Capillary-Isotachophoretic Analyses of Algal Acidic Polysaccharides and Their Application to a Survey of Heparinoid Active Sulfated Polysaccharides in Chlorophyta

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A capillary-isotachophoresis system for the analyses of algal acidic polysaccharides was developed on the basis of some improvements on the conventional methods of analysis for glucosaminoglucans. Under the newly-developed conditions the zone responsible for the heparinoid active component (a rhamnan sulfate) in *Monostroma nitidum* was successfully separated from the zones of other acidic polysaccharides in crude polysaccharide fractions, since only one of the zones on the isotachopherogram of the crude polysaccharide fraction was detected on the isotachopherogram of the purified active rhamnan sulfate. In the analyses of the crude polysaccharide fractions from some related species, the corresponding zones with the same potential unit value as the purified active rhamnan sulfate from *M. nitidum* were observed only in species with considerably high activity. These results suggested that this new system, with simple, rapid and microscale procedures, is useful in examining the molecular homogenity of purified active sulfated polysaccharides, as well as in surveying the distribution of similar molecular species in related algal species.

Keywords capillary-isotachophoresis; sulfated polysaccharide; rhamnan sulfate; heparinoid activity; Chlorophyta; *Monostroma nitidum*

Heparin is well known as an acidic polysaccharide with remarkable anti-coagulant activity. Earlier studies with regard to its structural–activity relationship have revealed that its ester sulfate or sulfoamino group, as well as the sugar constituents, are associated with the biological activity. Heparinoids that referred to sulfated polysaccharides having similar activities to heparin include some naturally occurring sulfated polysaccharides in addition to synthetic products such as dextran sulfates[1] and chitosan sulfate.[2] The widely distributed major components of cell wall mucilages of seaweeds are known as sources of naturally occurring sulfated polysaccharides. Among them, fucose-containing sulfated polysaccharides from some species of Phaeophyta are known to have tentative heparinoid activity.[3–5] On the other hand, in green algae examined so far although sulfated polysaccharides with a variety of molar properties have been detected, little is known about their biological activities. During a recent survey of the heparinoids activity in cell wall polysaccharides in Chlorophyta, an anti-coagulant active component higher than standard heparin was found in *Monostroma nitidum*, and its structure was characterized as a rhamnan sulfate after further purification procedures.[6,7] In the course of these investigations, the conventional capillary-isotachophoresis (CITP) systems for the analyses of hyaluronic acid[8] and chondroitin sulfate[9] were improved to offer a clear zone for this active polysaccharide with the electric charge of a sulfate group. Furthermore, a survey on distribution of the active sulfated polysaccharides in related Chlorophyta species was also performed on the basis of biological assay techniques for the anti-coagulant activities, along with this newly-developed CITP system using the purified active rhamnan sulfate from *M. nitidum* as an authentic sample in mixed charging. This paper deals with the results of these investigations.[10]

Materials and Methods

Materials All the Chlorophyta species treated in this study, *M. angicava*, *M. fusem*, *M. grevillei*, *M. groculandicum*, *M. latissium*, *M. pulchrum* and *M. zostericola* in addition to *M. nitidum*, were collected in the spring and summer of 1985—1987 at several places along the Pacific coast near Tokyo and Hokkaido.[7] After being washed throughly in sea water to remove macro-epiphyta, they were air-dried for extraction.

Extraction The dried green algae samples were dipped into a 10 × volume of distilled water at room temperature for 1 h, homogenized and refluxed for 2 h in a boiling water bath. The hot water extracts thus obtained were then dialyzed against running tap water for 3 d, and lyophilization of the non-dialyzable portions finally afforded the crude polysaccharide fractions.

Purification of the Active Rhamnan Sulfate The crude polysaccharide fraction of *M. nitidum* was subjected to ion-exchanging column chromatography with DEAE cellulose, and the elutes obtained by a stepwise and linear gradient increase of the ionic strength of KCl were collected to be examined for anti-coagulant activity, respectively. Then, the fraction with the most potent activity was further applied to gel-filtrating column chromatography. The active polysaccharide thus purified exhibited a single and symmetrical peak in high-performance liquid chromatography and gave a single band in zone electrophoresis using cellulose acetate strips. The details are described elsewhere.[7]

Biological Assay Method of the Anti-coagulant Activity Relative anti-thrombin activity (R-ATA) values to the standard heparin of the examined samples were measured by the method of Shimada *et al.*,[11] as described previously.[7]

CITP CITP was carried out employing a Shimadzu IP-2A isotachophoretic analyzer equipped with a PGD-2 potential detector. The leading electrolyte was 0.01 M HCl adjusted to pH 3.6 by β-alanine containing 30% (v/v) methanol (MeOH) and 0.1% (v/v) Triton X-100 at their final concentrations, respectively, and the terminating electrolyte was 0.01 M sodium acetate. An aliquot of the aqueous solutions from samples to be examined (0.1—1.0 mg/ml) was injected, and separation was run in a Teflon tube (0.5 mm × 4 cm + 1 mm × 15 cm) maintained at 20 °C. Migration current was first stabilized at 125 μA for 3 min, then at 75 μA until the voltage was elevated to reach 15 kV and at 25 μA thereafter. Chart speed was 20 or 40 mm/min. All the chemicals in relation to CITP were of analytical grade.

Others Chromatographic analyses for constitute monosaccharides, as well as determination of the sulfate ester contents of the crude polysaccharide fractions from examined species, were performed as described elsewhere.

Results and Discussion

In the previous conventional CITP systems for the analyses of glucosaminoglucans, such as hyaluronic acid[8] and chondroitin sulfate,[9] the chloride ion mainly in the aqueous media and caproate ion were used as the leading and terminating electrolytes, respectively. However, direct

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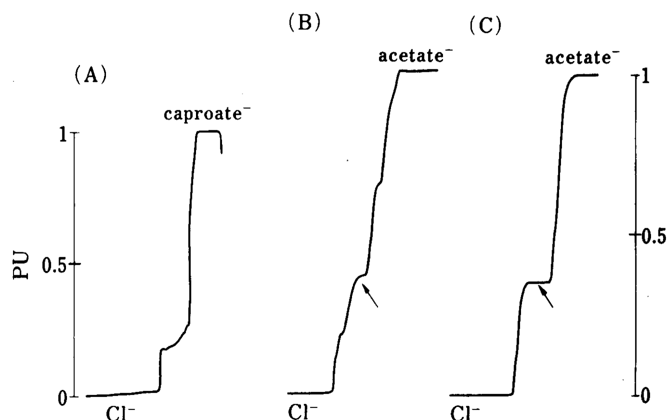

Fig. 1. Isotachopherograms of Acidic Polysaccharides of *M. nitidum*

(A) The crude polysaccharide fractions with an R-ATA value to heparin of 3.3 analyzed by the conventional system for glucosaminoglucans (leading electrolyte: 0.01 M HCl adjusted to pH 3.6 by β-alanine containing 0.1% (v/v) Triton X-100, terminating electrolyte: 0.01 M sodium caproate, sample: 40 μl of the 0.1 mg/ml aqueous solution of the crude polysaccharide fraction). (B) The crude polysaccharide fraction analyzed by the newly-developed conditions (leading electrolyte: 0.01 M HCl adjusted to pH 3.6 by β-alanine containing 30% (v/v) MeOH and 0.1% (v/v) Triton X-100, terminating electrolyte: 0.01 M sodium acetate, sample: 0.5 μl of the 1 mg/ml aqueous solution of the crude polysaccharide fraction. (C) The final purified active rhamnan sulfate with a R-ATA value to heparin of 6.0 (sample: 1.0 μl of the 1 mg/ml aqueous solution of the purified active rhamnan sulfate, other conditions: same as in (B)), the arrowed zone: the active rhamnan sulfate with a PU value of 0.35.

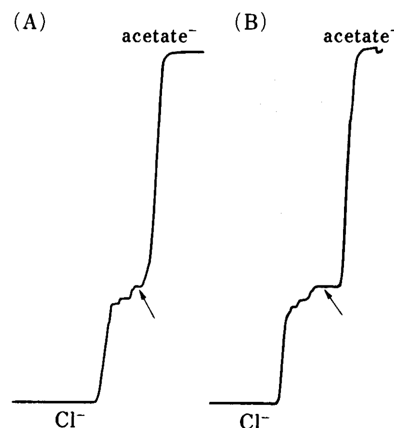

Fig. 2. The Identification of Acidic Polysaccharide by Mixed Analyses with the Purified Rhamnan Sulfate from *M. nitidium*

(A) The isotachopherogram of the crude polysaccharide fraction from *M. fusem* (sample: 2.5 μl from the 1 mg/ml aqueous solution). (B) The isotachopherogram obtained by mixed charging of the sample of (A) and the purified rhamnan sulfate from *M. nitidum*. The arrowed zone: active rhamnan sulfate with a PU value of 0.35, other conditions: same as in Fig. 1.

application of these systems to the analyses of the crude polysaccharide fractions from examined Chlorophyta species failed to achieve clear isotachophoretic separation, indicating that, as shown in Fig. 1A, under the conditions employed, the algal polysaccharides formed a mixed zone due to small differences in their potential unit (PU) values of near 0.2. The addition of MeOH to the leading electrolyte improved the resolution, but use of the MeOH-containing leading electrolyte caused a frequent occurrence of air bubbles, possibly due to MeOH addition. Yamamoto *et al.* found that the concentration levels of very hydrophilic polysaccharides were elevated in CITP beyond their solubilities.[12] In order to prevent the occurrence of this phenomenon, conditions for the MeOH concentration in the leading electrolyte and for the migration current were determined as described in Materials and Methods, and analyses of more than 5 μg (as the materials were injected) were also avoided. Furthermore, the use as a terminating ion of acetate ion, of which mobility as an anion is smaller than that of the conventionally employed caproate ion,[8,9] succeeded to give the appropriate PU value (0.35) to the subject substance in purification of the heparinoid active component from *M. nitidum*, as described below.

Under the newly-developed conditions, several zones were observed on the isotachopherogram of the crude polysaccharide fraction from *M. nitidum* (Fig. 1B), while in Fig. 1C, only one zone with a PU value of 0.35 was found on the isotachopherogram of the final purified active polysaccharide (a rhamnan sulfate), even after injection of twice the volume of the aqueous sample solution with the same concentration as in the former. This data seemed to support the results obtained using conventional techniques[7] (see Materials and Methods), suggesting that the heparinoid active rhamnan sulfate in *M. nitidum* was purified successfully to exhibit molecular homogenity in electrical charge, molecular weight, *etc.* So, in order to elucidate the distribution in related Chlorophyta species of similar active polysaccharide(s), the crude polysaccharide fractions from 7 *Monostroma* species other than *M. nitidum* were surveyed using the present CITP system for the component(s) responsible for the zone with a PU value of 0.35.

TABLE I. The Biological and Analytical Data of the Crude Polysaccharide Fractions from Chlorophyta Species Examined

Monostroma species	R-ATA values to heparin[a]	Sulfated ester contents (%)	Length of the PU = 0.35 zone[b] (mm)
M. nitidum	3.3	25.0	4.0
M. zostericola	2.1	23.8	0[c]
M. angicava	1.7	19.3	2.5
M. lattisium	1.3	16.5	2.0
M. pulchrum	0.5	6.2	0[c]
M. groculandicum	0.2	5.5	0[c]
M. fusem	2.4	16.7	1.5
M. grevillei	2.1	17.1	1.5

a) R-ATA values to heparin of the crude polysaccharide fractions. *b*) For 1 μg of the crude polysaccharide fractions injected to CITP. *c*) Not detected by the injection of 5 μg of the crude polysaccharide fractions.

The results indicated that the corresponding zone was clearly observed on the isotachopherograms of *M. angicava*, *M. fusem*, *M. grevillei* and *M. lattisium* in addition to *M. nitidum* in analyses of up to 25 μg of the crude polysaccharide fractions as the materials injected (as shown in Fig. 2, the identifications were done on the basis of elongation of the zone length in mixed charging with the purified active rhamnan sulfate from *M. nitidum* as the authentic sample). However, in the cases of *M. groculandicum*, *M. pulchrum* and *M. zostericola*, such zones could not be detected even in analysis of 5 μg of the materials (the crude polysaccharide fractions) injected (as described above, analyses of more than 5 μg of the materials injected were avoided in this system). The lengths of the PU = 0.35 zone for 1 μg of the materials injected, as well as the R-ATA values to heparin and the sulfate ester contents of the crude polysaccharide fractions, are summarized in Table I. As reported elsewhere, the R-ATA values to heparin correlated positively with the

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sulfate ester contents.[7] The data on Table I seemed to funther show that the high R-ATA values to heparin and large sulfate ester contents were generally associated with detection of the PU = 0.35 zone, since the zone was detected in only 5 species, including *M. nitidum*, having high R-ATA values to heparin of 1.3—3.3 as well as large sulfate ester contents of 16.5—25.0% (the zone lengths for 1 μg of the materials injected ranged from 1.5 to 4.0 mm. These values could not be detected in *M. groculandicum* (the R-ATA value to heparin: 0.2, the sulfate ester contents: 5.5%) and *M. pulchrum* (0.5 and 6.2%, respectively) even after injection of 5 μg of the crude polysaccharide fractions. The exception was *M. zostericola*, in which the zone was not detected despite a high R-ATA value to heparin (2.1) and a considerably high sulfate ester content (23.8%). This data suggested that *M. angicava*, *M. fusem*, *M. grevillei* and *M. lattisium* had molecular species of acidic polysaccharide(s) similar to that of the active rhamnan sulfate in *M. nitidum* and that the heparinoid activities in these 4 species were associated at least partly with the component(s) responsible for the PU = 0.35 zone. The data also seemed to indicate that *M. groculandicum* and *M. pulchrum*, as well as *M. zostericola*, did not contain a detectable amount of such component(s), and that *M. zostericola* had active polysaccharide(s) with different properties from the rhamnan sulfate in *M. nitidum*. Indeed, several zones with PU values other than 0.35 were observed on the isotachopherogram of *M. zostericola* (data not shown) and as revealed previously.[7] The major constituent monosaccharide in the crude polysaccharide fraction of *M. zostericola* is galactose rather than rhamnose, the major constituent of the five species mentioned above with high R-ATA values to heparin and large sulfate ester contents in addition to the PU = 0.35 zone. It was therefore assumed that the heparinoid activity in *M. zostericola* might be derived from acidic polysaccharide(s) other than rhamnan sulfate(s). This disagrees with the other five species that exhibit high activities.

The above-presented CITP system requires only up to 5 μl of the 1 mg/ml aqueous solutions of the crude polysaccharide fractions and one analysis takes less than 20 min. It is therefore considered that this system is suitable for rapid and microscale analyses in relation to the chemical studies of heparinoid active algel polysaccharides, especially in examining the molecular homogeneity of the purpose substances, as in the case of active rhamnan sulfate from *M. nitidum*. This system can also by valuable in surveying the distribution in related species of similar molecular species to the purified active polysaccharides, as in the cases of the component(s) responsible for the PU = 0.35 zone.

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