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A comparative study of cellulase and hemicellulase activities of brackish water clam *Corbicula japonica* with those of other marine Veneroida bivalves

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SUMMARY

Corbicula japonica **is a typical brackish water bivalve species belonging to the order Veneroida, and it is the most important inland fishery resource in Japan.** ***Corbicula japonica*** **has been suggested to assimilate organic matter from terrestrial plants, unlike** ***Ruditapes philippinarum*** **and** ***Mactra veneriformis*****, which selectively assimilate organic matter of marine origin. This led us to hypothesize that** ***C. japonica*****, despite being a suspension feeder, could assimilate cellulosic materials derived from terrestrial plants. In the present study, we measured cellulase and hemicellulase activities in the crystalline styles of** ***C. japonica*** **and other commercially important Veneroida bivalve species in Japan:** ***Ruditapes philippinarum*****,** ***Meretrix lamarckii*** **and** ***Meretrix lusoria.*** ***Corbicula japonica*** **demonstrated notably higher cellulase, xylanase and β-mannanase activities than the other marine bivalves, suggesting that this species possesses a far greater biochemical capacity to break down the structural polysaccharides of plant cell walls than the other species. In contrast, the β-1,3-glucanase and pectinase activities of** ***C. japonica*** **were similar to or even lower than those of the others. This is possibly due to the presence of these polysaccharides in the cell walls of diatoms, a principal food of most marine bivalves. Although direct evidence is lacking, the high cellulase, xylanase and β-mannanase activities of** ***C. japonica*** **may result from adaptation to an upstream estuarine environment where phytoplankton and diatoms are scarce, but plant-derived substances are abundant.**

Key words: cellulase, hemicellulase, bivalve, suspension feeder, estuarine.

INTRODUCTION

Order Veneroida (Class Bivalvia) includes many commercially important bivalve species in Japan, such as *Ruditapes philippinarum*, *Meretrix lusoria*, *Meretrix lamarckii* and *Corbicula japonica*. Because of this importance in fisheries, their feeding ecology has been extensively studied. They are classified as so-called 'suspension feeders' and it is generally accepted that they catch phytoplankton and detritus through filtration by holding their inhalant siphons above the sediment surface. However, because of this feeding mode, it is difficult to identify the substances that actually contribute to their growth or nutrition. In particular, the word 'detritus' is just a collective term for undefined organic matter existing in the environment, so it is difficult to specify what substances in the detritus are actually assimilated by these bivalves.

Recently, stable isotopic analysis has been extensively used to determine the food sources of animals including bivalves (Fry and Sherr, 1984). Using this method, Kasai and Nakata (Kasai and Nakata, 2005) showed that *C. japonica*, a typical brackish water bivalve species in Japan, assimilates terrestrial particulate organic matter (TPOM), while *R. philippinarum* and *Mactra veneriformis* selectively assimilate marine particulate organic matter (MPOM) (Kasai et al., 2004). MPOM is mainly composed of phytoplankton and benthic microalgae, while TPOM mainly consists of terrestrial plant residue. As the major part of plant residue is composed of structural polysaccharides from plant cell walls, such as cellulose and hemicelluloses, this result led us to hypothesize that *C. japonica*, despite being a suspension feeder, could efficiently assimilate cellulosic materials derived from terrestrial plants.

Cellulose is a dominant structural polysaccharide in plants composed of β-D-glucose units with β-1,4-linkages. Cellulose molecules aggregate into highly crystalline microfibrils and function as the main scaffold of plant cell walls. Cellulose fibers are cross-linked by other polysaccharides called 'hemicelluloses' to increase the physical strength of the cell wall. Hemicelluloses include xylan (β-D-xylose units with β-1,4-linkages), glucomannan (β-D-mannose units and β-D-glucose units with β-1,4-linkages), xyloglucan (β-D-glucose units with β-1,4-linkages, and β-D-xylose and β-D-glucose units with β-1,6-linkages), 1,3-1,4-β-glucan (β-D-glucose units with β-1,3- and β-1,4-linkages), and a relatively small amount of other polysaccharides composed of β-D-glucose, β-D-xylose, β-D-mannose and other sugar units with various linkages (McNeill et al., 1984). The scaffold of cellulose and hemicelluloses is filled with pectin (α-D-galacturonic acid units with mainly α-1,4-linkages), which functions as a cement-like substance in the cell wall.

Cellulose decomposition requires multiple enzymes. In general, cellulose is degraded to cellodextrin or cellobiose by the synergistic action of two cellulases: endoglucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91) (Tomme et al., 1995; Bayer et al., 1998). Degradation of cellodextrin or cellobiose into monomeric glucose units requires another enzyme, β-glucosidase (EC 3.2.1.21), that hydrolyzes non-reducing 1,4-linked-β-glucose (Henrissat et al., 1989). Recently, following the report of an endogenous cellulase gene in termites, which were previously considered to digest cellulose exclusively through symbiotic protists (Watanabe et al., 1998), endogenous cellulase genes have been found in many invertebrates such as insects, nematodes and mollusks (Watanabe and Tokuda, 2001). These findings contradict previously held

notions that cellulose can only be decomposed by microorganisms. We have also reported the occurrence of endogenous cellulase genes in *C. japonica* (Sakamoto et al., 2007; Sakamoto and Toyohara, 2009), and suggested the possibility that this clam assimilates cellulose. These findings support our hypothesis that *C. japonica* could assimilate cellulosic materials derived from terrestrial plants. This hypothesis predicts that *C. japonica* should have stronger cellulase and hemicellulase activities than other marine bivalves such as *R. philippinarum*. However, we have not previously reported a comparative study of cellulase and hemicellulase activities in *C. japonica* and other bivalve species.

Several studies have focused on the cellulase activities of bivalves and other marine invertebrates. For example, Kristensen (Kristensen, 1972) measured and compared carbohydrate-degrading enzyme activities in 19 marine invertebrates including four bivalve species and suggested that the invertebrates could not utilize structural polysaccharides such as cellulose because of their relatively low cellulase activity levels. On the other hand, Gianfreda and colleagues (Gianfreda et al., 1979) investigated cellulase and β-glucosidase (cellobiase) activities in 14 species of marine mollusks including four bivalves and concluded that the cellulase activity levels tend to reflect their herbivory to some extent, but the β-glucosidase activity levels do not. Despite these reports, biochemically convincing evidence for the utilization of plant-derived cellulose by bivalves is yet to be established. Even if the level of cellulase activity reflects the feeding habits of bivalves, the possession of cellulase activity should not be taken as being indicative of the ability to assimilate terrestrial plant-derived structural polysaccharides; this is because bivalves may contain cellulase in order to break down the cellulosic cell walls of phytoplankton such as green algae.

In the present study, we measured cellulase and hemicellulase activities in the crystalline style of *C. japonica* and other commercially important Veneroida bivalve species in Japan: *R. philippinarum* (inner-bay species), *M. lamarckii* (inner-bay species) and *M. lusoria* (pelagic species). The crystalline style is an extracellular structure existing in the stomach of bivalves, and is composed mainly of digestive enzymes including several carbohydrate-splitting enzymes (Gosling, 2003; Brock and Kennedy, 1992). By detailed comparison of the enzymatic activities in the crystalline style, we biochemically examined the possibility that *C. japonica* can assimilate plant-derived cellulose and hemicelluloses.

MATERIALS AND METHODS

Materials

The bivalve species investigated in this study were commercially obtained alive from the following locations in Japan: *C. japonica* Megerle von Mühlfeld 1811 from Shinji Lake, Shimane; *R. philippinarum* Adams and Reeve 1850 from Ise Bay, Mie; *M. lamarckii* Deshayes 1853 from the Kujukuri coastal plain, Chiba; and *M. lusoria* Roeding 1798 from Suou-nada Bay, Oita. *Corbicula japonica* was maintained in fresh water and the other species were maintained in artificial seawater for 2 days.

Preparation of enzyme samples

The crystalline styles of the bivalves were removed and homogenized in 50 mmol l^{-1} phosphate buffer (pH 7.0). The homogenates were centrifuged at 10,000 ***g*** for 10 min, and the supernatants were adjusted to protein concentrations of 1 or 5 mg ml^{-1} with the extraction buffer for use as the enzyme samples in experiments. Protein concentrations were determined by the method of Bradford (Bradford, 1976).

Plate assays

Substrate-containing agarose plates [1.5% agarose, 50 mmol l^{-1} acetate buffer pH 5.5, 0.1% carboxymethylcellulose (CMC, Sigma, St Louis, MO, USA)/birchwood xylan, Sigma/locust bean gum, Sigma] were prepared. Wells of 3.0 mm diameter were punched into the plates; the wells were then filled with 10 µl of the crystalline style enzyme sample of each species (5 mg ml^{-1}) and the plates were incubated overnight at 37°C. After incubation, the plates were flooded with Congo red (0.1%), left for 3 h to stain, and washed with 1 mol l^{-1} NaCl. Finally, for more sensitive detection of xylanase activity, the plates were turned blue by addition of 1 mol l^{-1} acetic acid.

Reducing sugar assays

Phosphoric acid swollen cellulose degrading activity

Phosphoric acid swollen cellulose (PASC) was prepared as described by Schülein (Schülein, 1997). A mixture of 5 µl crystalline style enzyme sample (1 mg ml^{-1}), 40 µl of PASC solutions of various concentrations and 5 µl of 1 mol l^{-1} sodium acetate buffer (pH 5.5) was prepared for each reaction sample. Blanks comprised the enzyme solution and the buffer. Each reaction was carried out in triplicate. A mixture without enzyme solution was also prepared to standardize the absorbance derived from the substrate. Reactions were carried out at 37°C for 20 min in 1.5 ml microtubes with continuous agitation. The incubated mixtures were centrifuged at 10,000 ***g*** for 1 min. The amount of reducing sugars was measured by the tetrazolium blue method (Jue and Lipke, 1985) using glucose as a standard. The absorbance values of the samples were measured using a UV-mini 1240 spectrophotometer (Shimazu, Kyoto, Japan). Enzyme activities were expressed per mg of protein in the enzyme solutions.

The following activities were measured using the method described above with slight modifications.

Endoglucanase (CMCase) activity

Endoglucanase activity was measured using CMC as the substrate. A mixture of 5 µl of enzyme sample (1 mg ml^{-1}), 40 µl of 1% CMC and 5 µl of 1 mol l^{-1} sodium acetate buffer (pH 5.5) was prepared for each reaction sample. The reactions were carried out for 10 min.

Xylanase activity

Xylanase activity was measured using birchwood xylan as the substrate. A mixture of 5 µl of enzyme sample (5 mg ml^{-1}), 40 µl of 1% suspended substrate solution and 5 µl of 1 mol l^{-1} sodium acetate buffer (pH 5.5) was prepared for each reaction sample. The reactions were carried out for 20 min. Xylose (Nacalai Tesque, Kyoto, Japan) was used as a standard.

β-Mannanase activity

β-Mannanase activity was measured using locust bean gum (Sigma) as the substrate. The substrate solution was prepared by autoclaving 1% locust bean gum followed by filtration through Whatman 3MM paper. The reactions were carried out for 10 min. Mannose was used as a standard.

β-1,3-Glucanase activity

β-1,3-Glucanase activity was measured using laminarin (Nacalai Tesque) as the substrate. The reactions were carried out for 10 min.

Pectinase activity

Pectinase activity was measured using polygalacturonic acid (Nacalai Tesque) as the substrate. The reactions were carried out

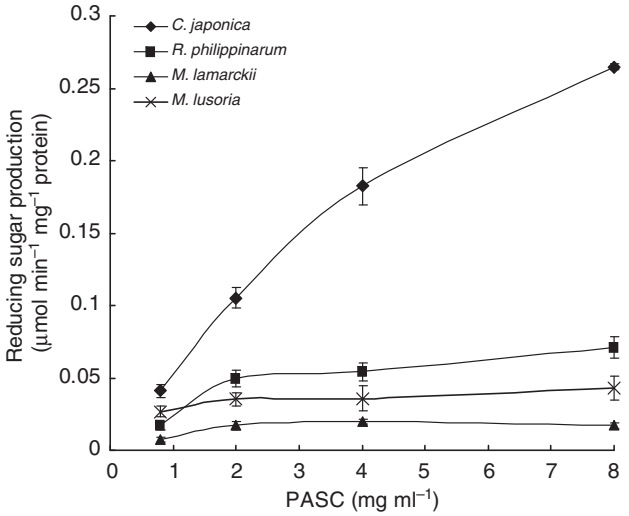

Fig. 1. Phosphoric acid swollen cellulose (PASC) degrading activity in the crystalline style extracts of various bivalves. Amounts of reducing sugar produced at various concentrations of PASC were measured. Bars represent means ± s.d.

for 10 min. Galacturonic acid (Fluka, Buchs, Switzerland) was used as a standard.

Calculation of kinetic parameters

The kinetic parameters (K_m and V_{max}) were calculated by linear regression from a Hanes–Woolf plot if the regression was significant ($P<0.05$) and the slope of the regression line was not negative. The measurement errors at the lowest substrate concentration greatly affect the regression. Thus, if the significant regression line was obtained by omitting the measured values at the lowest substrate concentration, the parameters were calculated without considering them.

RESULTS

PASC degrading activity

PASC degrading cellulase activity of the crystalline style extracts was measured. The measured values are expected to represent the total activity against amorphous insoluble cellulose, taking into account both endoglucanase and cellobiohydrolase. The contribution of β-glucosidase activity may be negligible as the crystalline style shows very low β-glucosidase activity (Sakamoto et al., 2007). As a result, incubation of PASC with the crystalline style extracts yielded reducing sugar for all four species (Fig. 1). The reducing sugar production of *C. japonica* was by far the highest at all substrate concentrations, followed by *R. philippinarum* and *M. lusoria*, while that of *M. lamarckii* was the lowest. The regression of the Hanes–Woolf plot was significant for all four species (Table 1). The V_{max} value of *C. japonica* cellulase activity was about 6–10 times higher than the V_{max} values of the other three species. The K_m value was also highest in *C. japonica*, followed by *R. philippinarum* and the two *Meretrix* species.

Endoglucanase (CMCase) activity

CMC is generally used as a soluble substrate to detect mainly endoglucanase activity, which breaks the internal bonds of cellulose. In the CMC plate assay, *C. japonica* generated the largest halo zone, followed by the other three species (Fig. 2A). Incubation of CMC with the crystalline style extracts yielded reducing sugar in all four species (Fig. 2B). The reducing sugar production of *C. japonica* was by far the highest at all substrate concentrations, followed by *R. philippinarum* and *M. lusoria*, while that of *M. lamarckii* was the lowest; this is highly consistent with the results obtained for PASC degrading activity. The regression of the Hanes–Woolf plot was significant for all four species (Table 1). The V_{max} value of *C. japonica* cellulase activity was about 4–6 times higher than the V_{max} values of the other three species. The K_m value was also the highest in *C. japonica*, followed by *M. lamarckii*, *R. philippinarum* and *M. lusoria*. However, the differences between *C. japonica* and the other three species with regard to these kinetic parameters of

Table 1. Kinetic parameters of cellulase and hemicellulases in the crystalline style extract of *Corbicula japonica*, *Ruditapes philippinarum*, *Meretrix lamarckii* and *Meretrix lusoria* calculated by Hanes–Woolf

	Parameter	*C. japonica*	*R. philippinarum*	*M. lamarkii*	*M. lusoria*
Total cellulase	R^2	0.87**	0.88**	0.91**	0.91**
	K_m (mg ml^{-1} PASC)	10.7	3.15	0.592	0.694
	V_{max} (μmol min^{-1} mg^{-1} protein)	0.634	0.099	0.045	0.019
Endoglucanase	R^2	0.85**	0.93**	0.63**	0.95**
	K_m (mg ml^{-1} CMC)	7.89	5.05	6.58	3.11
	V_{max} (μmol min^{-1} mg^{-1} protein)	2.00	0.512	0.325	0.392
Xylanase	R^2	0.85**	0.93**	0.88**	0.55*
	K_m (mg ml^{-1} birchwood xylan)	3.78	2.11	2.34	7.33
	V_{max} (μmol min^{-1} mg^{-1} protein)	0.043	0.028	0.030	0.040
β-Mannanase	R^2	0.94**	n.d.	n.d.	n.d.
	K_m (mg ml^{-1} locust bean gum)	1.62	–	–	–
	V_{max} (μmol min^{-1} mg^{-1} protein)	0.568	–	–	–
β-1,3-Glucanase	R^2	0.73**	0.743**	0.57**	0.85**
	K_m (mg ml^{-1} laminarin)	7.51	7.32	9.33	3.07
	V_{max} (μmol min^{-1} mg^{-1} protein)	1.05	1.64	3.42	1.99
Pectinase	R^2	0.80**	n.d.	n.d.	n.d.
	K_m (mg ml^{-1} polygalacturonic acid)	23.4	–	–	–
	V_{max} (μmol min^{-1} mg^{-1} protein)	3.01	–	–	–

CMC, carboxymethylcellulose; PASC, phosphoric acid swollen cellulose; $K_m$and V_{max}, kinetic parameters.
Asterisks indicate that the regression of the plot was significant (*$P<0.05$, **$P<0.01$).

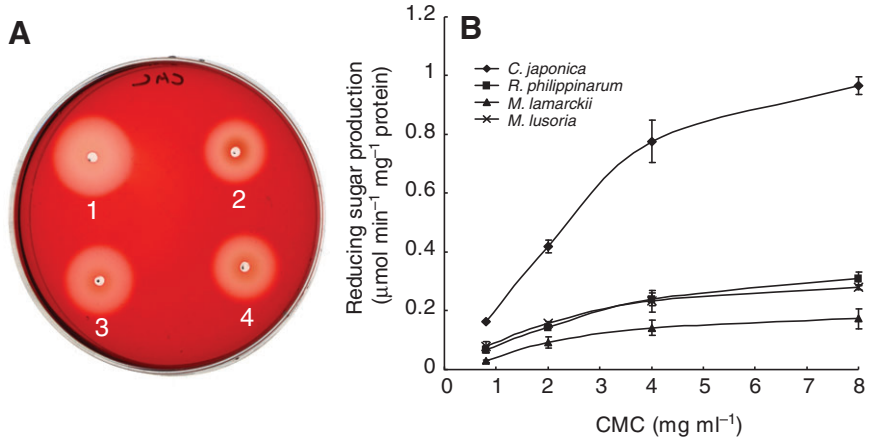

Fig. 2. Endoglucanase (CMCase) activity in the crystalline style extracts of various bivalves. (A) Carboxymethylcellulose (CMC) plate assay of the crystalline style extracts: 1, *C. japonica*; 2, *R. philippinarum*; 3, *M. lamarckii*; and 4, *M. lusoria*. (B) Amounts of reducing sugar produced at various concentrations of CMC were measured. Bars represent means ± s.d.

endoglucanase activity were relatively small compared with those of PASC degrading activity.

Xylanase activity

Xylanase activity was measured using birchwood xylan as the substrate. In the xylan plate assay, *C. japonica* generated a clear halo zone while the other bivalves showed faint halo zones (Fig. 3A). Incubation of birchwood xylan with the crystalline style extracts yielded reducing sugar in all four species (Fig. 3B). The reducing sugar production of *C. japonica* was the highest out of the four species except at the lowest substrate concentration. The regression of the Hanes–Woolf plot was significant for all four species (Table 1).

β-Mannanase activity

β-Mannanase activity was measured using locust bean gum as the substrate. Locust bean gum is composed mainly of galactomannan, a polysaccharide with a β-1,4-linked D-mannose main chain and α-1,6-linked D-galactose side chains. In the locust bean gum plate assay, *C. japonica* showed by far the largest halo zone (Fig. 4A) among these bivalves. Incubation of locust bean gum with the crystalline style extract of *C. japonica* yielded reducing sugar, while the other species generated little or no reducing sugar (Fig. 4B). The regression of the Hanes–Woolf plot was significant only for *C. japonica*; the plots of the other three species were not linear or the slope of the regression line became negative (Table 1). These data suggest that *R. philippinarum*, *M. lamarckii* and *M. lusoria*, unlike *C. japonica*, have only weak β-mannanase activity.

β-1,3-Glucanase activity

Laminarin consists mainly of β-1,3-glucan, and is therefore generally used as a substrate to detect β-1,3-glucanase activity. Incubation of laminarin with the crystalline style extracts yielded reducing sugar in all four species (Fig. 5). The reducing sugar production was high for *M. lamarckii* and *M. lusoria*, followed by *R. philippinarum* and *C. japonica*. The regression of the Hanes–Woolf plot was significant for all four species (Table 1). Although the differences among the four species with regard to the kinetic parameters were rather small, the V_{max} value of *M. lamarckii* was more than three times higher than that of *C. japonica*.

Pectinase activity

Pectinase activity was measured using polygalacturonic acid as the substrate. Incubation of polygalacturonic acid with the crystalline style extracts yielded reducing sugar for all four species, with little difference in the amount of reducing sugar produced (Fig. 6). The regression of the Hanes–Woolf plot was significant for *C. japonica*, but not for the other three species (Table 1).

DISCUSSION

Measuring digestive enzyme activity is a simple biochemical experiment; thus, it should be easy to estimate the substances that are actually assimilated by an organism by detecting digestive enzyme activities. However, it is actually difficult to compare the enzyme activities of different species, especially for small invertebrates, because the composition of digestive enzymes generally varies

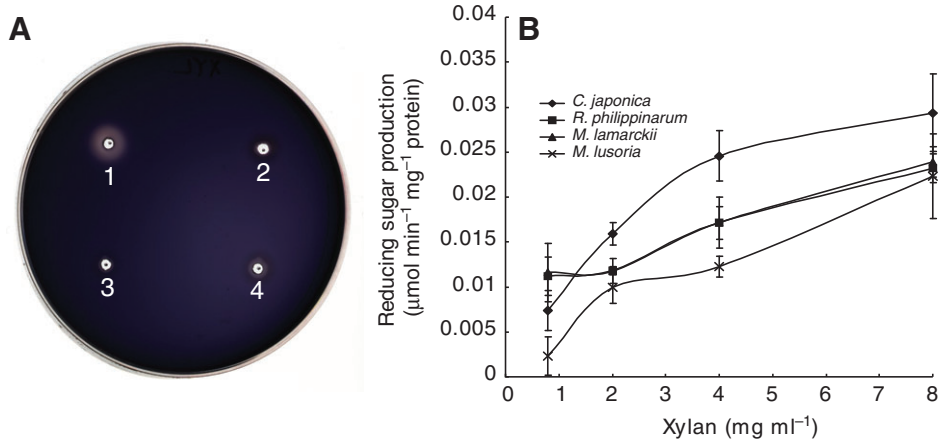

Fig. 3. Xylanase activity in the crystalline style extracts of various bivalves. (A) Xylan plate assay of the crystalline style extracts: 1, *C. japonica*; 2, *R. philippinarum*; 3, *M. lamarckii*; and 4, *M. lusoria*. (B) Amounts of reducing sugar produced at various concentrations of birchwood xylan were measured. Bars represent means ± s.d.

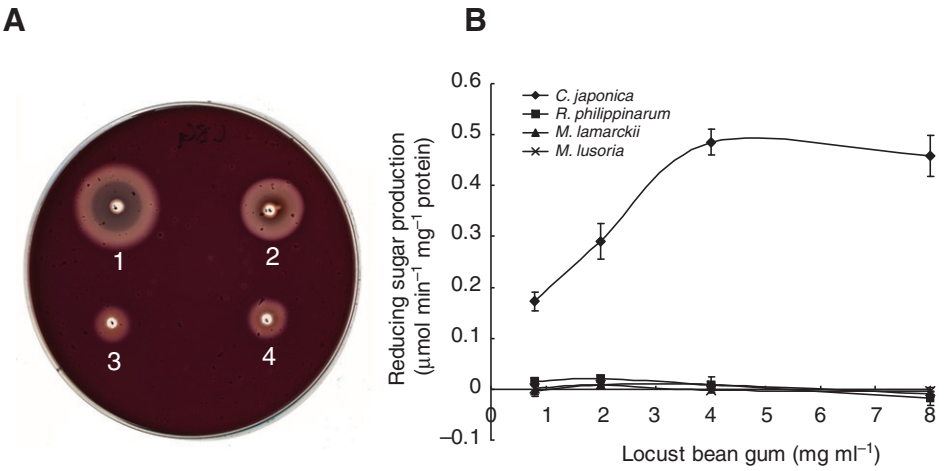

Fig. 4. β-Mannanase activity in the crystalline style extracts of various bivalves. (A) Locust bean gum plate assay of the crystalline style extracts: 1, *C. japonica*; 2, *R. philippinarum*; 3, *M. lamarckii*; and 4, *M. lusoria*. (B) Amounts of reducing sugar produced at various concentrations of locust bean gum were measured. Bars represent means ± s.d.

according to the physiological state of the organism, which depends on many factors such as time, season, maturity and the environment of its habitat. Thus, the concentration of a specific enzyme could not be standardized, even if the total protein concentration of the solution was standardized. For accurate comparison, it is necessary to conduct a detailed analysis of the enzymatic characteristics after enzyme purification in each species; however, such an analysis is impractical because of the laborious work of purification. In particular, decomposing cellulose and structural polysaccharides generally requires multiple enzymes (Watanabe and Tokuda, 2001), which makes analyzing enzyme activities more difficult. In addition, most such studies on invertebrates have used homogenates of digestive organs such as the digestive gland, stomach or intestine as enzyme samples (Gianfreda et al., 1979; Teo and Sabapathy, 1990; Crawford et al., 2005), but the effect of reaction product conversion to other substances by metabolic enzymes existing in such organs should not be neglected. For this reason, determining substrate reduction seems to be the best way to quantify digestive enzyme activity, but for polysaccharide-degrading enzymes such as cellulase, accurately determining substrate quantity is difficult because of limited methods.

Considering such problems, we cannot deny that experiments comparing digestive enzyme activity using crude extracts of digestive organs inevitably become a qualitative study rather than a quantitative one. To alleviate this defect as far as possible, we took the following measures. (1) Bivalve samples were acclimated in artificial seawater for 2 days under fasting conditions to prepare enzyme samples when the individuals were in their most basic digestive state. We anticipated that the variability of digestive enzyme composition between individuals might be somewhat standardized by this treatment. (2) Semi-quantitative plate assays that visualize the disappearance of substrates were conducted when possible, together with the reducing sugar assay most commonly employed. By taking this approach, we could estimate the enzyme activity in two ways: amount of the reaction product and decrease in the amount of substrate. (3) Biochemical parameters K_m and V_{max} were calculated to quantify the enzymatic activity. Strictly speaking, the polysaccharide-degrading reaction is not a simple bimolecular reaction, and thus does not follow Michaelis–Menten kinetics. However, we tried to broadly apply this mode of analysis to cellulase and hemicellulases. In this context, subtle differences among species

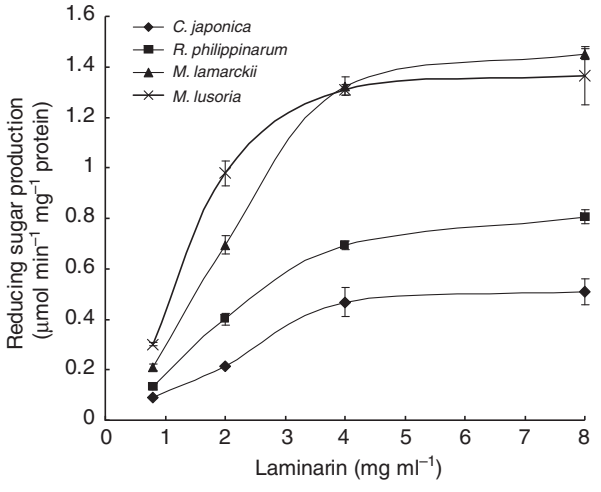

Fig. 5. β-1,3-Glucanase activity in the crystalline style extracts of *C. japonica*, *R. philippinarum*, *M. lamarckii* and *M. lusoria*. Amounts of reducing sugar produced at various concentrations of laminarin were measured. Bars represent means ± s.d.

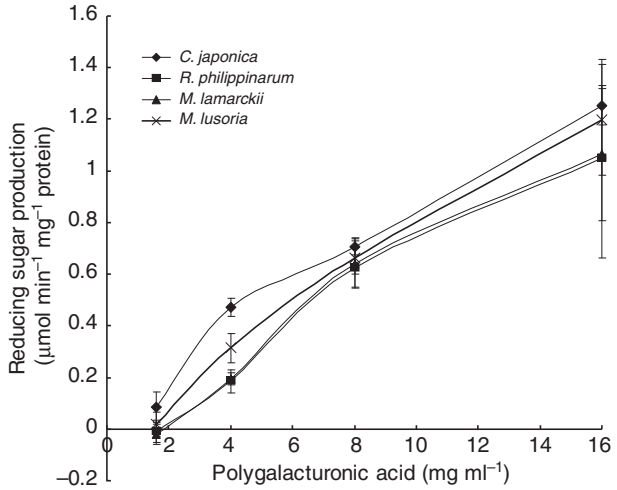

Fig. 6. Pectinase activity in the crystalline style extracts of various bivalves. Amounts of reducing sugar produced at various concentrations of polygalacturonic acid were measured. Bars represent means ± s.d.

with respect to such parameters are meaningless, but relatively big, severalfold differences should, to some extent, reflect actual differences in enzymatic activity.

In the cellulase assays, the V_{max} value of PASC degradation for *C. japonica* was about 6–10 times higher than the V_{max} values of *R. philippinarum*, *M. lamarckii* and *M. lusoria*, while the differences between the V_{max} values of *C. japonica* and the others were 4- to 6-fold for CMC degradation (endoglucanase) (Table 1). These data suggest that *C. japonica* possesses an efficient system for cellulose degradation, which possibly involves the cooperative action of endoglucanase and cellobiohydrolase. The relatively high K_m values of PASC degradation of cellulase and endoglucanase for *C. japonica* may reflect a high concentration of cellulose in the diet of *C. japonica*, whose habitat is farther upstream than that of the other species and thus richer in terrestrial plant-derived substances (Suberkropp et al., 1976; Kasai et al., 2004). Significant cellulase activity was detected in all four species (Fig. 1), which is reasonable because cellulase activity contributes to the degradation of cell walls of not only terrestrial plants but also phytoplankton with cellulosic cell walls. However, the remarkable intensity of *C. japonica* cellulase activity implies a special function of this enzyme in this species.

The xylanase activity of *C. japonica* also appears to be relatively high. Although the K_m and V_{max} values of *C. japonica* do not greatly differ from those of *R. philippinarum*, *M. lamarckii* and *M. lusoria* (Table 1), the crystalline style extract of *C. japonica* exhibited by far the largest halo zone among the four species in the xylan plate assay (Fig. 3A). To be precise, xylan is a hetero-polysaccharide with many side groups such as glucuronic acid and arabinose. The reducing sugar assay possibly reflects the decomposition of such side groups to some extent. On the other hand, the plate assay may mainly indicate breakdown of the main chain, because Congo red is known to specifically stain polysaccharides with β-1,4-linkages (Teather and Wood, 1982). If this is the case, *C. japonica* appears to possess a much higher ability to degrade the xylan main chain than the other bivalves. Whether *C. japonica* is able to assimilate xylose or not is still unclear, but xylan degradation should at least help to break down plant cell walls because xylan is generally the second most abundant structural polysaccharide in terrestrial plant cell walls.

As for β-mannanase activity, the crystalline style extract of *C. japonica* generated the largest halo zone in the locust bean gum plate assay (Fig. 4A), and reducing sugar production from this substrate was almost exclusively detected from *C. japonica* among the four species used in this study (Fig. 4B). This result is quite interesting, and raises the question of the major substrate of this enzyme in the natural environment. Several previous studies have focused on β-mannanases of mollusks (Yamaura et al., 1996; Ootsuka et al., 2006; Xu et al., 2002a; Xu et al., 2002b). In particular, the marine mussel *Mytilus edulis* and abalone *Haliotis discus hannai* are reported to possess endogenous β-mannanase genes (Xu et al., 2002b; Ootsuka et al., 2006). However, all such studies were more biochemical than biological, and put more emphasis on the enzyme itself than on the organism. It can be supposed that the abalone *H. discus*, an algophagous gastropod, should break down the β-1,4-mannan existing in red algae with β-mannanase (Otsuka et al., 2006), but the function of this enzyme in bivalves is unclear. Glucomannan, a heteropolysaccharide consisting of β-1,4-linked mannose and glucose units, is one of the major hemicellulose components in plant cell walls, especially in coniferous trees (Morrison, 2001), and thus a probable candidate for a substrate of *C. japonica* β-mannanase. *Corbicula japonica* may have β-mannanase activity to decompose such wood-derived substances.

In contrast to cellulase, xylanase and β-mannanase, the β-1,3-glucanase activity of *C. japonica* was indicated to be the lowest of the four species (Fig. 5). Although the variation in K_m and V_{max} values among the species was relatively small, the V_{max} values of *M. lamarckii* and *M. lusoria* were about 3-fold and 2-fold higher, respectively, than the V_{max} value of *C. japonica* (Table 1). Polysaccharides containing the β-1,3-glucoside linkage, putative substrates of β-1,3-glucanase, occur widely in nature, though they are often called by different names according to their origin. For instance, the cell walls of grasses contain a relatively large amount of β-1,3-1,4-glucan (Carpita, 1996). Higher plants and bacteria are known to produce linear β-1,3-glucans called 'callose' and 'curdlan', respectively (Currier 1957, Harada et al., 1968). Moreover, β-1,3-glucan is a major component of fungal cell walls. Laminarin, which was used as the substrate of β-1,3-glucanase in this experiment, is the name of a β-1,3-1,6-glucan produced by heterokonts including brown algae, diatoms and so on as a reserve polysaccharide. It is widely thought that diatoms are a major food source for bivalves. Relatively high activity levels of β-1,3-glucanase in all four species tested may reflect the assimilation of laminarin in diatoms.

Although the kinetic parameters could be calculated only for *C. japonica*, pectinase activity was detected in all four species at nearly equal levels (Fig. 6). Pectin is a component of plant cell walls, and also of the siliceous cell walls of diatoms (Reimann et al., 1965). Thus, the ability to degrade pectin is likely required to break down both plant cell walls and diatom cell walls.

Conclusions

In the present study, we measured cellulase and hemicellulase activities in the crystalline style of Veneroida bivalves, and showed that *C. japonica* possesses relatively high activities of cellulase, xylanase and β-mannanase, while its β-1,3-glucosidase activity was relatively low. These results suggest that *C. japonica* is able to decompose the structural polysaccharides in plant cell walls, such as cellulose, xylan and glucomannan, more efficiently than *R. philippinarum*, *M. lamarckii* and *M. lusoria.* In the natural environment, such structural polysaccharides are likely to exist in a more complicated form composed of cellulose, hemicelluloses and other persistent materials such as lignin. *Corbicula japonica*, possessing high hemicellulase activities as well as a cellulase activity, may have a stronger ability to decompose plant cell walls than that indicated by individual measurement of such enzyme activities because of the synergistic effect of cellulase and hemicellulase (Murashima et al., 2003). However, needless to say, possession of cellulase and hemicellulase activities does not by itself directly indicate the assimilation of terrestrial plant residues. For example, cellulose is also a major component of the cell walls of oomycetes (Helbert et al., 1997), and β-1,3-glucan and β-1,4-mannan are found in the cell walls of some types of fungi (Ruiz-Herrera, 1992). *Corbicula japonica* might mainly assimilate oomycetes and fungi colonizing plant residues rather than the plant residue itself.

All we can say from this study is that *C. japonica* appears to have a far greater biochemical capacity to decompose structural polysaccharides derived from terrestrial organisms such as vascular plants, oomycetes or fungi than *R. philippinarum*, *M. lamarckii* and *M. lusoria*. As for the oyster *Crassostrea virginica*, the partial contribution of plant-derived substances to its diet is suggested by a radioactive isotopic analysis (Crosby et al., 1989). Microbial biomass is also suggested to be important in converting refractory detritus into more palatable materials for detritivores (Crosby et al., 1990; Raghukumar, 2004). We suppose that *C. japonica* mainly assimilates a complex of such microorganisms and plant-derived

materials, some type of 'fermented food' made from terrestrial plant residues. The amount of such terrestrial plant residues increases on ascending a river upstream (Suberkropp et al., 1976; Kasai et al., 2004), and it is plausible that the high cellulase and hemicellulase activity of *C. japonica* is a result of an adaptation to such an environment. Enzymatic studies of cellulase and hemicellulases may not be able to provide direct evidence for cellulose and hemicellulose assimilation. However, in the field of experimental biology, they can become a strong tool to help understand the digestive physiology of bivalves and other animals when used in conjunction with other techniques such as stable isotopic analysis.

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