily composed of cellulose[3, 13-14]. Therefore, these animals should be equipped with the enzymes necessary for the breakdown of substances such as cellulose, mannan, xylan, and laminarin[15]. The results of stable isotopic analysis suggest that the brackish bivalve *Latermula marilina* and the polychaete *Hediste* spp. assimilate plant detritus[10]. Similarly, the brackish bivalve *Corbicula japonica* assimilates terrestrial organic matter through the utilization of multiple endogenous cellulases[16-20]. Niiyama and Toyohara detected several plant-degrading enzymes in various benthic animals, including

crustaceans and mollusks[19]. In addition, the benthic mollusks *Aplysia kurodai*, *Haliotis discus hannai*, and *Mytilus edulis* are reported to possess several enzymes necessary for digesting cellulose, mannan, and alginic acid in seaweeds[21-24].

The distribution of benthic animals in brackish water areas is restricted by factors such as elevation, salt content, substratum, and food[25-28]. Benthic animals possess digestive enzymes for plant-derived carbohydrates[15, 29], and therefore, the activity level of these enzymes may determine the distribution of related benthic animals in estuaries. In the present study, we used distribution surveys, stable isotope analysis, and measurement of enzyme activity to investigate the relationship between feeding characteristics and ecological distribution of four related gastropod species (*Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*) inhabiting the mouth of the Tanaka River in Mie Prefecture.

2.2.Materials and Methods

2.2.1.Study sites and sample collection

The Tanaka River runs through the north of central Mie Prefecture and into the west side of Ise Bay. The estuary includes an oval tidal flat with an area of approximately 3600 m^2, which stretches 200 m in an east-west direction and 300 m in a north-south direction[30-31]. This tidal flat is divided into a reed bed containing accumulated litter, and an adjacent field without reeds (**Fig. 2-1**). The southern part of the field is closest to the reed bed and is composed of clay soil, and the northeastern part is composed of sandy clay soil. A fish culture pond is located to the west of the estuary, and the river flows out through a gate in the northwestern part of the estuary. The pulmonates *Ellobium chinense* and *Laemodonta siamensis*, as well as >20 gastropod species, inhabit the estuary, mainly in the reed bed[30, 32]. Tidal range in the estuary varies between −0.04 m and 2.35 m between May and September[33].

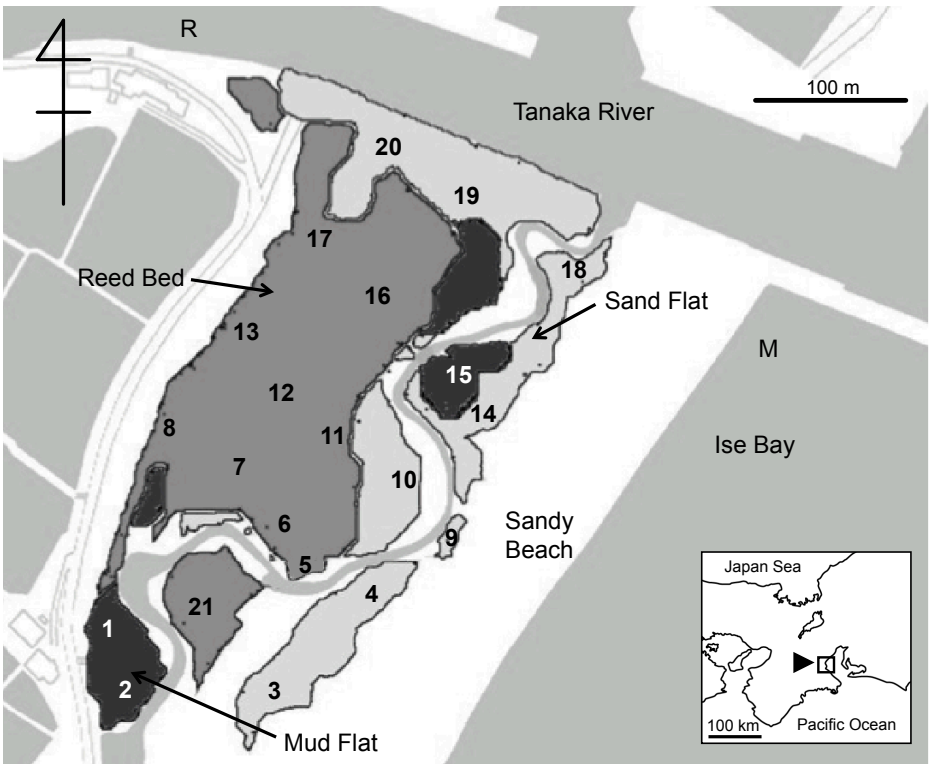

Fig. 2-1 Sampling sites in the estuary of the Tanaka River, Mie Prefecture, Japan. Charcoal gray, reed bed; black, mud flat; gray, sand flat.

For the analysis of species distribution, samples were collected at 20 stations. For enzyme activity analysis, *Cerithidea rhizophorarum* was sampled at sites 6, 13, 17, and 21; *Cerithidea cingulata* was sampled at sites 10 and 17; *B. multiformis* was sampled at site 20; and *B. attramentaria* was sampled at site 19. For stable isotopic analysis, MPB was collected at sites 2 and 15. Sediment was sampled at sites 2, 10, 15, and 17; riverine particulate organic matter (RPOM) was sampled at point "R"; and marine particulate organic matter (MPOM) was sampled at point "M". The marsh plant, *Phragmites australis*, was sampled at sites 8 and 17, and *Carex scabrifolia* was sampled at site 2.

2.2.2.Preparation of enzymatic extracts

Gastropods were collected for the measurement of enzyme activity in October 2010. The 21 sampling sites are indicated in Figure 1. The specific collection sites for each species were determined according to environmental characteristics as follows: *Cerithidea rhizophorarum* was collected from reed-bed sites 6, 13, 17, and 21;

Cerithidea cingulata was collected from sand-flat site 10 and reed-bed site 17; *B. multiformis* was collected from sand-flat site 20; and *B. attramentaria* was collected from sand-flat site 19. Five individuals of each gastropod species were collected and transported on ice to the laboratory at Kyoto University.

Gastropods were dissected on ice to isolate the midgut and crystalline style, which were homogenized with 5 mL of phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 5.5 mM KH_2PO_4 at pH 7.4). The homogenates were centrifuged at 9,100 × *g* for 10 min, and 1 mL of each supernatant was used as enzyme solution. The protein concentration of each enzyme solution was determined according to the method of Bradford[34], by measuring the absorbance at 595 nm (UV mini 1240 spectrophotometer; Shimadzu Corporation). Bovine serum albumin was used as the standard for protein concentration assays.

2.2.3.Enzyme activity assay

2.2.4.Cellulase activity

Enzyme solution (5 μL), 5 μL of 1 M sodium acetate (AcNa) buffer (pH 5.5), and 40 μL of 1% carboxymethyl cellulose (CMC; Sigma) were mixed and incubated at 37°C for 10 min with shaking. After incubation, enzyme reactions were terminated by heating at 100°C for 3 min. Next, 1 mL of blue tetrazolium was added and each sample was heated at 100°C for 3 min. After cooling on ice, the absorbance at 660 nm was measured to determine the amount of reducing sugar produced[35].

Two types of control were prepared to account for the amount of reducing sugar contained in each enzyme solution and in CMC. To determine the amount of reducing sugar in the enzyme solutions, 5 μL of each enzyme solution, 5 μL of 1 M AcNa buffer (pH 5.5), and 40 μL of distilled water were mixed and measured. To determine the amount of reducing sugar in CMC, 5 μL of distilled water, 5 μL of 1 M AcNa buffer (pH 5.5) and 40 μL of 1% CMC were mixed and measured. The enzyme activity was expressed as the amount of glucose released per min per μg of protein. The control values were subtracted from the sample values, to obtain the enzyme activity for each animal collected.

2.2.5.Hemicellulase activity

Mannanase, xylanase, and laminarinase activities were measured by using the

same method as for cellulase, but with CMC replaced by 1% locust bean gum (Sigma), xylan (Sigma), and laminarin (Nacalai Tesque), respectively.

2.2.6.Zymographic analysis

SDS-PAGE zymographic analysis was performed according to the method of Beguin[36]. Enzyme solutions (protein content per 5 μl: *B. multiformis*, 7 μg; *B. attramentaria*, 7 μg; *Cerithidea cingulata*, 4 μg; *Cerithidea rhizophorarum*, 0.25 μg) were applied to 7.5% polyacrylamide gels containing 0.5% w/v CMC. After electrophoresis, each gel was rinsed twice for 30 min in 0.1 M AcNa buffer (pH 5.5) containing 0.1% Triton X-100, and was then incubated in 0.1 M AcNa buffer (pH 5.5) at 37°C for 24 h. Gels were stained with 0.1% Congo Red for 30 min and de-stained with 1 M NaCl. Enzyme activity was detected as de-stained bands in the gel.

2.2.7.Stable isotopic analysis

Stable isotope ratios increase at each step in the food chain ($\delta^{13}C$, 0‰ to +1‰; $\delta^{15}N$, +3‰ to +4‰) and can be used to estimate the trophic level[37-38].

Samples of four gastropod species were collected by hand at the same locations and at the same time as the samples collected for the measurement of enzyme activity (**Fig. 2-1**), and were transported to the laboratory on ice. Foot tissue was removed, washed with deionized water, freeze-dried (24 h), and powdered by using a mortar. Lipids were removed with chloroform-methanol solution (2:1 by volume).

Potential food sources were sampled several times from 2009 to 2010. MPOM and RPOM samples were collected (**Fig. 2-1**) in May 2009, August 2009, February 2010, and March 2010. At each station, surface water was sampled by collection in a polypropylene bottle (n = 1), and was pre-filtered through a 0.125-mm mesh. The POM in each sample was then concentrated onto a pre-combusted Whatman GF/F glass fiber filter (500°C, 2 h). Sediment organic matter (SOM) was collected with a spatula from the top 1 cm at sites 2, 7, 15, and 17 in May and September 2009 and in March 2010, and was placed in three plastic tubes. MPB was collected at sites 2 and 15 (**Fig. 2-1**) in May 2009, September 2009, and March 2010, when visible diatom mats developed. In the field, surface sediments (0–5 mm deep) were placed on three polyethylene trays, and MPB samples were extracted in the laboratory by exploiting their positive phototaxis under two fluorescent lamps[39]. Samples were then suspended in deionized water and

concentrated onto a pre-combusted GF/F. All filters and sediment samples were acidified with 1 M HCl to remove carbonates, and were then freeze-dried (24 h). In May 2009, two species of marsh plants were collected—*Phragmites australis* was collected from sites 8 and 17, and *Carex scabrifolia* was collected from site 2 (three replicates of each). Samples were cleaned by using a brush dipped in deionized water, freeze-dried (24 h), and powdered.

$\delta^{13}C$ and $\delta^{15}N$ were determined by using a mass spectrometer connected to an elemental analyzer (Flash EA1112 and Delta XP; Finnigan Mat). Isotope ratios were expressed in delta notation:

$\delta^{13}C$ or $\delta^{15}N$ (‰) = $(R_{sample}/R_{standard} - 1) \times 1000$

where R is the $^{13}C/^{12}C$ or $^{15}N/^{14}N$ ratio for $\delta^{13}C$ or $\delta^{15}N$, respectively. Pee Dee belemnite and atmospheric N_2 were used as references for $\delta^{13}C$ and $\delta^{15}N$, respectively. D-Alanine and L-histidine were used as working standards. In the analyses, the C/N molar ratio was also calculated for plants, MPB, POM, and SOM samples, based on their total organic carbon and total nitrogen contents.

2.2.8.Spatial distribution of gastropods

The investigation of species distribution was conducted during the ebb tide on May 18 and 21, 2010. In order to include the various environments of the estuary, 20 sites were located at even intervals along five transect lines (thirteen sites on the sand flat or mud flat, and seven sites on the reed bed; **Fig. 2-1**). Gastropods were collected in three quadrats (50 cm × 50 cm) laid randomly at each of the 20 sites. All visible gastropods within the quadrats were collected and taken to Mie University, fixed in 80% alcohol, identified, and counted.

2.2.9.Physical environmental data

Elevation and plant coverage were measured for each of the 20 sites. Elevation was calculated by using coordinates obtained from a GPS survey. Elevation was defined as follows:

Elevation (m) = $(e - T) + 1.3 \times T$

where e is the measured elevation at the estuary and T is the mean sea level of Tokyo Bay. The tide level of the intertidal zone ranged from −0.04 m to 2.35 m at Toba City, Mie, between May 2010 and September 2010[33].

Plant cover was estimated by using the same quadrat method as for the collection of gastropods. In September 2010, the vegetation inside each quadrat was recorded, and the amount of sediment covered by vegetation was expressed as subjective values from 0 to 10 according to visual estimation.

2.2.10.Ignition loss of organic matter in sediment

On the same day that plant coverage was recorded, sediment samples were collected from each quadrat by using a core sampler (vinyl chloride pipe, diameter 6 cm, height 10 cm). After removing gravel (major axis longer than 2 mm), plants, benthos, artifact fragments such as broken pieces of glass bottles or plastic goods, and sediment were weighed and dried to a constant weight at 110°C in a drying oven. Approximately ten gram of each sample was well ground, and coarse particles were removed by passing the sample through a 1-mm mesh with shaking. Three grams of each sample were weighed, placed in an oven, and ignited for 2 h at 850°C. The percentage of organic matter content was calculated as the ratio of the sample weight lost after ignition to the sample weight before ignition. Sample sediment measurements were conducted in triplicate, and values were expressed as the average ± SEM.

2.2.11.Statistical analysis

Differences in enzyme activity level among sites or species, stable isotope ratio among sites or species, and distribution among sampling sites were statistically tested by using one-way ANOVA followed by post-hoc tests (Tukey's HSD) in SPSS (IBM, version 22).

2.3.Results

2.3.1.Enzyme activities of gastropods

All of the investigated gastropods showed cellulase, mannanase, xylanase, and laminarinase activities (**Fig. 2-2**). The level of enzyme activity differed between species and among sampling sites.

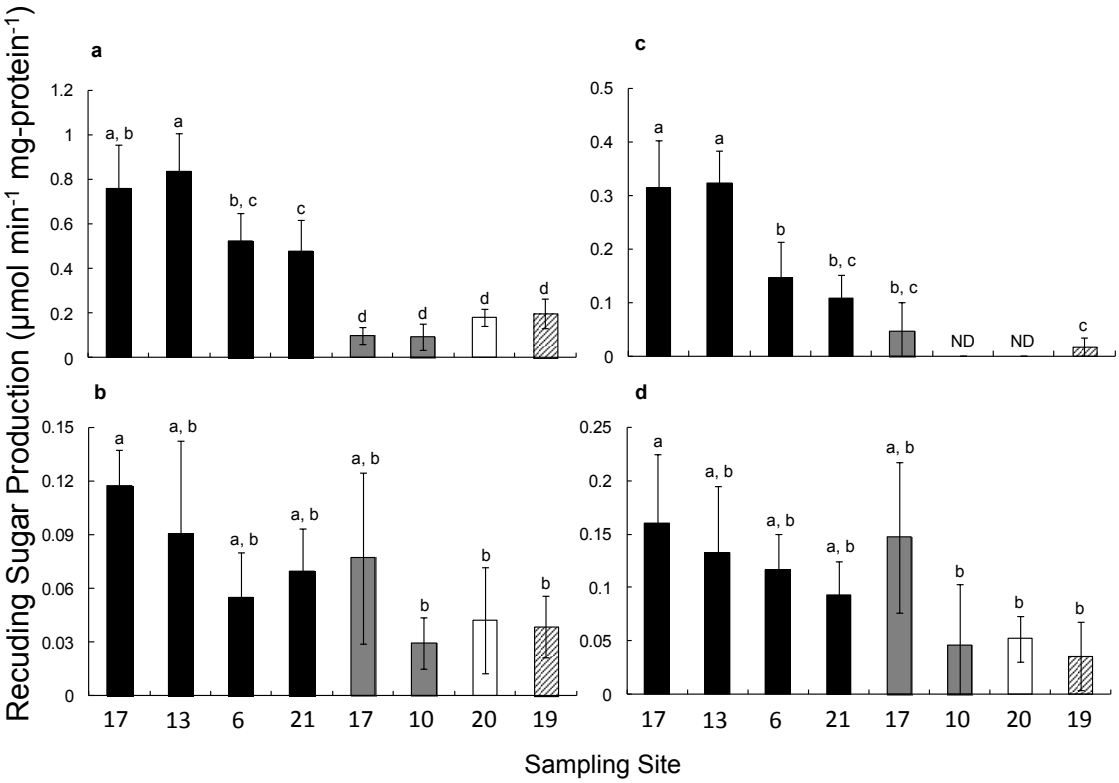

Fig. 2-2 Cellulase and hemicellulase activities in the digestive glands of *Cerithidea rhizophorarum* after incubation of tissue extracts with appropriate substrates. a, cellulase activity; b, mannanase activity; c, xylanase activity; d, laminarinase activity. Bars represent SEM. ND, not detectable. Black, *Cerithidea rhizophorarum*; gray, *Cerithidea cingulata*; white, *Batillaria multiformis*; striped pattern, *B. attramentaria*. Values followed by the same letters do not differ significantly (Tukey's HSD, $p < 0.05$).

For *Cerithidea rhizophorarum*, individuals collected from reed-bed site 21 showed the lowest level of cellulose activity. In addition, individuals collected from reed-bed site 13 showed significantly higher cellulose activity than did those collected from reed-bed sites 6 and 21 (Tukey's HSD, $p < 0.05$). The cellulase activity of *Cerithidea rhizophorarum* was significantly higher than those of the other three species ($p < 0.05$). However, there were no significant differences among the level of cellulase activity of *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*.

For gastropods collected from reed-bed site 6, the mannanase activity of *Cerithidea rhizophorarum* was lower than was that of the other three species; however,

this difference was not statistically significant. The mannanase activity level of *Cerithidea rhizophorarum* collected from reed-bed site 17 differed significantly from that of *Cerithidea cingulata* collected from sand-flat site 10, and also from those of *B. multiformis* and *B. attramentaria* (Tukey's HSD, $p < 0.05$).

The xylanase activity of *Cerithidea rhizophorarum* was lowest at reed-bed site 21. Similar to the cellulase activity, the xylanase activities of individuals collected from reed-bed sites 13 and 17 were significantly higher than those of individuals collected from reed-bed sites 6 and 21 (Tukey's HSD, $p < 0.05$). In addition, the xylanase activity level of *Cerithidea rhizophorarum* collected from reed-bed sites 17 and 13 differed significantly from that of *Cerithidea cingulata* collected from reed-bed site 17, and also from that of *B. attramentaria*. No xylanase activity was detected in *Cerithidea cingulata* collected from sand-flat site 10, or in *B. multiformis*.

The laminarinase activity level was similar among the investigated sites and species. The laminarinase activity level of *Cerithidea rhizophorarum* collected from reed-bed site 17 differed significantly from that of *Cerithidea cingulata* collected from sand-flat site 10, and also from those of *B. multiformis* and *B. attramentaria*.

The results of zymographic analysis to detect the molecular weight of cellulases in the midguts and crystalline styles of gastropods (**Fig. 2-3**) revealed that multiple cellulases were present in all of the gastropods. Activity bands representing approximately 44 kDa, 80 kDa, and 100 kDa were observed for *B. multiformis* and *B. attramentaria*. Activity bands representing approximately 34 kDa were observed for *Cerithidea rhizophorarum* and *Cerithidea cingulata*.

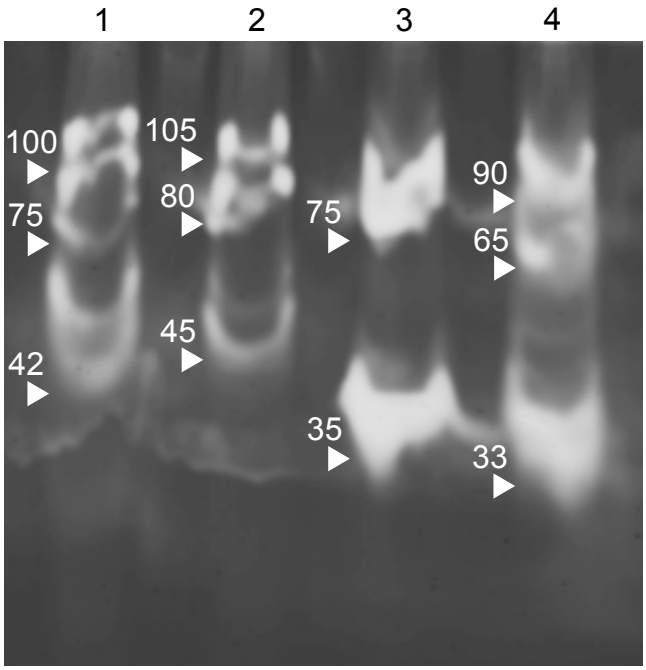

Fig. 2-3 Detection of deduced molecular weight of cellulases by using zymographic analysis. 1, *Batillaria multiformis*; 2, *B. attramentaria*; 3, *Cerithidea cingulata*; 4, *Cerithidea rhizophorarum*. White triangle indicates the site of each band. Numbers denote the deduced molecular weight (kDa).

2.3.2.Stable isotope ratio and statistical analysis

Figure 2-4 shows the results of stable isotope ratio analysis. The sediment $\delta^{15}N$ values were similar among the sites (8.4–9.2‰), whereas the sediment $\delta^{13}C$ values varied markedly (–25.9‰ to –21.0‰). The MPB $\delta^{13}C$ value (–17.8 ± 0.9‰) was similar to the *Cerithidea rhizophorarum* $\delta^{13}C$ value (–19.0‰ to –17.7 ‰), but differed from the *Cerithidea cingulata* (–14.1‰ to –13.5 ‰), *B. multiformis* (–14.6 ‰), and *B. attramentaria* (–13.4 ‰) $\delta^{13}C$ values. The $\delta^{15}N$ and $\delta^{13}C$ values for *P. australis* and *C. scabrifolia*, which are major salt marsh herbaceous plants, were approximately equal. The sand-flat sediment $\delta^{15}N$ and $\delta^{13}C$ values were similar to the MPOM $\delta^{15}N$ and $\delta^{13}C$ values, whereas the reed-bed sediment $\delta^{15}N$ and $\delta^{13}C$ values differed from the MPOM $\delta^{15}N$ and $\delta^{13}C$ values. The C/N ratios of sediment (12.2–15.3) and reeds (13.3) were high. The RPOM $\delta^{15}N$ and $\delta^{13}C$ values were lower than all of the other $\delta^{15}N$ and $\delta^{13}C$ values.

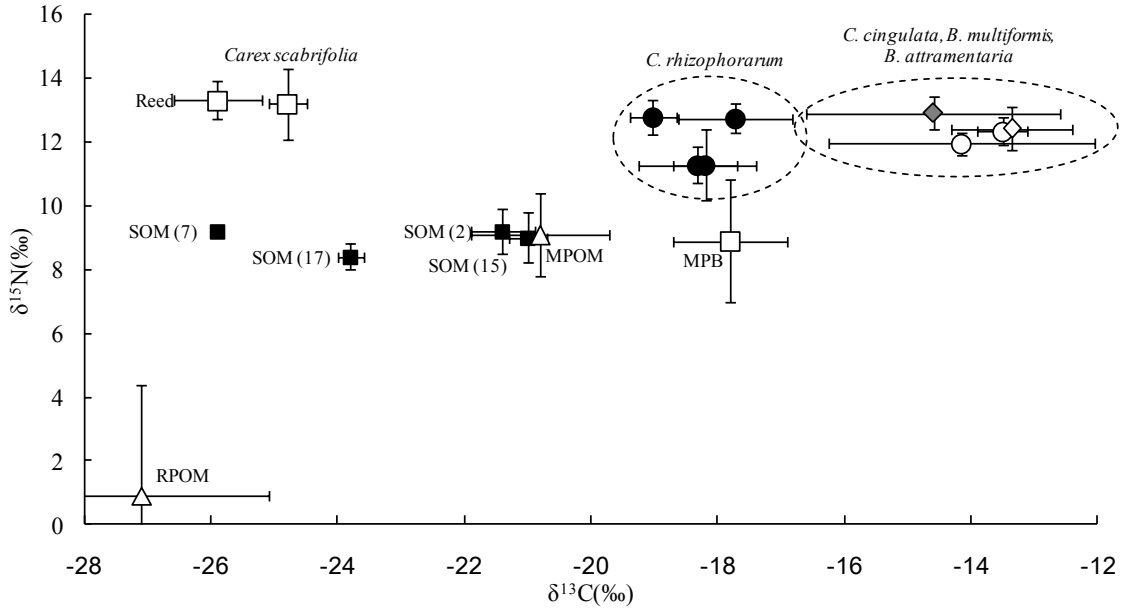

Fig. 2-4 Stable isotope ratios of *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, *B. attramentaria*, sediment organic matter (SOM), plants, microphytobenthos (MPB), and particulate organic matter (POM). All samples were collected at the Tanaka River estuary. Black circle, *Cerithidea rhizophorarum*; white diamond, *B. multiformis*; gray diamond, *B. attramentaria*; white circle, *Cerithidea cingulata*; black square, SOM; white square, plants and MPB; white triangle, POM. Bars represent SEM.

The average $\delta^{15}N$ value for each gastropod species ranged from 11.9‰ to 13.0‰; these values did not differ significantly between sites or species (Tukey's HSD, $p < 0.05$). On the other hand, the $\delta^{13}C$ value of *Cerithidea rhizophorarum* differed significantly from those of the other gastropods (–19.0‰ to –17.7‰ vs. –14.6‰ to –13.4‰; Tukey's HSD, $p < 0.05$). The $\delta^{13}C$ value of *Cerithidea cingulata* at reed-bed site 17 was more similar to that of *Cerithidea cingulata* at sand-flat site 10 than to that of *Cerithidea rhizophorarum* in any of the reed-bed sites.

In comparison with *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, the $\delta^{13}C$ value of *Cerithidea rhizophorarum* was significantly lower, whereas the activity levels of cellulase and xylanase were significantly higher (**Fig. 2-5**; Tukey's HSD, $p < 0.05$); further, the number of *Cerithidea rhizophorarum* individuals in the reed bed was significantly higher (**Fig. 2-5**; Tukey's HSD, $p < 0.05$).

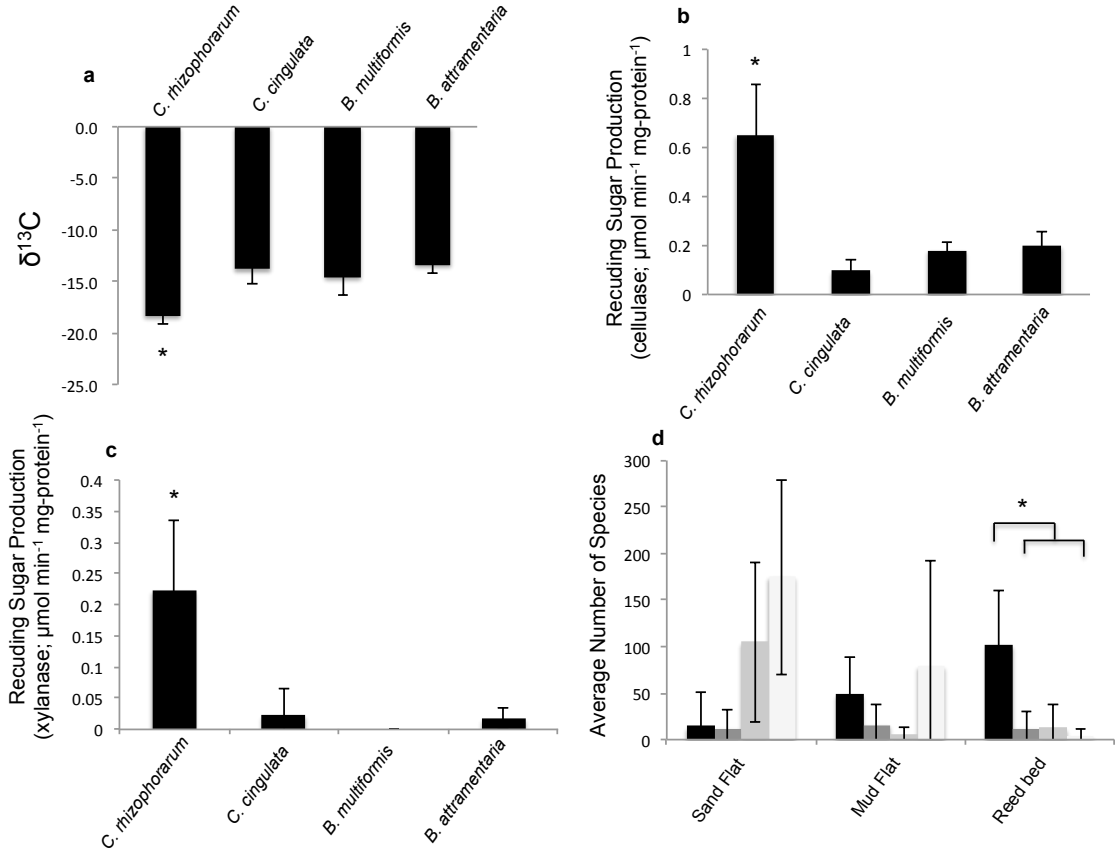

Fig. 2-5 Comparison of δ ^{13}C value, cellulase activity, xylanase activity, and spatial distribution among 4 species of gastropods. Bars represent SEM. (a), δ ^{13}C value; (b), cellulase activity; (c), xylanase activity; (d), spatial distribution. Error bars represent SD; asterisks indicate significant differences. Bars in (d): black, *Cerithidea rhizophorarum*; dark gray, *Cerithidea cingulata*; gray, *Batillaria multiformis*; white, *B. attramentaria*.

2.3.3.Spatial distribution of gastropods

Figure 2-6 shows the total populations of gastropods in each of the investigated sites. *Cerithidea rhizophorarum* was observed in six sand-flat sites and in nine reed-bed sites. The population density of this species was highest in the reed bed (mean population, 101 gastropods per m^2) than in the sand flat or mud flat. The population density was highest in reed-bed site 5 (213 gastropods per m^2), which was located at the edge of the reed bed; this was followed by the sites in the center of the reed bed (reed-bed site 17, 156 gastropods per m^2; reed-bed site 16, 137 gastropods per m^2; and

reed-bed site 6, 133 gastropods per m^2). The population density was also relatively high in mud-flat sites 1 and 2. No *Cerithidea rhizophorarum* individuals were observed in sand-flat sites 9, 10, 15, 19, and 20, all of which were located in the tidal flats.

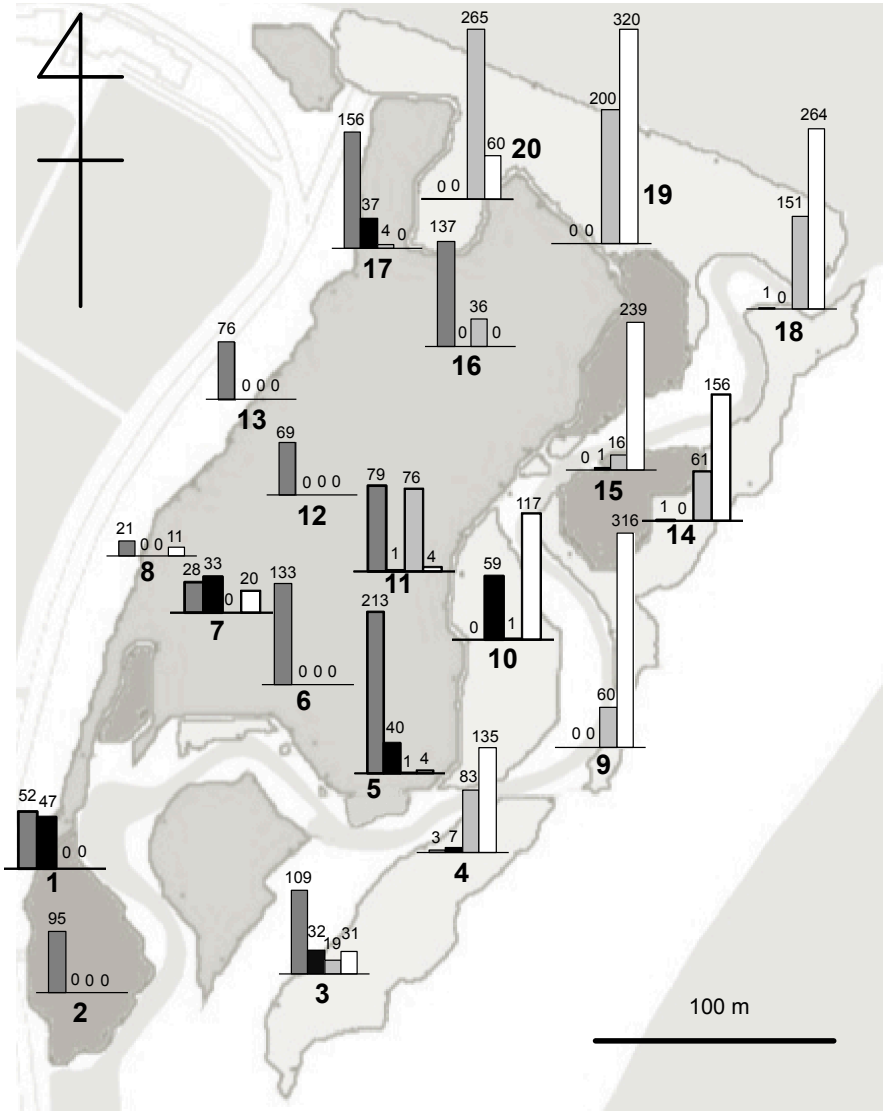

Fig. 2-6 Distribution of gastropods in the Tanaka River estuary. Charcoal gray, *Cerithidea rhizophorarum*; black, *Cerithidea cingulata*; gray, *B. multiformis*; white, *B. attramentaria*. Population is the sum of 3 quadrats (50 cm × 50 cm); 3 core samples were taken at each site.

Cerithidea cingulata was observed in five sand-flat or mud-flat sites, and in four reed-bed sites. The gastropod population density was high at reed-bed site 5, mud-flat site 1, and sand-flat site 10. The mean population density in these sites ranged from 40 gastropods per m^2 to 59 gastropods per m^2. *Cerithidea cingulata* was observed in fewer sites than were *Cerithidea rhizophorarum*, *B. multiformis*, and *B. attramentaria*. In addition, the total population of *Cerithidea cingulata* was lower than those of the three other investigated species. *Cerithidea cingulata* tended to be most abundant near the water gate (mud-flat site 1 and reed-bed site 7), along the waterway (sand-flat site 10), and close to the river (reed-bed site 17).

Batillaria multiformis was observed in nine sand-flat or mud-flat sites, and in four reed-bed sites. Within these sites, this species tended to be distributed in sandy and sandy-muddy areas. The population density of *B. multiformis* was highest in sand-flat sites 18, 19, and 20; at these sites, the average population density ranged from 151 gastropods per m^2 to 265 gastropods per m^2, and accounted for 70% of the total population.

Batillaria attramentaria also tended to be distributed in sandy and sandy-muddy areas, and was observed in nine sand-flat or mud-flat sites, and in four reed-bed sites. The overall population density of this species was 1677 gastropods per m^2, which was the highest among the four investigated species. The population density was highest along the waterway (sand-flat sites 4, 9, 14, 18, and 19; and mud-flat site 15). Together, these six sites accounted for >50% of the total population. *Batillaria attramentaria* was rarely observed in the reed bed.

2.3.4.Physical environmental data

Figure 2-7 summarizes the physical environmental data for the study sites. The elevation of the 20 sites ranged from 1.0 m to 1.88 m. The mean elevation in the reed bed was 1.3–1.81 m, indicating that the reed bed was located in the intertidal to upper tidal zone. The reed bed was at a higher elevation than were the sand and mud flats (data not shown).

The plant coverage of the sand and mud flats was 0.0–3.0 (mean = 0.4). The plant coverage of the reed bed was 2.0–3.7 (mean = 2.8; data not shown). On the tidal flat, *Carex scabrifolia* was observed at mud-flat site 1, *Phragmites australis* was observed at reed-bed site 5 and sand-flat site 8, and *Gracilaria vermiculophylla* was observed at

sand-flat site 10. On the reed bed, *Phragmites australis* was observed at all of the study sites.

The sediment in the sand and mud flats contained 0.89–4.48% organic matter (mean = 2.28%). In the reed bed, the sediment contained 1.53–3.61% organic matter (average = 2.39%; data not shown). The percentage organic matter was slightly higher in the reed bed than in the sand or mud flats. Mud-flat areas (sites 1 and 2) contained high percentages of organic matter (3.58% and 3.05%, respectively). These sites were located near a sluice, where fresh water containing domestic wastewater entered the river. In the reed bed, the percentage of organic matter was particularly high at site 11 (3.61%), which was located on the boundary between a reed bed and a mud flat.

The physical environmental data indicate an increasing tendency of reed-bed spread, plant coverage, and organic matter at higher elevation.

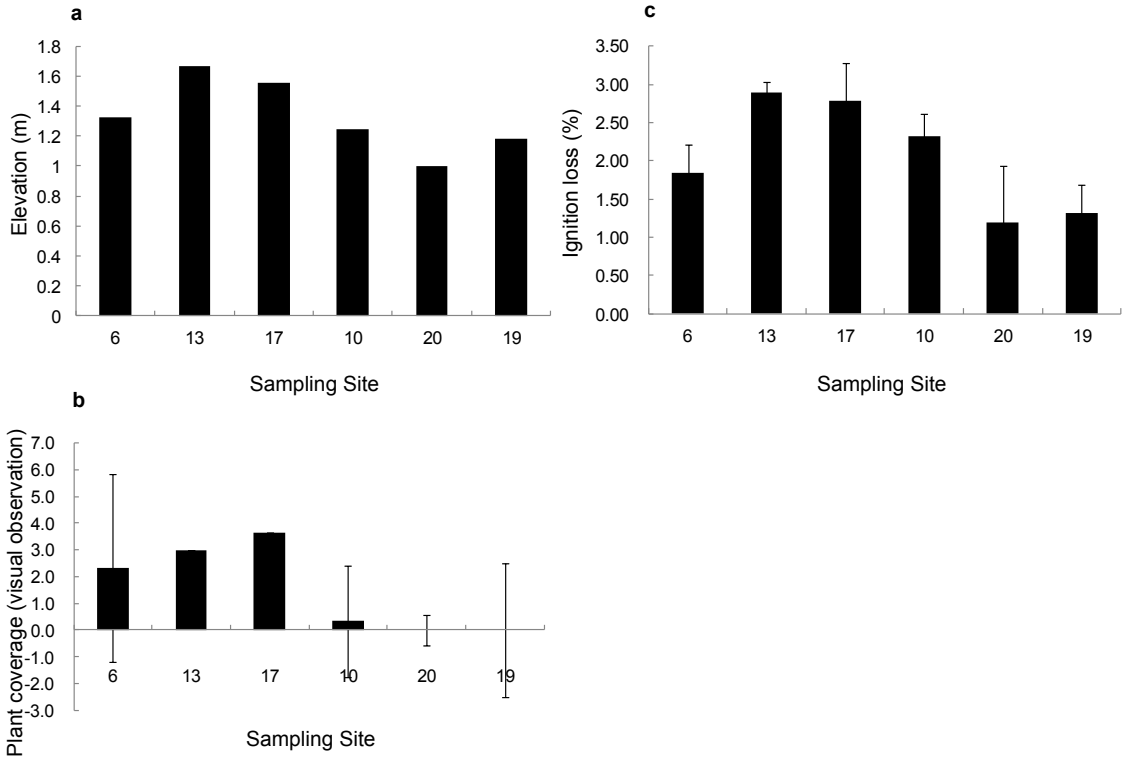

Fig. 2-7 Environmental characteristics of the sampling sites. (a) elevation; (b) plant cover; (c) ignition loss of organic matter in sediment. Values are mean ± SEM.

2.4.Discussion

Recent studies on benthic animals have focused either on their plant-digesting enzymes[15, 24, 29] or on their species distribution[25-27]. However, to the best of our knowledge, the relationship between enzyme activity level and species distribution has not previously been investigated. In the present study, we examined the relationship between digestive enzyme activities and the distribution of four species of gastropods (*Cerithidea rhizophorarum* , *Cerithidea cingulata*, *Batillaria multiformis*, and *B. attramentaria*) inhabiting the Tanaka River estuary (Mie Prefecture, Japan), to clarify the species-specific dis tribution of related gastropods.

We successfully quantified the activities of cellulase and several hemicellulases by measuring the production of reducing sugars. Our results indicated that the investigated gastropod species were able to digest cellulose, mannose, xylan, and laminarin (**Fig. 2-2**). Among the four species evaluated, *Cerithidea rhizophorarum* showed particularly high activity for all of the investigated enzymes, implying that this species more efficiently secretes these enzymes than do *Cerithidea cingulata*, *Batillaria multiformis*, and *B. attramentaria*.

The levels of xylanase and cellulase activities differed markedly according to species and sites. Xylan concentrations are particularly high in terrestrial plants; on the other hand, cellulose is included in all plants including marine algae[40-41]. Therefore, significant differences in xylanase activity between gastro pods are likely related to the consumption of terrestrial plants. For example, *Cerithidea cingulata* collected from reed-bed site 17 showed xylanase activity, whereas *Cerithidea cingulata* collected from sand-flat site 10 did not. Further, *Cerithidea rhizophorarum* individuals collected from reed-bed sites 17 and 13 showed significantly higher xylanase activity than did those collected from sand flats (**Fig. 2-2**). In other words, *Cerithidea cingulata* and *Cerithidea rhizophorarum* collected from the reed bed showed high xylanase activity, and probably ingested and assimilated organic matter derived from reeds in the sediment.

Mannan is a hemicellulose found in some species of marine algae[41-42]. Laminarin is a hemicellulose used for storage of carbohydrates by marine algae. Brown algae such as *Laminaria hyperborea* and *Saccharina longicruris* contain large amounts of laminarin, whereas red algae contain relatively low amounts[43]. In the present study, we observed no marked differences in the levels of mannanase and laminarinase activities among species and sites (**Fig. 2-2**). However, all of the gastropods examined

showed detectable activities of mannanase and laminarinase. In accordance with previous findings, our results suggest that gastropods ingest algae and benthic diatoms entering the estuary[9, 11, 44].

The results of zymographic analysis indicated that the four species of gastropods examined possess multiple cellulases with different molecular weights. The molecular weights of cellulases in *Cerithidea rhizophorarum* and *Cerithidea cingulata* were similar (approximately 34 kDa); however, *B. multiformis* and *B. attramentaria* cellulases had molecular weights of approximately 44 kDa, 80 kDa, and 100 kDa (**Fig. 2-3**). Thus, related gastropod species showed similar cellulase patterns *Cerithidea rhizophorarum* and *Cerithidea cingulata* belong to the family Potamididae, whereas *B. multiformis* and *B. attramentaria* belong to the family Batillariidae[29, 45]. Phylogenetic relationships are not necessarily correlated with digestive enzyme characteristics, but may explain our observed pattern of results. For example, the amino acid sequences of cellulases in *Haliotis discus hannai* and *Haliotis discus discus* tend to be highly homologous[32, 46].

In the present study, we further showed that the cellulase activity levels differed significantly among species (**Fig. 2-2**), resulting in different band intensities when the same amount of protein was applied. When the amount of protein required to obtain the clearest band for *B. multiformis* was used for all of the investigated species, the band for *Cerithidea rhizophorarum* was smeared (data not shown). Therefore, we optimized the amount of protein used in the zymographic analysis for each species, in order to obtain clear bands (**Fig. 2-3**). We showed that the largest amount of protein was required by *Batillaria multiformis*, followed by *B. attramentaria*, *Cerithidea cingulata*, and *Cerithidea rhizophorarum*. Our results suggest that differences in enzyme activity level among species are not derived from differences in the enzyme itself, but from the amount of enzyme secreted.

We evaluated several potential food sources for the four investigated species of gastropod. We determined the highest δ^{13} C value for MPB (annual mean = −17.8‰), followed by MPOM (annual mean = −20.8‰), *C. scabrifolia* (annual mean = − 24.8‰), *P. australis* (annual mean = −25.9‰), and RPOM (annual mean = −27.1‰). The δ^{13} C of the four gastropod species ranged from −19.0‰ to −13.4‰, which was more than 5‰ higher than the reed δ^{13} C (−25.9‰), but similar to the MPB δ^{13} C and the MPOM δ^{13} C. Our results indicate that organic matter from reeds is not

usually utilized as a source of nutrients by gastropods. On the other hand, the δ^{13} C of gastropods collected from reed beds was approximately 5‰ lower than that of gastropods collected from sand and mud flats, indicating that reeds contribute, at least partially, to the diet of reed-bed inhabiting gastropods. This may be explained by a relatively low availability of MPB because of plant cover, and also an abundance of organic matter from reeds. We further observed that reed beds had a high δ^{13} C value (−25.9 to −23.87‰ at sites 7 and 17), but a low C/N ratio (12.2–15.3). Similarly, reeds had a low δ^{13} C value (−25.9‰), but a high C/N ratio (13.3), indicating the presence of abundant organic matter derived from reeds within the reed-bed sediment.

Several studies have shown that the main carbon sources of macrobenthos are benthic diatoms, epiphytic algae, and phytoplankton, whereas the contribution of terrestrial organic matter is low[44, 47-49]. Kurata *et al*. reported that *Assiminea japonica* and *Angustassiminea castanea castanea* inhabiting salt marshes utilize phytoplankton and benthic diatoms. Choy *et al*. reported that two species of Batillariidae (*Batillaria attramentaria* and *B. exarata*) mainly assimilated algae and bacteria[50]. Similarly, the gastropods examined in the present study probably eat benthic diatoms and phytoplankton, rather than terrestrial plants. Interestingly, however, in comparison with *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, *Cerithidea rhizophorarum* showed a significantly low δ^{13} C value, high cellulase and/or xylanase activities, and dominance in reed beds (**Fig. 2-5**). Taken together, these results suggest that *Cerithidea rhizophorarum* depends more heavily on reeds for its nutrition than do *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*.

In the present study, we further observed that *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* tended to have isolated habitats (**Fig. 2-6**). *Cerithidea rhizophorarum* had a tendency to dominate reed beds, whereas relatively few individuals were found in the sand and mud flats. Interestingly, at sand-flat site 3, *Cerithidea rhizophorarum* was the most common species. This site was in a sand-flat habitat, but was located very close to the edge of the reed bed, and had a similar biota to that found inside the reed bed.

Wada and Nishikawa reported that, among *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, *Cerithidea rhizophorarum* was most likely to be unevenly distributed in the upper intertidal zone[51]. The authors concluded that this tendency was strongly affected by the presence of vegetation. The

results of our present study are in accordance with the findings of Wada and Nishikawa [51], because the reed bed had relatively high elevation, plant coverage, and organic matter (**Fig. 2-7**). *Cerithidea rhizophorarum* is reported to be more tolerant of aridity than are *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*[52]. This attribute may further explain why this species was widely distributed in the reed bed. *Cerithidea rhizophorarum* individuals collected from reed beds (sites 13 and 17) showed higher enzyme activities than did those collected from other sites (**Fig. 2-2**). Sites 13 and 17 tended to show relatively high, although not markedly high, levels in environmental parameters (elevation, plant coverage, and organic matter). Enzyme activity level can vary according to the substratum environment, even within the same reed bed; therefore, further detailed environmental research to confirm the species differences observed in the present study is required.

Cerithidea cingulata, *B. multiformis*, and *B. attramentaria* were rarely observed in reed beds, but were dominant in the sand and mud flat habitats. According to Wada and Nishikawa[51], *Cerithidea cingulata* shows a preference for muddy substrata in the middle intertidal zone. The sites where *Cerithidea cingulata* was found in the present study had higher elevation in the intertidal zone than did the sites where *B. multiformis* and *B. attramentaria* were dominant (**Fig. 2-7**). This elevation characteristic of *Cerithidea cingulata* is similar to that of *Cerithidea rhizophorarum*. Therefore, *Cerithidea cingulata* and *Cerithidea rhizophorarum* may prefer similar physical environments. These two species were generally found at the same sites in the present study (**Fig. 2-6**). It is possible that the observed differences in the number of these two species were derived from the considerable gap between their enzyme activities, and therefore the resulting differences in dietary habits (**Figs 2-2** and **2-3**).

In comparison with *Cerithidea cingulata* and *Cerithidea rhizophorarum*, *B. multiformis* and *B. attramentaria* showed a preference for sites with lower elevation, less plant coverage, and smaller amounts of organic matter (**Fig. 2-7**). The enzyme activities of *B. multiformis* and *B. attramentaria* were significantly lower than those of *Cerithidea rhizophorarum* (**Figs 2-2** and **2-3**). These differences in enzyme activity level and food habits likely determine substratum preference, thereby contributing to the observed habitat distribution. *Batillaria multiformis* and *B. attramentaria* preferred similar habitats, but showed differing density distributions *B. multiformis* preferred the riverbank, whereas *B. attramentaria* preferred sites along the waterway (**Fig. 2-6**).

According to Magi *et al*.[53], the distribution of these two species is regulated by distribution preferences, and substrate and salt tolerance. Further detailed research to facilitate detailed elucidation of their habitat requirements is required.

Habitat isolation of related species has been extensively studied. For example, the mytilid mollusks *Musculista senhousia* and *Xenostrobus securis* show habitat segregation in the larval stage, based on bottom sediment characteristics[54]. *Batillaria multiformis* and *B. attramentaria* prefer sand and mud substrata, whereas *Cerithidea rhizophorarum* prefers elevated locations[51, 53]. However, research on mollusk habitats has mainly focused on the relationship between species distribution, and ecology or gene diversity. Few studies have compared digestive enzymes, feeding habits, and habitat isolation of related species inhabiting a relatively small area.

In the present study, *Cerithidea rhizophorarum* showed relatively high cellulase and xylanase activities (**Figs 2-2** and **2-3**). In addition, the results of stable isotopic analysis indicated that *Cerithidea rhizophorarum* assimilated more terrestrial plant material than did *Cerithidea cingulata*, *B. multiformis*, or *B. attramentaria* (**Figs 2-4** and **2-5**). Further more, our species distribution investigation revealed that *Cerithidea rhizophorarum*, *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* were distributed differently inside and outside the reed bed (**Figs 2-6** and **2-7**). It is possible that habitat is determined not only by environmental characteristics such as substratum, but also by the activity of digestive enzymes. Based on the results of our present study, and also on the findings of several previous studies, we draw the following conclusions. (1) *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria* have lower abilities to digest cellulose than does *Cerithidea rhizophorarum*, and therefore cannot settle inside reed beds, which contain abundant reed-derived carbohydrates that are difficult to degrade. Thus, as previously reported, these three gastropod species feed mainly on benthic diatoms or seaweed in tidal flats[9, 11, 44]. (2) In order to degrade plant-derived carbohydrates from reeds, *Cerithidea rhizophorarum* secretes cellulase and xylanase more efficiently than do *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*. Therefore, *Cerithidea rhizophorarum* is able to inhabit reed beds that are inhospitable to the other three gastropod species.

Thus, the evaluation of digestive enzyme activities can clarify the detailed habitat use of gastropods inhabiting the same area. *Cerithidea rhizophorarum* likely uses reed beds as its habitat, because it can degrade plant-derived carbohydrates, which

other gastropods cannot. Reed bed areas have been shown to support many rare benthic animals[7, 30]. The results of our present study indicate that the physiological characteristics of polysaccharide degradation are important factors for the expansion of benthic animals into reed beds. Various mollusks inhabit the estuary environment, including many endangered species, e.g., *Ellobium chinense*[8, 30]. *Batillaria multiformis*, *Cerithidea cingulata*, and *Cerithidea rhizophorarum* are major gastropods inhabiting estuaries of Japan, but are listed as near threatened species in the Red Data Book[7]. Populations of these species are decreasing markedly, because of the destruction, burial, and pollution of their habitats through land reclamation[7, 55]. Therefore, clarification of food habits and ecological distribution is essential for the preservation of Japanese domestic biodiversity.

In the present study, the observed differences in digestive enzyme activity level may determine the detailed distribution of related species inhabiting the same environment. For example, *Cerithidea rhizophorarum* assimilated larger amounts of terrestrial plant materials than did *Cerithidea cingulata*, *B. multiformis*, and *B. attramentaria*, and therefore tended to dominate reed beds. We believe that our findings will facilitate a detailed understanding of estuarine environments and the preservation of benthic animals.

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CHAPTER 3

Contribution of Environmental Cellulase to the Breakdown of Hard-degradable Polysaccharides in Intertidal Zone

3-1: Discovery of Environmental Cellulase

3.1.1.Introduction

Cellulose is the main component of the cell walls of plants. Cellulose that is transported from forests to wetlands by rivers is assumed to be utilized by a variety of organisms as a carbon source[1-6]. However, the details of the degradation process of cellulose in wetlands remain unknown.

Cellulose is a high molecular weight polysaccharide comprised of glucose bound by β-1, 4 linkage that is biochemically stable when compared with starch, in which glucose is bound by α-1, 4 linkage and α-1, 6 linkage. Accordingly, a series of enzymes such as endo-β-glucanase, cellobiohydrolase, and β-glucosidase, which are collectively designated as cellulases, are required for the enzymatic breakdown of cellulose[7].

Until recently, it has been assumed that herbivores digest cellulose using cellulases derived from the symbiotic microorganisms[8]. Moreover, cellulases in invertebrates, including insects, were long been assumed to originate from symbiotic microbes before demonstration of the endogenous origin of termite cellulase[9]. During the last decade, cellulase genes have been reported in the aquatic organisms such as crayfish[1], mussel[2], abalone[3], bivalve[4], and sea urchin[5].

In addition to these macrobenthos, other organisms such as meiobenthos, fungi, and bacteria are expected to be involved in the degradation of cellulose in the sediments of wetlands[6, 10-11]. In the present study, we attempted to identify organisms contributing to the degradation of cellulose in sediments. The results revealed that cellulases derived from organisms are bound to the components of sediments such as clay and plant residues. Overall, the results of the present study suggest that sediments in wetlands function as a bioreactor to degrade cellulose.

3.1.2.Materials and Methods

3.1.2.1.Collection of sediments

Sediments from the Tanaka River (Mie Prefecture) were collected on October 20, 2009 and September 8, 2010. Sediments from the Yodo River (Osaka Prefecture) were collected on September 23, 2009. Sediments from the Chikugo River (Fukuoka Prefecture), Hamado River (Kumamoto Prefecture), and Midori River (Kumamoto Prefecture) were collected on October 29, 2010. We selected one collecting site within 50 m from the river mouth and transported these samples at 4°C back to the laboratory at Kyoto University. Sediment samples were stored at 4°C until analyses. Macrobenthos such as bivalves and crustaceans were removed prior to collection of the sediments. Upon arrival at the laboratory, meiobenthos such as nematodes and oligochaetes were carefully removed using tweezers in conjunction with microscopic observation (OLYMPUS-SZX12, OLYMPUS, Tokyo, Japan). To remove the meiobenthos completely, the sediments were further filtered through a 63 μm mesh and the pass-through fraction was used for the subsequent experiments.

3.1.2.2.Measurement of cellulase activity of sediments

The cellulase activities of sediments collected from rivers were estimated by measuring reducing sugar released from carboxymethyl cellulose (CMC, Sigma, St. Louis, MO, USA) according to the method described by Somogyi-Nelson [12]. Briefly, 5.0 g of sediment were mixed with 0.5 ml toluene, 10 ml of 0.2 M sodium acetate buffer (pH 5.9) and 10 ml of 1% CMC solution. The CMC was dissolved in water to give a 1% solution. For the blank, water was used instead of CMC solution. Following incubation at 30°C for 24 h, the reaction mixture was centrifuged for 10 min at 2,500×*g*, and 1 ml of the supernatant was then transferred to another tube and combined with 1 ml of Somogyi solution (1.2% $KNaC_4H_4O_6 \cdot 4H_2O$, 2.4% Na_2CO_3, 0.4% $CuSO_4$, 1.6% $NaHCO_3$, 18% Na_2SO_4). The mixture was then incubated at 100°C for 10 min. Next, the samples were cooled with cold water, then 1 ml of Nelson solution (1.2% $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.15% $Na_2HAsO_4 \cdot 7H_2O$, 4.2% H_2SO_4) and 18 ml of water were added. The absorbance at 600 nm was then measured using a spectrophotometer (UV-mini-1240, Shimadzu, Kyoto, Japan) and the activity was represented as the reducing sugar released by 1 g of sediment for 1 h[12]. Unless otherwise specified, reagents of specific grades were purchased from nacalai tesque (Kyoto, Japan).

3.1.2.3.Effect of chloramphenicol on cellulase activity of sediments

To determine if cellulase activity detected in the sediments was derived from microorganisms, the effects of antibiotics on the cellulase activity of the sediments were investigated. As a preliminary experiment, the effects of ampicillin, kanamycin, tetracycline, and chloramphenicol on the growth of microorganisms in the sediment were tested. Sediments collected from the Midori River were mixed with 400 mg, 40 mg, or 4 mg of ampicillin, kanamycin, tetracycline, or chloramphenicol per gram of wet sediment, after which they were incubated at 37°C for 24 h. The effects of antibiotics were evaluated by plating the incubated sediments on LB (Luria Bertani medium) plates and incubating for five days. Only chloramphenicol (400 mg and 40 mg per 1g sediment) completely inhibited the growth of the microorganisms including bacteria and fungi. Thus, we selected chloramphenicol to sterilize microorganisms in the sediments.

A solution of 2 ml of chloramphenicol dissolved in 50% ethanol to give 100 mg/ml was added to 5 g of per gram of wet sediment collected from the Tanaka and Midori Rivers, which gave 40 mg chloramphenicol per gram of wet sediment in a final concentration. These samples were then incubated for 24 h at 30°C. As a control, 2 ml of 50% ethanol in water was added. After incubation, the sediment was spread on a LB plate to validate the sterilizing effect of chloramphenicol on microorganisms.

Meanwhile the sediment was spread on CMC agar plates containing 1% CMC, 0.15% $Ca(NO_3)_2$, 0.05% $MgSO_4$, 0.05% K_2HPO_4 and 1.5% agar and the samples were then incubated at 30°C for three days to detect the remaining cellulase activity of the sediment (data not shown). Then, the remaining cellulase activities of the two rivers were quantified by Somogyi Nelson method described above[12].

3.1.2.4.Evaluation of cellulase binding ability of sediments

Sediments collected from all rivers were autoclaved at 121°C for 15 min to inactivate sediment-bound cellulases after vigorous washing with water. After cooling, the following procedures were conducted in the clean bench. Two grams (dry weight) of autoclaved sediment and 6.25 mg of *Aspergillus niger* cellulase (MP Biomedicals, California, USA) dissolved in 5 ml H_2O were mixed and shaken for 1 h at room temperature to bind the cellulase to the sediments. After centrifugation at 2,500×*g* for

10 min, the pellet was vigorously washed twice with excess water to remove the unbound cellulase. The cellulase activities of the sediments were measured as described above to determine cellulase binding ability. The activity was expressed as the reducing sugar released per dry weight of sediments.

3.1.2.5.Comparison of cellulase binding ability between clay and plant residues

Sediments collected from the Midori River were separated into clay and plant residues using tweezers in conjunction with microscopic observation. To inactivate the originally bound cellulase, separated clay and plant residues (approximately 5 g in wet weight) were autoclaved for 121°C for 15 min. Next, the commercially obtained fungal cellulase and autoclaved clay or plant residues were mixed for 1 h at room temperature as described above. After washing vigorously with an excess amount of water, the cellulase activities bound to clay and plant residues were measured, respectively. The activity was expressed as the reducing sugar released per dry weight of clay or plant residues.

3.1.2.6.Correlation between organic matter content and cellulase binding ability

Sediments were heated at 600°C for 3 h using a mantle heater (KCA-10A, Koito, Tokyo, Japan). The organic matter content was determined based on the loss on ignition values. The correlation between the cellulase binding ability determined in Table 3 and the organic matter content was then estimated.

3.1.2.7.Statistical analyses

All data were statistically analyzed by ANOVA followed by Post Hoc.

3.1.3.Results

3.1.3.1.Cellulase activity of sediments from five rivers

As shown in **Table 3-1-1**, sediments collected from all five rivers exhibited cellulase activities. However, the activity levels differed among rivers. Specifically, sediments from the Yodo River showed significantly higher activity than those from the Hamado River, Midori River and Tanaka River, while those from the Chikugo River had higher values than those from the Midori River and Tanaka River. Sediments from

the Hamado River showed significantly higher activity than those from the Tanaka River. However, the activity levels differed among rivers so far as compared by using the data obtained from the collecting sites of each river.

3.1.3.2.Effect of chloramphenicol on the cellulase activity of sediment

As shown in **Table 3-1-2**, chloramphenicol exhibited no effect on the cellulase activity of sediment from the Midori River, while it showed a partially inhibiting effect on that of the Tanaka River. These findings suggest that part of the cellulase activity of the Tanaka River was derived from microorganisms sensitive to chloramphenicol. It should be stressed that a substantial amount of the activity of sediments from both rivers remained, even in the presence of chloramphenicol, suggesting that these activities were derived from cellulases extracellularly secreted from microorganisms and /or benthic animals.

Table 3-1-1 Cellulase activity of the sediments collected from five rivers

Collecting sites	Chikugo River	Midori River	Hamado River	Yodo River	Tanaka River
Cellulase activity (nmol/g h)	$59.2 \pm 26.0^{a,d}$	$22.5 \pm 3.4^{b,c}$	$33.6 \pm 8.45^{a,c}$	77.6 ± 7.1^{d}	12.9 ± 8.2^{d}

Values with different superscripts are significantly different ($p < 0.05$). Values are mean ± SEM (n = 3).

Table 3-1-2 Effect of chloramphenicol on cellulase activities of the sediments from Tanaka River and Midori River

Collecting sites	Tanaka River		Midori River	
	Cholramphenicol		Chloramphenicol	
	+	-	+	-
Cellulase activity (nmol/g h)	27.4 ± 1.7^{a}	43.1 ± 4.7^{b}	18.8 ± 2.0^{a}	28.3 ± 7.7^{a}

Concentration of chloramphenicol was 40 mg/g sediment. Values are mean ± SEM (n = 3). The statistical analyses on both rivers were performed independently. Values with different superscripts are significantly different ($p < 0.05$).

Table 3-1-3 Binding of fungal cellulase to the autoclaved sediments collected

Collecting sites	Chikugo River	Midori River	Hamado River	Yodo River	Tanaka River
Cellulase-binding ability (μg/g)	29.0 ± 5.4a	19.7 ± 4.3b	29.2 ± 9.13a,b	16.6 ± 10.6b,c	11.6 ± 4.0c

Values are mean ± SEM (n = 3). Values with different superscripts are significantly different ($p < 0.05$).

3.1.3.3.Binding of fungal cellulase to the sediments

The finding above suggested us that cellulases secreted from organisms would directly bind to sediments under natural condition. To validate this, we examined the binding ability of sediments to commercial available fungal cellulase. As shown in **Table 3-1-3**, sediments collected from all five rivers showed fungal cellulase binding ability (**Table 3-1-3**). The sediments from Tanaka River showed significantly lower binding ability than those from Hamado River and Chikugo River, while those from Yodo River showed lower binding ability than those from Chikugo River.

The finding clearly suggested that sediment has the ability to bind cellulase. Sediments are mainly composed of clay and plant residues. Thus, we subsequently compared the cellulase binding ability between clay and plant residues using sediment collected from the Midori River. The result revealed that 11.7 ± 2.3 μg/g of cellulase was bound to clay, while 419.5 ± 47.1 μg/g of cellulase was bound to plant residues. These results suggest that plant residues have significantly higher ($p < 0.05$) cellulase binding ability than clay.

3.1.3.4.Correlation between organic matter content and cellulase binding ability

As shown in **Fig. 3-1-1**, the organic matter content varied among rivers. Organic matter in sediment was assumed to primarily consist of plant residues. For example, sediment from the Midori River included a large content of plant resides (14.2%), while that of the Tanaka River included a small amount of plant residues (1.3%). A strong correlation between cellulase binding ability and the organic matter content in the sediments was also observed ($R = 0.66$), suggesting that plant residues can function as an efficient binder of cellulase in the sediment.

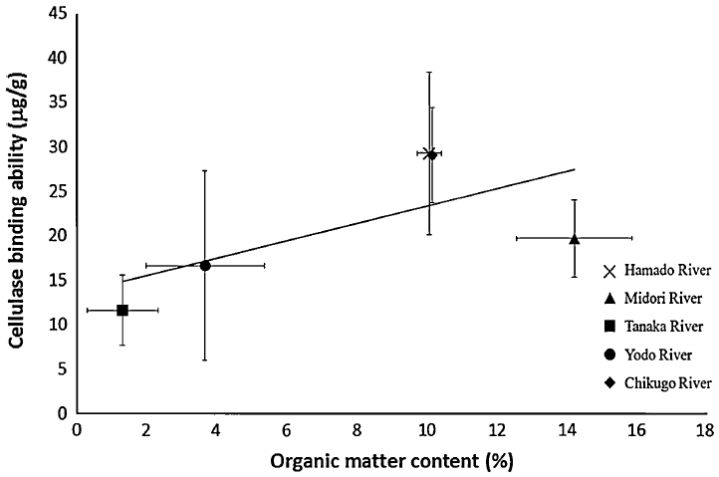

Fig. 3-1-1 Correlation between the organic matter content and the cellulase-binding ability of the sediments from various rivers. A strong correlation was demonstrated between them (R = 0.66)

3.1.4.Discussion

A significant amount of cellulase activities remained in the presence of chloramphenicol (**Table 3-1-2**). When sediments incubated in the presence of chloramphenicol were inoculated onto agar plates containing LB medium without chloramphenicol, no colonies of microorganisms (fungi or bacteria) were observed during incubation for five days (data not shown), suggesting that microorganisms in the sediment were completely attenuated by chloramphenicol. Based on these findings, we assumed that sediment-complex-binding cellulases secreted from microorganisms and/or benthic animals function as a bioreactor independent of organisms under natural conditions. Actually, we found that cellulases are secreted in feces as an active form in our preliminary experiment (data not shown). We are now pursuing the characterization of cellulases in feces of various macrobenthos.

High cellulase activities of sediments in the Yodo River and Chikugo River were detected, while low activity was observed for the Tanaka River (Table 1). This is because sediments from the Tanaka River showed low cellulase binding ability, possibly due to the low content of plant residues (**Table 3-1-3** and **Fig. 3-1-1**). The cellulase activity in sediment from the Midori River was significantly lower than that of

the Yodo River (**Table 3-1-1**), although the cellulase binding ability of the sediment from the Midori River was not significantly different from Yodo River (**Table 3-1-3**). As described above, these results suggest that a significant amount of cellulase was secreted extracellularly from microorganisms and/or benthic animals. Accordingly, these findings strongly suggest that the low cellulase activity in sediment of the Midori River could be ascribed to the low level of cellulase supplied by microorganisms and/or benthic animals.

The results shown in Table 3 suggest that river sediments have cellulase binding ability but that the level differed among rivers. As shown in **Fig. 3-1-1**, the cellulase binding ability of sediments depends on the content of plant residues. Indeed, there was a strong correlation between the organic matter content in the sediments and cellulase binding abilities ($R = 0.66$). Organic materials in sediments were considered to be almost entirely a result of plant residues because no weight loss was observed before and after heat treatment of sediments from which the plant residues were removed by tweezers as possible as carefully (data not sown). The difference in the level of cellulase activity of the sediments of various rivers could be ascribed to the content of plant residues as well as the content of cellulase supplied by microorganisms and/or benthic animals.

To confirm that plant residues had a higher binding ability than clay, we separated plant residues and clay from the sediment of the Midori River and compared the cellulase binding abilities. Results indicate that the plant residues exhibited approximately 36 times higher cellulase binding than clay. These findings strongly suggest that plant residue functions as an efficient binder of cellulase in the sediment.

Overall, this is the first study to demonstrate that sediments composed of clay and plant residues bind cellulase and act as a bioreactor independent of organisms. We are now identifying the organisms that secrete cellulases and will report these results in the near future.

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3-2: Molecular Mechanism of the Environmental Cellulase System

3.2.1.Introduction

Wetlands cover only 2 to 6 % proportion of the surface of land on the earth[1, 2], but contain a range of 3 to 68 % of global carbon[3, 4]. Among the 60 GtC synthesized by forests, a massive fraction is released into rivers and accumulates in wetland in the form of cellulose, the most abundant organic carbon source on earth[5]. Decomposition of cellulose has been considered so far to rely mainly on microbes[6]. However, recent studies revealed that various invertebrates in wetlands produce endogenous cellulase[6, 7]. Are these invertebrates negligible regarding the decomposition of cellulose in wetlands? We previously showed that wetland sediments contain cellulase activities in the absence of organisms. Based on the facts, we hypothesize that wetlands function as a field of cellulose decomposition. In the present study, we found the "environmental cellulase" produced by an invertebrate bivalve, and discuss its significance.

3.2.2.Methods

3.2.2.1.Materials.

Unless otherwise specified, all reagents were GR grade purchased from Nacalai Tesque. Sediment samples were collected in river estuaries, ponds, lakes and lands in Hokkaido, Honshu, and Okinawa Islands in Japan. Reed leaves and *Corbicula japonica* were collected from Tanaka River Estuary (TRE). All samples were transported to the laboratory at 4℃. Sediments were continously stored at 4℃ until use. Reed leaves were dried and autoclaved in a package before use. *C. japonica* were cultured at 20℃ in 50% artificial seawater (REI-SEA Marine II, IWAKI), and fed diatoms (*Chaetoceros constrictus*, *Chaetoceros didymus*, *Chaetoceros socialis*, *Chaetoceros socillis* mixture) until use.

3.2.2.2.Cellulase activity measurement.

Reducing sugar production was measured according to the tetrazolium method. Glucose concentrations were measured by using an EnzyChrom Glucose Assay Kit (BioAssay Systems) following the manufacturer's protocol.

3.2.2.3.Recombinant protein expression and purification.

The Carbohydrate Binding Module (CBM) sequence of *Cjcel9A* (GenBank: BAF38757.1) was amplified using primers 5'-GAGGGATCCC$(CAC)_6$ GCACCAGTAACTATC-3' and 5'-GAGGGTACCCCTGGACCTACAGACCT-3' (the underlined sequence is complementary to the *Cjcel9A* CBM), inserted into pEGFP plasmid (BD Biosciences Clontech), and transformed into the *XL-blue* strain of *E.coli* for expression. As a control, a His_6-tag was also inserted into the 5' end of the GFP region of pEGFP plasmid for further GFP purification. After pre-culturing in 2 ml of LB medium containing 100 μg ml^{-1} ampicillin at 37℃ for 16 h, the *E.coli* was transferred to 200 ml of fresh LB medium and incubated at 37℃ for another 4 h, and then isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 100 μg ml^{-1}. The cells were collected, re-suspended in 2 ml of PBS, lysed by sonication on ice, and centrifuged at 8,000 *x* g for 20 min. The supernatant was collected as a crude extract of the recombinant proteins and further purified using a His-trap™ FF column (GE Healthcare) following the manufacturer's protocol and stored at -80℃ in the presence of 1 mM NaN_3.

3.2.2.4.Immunological detection.

The purified His_6-CBM-GFP recombinant protein was used as the antigen in the purification of the antibody against Cjcel9A of *C. japonica* produced in our previous study[9]. Five milliliters of the antiserum was concentrated using a 5000 kDa molecular sieve (Amicon Ultra-15 5,000 MWCO, Millipore) following the manufacturer's protocol and then purified using a HiTrap NHS-activated HP column (GE Healthcare) following the manufacturer's protocol. Two and a half milligrams of His_6-CBM-GFP desalinized using a Prepacked Disposable PD-10 column (GE Healthcare) had been pre-immobilized on this NHS column.

Ten micrograms of purified His_6-CBM-GFP or His_6-GFP were used for the test of the anti-Cjcel9A antibody recognition domain.

For Western Blotting, the samples were separated by electrophoresis on a 7.5% acrylamide gel in the presence of 2-mercaptoethanol and then transferred to a polyvinylidene difluoride (PVDF, GE Healthcare) membrane. The primary anti-Cjcel9A antibody was diluted 1:1,000, and the secondary anti-rabbit IgG (H&L) HRP-linked antibody (Cell Signaling Technology) 1:10,000. High performance

autoradiography film (Amersham® Hyperfilm®, GE Healthcare) was used for imaging.

For immunofluorescence, the primary anti-Cjcel9A antibody was diluted 1:1,000. and the secondary Alexa Fluor 546 antibody (Life Technologies) 1:500.

3.2.2.5.“Environmental” cellulase experiments.

To detect the “environmental” cellulase activity in TRE, the sediments collected in the four sites in TRE were sieved using a 45-μm sieve to remove all macrobenthos (organisms cataloged as larger than 2 mm) and meiobenthos (larger than 45 μm and smaller than 2 mm). Then, approximately 5 g (wet weight) of sediment was mixed with chloramphenicol to a final concentration of 40 mg g^{-1} sediment (wet weight) in order to suppress the microbe activity as described in our previous report[10]. Meanwhile, 10 ml of 0.2 M sodium acetate buffer (pH 5.9) and 10 ml of 1% carboxymethyl cellulose (CMC, Sigma) were mixed with the sediment. After incubation at 20 ℃ for 24 h, the reaction mixture was centrifuged at 2500 *x* g for 10 min, and 1ml of the supernatant was transferred to a new tube and the reducing sugar released was measured according to the tetrazolium method[11]. The remaining reaction mixture was dried and weighed. The cellulase activity was calculated as unit g^{-1} of dry sediment, with 1 unit defined as the enzyme activity that could produce reducing sugar with the same reducing power as 1 μg of glucose in 1 min at 20 ℃.

In order to examine the presence of “environmental” Cjcel9A cellulase in natural sediments, sediment samples approximately 5 cm wide and1 cm deep surrounding 10 *C. japonica* living on the surface sediment in TRE were collected. All the sediment samples were mixed together, and then mixed with 500 ml of autoclaved artificial seawater and allowed to stand for 1 h. The supernatant (upper phase, mostly seawater) and the precipitate (lower phase, mostly sediment) were separated, and each was supplemented with SDS at a final concentration of 0.1% and mixed vigorously. After the mixture was left standing for 1 h, 100 ml of the supernatant of each phase was centrifuged at 8000 *x* g for 5 min. The supernatant of each was concentrated using a 5000 kDa molecular sieve (same type used in antibody purification) to approximately 0.5 ml, and then used for Western Blotting.

To separate the plant residue from sediment, tweezers were used to pick out the visible plant residues from the sediment collected from TRE under an optical microscope. Approximately 5 g of sediment (wet weight) and the plant residues that had

been removed from the sediment were respectively mixed with 10 ml of 0.2 M sodium acetate buffer (pH 5.9) and 10 ml of 1% CMC, and incubated for 24 h at 20 ℃. The cellulase activity was measured as described in the cellulase activity measurement section above.

For the reconstructive experiment investigating the secretion of *Cjcel9A* cellulase, reed leaves or α-cellulose were immersed inside a sieve (45 μm, ϕ = 75 mm) in a culture tank (length: 36 cm *x* width: 28 cm *x* depth:15 cm) containing 2 L of artificial seawater and 25 *C. japonica* outside the sieve. Before the experiment, all apparatuses were autoclaved, and the shells of *C. japonica* were sterilized using 70% ethanol. So that only water-soluble secreted cellulase would bind to reed leaves or α-cellulose, *C. japonica* were separated from reed leaves or α-cellulose through a sieve. After 24 h, the leaves or α-cellulose fiber was collected, washed with 1 ml of artificial seawater 5 times before use in further experiments. Fifteen milligrams of autoclaved reed leaves was used to examine the presence of bound cellulase. After the incubation, 1 mg of reed leaves was mixed with 100 μl of 0.2 M NaAc buffer (pH 5.9) in the presence of 10 mM NaN_3, incubated, and measured for reducing sugar production (n = 3). Significance of differences was tested using the *t*-test.

Also for the reconstructive experiment. 5 mg of α-cellulose was used to detect the existence of secreted cellulase using Western blotting or immunofluorescence. Finally, 25 mg of α-cellulose was used to detect the existence of bound cellulase by measuring reducing sugar production. After the incubation, for each sample to be tested, 1mg of α-cellulose was mixed with 100 μl of 0.2 M AcNa buffer (pH 5.9) in the presence of 10 mM NaN_3 and incubated, and reducing sugar production and glucose production were measured (n = 5).

To test the binding ability of *C. japonica* cellulase for various kinds of cellulose, approximately 10 crystalline styles were homogenized in 100 μl of PBS. After centrifugation at 8000 x g for 5 min, the protein concentration was measured by the Bradford method. One milliliter of PBS, 1mg of cellulose (α-cellulose, filter paper or reed leaves) and 0.25 mg protein of crystalline style extract were mixed and incubated at 4℃ for 1 h. After the incubation, the cellulose was washed with artificial seawater 5 times. and mixed with 20 μl of sample buffer (Laemmli buffer) for Western Blotting described above.

3.2.2.6.Correlation between cellulase binding ability and soil texture of the sediments.

To measure *C. japonica* cellulase binding ability to the sediments collected from various sampling sites in Japan, 100 mg of each sediment sample (dry weight) was supplemented with 1 ml of 100 unit ml^{-1} cellulase extract from crystalline style prepared as described above and mixed at 4 ℃ for 3 h. After discarding the supernatant, the sediment was washed with 1 ml of artificial seawater 5 times. After the washing, the sediment was mixed with 1 ml of 1% CMC solution. The mixture was incubated at 25 ℃ for 3 h and the reducing sugar content was measured according to the tetrazolium method described above. The cellulase binding ability of sediment was expressed as the bound cellulase activity (unit) per gram of sediment.

To measure the organic matter content of the sediments, approximately 5 g of sample sediment of each site was added to a pre-weighed melting pod (m_{mp}, g) and weighed totally as m_{before} (g). After it was heated at 600 ℃ for 3 h, the melting pod with sediments was weighed again as m_{after} (g). The organic matter content was defined as the ignition loss rate (ILR).

ILR (Organic matter content, %) = $(m_{before} - m_{after}) / (m_{before} - m_{mp}) \times 100$

To measure the pH value of the sediments, 5 g of TRE sediment was shaken with 25 g of water for 1 h at room temperature.

The characteristics of the sediments were tested according to the methods reported previously[12].

The metal content (ferrous, aluminum and silica) of sediments was measured by both oxalate extraction assay and pyrophosphate extraction assay according to the method report previously[12].

3.2.2.7.Functional analyses of CBM.

To investigate the affinity of the CBM of Cjcel9A to various carbohydrates (Fig. 2a), 40 ml of artificial seawater and 1 ml of 9 μM His_6-CBM-GFP were mixed with 1 mg of each of 4 kinds of candidate substrate, namely, α-cellulose, reed leaves, xylan and filter paper. After shaking at 4 ℃ for 1 h, the substrate was collected and washed with 40 ml 1M NaCl 3 times before fluorescence microscopy. As controls, substrate mixed with His_6-GFP or His-tag column elution buffer were also observed. The observation in Extended Data Fig. 8d-g was performed by the same method.

To measure the Km value of CBM of *Cjcel9A*, 1 mg of α-cellulose was mixed with His_6-CBM-GFP at 10 different final concentrations (6, 3, 1.5, 0.75, 0.57, 0.38, 0.28, 0.19, 0.14 and 0.09 μM) in PBS. The α-cellulose was then collected and washed with 1 ml of 1M NaCl 5 times. After samples were re-suspended in 100 μl of artificial seawater, they were transferred to a flat-bottom fluorescence 96-well plate and the intensity of fluorescence was measured by using an image analyzer (FLA-9000, FUJIFILM). The concentration of bound His_6-CBM-GFP was calculated by reference to a standard curve of the fluorescence intensity of pure His_6-CBM-GFP. The difference of binding ability between α-cellulose and filter paper was measured by the same method described above. The final concentration of His-CBM-GFP or His-GFP was 0.75 μM.

3.2.2.8.Immuno-scanning electron microscopy.

The preparation for the immune-scanning electron microscopy (immune-SEM) was the same as for the immunofluorescence experiment except for the substitution of Immune Gold Conjugate EM. Goat Anti-rabbit IgG: 15 nm (BBInternational) for the Alexa Fluro 546 antibody. Before samples were subjected to scanning electron microscope (TM3000, Hitachi), they were immobilized by adding 1 ml of reagent containing 2% glutaraldehyde and 4% paraformaldehyde and incubated at 4℃ for 1 day, and then dried at room temperature for 1 day.

3.2.2.9.X-ray crystallography.

To determine the crystal structure of cellulose, 0.5 g of α-cellulose and filter paper were subjected to X-ray diffraction measurement by the reflection mode using a Rigaku Ultima IV diffractometer with Nickel-filtered Cu Kα radiation at 40 kV and 40 mA.

3.2.2.10.Search for cellulase secretion trigger.

Glucose, cellobiose, cellotriose, cellotetraose, cellopentaose, cellohexaose, α -cellulse, CMC, xylan (Birchwood) and pectin (Citrus) were added to the total 3 ml of culture solution (aritificial seawater) of a single *C. japonica* (n=3) at the final concentration of 1, 10, 100 and 1000 μg/ml. After 24 h, the cellulase activity in the culture was measured by the tetrazolium method described above, together with the control group of *C. japonica* that culture with only culture solution (n = 12). Statistical

analyses were carried out using IBM SPSS, ver.22 (Levene Statistic analysis followed by One-way ANOVA, then Scheffe Post Hoc analysis).

3.2.2.11.Glucose uptake.

C. japonica (2, 4 and 6 individuals per group, n = 3) were cultured in 100 ml of artificial seawater containing 1mM glucose and 1mM NaN_3. Glucose uptake of *C. japonica* was evaluated from the decrease of glucose concentration during the culture period. Glucose concentration was measured by using the tetrazolium method.

3.2.3.Results and Discusstion

Tanaka River Estuary (TRE) located in Mie Prefecture is one of Japan's untouched wetlands[13]. Different kinds of invertebrates were observed in the sediments derived from four different areas of TRE, while all of the sediments contained environmental cellulase activities (**Fig. 3-2-1** and **Fig. 3-2-2**). These activities were considered to derive from the microorganisms and/or cellulase-possessing invertebrates. We choosed a dominant bivalve species in the sandy field of TRE, namely *Corbicula japonica*, for investigating whether its cellulase is involved in the environmental cellulase activity, because several cellulase genes of this animal have been cloned[14, 15]. If ture, it is reasonable to believe that the same thing happens on other cellulase-possesing invertebrates inhabiting TRE.

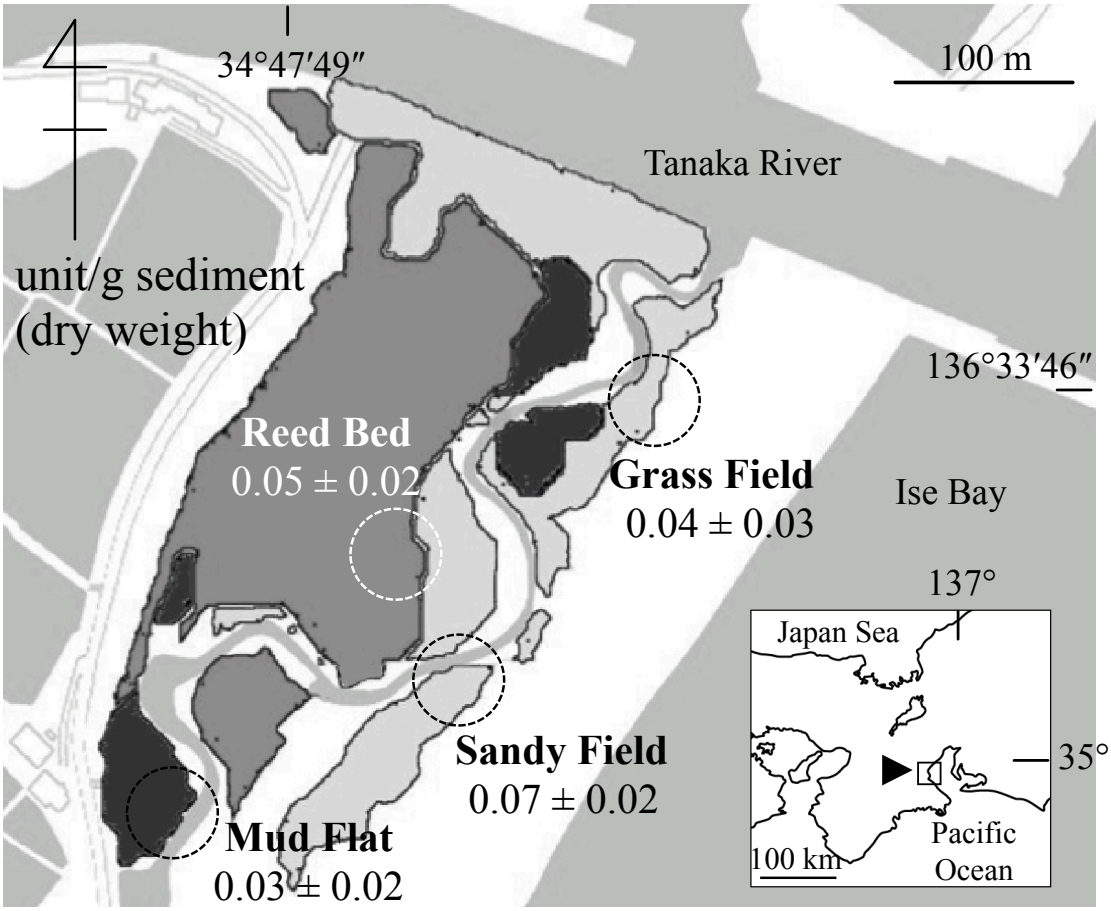


Fig. 3-2-1 "Environmental" cellulases of *C. japonica*. "Environmental" cellulase activities in the sediment of TRE.

Fig. 3-2-2 Sites ivestigated in TRE. Sediment of Sandy field contains more sands compares to other sites. Reed bed is grown with reeds while Grass field is grown with grasses. Sediment of Mud flat contains more clays and are more black compares to other sites.

In order to detect whether one of the *C. japonica* cellulase (Cjcel9A) was immobilized in the sediment or not, sediment inhabited by *C. japonica* was collected and washed. The sample was separated into the upper phase (mainly seawater) and the lower phase (mainly sediment including organic matters). We raised an antibody against Cjcel9A (**Fig. 3-2-4**) and carried out Western blot assay. The Cjcel9A cellulase was immunologically detected in the lower phase (**Fig. 3-2-3** Lane 2), indicating that the cellulases such as Cjcel9A are immobolized in the sediment.

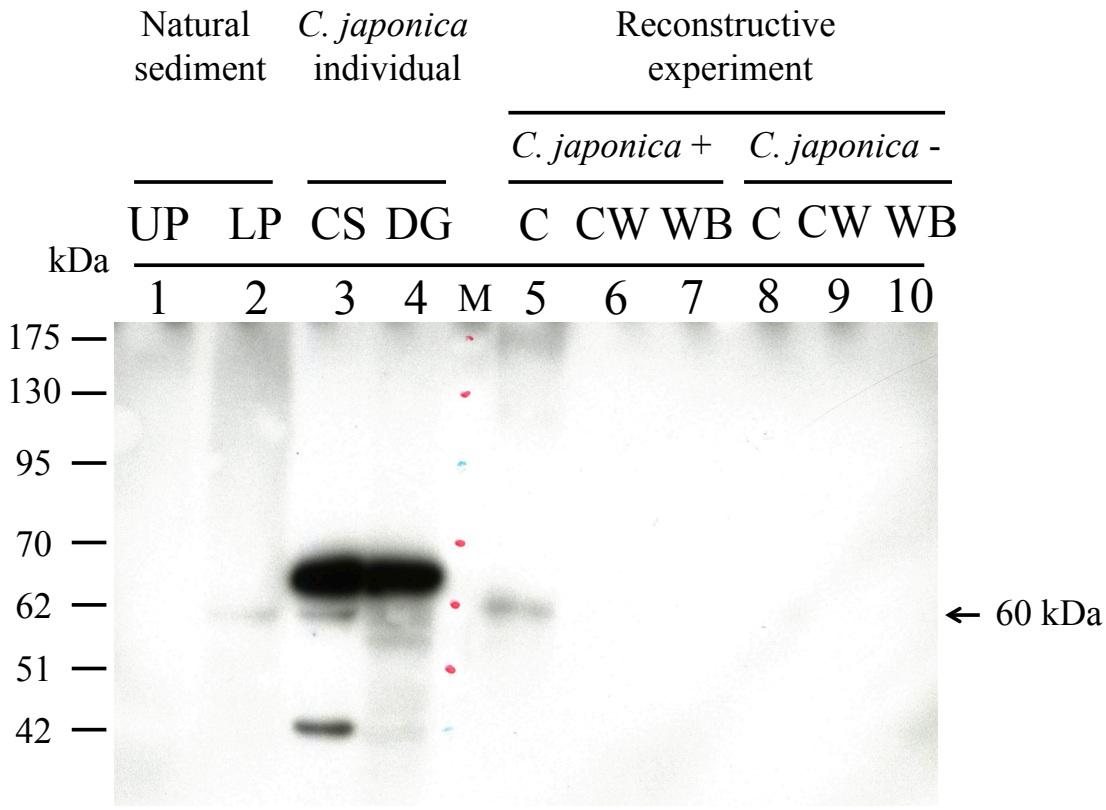


Fig.3-2-3 Immunological detection by Western blotting of "environmental" *Cjcel9A* cellulase. UP, upper phase of sediment. LP, lower phase of sediment. CS/DG, crystalline style/ digestive glands of *C. japonica*. C, cellulose. CW, culture water. WB, washing buffer of α-cellulose/leaf.

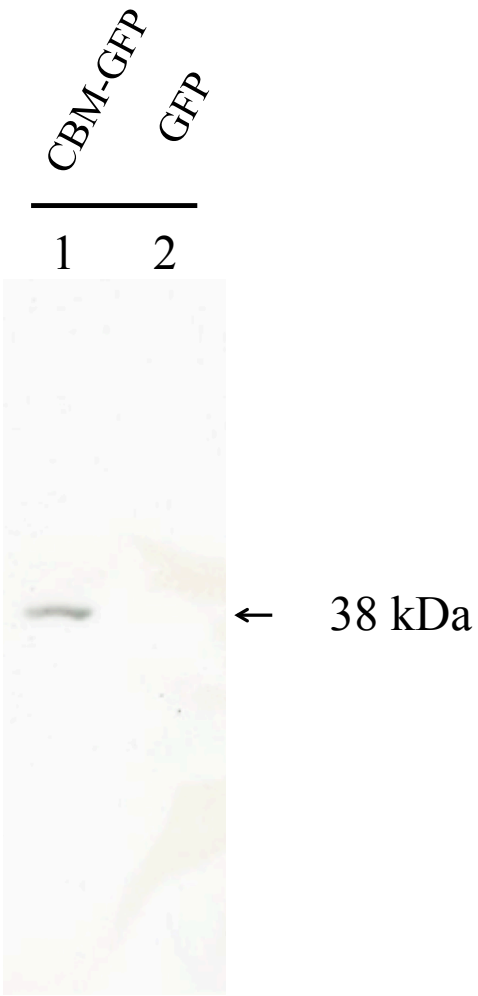


Fig. 3-2-4 Purified Cjcel9A antibody recognizes CBM of *Cjcel9A* not GFP tag.

Wetland sediment is constantly washed by water flow. Then how, and where, are the cellulases immobilized in this sediment to avoid being washed away? The sediment is mainly composed of minerals and organic matter[16]. The sediment inhabited by *C. japonica* has an organic matter content of 1.5%, mostly consisting of plant residues. We separated the visible plant residues from the sediments, and measured the cellulase activities of both the plant residues and the rest of the sediment. Enzymatic activity assays showed that the cellulase activity in the sediment was originated from mostly the plant residues (over 70%) (**Fig. 3-2-5**), indicating that environmental cellulase was mainly immobilized on plant residues.

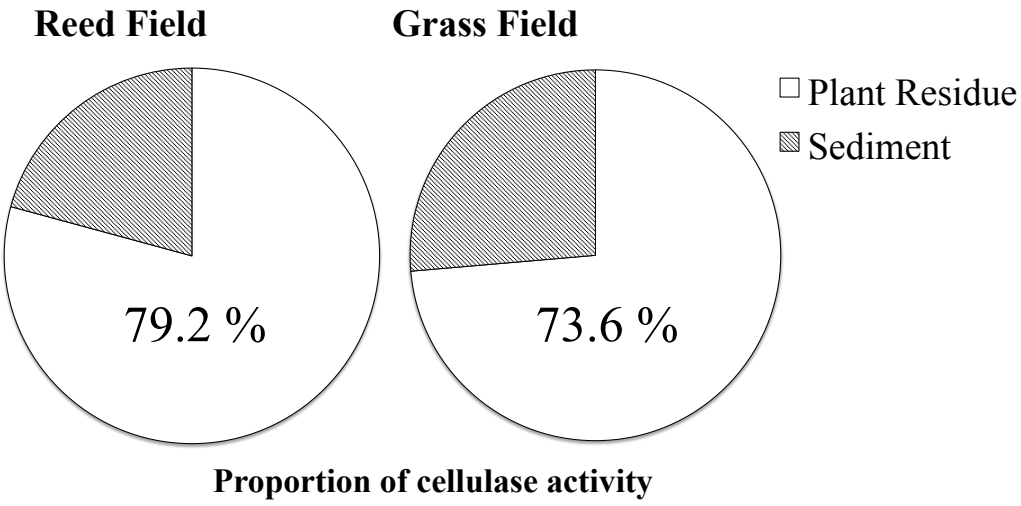


Fig.3-2-5 Porportion of cellulase activity of the plant residues and the rest sediment.

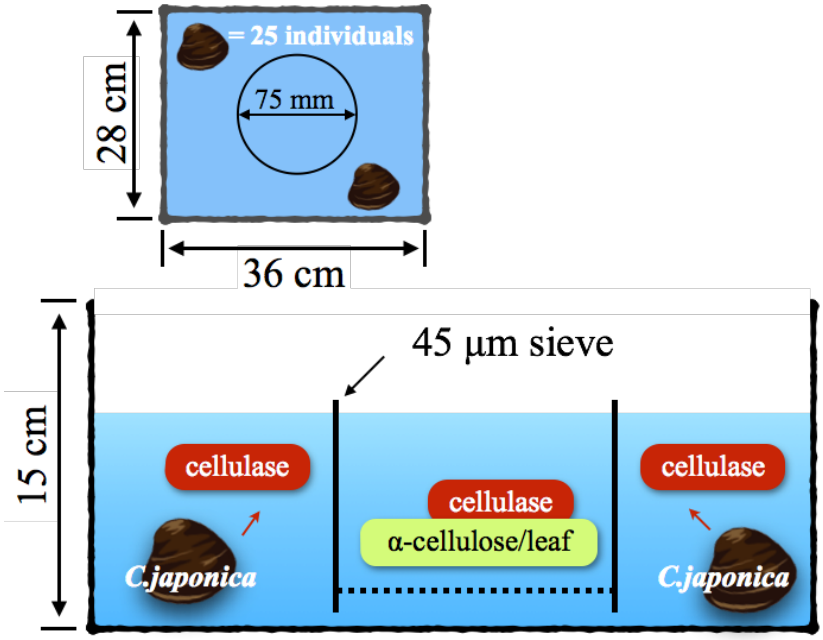


Fig.3-2-6 Scheme of the reconstructive experiment.

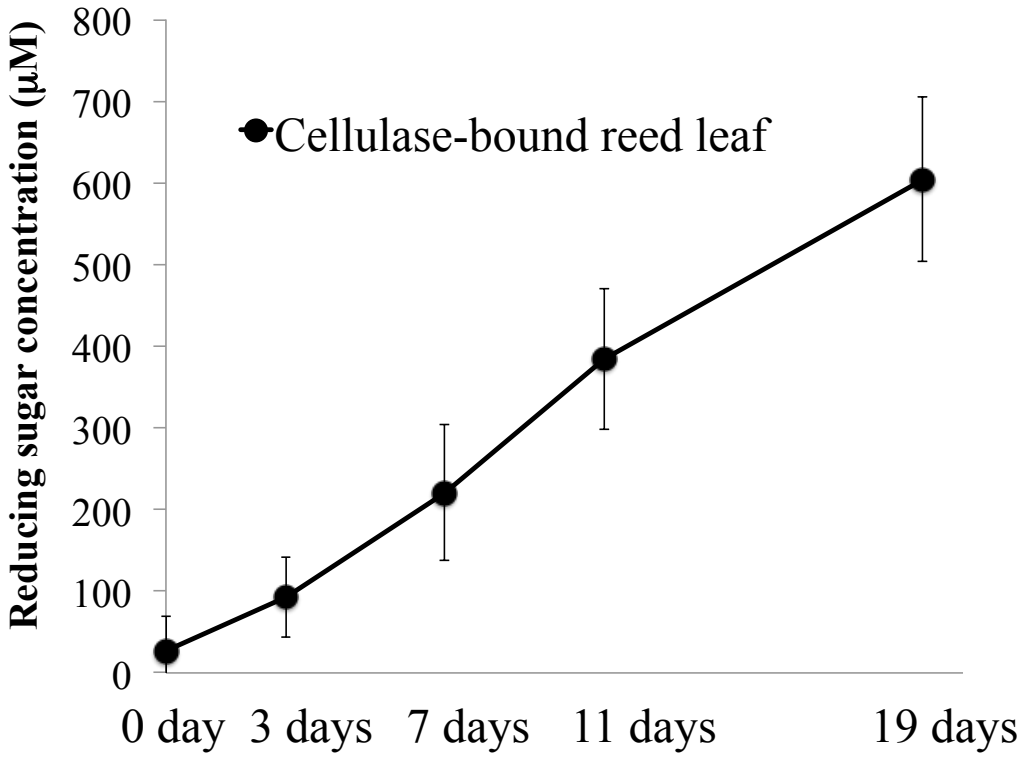


Fig.3-2-7 Reducing sugar production of the leaves collected in **Fig.3-2-6**. Bars represent SEM (n = 3).

Based on these results, we hyprothized that plant residue in the sediment functions as a scaffold for the secreted cellulases. Cosidering the fact that cellulose is the substrate of cellulases, the cellulase and cellulose may form a complex that is immobilized in the sediment. A reconstruction experiment was then designed to evaluate our hyprothesis: whether *C. japonica* secretes cellulase, and if ture, whether the secreted cellulase immobilize on the plant residues or celluloses (**Fig.3-2-6**). As for activity assay, cellulase activity was detected in the plant residues that were enzymatically deactivated via autoclave and then co-cultured with *C. japonica* (**Fig. 3-2-7**); As for Westen Boltting, no signal of Cjcel9A was detected on reed leaves. This may have occurred because the cellulose contained in the reed leaves collect in the TRE sediment was already decomposed, or not yet fully exposed for enzyme attack. In order to boost the signal, we substituted α-cellulose (a chemically pure cellulose) for reed leaves. As a result, a signal of Cjcel9A was detected on α-cellulose (**Fig. 3-2-3** Lane 5,

Fig. 3-2-8). Interestingly, although there are multiple forms of Cjcel9A in the enzyme storage organ called the “cry stalline style” and the digestive gland mixture of *C. japonica*, only one form of Cjcel9A (60 kDa) was secreted by *C. japonica* (**Fig. 3-2-3** Lanes 2 and 5). The cellulase corresponding to the major signal (67 kDa) might be a precursor of Cjcel9A (**Fig. 3-2-9**). Moreover, oligosaccharides and glucose were produced from the α-cellulose co-cultured with *C. japonica* (**Fig. 3-2-10** and **3-2-11**). Thus, taken together, these results indicate that *C. japonica* can secrete the cellulose Cjcel9A, which can bind to celluloses in the environment and that decomposition of cellulose is fully operationally independent of the presence of *C. japonica*.

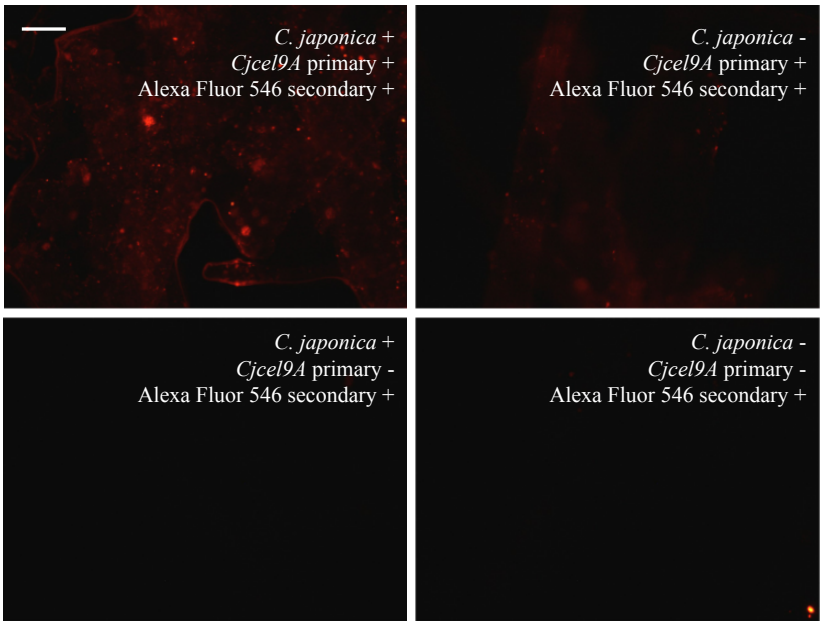


Fig.3-2-8 Immunofluorescence assay of the α-cellulose collected in the reconstructive experiment **Fig.3-2-6**. Scale bar, 100 μm.

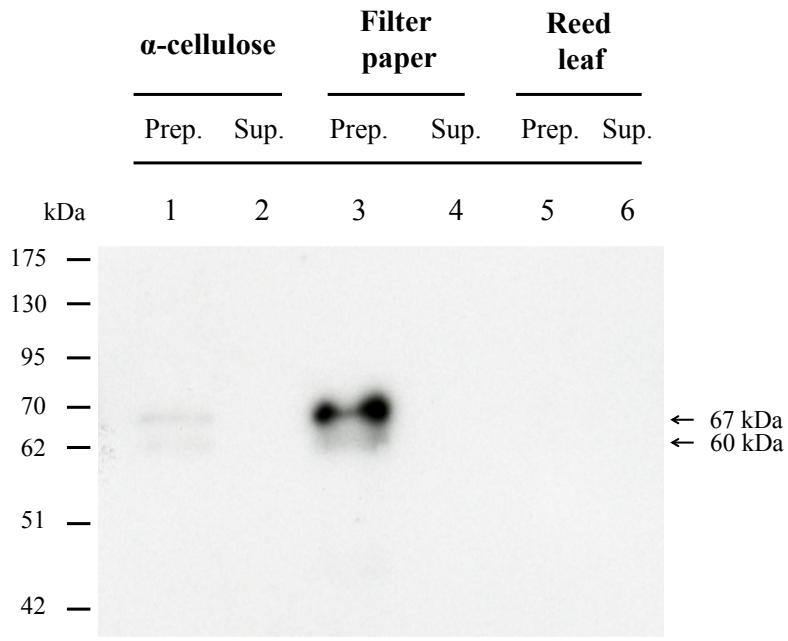


Fig.3-2-9 Cellulose-binding ability of *Cjcel9A* cellulase in crystalline style of *C. japonica*. Two forms of *Cjcel9A* were capable of cellulose binding (Lane 1, 3). Prep., precipitate (three kinds of cellulose). Sup., supernatant (buffer).

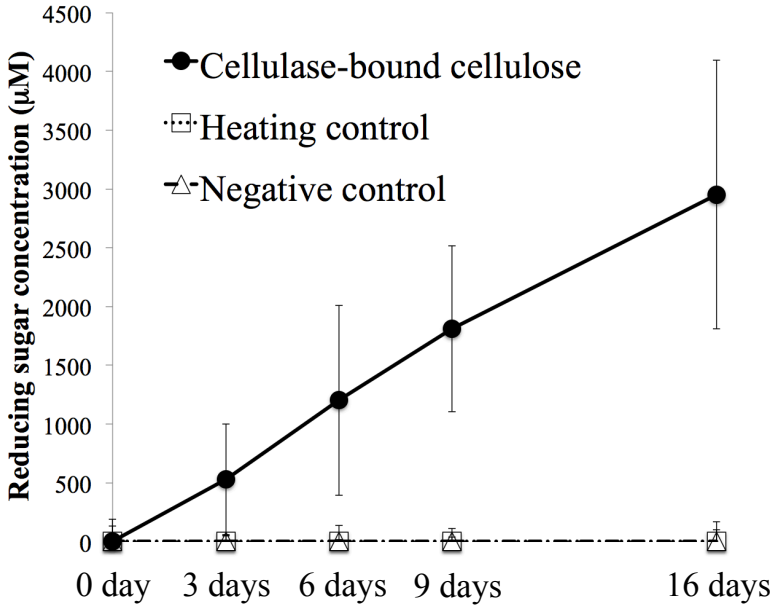


Fig.3-2-10 Reducing sugar production of the α-cellulose collected in **Fig.3-2-6**. Heating control, cellulose collected was heated at 100℃ for 2 hours before the incubation. Negative control, cellulose without *C. japonica* culturing. All values are mean ± SEM.

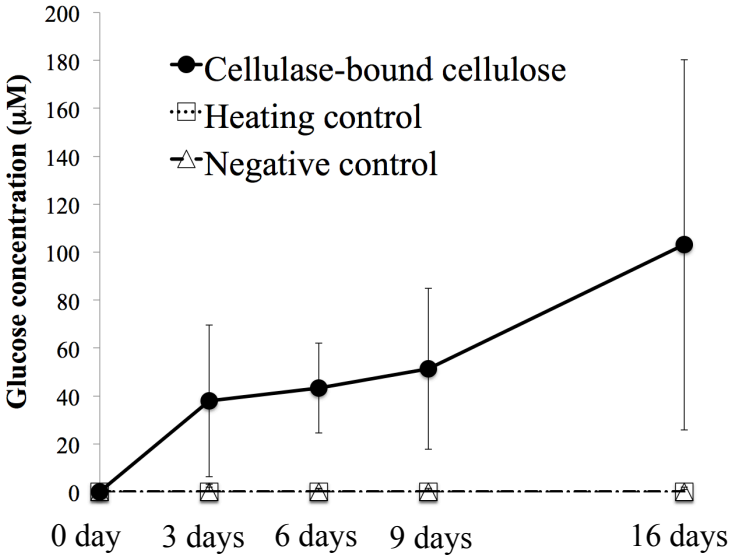
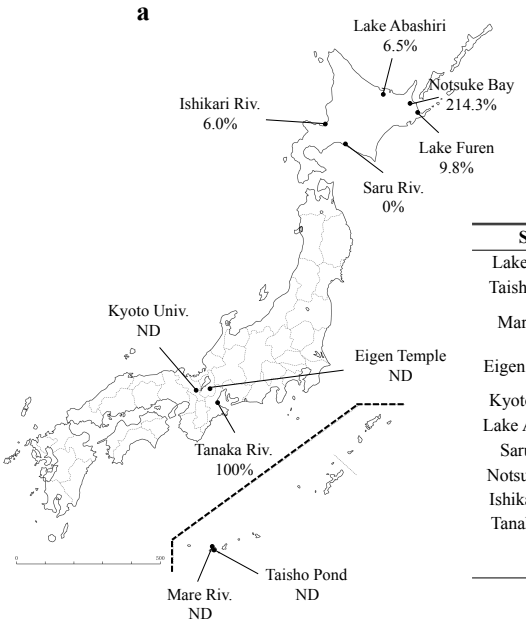


Fig.3-2-11 Glucose production of the α-cellulose collected in **Fig.3-2-6**. Heating control, cellulose collected was heated at 100℃ for 2 hours before the incubation. Negative control, cellulose without *C. japonica* culturing. All values are mean ± SEM.



b

Site	Soil Texture	Code	Sampling Date
Lake Furen	Clay Loam	CL	Aug.2010
Taisho Pond	Sandy Loam	SL	Dec. 2010
Mare Riv.	Sandy Clay Loam	SCL	Nov. 2011
Eigen Temple	Silty Clay Loam	SiCL	Sept.2010
Kyoto Univ.	Loam	L	Sept.2010
Lake Abashiri	Sand	S	Aug.2010
Saru Riv.	Sand	S	Aug.2010
Notsuke Bay	Sand	S	Aug.2010
Ishikari Riv.	Sand	S	Aug.2010
Tanaka Riv.	Sand	S	Aug. 2010 for Fig.1a, c Sept. 2010 for Extended Data Fig. 5 July 2014 for Fig. 1b

Fig.3-2-12 Sediments collected for cellulase binding ability assay. **a,** locations and natural cellulase activities of the collection sites. **b,** soil texture and sampling dates.

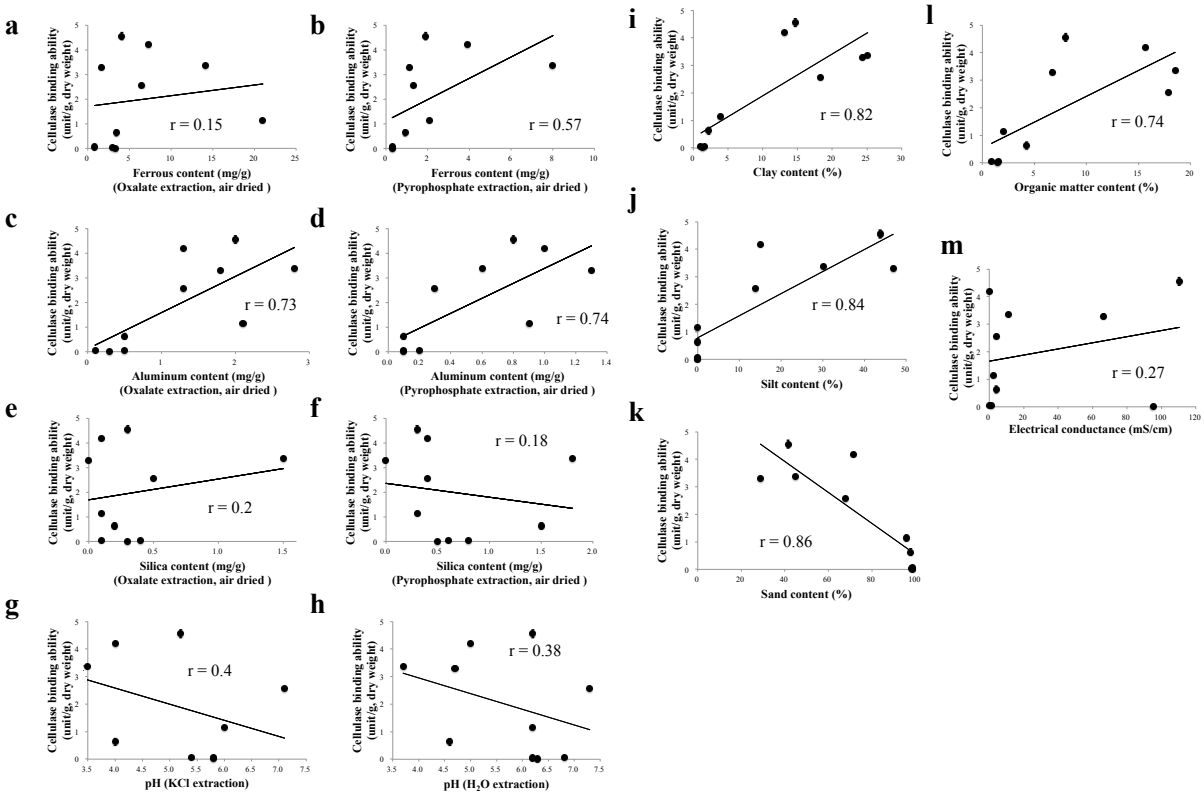


Fig. 3-2-13 Correlations between the *C. japonica* cellulase binding abilities and soil features of various sediments of Japan. Values of cellulase binding abilities are mean ± SEM, values of soil texture are from single measurement.

In addition, we collected various different types of sediments in Japan (**Fig. 3-2-12**) to investigate whether the sediments from other wetlands have the same capability of holding cellulase. Cellulase of *C. japonica* was prepared and mixed with the sediments collected. The correlation coefficient between the cellulase-binding ability and the soil features of each sediment sample was examined (**Fig. 3-2-13**), and the results showed that the binding ability was positively correlated with the organic matter content (**Fig. 3-2-13l**), suggesting that cellulase in the sediments matter content (**Fig. 3-2-13l**), suggesting that cellulase in the sediments mainly bound to cellulose in plant residues. The binding ability was also positively correlated with the particle size of the sediments (**Fig. 3-2-13i-k**), indicating that the cellulase may also partially bind to the sediment itself. Finally, is the binding ability was correlated with the aluminum content of the sediment (**Fig. 3-2-13c, d**). However, this correlation may have been indirect, because aluminum forms complexes with plant residues in the sediment[17].

There is a specific amino acid sequence, called the carbohydrate-binding module (CBM), located beside the catalytic domains of cellulases and possessing carbohydrate-binding activity. CBMs have been well studied in microbes, in which they have been shown to increase the efficiency of cellulose decomposition[18]. In fact, Cjcel9A has a CBM inside the N terminal of its catalytic domain[15]. We hypothesized that the CBM could function as an "anchor" in wetland sediments. By using GFP as a fluorescent reporter (**Fig. 3-2-14**), Cjcel9A CBM was observed to bind to both α-cellulose and reed leaves (**Fig. 3-2-15**). This result confirmed our former suggestion that the Cjcel9A is capable of binding to untreated reed leaves. On the other hand, a sequence database (http://www.ncbi.nlm.nih.gov) showed that this CBM possibly binds one of the hemicelluloses, xylan, but the result of a binding test was negative (**Fig. 3-2-15**).

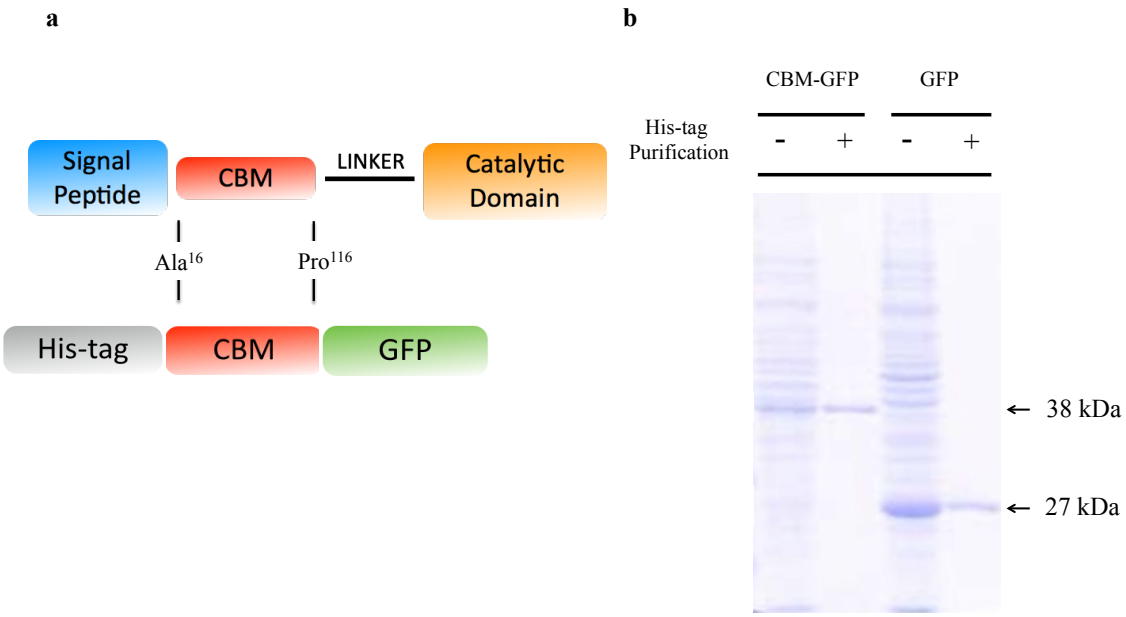


Fig. 3-2-14 Recombinant His_6-CBM-GFP and His_6-GFP expression and purification in *XL-1 blue E. coli*.

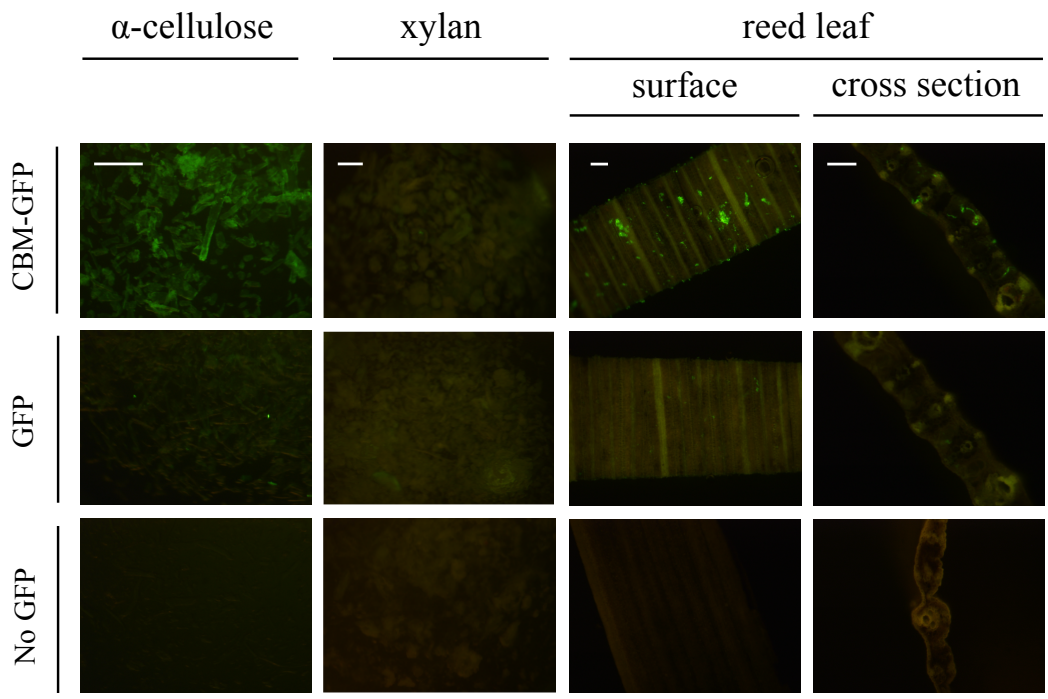


Fig. 3-2-15 Affinity of *Cjcel9A* CBM. Top panels, His_6-CBM-GFP bound to α-cellulose and reed leaves but not xylan. Middle panels, GFP controls. Bottom panels, autofluorescence controls. Exposure time, 1 s. Scale bars (upper left, 200 μm) shared in same column.

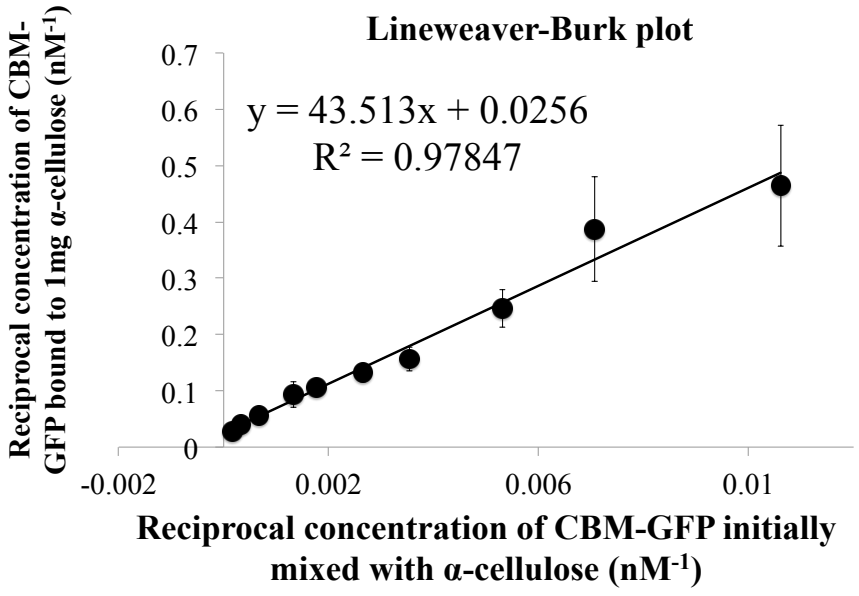


Fig.3-2-16 Lineweaver-Burk plot of *Cjcel9A* CBM against α-cellulose. Values are mean ± SEM.

To estimate the binding ability of the Cjcel9A CBM, Lineweaver-Burk plot were determined. The Km value of 1.7 μM was considerably low (**Fig. 3-2-16**), compared to those of other polysaccharide-hydrolyzing enzymes, such as a α-glucosidase from *Aspergillus nigher*[19]. One molar NaCl was used to wash off the unbound cellulase, indicating that the Cjcel9A CBM is fully functional even in a condition of high ion concentration, for example, that in brackish water.

To investigate the unequal binding intensities of Cjcel9A CBM to α-cellulose and plant residues (**Fig. 3-2-15**), immune-SEM was carried out (**Fig. 3-2-17**). Unexpectedly, the results showed that the Cjcel9A CBM bound to specific regions of α-cellulose. This specific binding of the CBM was possibly due to the recognition of crystallized cellulose, because the CBM binds to filter paper ubiquitously and more strongly than to α-cellulose due to the higher level of crystallization of cellulose in filter paper (**Fig. 3-2-18**). Although reed leaves contain highly crystallized cellulose[5], we suggest the same two reasons for both the unubiquitous binding of the Cjcel9A CBM to reed leaves and the negative result in Western blot discribed above. Taken together, our results revealed that the CBM is crucial for the immobilization of secreted Cjcel9A cellulase of *C. japonica*.

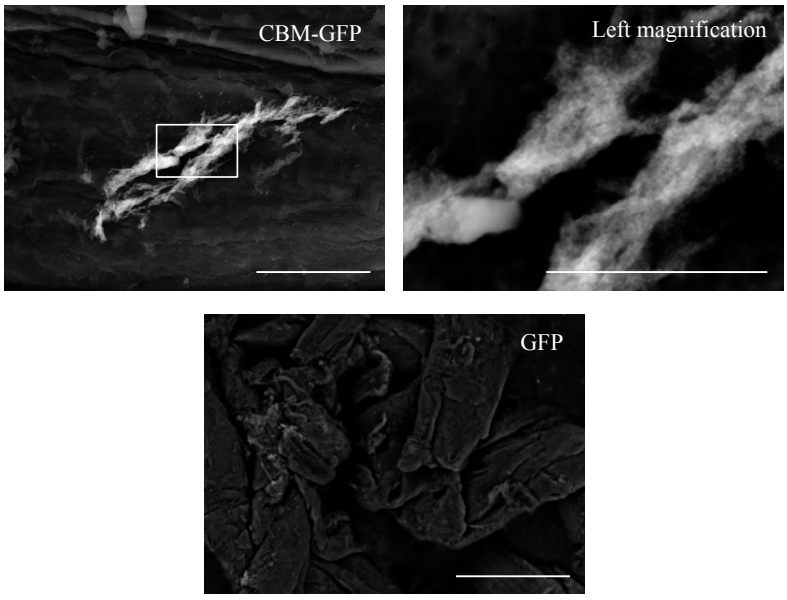


Fig.3-2-17 Immune-scanning electron microscopy of α-cellulose-bound CBM-GFP. Scale bars, 100 μm.

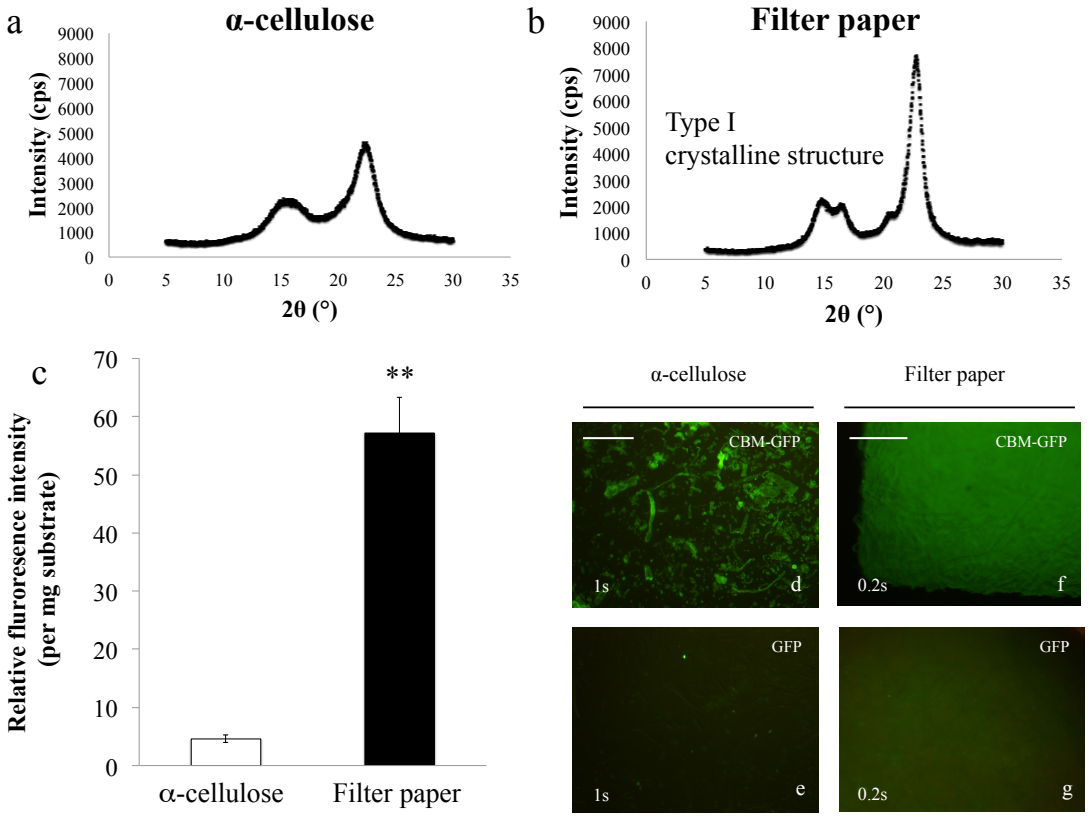


Fig. 3-2-18 Relationship between cellulose affinity and crystalline structure of cellulose. **a, b,** X-ray crystallographic assay of α-cellulose (**a**) and filter paper (**b**). **c,** Affinity of *Cjcel9A* CBM for filter paper was significantly higher than that of α-cellulose (**, $P = 0.0000000612$, *t*-test). Values are mean ± SEM. **d, f,** Fluorescence microscopy. **e, g**, GFP controls. Bottom left corners, exposure time. Exposure time (bottom left) and scale bars (left-up, 200 μm) are the same in the same column.

Figures 3-2-10 and **3-2-11** showed that the secreted cellulase could produce oligosaccharides and glucose. To examine whether *C. japonica* regulated the amount of secreted cellulases in response to oligosaccharides and/or glucose, we investigated the level of cellulase secretion of *C. japonica* in the presence of glucose, oligosaccharides, α-cellulose, CMC, and hemicellulose at four different concentrations (**Fig. 3-2-19**). The results showed that cellulase secretion increased most markedly in the presence of glucose, especially at the concentration 100 mg ml^{-1}. α-Cellulose had the same cellulase secretion-inducing effect irrespective of its concentration. It is possible that the glucose released from α-cellulose (**Fig. 3-2-11**) eventually triggered the secretion of cellulase. In contrast, oligosaccharides had no effect, possibly because *C. japonica* does not have enzymes able to digest these oligosaccharides. Our findings suggest that *C. japonica* might use glucose sensors to monitor the environment in order to control the level of its cellulase secretion and regulate the "environmental cellulase" system positively as a final outcome. Also, *C. japonica* is able to utilize the glucose produced in the environment (**Fig. 3-2-20**).

C. japonica is the first invertebrate model shown to be able to secrete its endogenous cellulase into the environment. the secreted cellulase uses its CBMs to anchor the cellulase on sediment cellulose as its scaffold, forming a cellulose decomposition system in wetland sediments (**Fig. 3-2-21**). The estimated contribution of *C. japonica* to environmental cellulase is approximately 15 ~ 75 % (**Fig. 3-2-22**). Although this rate is greatly influenced by the number of the *C. japonica*, the contribution of *C. japonica* to the environmental cellulase should not be ignored at least in their inhabit.

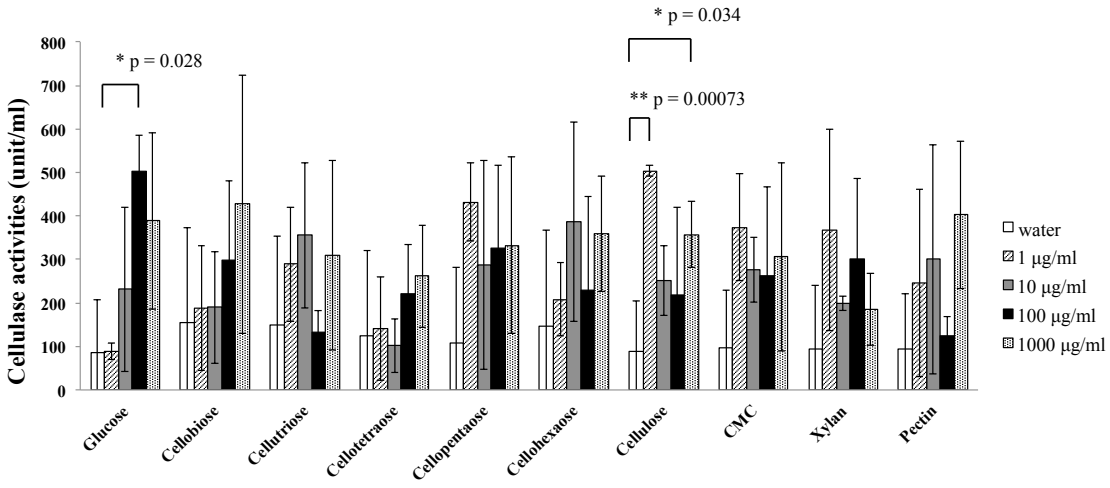


Fig. 3-2-19 Investigation of possible inducer that triggers cellulase secretion of *C. japonica* individuals. Glucose and cellulose triggered cellulase secretion. Values are mean ± SEM.

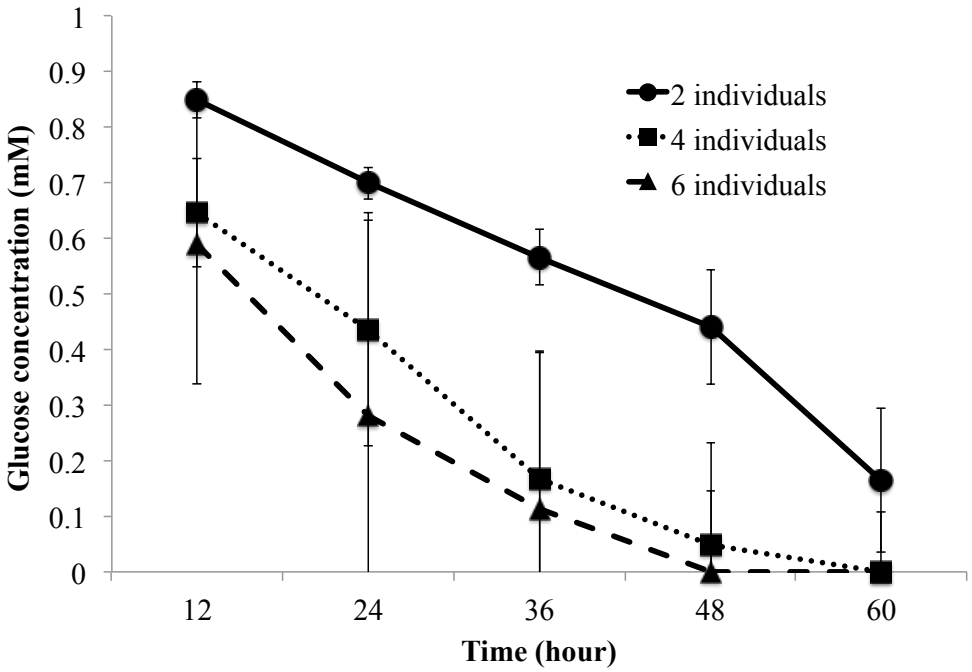


Fig. 3-2-20 Glucose uptake by *C. japonica* individuals. The concentration of glucose dissolved in culture water continually decreased in the presence of *C. japonica*. Values are mean ± SEM.

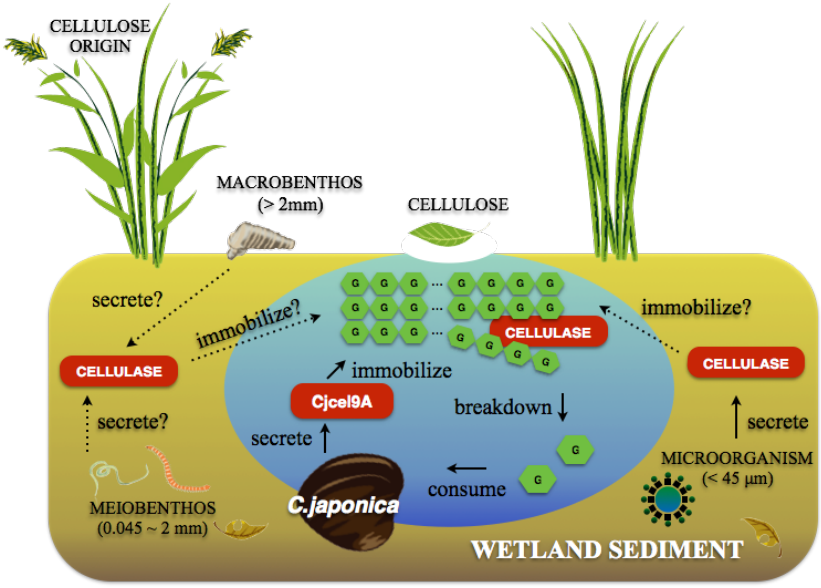


Fig. 3-2-21 "Environmental" cellulose decomposition system in wetland sediments. Scheme of the "environmental" cellulose decomposition system in wetland. *Corbicula japonica* exemplifies the bivalves that secrete cellulase to the environment. Secreted cellulase forms a immobilized complex. Cellulose is continually being decomposed into oligosaccharide and glucose. Glucose is comsumed by *C. japonica* and amplifies its cellulase secretion. Other organisms are also considered to provide "environmental" cellulase (dotted arrows), making wetland a cellulose-decomposition factory.

Rate of *C. japonica* cellulase activity in sediment

$$= \frac{ECS_{C.\ Japonica} \times D_{C.\ japonica}}{A \times D_{sediment} \times RD_{sediment}} \div CA \times 100\ \%$$

$= 15 \sim 75\ \%$

$ECS_{C.\ japonica}$: efficiency of cellulase secretion ≈ 300 ~ 1500 unit per *C. japonica*
$D_{C.\ japonica}$: density of *C. japonica* in sediment ≈ 5 individuals m^{-2} (sandy field of TRE)
A: area of sediment = 1 m^2
$D_{sediment}$: depth of sediment with cellulase activity ≈ 0.1 m
$RD_{sediment}$: relative density of sediment ≈ 2 x 10^6 g m^{-3}
CA: average cellulase activity ≈ 0.05 unit g^{-1}

Fig. 3-2-22 Rate of *C. japonica* cellulase activity in sediment

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