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Correlation between Thallus Stiffness and Molecular Size of Water Soluble Polysaccharides in Laver (Culture Strain of Red Alga *Porphyra yezoensis*)

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The thallus stiffness of laver (culture strain of red alga *Porphyra yezoensis*) was analyzed in relation to its polysaccharide components. The thallus stiffness increased gradually during thalli culture. The content of polysaccharides in the water-soluble fraction (water-soluble polysaccharides) was the highest of all polysaccharide fractions examined and increased with culture duration. Column chromatography analyses showed that the mean molecular sizes of water-soluble polysaccharides were remarkably different among algal samples of different thallus stiffness : larger molecular sizes in stiffer thalli and smaller molecular sizes in softer thalli. These results suggest that the molecular sizes of the water-soluble polysaccharides in thalli are related to the polysaccharide content and thalli stiffness.

1 Introduction

Softness／stiffness of the thallus is an important factor for evaluating the commercial value of laver (*Porphyra yezoensis*) products. In general, commercially high grade products of "Itanori", Japanese trade name for laver products, consist of softer thalli and, in contrast, commercially cheap "Itanori" is in most cases considerably stiffer.[1] Therefore, many efforts have been made to produce soft thalli in Japanese culture sea-farms.

Several investigators have reported on the polysaccharide components of red algae. Su and Hassid[2] showed that intercellular polysaccharide in the water extract fraction of the red alga *Porphyra perforata* consisted chiefly of DL-galactan. Gretz *et al.*[3] demonstrated that cellulose was present in the conchocelis but absent in the thallus of *Bangia fuscopurpurea*. Mukai *et al.*[4] showed that the cell wall of *Porphyra tenera* thallus contained predominantly mannose. However, few biochemical studies have been undertaken on the softness／stiffness of laver thalli in relation to carbohydrate components. In the present study, we analyse

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the softness/ stiffness of thallus concerning with the change of content and composition of thallus polysaccharide during laver growth.

2 Materials and Methods

2. 1 Algae

The culture strain of *Porphyra yezoensis*, Fukuoka-1, used in this study was procured from the Fukuoka Fisheries and Marine Technology Research Center-Ariake Institute (Fukuoka, Japan). The alga was cultured using "nori-nets" at sea farms in Ariake Bay, Japan. When it grew about 3 cm in length, it was harvested, sun-dried for about 3 h and then stored at −20℃. The frozen thalli were thawed and incubated in sea water for several hours at 15℃ in the dark. Thalli were then released from " nori-nets", and 20 to 30 individual thalli were selected, picked up and cultured in one liter SWM-III culture medium[5] containing streptmycin and penicillin G (final concentrations of 100μg/ml) at 15℃ under 12 h : 12 h day-night regime with continuous aeration. Thalli were harvested at about one and 4 day after culture initiation. Five to 7 individuals of the 4 day cultured thalli were transferred to a new culture medium and continuously cultured for an additional 14 days, exchanging the culture medium at 3 days intervals.

2. 2 Measurement of thallus stiffness

The thallus stiffness was measured manually using the method reported by Yamashita[6] with slight modifications. The 23G needle, which was slightly blunted, was set vertically on the thallus surface, and small glass beads, approximately 150 mg in weight, were placed gently on top of the needle one by one until the needle penetrated the thallus. When the needle penetrated, all of the glass beads on top of the needle were weighed. The degree of thallus stiffness was expressed as this weight.

2. 3 Fractionation of thallus polysaccharides

Thalli were dried completely in a desiccator and powdered with a stainless pot mill for 3 min. The 0.2 to 0.8 g of algal powder was suspended in approximately 200 ml of distilled water and incubated for 5 h at 60℃ wit occasional shaking. After cooling, the suspension was centrifuged at 10,000 rpm for 15 min. The supernatant was recovered, added by 4 volumes of ethanol and centrifuged at 10,000 rpm for 10 min. The precipitated materials were dissolved in distilled water, added by 4 volumes of ethanol and again centrifuged. The above ethanol precipitation and water dissolution of polysaccharides was repeated two times further. After the final ethanol presipitation, the precipitated materials were washed once with ether, dried and dissolved with 20 ml of 10 mM Na-phosphate buffer (pH 7.5) (water-soluble polysaccharides, Fraction 1 polysaccharides). The pellet obtained after the initial centrifugation of the powder suspension was added by 50 ml of 24% KOH solution, stood at room temperature for more than 20 h and then centrifuged at 10,000 rpm for 10min. The supernatant was recovered and neutralized by the addition of 2 M acetate and centrifuged to separate the neutral-soluble and insoluble

materials. The neutral-soluble, insoluble and the 24% KOH-insoluble materials were respectively suspended with 20 ml of distilled water, precipitated with ethanol and suspended again in distilled water. This ethanol precipitation and water suspension was repeated for an additional two times as described above. These materials were finally suspended in 10 ml of 10 mM Na-phosphate buffer (pH 7.5). The neutral-soluble, insoluble and the KOH-insoluble polysaccharides are referred to as Fraction 2, 3 and 4 polysaccharides, respectively.

2. 4 Column chromatography of water soluble polysaccharides

12 mg of water-soluble (Fraction 1) polysaccharides in 10 m M Na-phosphate buffer (pH 7.5) containing 0.2 M NaCl was applied to a column (2.5×20 cm) of DEAE-Sephadex A-50 (Pharmacia Fine Chemicals Co. Sweden) equilibrated with the same buffer containing 0.2 M NaCl. After washing the column with 10 mM Na-phosphate buffer containing 0.2 M NaCl, the absorbed materials were eluted with a linear salt gradient produced by mixing 300 ml each of 0.2 M NaCl in 10 mM Na-phosphate buffer (pH 7.5) and 1.0 M NaCl in the same buffer. Fraction of 5 ml of each fraction was collected and a part of each fraction was assayed for carbohydrate.

The molecular sizes of Fraction 1 polysaccharides were assayed by gel-filtrations. 5 mg of Fraction 1 polysaccharides in 0.5 ml 10 mM Na-phosphate buffer-20 mM NaCl was applied to a column (2×40 cm) of Sephacryl S-200 (Pharmacia) equilibrated with the same buffer/NaCl as described above, followed by elution with the same buffer/NaCl. Two ml of each fraction was collected and a part of each fraction was assayed for carbohydrate. The column had been calibrated using protein molecular marker kit (Bio-Rad Laboratories, California), reading OD. 280 nm with a UV detector (Hitachi Co. Ltd.).

2. 5 Enzyme digestion of the polysaccharides

The cell wall-lytic enzyme was partially purified from abalone mid-gut glands as described previously.[7] 1.5 mg of the Fraction 1 polysaccharides extracted from 18 day-cultured thalli was mixed with 200 μl of the partially purified wall-lytic enzyme in 20 mM acetate buffer (pH 6.0) and incubated at 37℃ for 6 h. The products of enzyme digestion were assayed on a DEAE-Sephadex A-50 column chromatograph using the method described above. Two ml of each fraction was collected and a part of each fraction was assayed for carbohydrate. 1.0 mg of normal, enzyme-untreated Fraction 1 polysaccharides from 18 day-cultured thalli was also chromatographed for comparison, using the method described above.

2. 6 Quantitative analysis of carbohydrate

The amount of carbohydrate in each column chromatographic fraction was assayed by the phenol-sulfuric acid method[8] and shown as absorbances at 480 nm. The net contents of carbohydrate in various fractions of polysaccharides were also determined by the same method described above using xylose as a standard of content and

absorbance at 480 nm.

3 Results and Discussion

The stiffness was measured and compared among the three algal samples. Fig. 1 shows the comparison of thallus stiffness among the three samples obtained 1, 4 and 18 days after culture initiation (referred to as 1, 4 and 18 day-thalli, respectively). The results indicated that 18day-thalli were stiffest, with 1 day-thalli the softest of the three samples examined. The mean stiffness of 18 day-thalli was approximately ten times greater than that of 1 day-thalli. These findings suggest that the laver grew stiffer with the progress of culture time in our culture system. The correlation between thallus stiffness and polysaccharide contents was also examined. The polysaccharide components were fractionated into four fractions : Fraction 1 (water-soluble polysaccharides), 2, 3 and 4. Table 1 shows that the polysaccharide contents are different among the three algal samples for all fractions and that Fraction 1 polysaccharides are the most abundant in all samples. The contents of Fraction 1 polysaccharides were remarkably different among the algal samples : more than three times higher in 18 day-thalli than in 1 day-thalli, indicating that the content of Fraction 1 polysaccharides increased in parallel with thalli stiffness. These results suggest that the polysaccharide content of the water-soluble fraction is related to laver thalli stiffness.

Differences in the polysaccharide components and/or compositions in the water-soluble fraction (Fraction 1) among the three algal

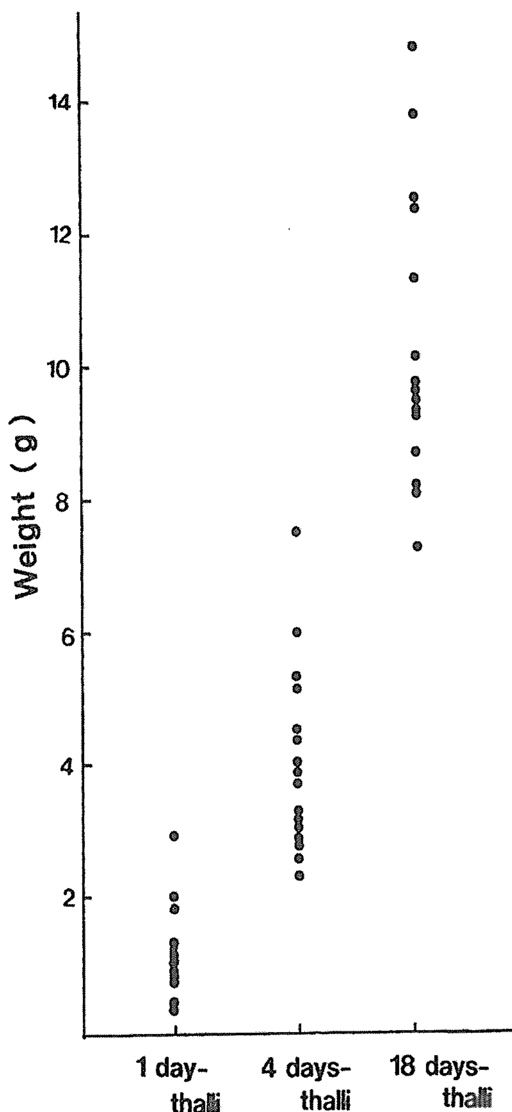

Fig. 1. Comparison of stiffness among thalli cultured for three different periods.
The frozen thalli, approximately 3 cm in length, were thawed and cultured for 1, 4 and 18 days. 10 to 20 individuals of thallus were picked up at random from each algal sample and their stiffness was individually measured. The degree of stiffness is represented as the weight required to break through a thallus, and is shown with black dots in the figure. 1 day-thalli ; 1 day-culture thalli, 4 days-thalli : 4 day-culture thalli, 18 days-thalli ; 18 day-culture thalli.

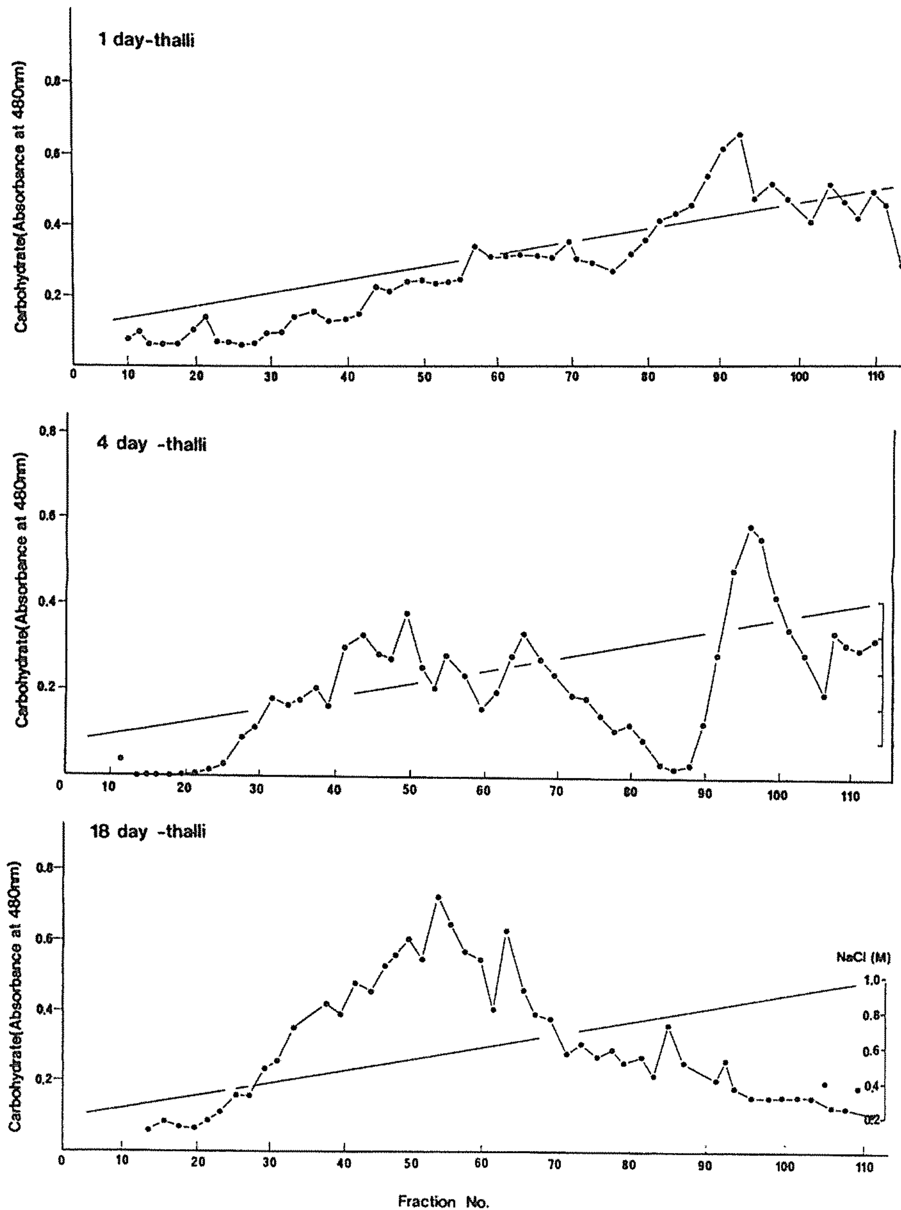

Fig. 2. DEAE-Sephadex A-50 column chromatography of the water-soluble polysaccharides from thalli cultured for three different periods.

Three samples of thalli were obtained as described in Fig. 1. Approximately 12 mg of water-soluble polysaccharides were prepared from each of the samples, applied onto a DEAE-Sephadex A-50 column equilibrated with 50 mM Na-phosphate buffer (pH 7.5), washed with the same buffer and eluted with a linear salt gradient in the same buffer. The carbohydrate contents in each fraction were determined and represented as absorbance at 420 nm after the phenol-sulfuric acid reactions.

samples were examined by column chromatography analysis. Fig. 2 shows elution patterns of Fraction 1 polysaccharides from the three algal samples. The elution patterns were considerably different from each other. Polysaccharides from 1 day-thalli were eluted mainly at positions of more than 0.8 M concentrations of NaCl, whereas those from 18 day-thalli were eluted mainly at 0.5 M NaCl. Polysaccharides from 4 day-thalli were eluted at positions between 0.4 and 0.6 M NaCl. Based on the elution pattern difference among the three algal samples, the following two possibilities were considered : 1) there was a difference in polysaccharide components and 2) a difference existed in the molecular sizes of the major polysaccharide components involved in the water soluble fractions. First, the composition of polysaccharide components was analyzed with acrylamide gel electrophoresis and PAS staining, and the electrophoretic patterns obtained were compared among these samples. However, no difference could be detected in these patterns among the three samples, suggesting that the eluiton pattern differences found in Fig. 2 did not arise from the difference of polysaccharide components among the three samples (data not shown). The molecular sizes of polysaccharide components were then compared among the three samples by gel filtration analysis. Fig. 3 shows that the elution volumes of the major polysaccharide components were slightly different among the three algal samples although the molecular weights of these components could not be determined. Major components in 18 day-thalli eluted slightly faster while in 1 day-thalli eluted the slowest of the three samples, suggesting that the molecular sizes of the major polysaccharide components in Fraction 1 were different among the three algal samples; highest in 18 day-thalli and lowest in 1 day-thalli. Therefore, it is possible that the difference in elution patterns shown in Fig. 2 and the change in polysaccharide content in the water-soluble fraction shown in Table 1 were caused by the difference in molecular size of major polysaccharide components among the three algal samples.

To confirm the above possibility, Fraction 1 polysaccharides from 18 day-thalli were then degraded by partial enzyme digestion, and the products of the digestion were assayed with DEAE-sephadex column chromatography. The polysaccharide-lytic enzyme used for this

Table 1. Comparison of carbohydrate contents in four fractions among the three thalli samples shown in Fig.1.

	Carbohydrate (μg/mg thallus powder)		
	1 day-thalli	4 day-thalli	18 day-thalli
Fraction 1	30±3	62±3	94±3
2	8±2	7±1	9±2
3	12±0	12±1	15±2
4	12±1	13±6	6±1

Values are means±standard deviation for three experiments.

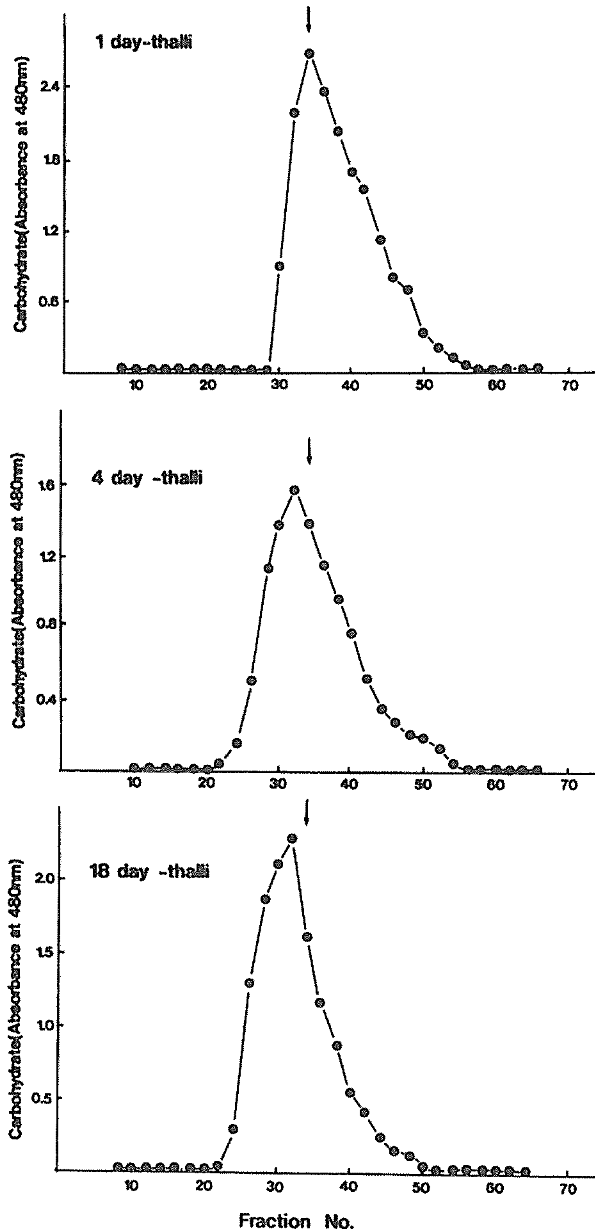

Fig. 3. Gel filtrations of the water-soluble polysaccharides from thalli cultured for three different periods. The water-soluble polysaccharides, identical with those in Fig. 2, were applied onto a column of Sephacryl S-200, followed by elution with 10 mM Na-phosphate buffer (pH 7.5) containing 20 mM NaCl. The 2 ml portion of eluted solution was collected in each tube and the carbohydrate contents were measured for each tube. Arrows indicate the positions of 260 nm-absorbing materials, which were probably partially degraded ribonucleic acids derived from rRNA and tRNA.

enzyme digestion was prepared as reported previously.[7] Fig. 4 shows that the control polysaccharides, treated with no enzyme, from 18 day-thalli are eluted at a position of approximately 0.5 M NaCl, similar to that shown in Fig. 2, whereas products of partial digestion are eluted at positions of approximately 0.8 M NaCl. This result suggests that a part of polysaccharide components eluted at 0.5MNaCl were partially degraded by the enzyme to smaller sized molecular components and eluted at approximately 0.8 M NaCl. Therefore, we suggest that in our experiments, the elution patterns of polysaccharides subjected to DEAE-Sephadex A-50 column chromatography are easily influenced by the molecular sizes of polysaccharide components. Thus, the elution pattern difference shown in Fig. 2 were caused by the change of molecular sizes of polysaccharide components during the growth of thalli.

From the data presented, we conclude that thallus stiffness gradually increases during growth and that in parallely, molecular sizes of water-soluble polysaccharide thallus components increase with the progress of culture time. The high polymerization of polysaccharide molecules may relate to laver thallus stiffness. However, whether such factors as proteins, water-insoluble polysaccharides and polysaccharide sugar composition also affect laver thallus stiffness remains to be investigated.

Acknowledgment

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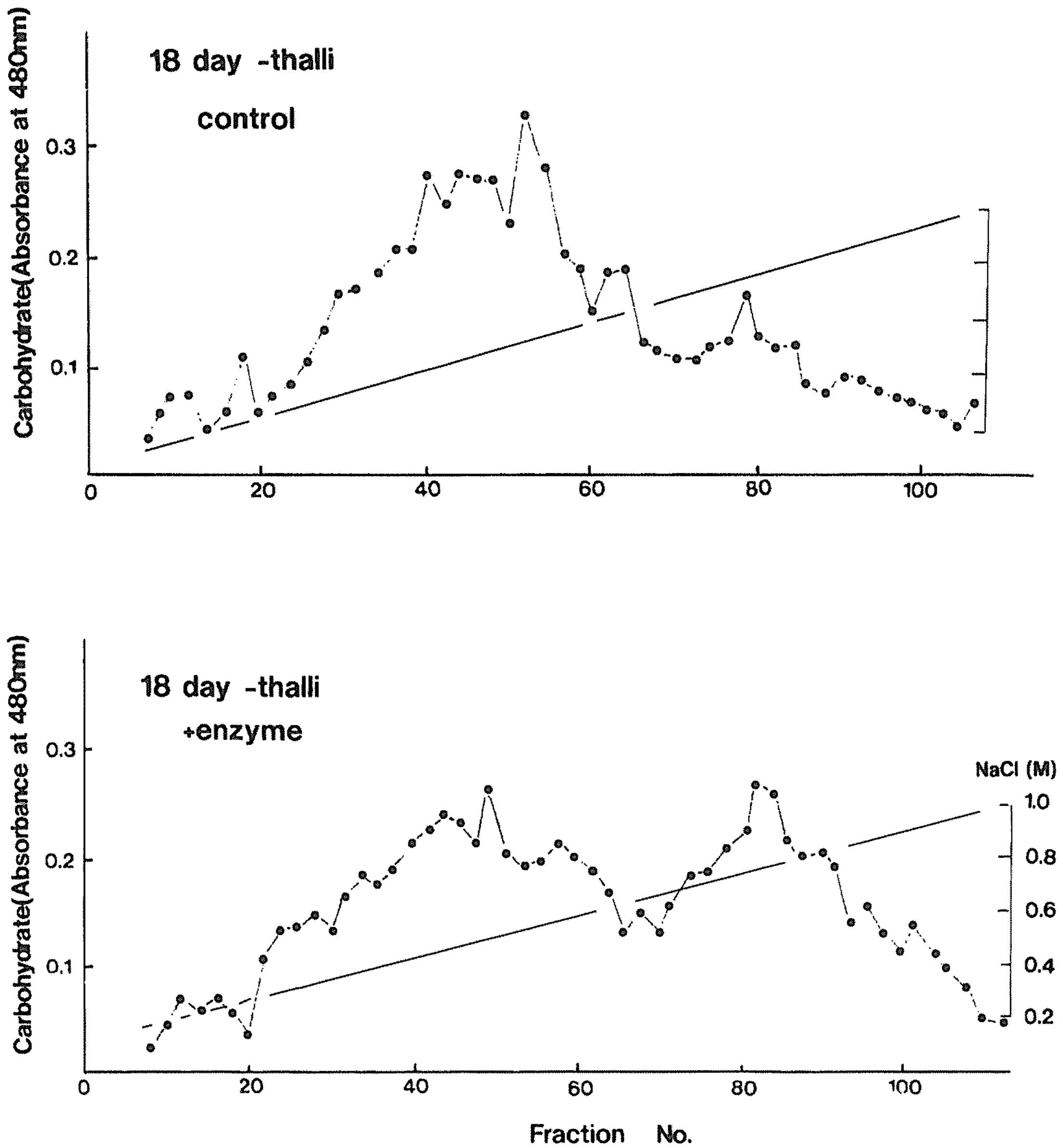

Fig. 4. Change in the elution pattern of the water-soluble polysaccharides by the enzyme treatment.
A solution of the water-soluble polysaccharides from 18 day-culture thalli was divided into two portions. Partially purified polysaccharide-degraded enzymes extracted from abalone mid-gut glands were added to one portion. 0.5 ml of 20 mM Na-phosphate buffer was added to the other portion. Both were incubated at 35 ℃ for 4 h, applied to a DEAE-Sephadex A-50 column and eluted as described in Fig. 2. The carbohydrate contents in each fraction were determined as described in Fig. 2.

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ノリ（海苔）葉体の硬さと多糖質との関連性

水上　譲・鬼頭　鈞・村瀬　昇・小林正裕

養殖スサビノリ（海苔）葉体の硬さは培養時間が長くなるにしたがって増大し、それにともない、葉体の主要な多糖質である水溶性画分多糖質の量も増加した。また、葉体の硬さの違いによって多糖質成分の分子サイズ（分子量）が異なり、柔らかい葉体では比較的低分子量な多糖質成分が、硬い葉体ほど高分子量な多糖質成分が多く認められた。このような分子サイズの変化は多糖質分解酵素を用いた *in vitro* 実験によっても示唆され、葉体の硬さや多糖質量の変化には水溶性多糖質成分の分子サイズの変化が関与しているものと推察された。