Distribution of Polysaccharide Digestive Enzymes in a Marine Gastropod *Dolabella auricularia**¹

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The gastric teeth and midgut gland of a marine gastropod *Dolabella auricularia* were investigated with respect to the distribution of polysaccharide saccharification activities and glycosidase activities. Crude enzymes from both organs were obtained by extraction with a buffer (pH 7.5) containing 0.5 M NaCl and their activities were measured for twelve kinds of polysaccharides and seven kinds of PNP-glycosides.

The total enzyme activities (Units/organ) for saccharification of soluble starch, CM-cellulose, alginic acid, β -1,4-mannan, and β -1,3-glucan were found to be relatively high in both organs. The specific activities (Units/mg protein) of these enzymes in the gastric teeth exhibited two- to eight-fold of those in the midgut gland.

As for glycosidases, their activities were mainly distributed in the midgut gland, when compared to the gastric teeth in which only β -glucosidase, β -mannosidase, and α -glucosidase activities were detected.

Dolabella auricularia, a herbivorous marine gastropod, has been shown to have polysaccharide digestive enzymes in the alimentary tract; a high cellulase activity was detected in the gastric teeth which are a characteristic digestive organ,¹⁾ and and purification and properties of cellulases from the gastric teeth have been reported.^{2,3)} Likewise, cellulase and alginate lyase activities have also been detected in the midgut gland of this species.^{4,5)} Although the distribution of polysaccharide digestive enzymes in the digestive organs of the mollusks was investigated mainly in bivalves,^{6,7)} and high activities of these enzymes were detected in crystalline style. On the other hand, the polysaccharide digestive activities of gastropods were mostly assayed with the extracts of midgut gland, and little information is available on the distribution of these enzymes among the digestive organs.⁸⁾ In the present study, distribution of these enzyme activities in *D. auricularia* was investigated from the phylogenetic aspects of polysaccharide digestion.

Materials and Methods

The gastropods weighing an average of 968 g per six individuals were collected from Suzaki, Shimoda, Shizuoka, Japan. Gastric teeth and midgut gland were removed, pre-washed with filtered sea water, and then washed thoroughly with distilled water. The final average weights of the gastric teeth and midgut gland obtained were 10.6 and 46.2 g, respectively. These organs were immediately stored at -20°C until use.

Substrates

Twelve kinds of polysaccharides were used as substrates. CM-cellulose (Cellogen BS, $\overline{\text{DS}}=0.63$) was purchased from Daiichi Industrial Pharmaceutical Co., Tokyo, Japan. Swollen cellulose was prepared from absorbent cotton by the method of Walseth.⁹⁾ Oat-spelt xylan (β -1,4-xylan) was purchased from Seikagaku Kogyo Co., Ltd. Bryopsis xylan (β -1,3-xylan) was prepared from the cell wall preparation of *Bryopsis maxima* by

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Abbreviations: ACES, *N*-(2-acetamide)-2-aminoethane sulfonic acid; GTA buffer, 3,3-dimethylglutaric acid- Tris(hydroxymethyl)aminomethane-2-amino-2-methyl-1,3-propanediol wide range buffer solution; CM-, carboxymethyl-; PNP-, *p*-nitrophenyl-; cp, centipoise; M/G, D-mannuronic acid to L-guluronic acid ratio.

the method of Iriki *et al.*¹⁰⁾ *Codium* mannan (β -1,4-mannan) was prepared from the cell wall preparation of *Codium latum* by the method of Iriki and Miwa.¹¹⁾ *Ascophyllum* alginate (D-mannuronic acid rich alginate: 100 cp, M/G=1.80) and *Hyperborea* alginate (L-guluronic acid rich alginate: 100 cp, M/G=0.42) were purchased from the Protan A/S, Drammen, Norway. Fucoidan from *Fucus vesiculosus* and λ -carrageenan from *Gigartina aciculaire* and *G. pistillata* were purchased from Sigma Chemical Company, St. Louis, USA. Agarose ME-12 was supplied by Ina Food Industry Co., Ltd., Ina, Nagano, Japan. Soluble starch was purchased from Wako Pure Chemical Industries, Ltd. Pachyman (β -1,3-glucan) used as an analog of laminaran was prepared from *Poria cocos* (Bukuryo) by the method of Cirelli and De Lederkremer.¹²⁾

Seven kinds of PNP-glycosides used for glycosidase assay were purchased from Sigma Chemical Company, St. Louis, USA. All other chemicals were analytical reagent grade.

Extraction of Enzymes

The gastric teeth were cut into thin slices with a razor. The slices were ground in a cold mortar with the equal weight of quartz sand and the eight volumes of 0.1 M ACES-NaOH buffer (pH 7.5) containing 0.5 M NaCl. The mixture was centrifuged at $10,000 \times g$ for 30 min, and the supernatant obtained was used as the crude enzyme solution. The midgut gland was cut into small pieces in the four volumes of 0.1 M ACES-NaOH buffer (pH 7.5) containing 0.5 M NaCl, and was homogenized by a Potter-homogenizer. The homogenate was centrifuged at $15,000 \times g$ for 30 min, and the supernatant was used as the crude enzyme solution.

Enzyme Assays

1. Polysaccharide saccharification activity: The reaction mixture consisted of 500 μ l of 1.0% substrate solution, 250 μ l of 100 mM GTA buffer (pH 2.0–11.0), and 50 μ l of the enzyme solution in the total volume of 1.0 ml. In the case of agarose, 500 μ l of 0.1% substrate solution was used. After incubation for an appropriate period at 30°C, the reducing sugar liberated was determined; one unit of enzyme activity was defined as the amount of enzyme which catalyzed the liberation of reducing sugar equivalent to 1.0 μ mol of standard monosaccharide per min. The standard monosaccharides were D-glucose for CM-cel-

lulose, swollen cellulose, soluble starch, and pachyman, D-xylose for β -1,4-xylan and β -1,3-xylan, D-mannose for β -1,4-mannan, D-mannuronic acid lacton for D-mannuronic acid rich alginate and L-guluronic acid rich alginate, L-fucose for fucoidan, and D-galactose for λ -carrageenan and agarose.

2. Glycosidase activity: The reaction mixture consisted of 50 μ l of 10 mM PNP-glycoside, 100 μ l of 1.0 M Na-citrate buffer (pH 2.0–7.0), and 50 μ l of the enzyme solution in the total volume of 200 μ l. After incubation at 30°C for an appropriate period, 4.0 ml of 0.1 M sodium carbonate solution was added to the mixture, and the liberated *p*-nitrophenol was determined at 420 nm. One unit of the enzyme activity was defined as the amount of enzyme which liberated 1.0 μ mol of *p*-nitrophenol per min.

Chemical Analysis

Concentration of the substrate in the reaction mixture was determined by the phenol-sulfuric acid method for neutral sugar¹³⁾ and by the carbazole-sulfuric acid method for uronic acid.¹⁴⁾ Reducing sugar liberated with enzyme reaction was determined by the method of Somogyi¹⁵⁾ and Nelson.¹⁶⁾

The amount of protein in the enzyme solution was determined by the method of Bradford.¹⁷⁾ Crystalline bovine serum albumin was used as a standard.

Results and Discussion

Optimum pH

Enzyme activities in the crude enzyme solutions from both organs were determined at various pH values. The optimum pHs are shown in Tables 1 and 2.

1. Polysaccharide saccharification activity: The optimum pH for activities of these enzymes, except for β -1,3-xylanase in the midgut gland and alginate lyase in both organs, ranged from 4.89 to 6.69, which were within the pH range of the digestive fluids (Fig. 1); those for β -1,3-xylanase (pH 3.4) and alginate lyases (pH 8.34–8.52) fall outside this pH range.

The saccharification activities for fucoidan, λ -carrageenan, and agarose were very low and so profiles of the activities at various pHs could not be determined.

2. Glycosidase activity: The optimum pHs for glycosidase activities in the gastric teeth were

Table 1. Polysaccharide saccharification activities in the digestive organs of *Dolabella auricularia*

Substrate	Gastric teeth			Midgut gland		
	Optimum pH	Activity		Optimum pH	Activity	
		Total (unit)	Specific (unit/mg protein)		Total (unit)	Specific (unit/mg protein)
Cell wall polysaccharide						
CM-cellulose	6.25	1009	3.18	6.48	394	0.39
H ₃ PO ₄ -swollen cellulose	4.89	108	0.34	6.48	51	0.05
β -1,4-Mannan	5.64	225	0.71	5.11	181	0.18
β -1,4-Xylan	5.32	7	0.02	5.58	49	0.05
β -1,3-Xylan	5.50	3	0.01	3.90	12	0.01
Intercellular polysaccharide						
D-Mannuronic acid rich alginate	8.52	301	0.95	8.52	226	0.22
L-Guluronic acid rich alginate	8.34	161	0.51	8.41	192	0.19
Fucoidan	(6.50)* ¹	4	0.01	(6.50)* ¹	9	0.01
λ -Carraggenan	(6.50)* ¹	U.D.* ²	U.D.* ²	(6.50)* ¹	6	0.01
Agarose	(6.50)* ¹	U.D.* ²	U.D.* ²	(6.50)* ¹	U.D.* ²	U.D.* ²
Storage polysaccharide						
Soluble starch	6.21	5108	16.07	6.69	3751	3.71
Pachyman (β -1,3-glucan)	5.20	33	0.10	5.01	49	0.05

*¹ The buffer solution of pH 6.5 was used for the enzyme assay, since optimum pHs could not be determined due to the very low activity of the enzymes.

*² Undetectable.

Table 2. Glycosidase activities in the digestive organs of *Dolabella auricularia*

Substrate	Gastric teeth			Midgut gland		
	Optimum pH	Activity		Optimum pH	Activity	
		Total (unit)	Specific (milliunit/mg protein)		Total	Specific (milliunit/mg protein)
PNP- β -D-glucoside	5.17	2.61	8.22	5.17	44.36	43.86
PNP- β -D-xyloside	5.17	0.08	0.26	3.73	10.10	9.99
PNP- β -D-mannoside	5.17	47.32	148.86	5.15	130.55	129.09
PNP- β -D-galactoside	5.56	1.11	3.48	3.23	51.03	50.46
PNP- α -D-galactoside	(5.17)*	0.03	0.10	4.16	26.59	26.29
PNP- α -L-fucoside	(5.17)*	0.02	0.05	4.16	22.27	22.02
PNP- α -D-glucoside	5.56	11.91	37.47	5.57	11.48	11.35

* The buffer solution of pH 5.17 was used for the enzyme assay, since optimum pHs could not be determined due to the very low activities of the enzymes.

from pH 5.17 to 5.56 which fall within the pH range of the digestive fluids, while the activities for β -xylosidase, β -galactosidase, α -galactosidase, and α -fucosidase in the midgut gland were high in the more acidic conditions (pH 3.23–4.16). The optimum pHs of α -galactosidase and α -fucosidase in the gastric teeth could not be estimated due to their very low activities.

Distribution of Polysaccharide Digestive Enzymes

The average values of total and specific activities of enzymes in individual specimens are summarized in Tables 1 and 2.

Among enzymes for saccharification of cell wall polysaccharide, cellulase activities were rich in the both organs; particularly, the gastric teeth showed a high activity when compared to the midgut gland. The comparable activities of β -1,4-mannanase were detected in both organs. On the other hand, the activities of β -1,4-xylanase and β -1,3-xylanase were lower than those of cellulases and β -1,4-mannanase, and were mainly distributed in the midgut gland.

Among enzymes for saccharification of intercellular polysaccharide, alginate lyases acting upon D-mannuronic acid or L-guluronic acid rich al-

Fig. 1. The $\bar{x}$ values of pH in the digestive fluid of a gastropod *D. auricularia*.

A, gizzard; B, midgut gland.

Total number of the specimens, 40; average body weight, 857 g (M.D.% = ± 15.5), average weight of gastric teeth, 8.5 g (M.D.% = ± 21.5); and average weight of midgut gland, 41.7 g (M.D.% = ± 16.0).

ginate exhibited high activities, and the comparable activities were measured in both organs. Fucoidan saccharification activity was lower than alginate lyase activities. The activities for galactans such as λ -carrageenan and agarose were virtually undetectable in both organs (Table 1).

Among enzymes for saccharification of storage polysaccharide, the amylase activity was found to be extremely high when compared to the β -1,3-glucanase activity.

These results indicate that several polysaccharide saccharification activities were widely distributed in both organs. However, the specific activities of these enzymes except for xylanases in the gastric teeth were two- to eight-fold of those in the midgut gland (Table 1).

Among glycosidases, the activities possessing the optimum pHs from 5 to 6 were detected for β -glucosidase, β -mannosidase, and α -glucosidase at a significant level in the gastric teeth. On the other hand, relatively high activities of β -xylosidase, β -galactosidase, α -galactosidase, and α -fucosidase acting in acidic conditions were

detected in the midgut gland (Table 2). These results suggested that the glycosidases in the midgut gland did not participate in polysaccharide digestion; the latter four enzymes might have originated from lysosome of the glands.

The results from this study suggested that the gastric teeth are the main site for polysaccharide digestion in *D. auricularia*. Polysaccharide digestive enzymes in many bivalves were mainly found in the crystalline style.⁷³ It is considered morphologically that the gastric teeth corresponds to the gastric shield, but not to the crystalline style of bivalves.¹⁶⁾ However, the distribution of these enzymes in the gastric teeth indicates that the function of this organ resembles that of the crystalline style of bivalves.

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References

- 1) Y. Yokoe and I. Yasumasu: *Comp. Biochem. Physiol.*, **13**, 323-338 (1964).
- 2) H. Anzai, K. Nisizawa, and K. Matsuda: *J. Biochem.*, **96**, 1381-1390 (1984).
- 3) H. Anzai, N. Uchida, E. Nishide, K. Nishizawa, and K. Matsuda: *Agric. Biol. Chem.*, **52**, 633-640 (1988).
- 4) H. Anzai and K. Nisizawa: *Nippon Suisan Gakkaishi*, **48**, 1317-1322 (1982).
- 5) Y. Hashimoto and K. Onoma: *Nippon Suisan Gakkaishi*, **15**, 253-258 (1949).
- 6) V. V. Sova, L. A. Elyakova, and V. E. Vaskovsky: *Comp. Biochem. Physiol.*, **32**, 459-464 (1970).
- 7) T. Onishi, M. Suzuki, and R. Kikuchi: *Nippon Suisan Gakkaishi*, **51**, 301-308 (1985).
- 8) S. Horiuchi and C. E. Lane: *Comp. Biochem. Physiol.*, **17**, 1189-1197 (1966).
- 9) J. W. Green: in "Method in Carbohydrate Chemistry" Vol. III (ed. by R. L. Whistler), Academic Press, New York, 1963, pp. 95-103.
- 10) Y. Iriki, T. Suzuki, K. Nisizawa, and T. Miwa: *Nature*, **187**, 82-83 (1960).
- 11) Y. Iriki and T. Miwa: *Nature*, **185**, 178-179 (1960).
- 12) A. F. Cirelli and R. M. De Lederkremer: *Carbohydr. Res.*, **48**, 217-223 (1976).
- 13) M. Dubois, K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith: *Anal. Chem.*, **28**, 350-356 (1956).

- 14) T. Bitter and H. M. Muir: *Anal. Biochem.*, **4**, 330-334 (1968).
- 15) M. Somogyi: *J. Biol. Chem.*, **195**, 19-23 (1952).
- 16) N. Nelson: *J. Biol. Chem.*, **153**, 375-380 (1944).
- 17) M. Bradford: *Anal. Biochem.*, **72**, 248-254 (1976).
- 18) Y. Hashimoto, T. Hibiya, and E. Iwai: *Nippon Suisan Gakkaishi*, **19**, 67-70 (1953).