Distribution of heparinoid-active sulphated polysaccharides in some Indian marine green algae

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Sixteen species of Indian marine green algae belonging to the genus Caulerpa, Cladophora, Bryopsis, Boodlea, Chaetomorpha, Ulva, Enteromorpha and Valoniopsis were screened for blood anticoagulant activity. Cold and hot water sulphated polysaccharides extracts were prepared, their chemical constituents i.e. sulphate, sugar, protein and uronic acids were estimated and molecular weight (MW) range was determined. Blood anticoagulant activity was evaluated by prothrombine time (PT) test. It was found that activity was associated with sugar and sulphate contents and MW. Extracts possessing comparatively high molecular weight ($12-14 \times 10^5$) with higher sugar ($\geq 20\%$) and sulphate ($\approx 8\%$) and lower protein ($\approx 12\%$) and low uronic acid ($\approx 2\%$) contents showed maximum activity. Species of Caulerpa exhibited highest activity which is comparable to heparin i.e. they contained 93-151 heparin units/mg (IU/mg), species of Ulva and Enteromorpha showed feeble activity ($\approx 6.0$ IU/mg) and all other species had moderate activity with $\approx 60$ IU/mg. Thus, SPS isolated from different Chlorophycean algae had different chemical composition, MW range and blood anticoagulant activity which were comparable within species level, but those differed in degrees from one genus to another.

Marine algae are used as food, feed and medicine$^{1,2}$. In folk medicines seaweeds have been used for a variety of remedial purposes such as in eczema, gallstone, gout scrofula, cooling agent for fever, menstrual trouble, renal trouble, scabies etc.$^{1,2}$. Marine animals and plants are reported to have wide spectrum of interesting biological properties and the compounds isolated from marine source have the chemical structures which are not commonly found in terrestrial counter parts. Sulphated polysaccharides (SPS), reported to have wide pharmacological properties$^3$ are commonly found in marine algae and higher animals and scarcely present in microbes and absent in higher plants. Heparin, a highly sulphated polysaccharide present in mammalian tissues, is used as blood anticoagulant both in the laboratory and therapeutically$^4$. The sulphated polysaccharides from marine algae are of highly diverse nature and there exist similarities between their structure with that of heparin. The anticoagulant activity of SPS from marine algae was first reported by Chargaff$^{5}$ et al.$^5$. Heparin has some disadvantages, i.e. it is extracted from internal organs of higher animals and purified, hence its recovery is difficult, and it exhibits haemorrhagic side effects, hence, there is a need for novel/alternative anticoagulants. Anticoagulant activity associated with the SPS of red and brown algae have been widely reported, Deacon-Smith et al.$^6$ conducted a study of the anticoagulant effects of British marine algae, and reported such activity in green algae for the first time and subsequently many green algae were reported for their blood anticoagulant activity$^7,9$. Distribution of anticoagulants in marine algae, their chemistry, potency and mechanism of action has recently been reviewed$^{10}$. Attempts on clinical use of algal anticoagulants have been made and the findings suggest that the SPS might be used in the anticoagulation treatment of refractory nephrosis$^{11}$. Heparin analogues (heparinoids) that are inhibitory to thrombin activities have been reported from marine green algae (Chlorophyta) of Indian coasts for the first time$^{12,13}$. Thirteen species of marine algae belonging to the family Codiaceae were screened for blood anticoagulant activity. It was observed that blood anticoagulant activity (cf. Codium spp.) is generally higher with samples containing higher sugar and sulphate contents$^{11}$. Sulphated arabinin, the most active component was isolated from Codium dwarkense and C. tomentosum$^{11,14,15}$. In continuation of the research on the identification of Indian marine algae possessing promising anticoagulant activity, 16 species of green algae were screened and the results are reported in this paper.

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Material and Methods

Plant material—Sixteen species of Indian marine green algae i.e., Caulerpa racemosa, (Forssk.) Weber v Bosse, C. taxifolia (Vahl.) Ag., C. scapelliformis (R.Br.) Web. v. Bose, C. veravalensis Thivy and Chauhan, C. peltata Lamour. of Caulerpaceae, Cladophora fascicularis (Mertens) Kuetz., Chaetomorpha media Boerg., C. torta (Farlow) McClatchie of Cladophoraceae, Boodlea composita (Harv. Et Hook.f.) Brand and Valaniopsis pachynema (Martens) Boerks., of Valoniaceae, Bryopsis plumosa (Huds.) Ag., of Protosiphonaceae, Ulva fasciata Delile, U. reticulata Forssk., U. lactuca Linnaeus, Enteromorpha elathrata (Roth) J.Ag. and E. compressa (Linn.) Grev. of Ulvaceae were collected from Okha (lat 22° 28' N, long. 69° 05'E), Veraval and Diu (lat. 20° 54'N, long. 70° 22'E)/Porbandar (lat. 21° 38'N, long. 69° 37'E.) and Mandapam (lat. 08° 45' N, long. 78° 12'E) coasts. Algal species were collected once from the lower intertidal zones during the period October 1998 to April 2000. Voucher specimen of each species have been deposited with CSMCRI Herbarium, Bhavnagar, India. Algae were thoroughly cleaned by removing contamination of other algae, epiphytes and animal castings, materials were quick-washed with tap water and allowed it to return to room temperature. The cold water extract was first filtered through muslin cloth and then with filter paper (Whatman 541 with celite). Extract was concentrated to 1/10 of its original volume under reduced pressure. The concentrated extract was precipitated with acetone (4 volumes v/v), dehydrated and then dried at 40°C till acetone-free. The dried product was dissolved in minimum distilled water and dialysed till it was chloride-free. The dialysate was centrifuged at 8000 rpm for 30 min to remove water insoluble matter and lyophilised to yield crude cold water extract. The algal residue of cold water extraction was soaked in sufficient distilled water and heated at 80 °C for 3 h and hot water extract was obtained following the same procedure used for the cold water extraction.

Chemical analysis — Yield of the crude sulphated polysaccharide (SPS) extracts was calculated on the basis of the dry weight of algal sample. The SPS sample was ignited to ash at 550°C for 6 h and percentage of ash content was calculated based on the weight of oven dried (110°C for 2 h) sample. Total sugar was estimated by Dubois et al. 17 method. Protein content was estimated by Lowry et al. 18 method using albumin as a standard. Sulphate content of the SPS sample was estimated by hydrolysing the sample with 1.0 N HCl and measured by BaCl2-gelatin method 19 whereas uronic acid content was determined by the method of Knutson & Jeans 20 using galacturonic acid as a reference. Infrared spectra were recorded using KBc pellets on a Perkin Elmer spectrum GX FT-IR system. CHNS (carbon, hydrogen, nitrogen and sulphur) analysis were done on Perkin-Elmer Series II-2400 CHNS/O Analyzer.

Molecular weight determination — Gel filtration chromatography was used to determine the molecular weight and it was carried out on a Seralose CL-4B (SRL) column (45 x 2.5 cm) eluted with 0.2 M NaCl containing 0.02% sodium azide at 25°C at a flow rate of 4.4 ml/h. Molecular weight of the sample was determined from standard graphic curve on logarithmic scale [log (MW) against Ve/Vo where Ve is elution volume of the standard marker and Vo is elution volume of blue dextran i.e. bed volume (10.5 ml). Polymeric dextrans having different molecular weights (16.0x10^4, 6.6 x10^5, 1.62x10^5, 8.7x10^4, 6.6 x10^4, 1.95x10^4) and blue dextran (2.0x10^6) were used as markers. Molecular weight range of the polysaccharide samples were calculated using the initial and final elution volume values (Ve’s) of the peak obtained in the gel column.

Evaluation of blood anticoagulant activity—Blood was collected from healthy volunteers and normal human plasma was prepared in the following manner: Blood was collected using a disposable polypropylene syringe and anticoagulated using 3.8% tri-sodium citrate in a polypropylene container (9 parts of blood to 1 part of tri-sodium citrate solution) and it was centrifuged immediately at 4000 rpm for 15 min. Plasma was separated and pooled. Algal SPS samples were prepared in normal saline (0.85% NaCl) solution and anticoagulant activities were measured by Prothrombin time test (PT) 21. Liquiplastin [TULIP Diagnostics (P) Ltd, Goa, India] was used as a source of thromboplastin. Clotting tests and control were performed in duplicate, and the average of the
Results

Chemical composition and blood anticoagulant activity of cold and hot water extracts of sixteen green algal species are presented in Table 1. Freeze-dried SPS of all species were colorless to brownish in color, the texture of colorless SPS products were fibrous and fluffy and colored ones were powdery in nature. All the texture of colorless SPS products were fibrous and they ranged between 8.8-16.3 and 4.8-14.8% respectively and minimum was in Valoniopsis pachynema i.e. 4.7-6.2%. Trace of uronic acid was found in extracts of all species and it ranged between 2-4%. Species of Ulva and Enteromorpha contained 7.0 - 15.0 % and Caulerpa species had 10.0 - 17 % protein. Maximum protein was observed in cold and hot water extracts of Bryopsis plumosa (Table 1). Molecular weight of green algal SPS studied here had ranged between 0.19 - 15 × 10^5 D. Cold water extracts of all green algae had low MW (0.19 - 8.15 × 10^5 D) than those of hot water extracts (1.0 - 15.0 × 10^5 D). Wide range of MW distribution was observed in hot water extracts of Ulva fasciata, Ulva lactuca, Valoniopsis pachynema, Bryopsis plumosa, Cladophora fascicularis and Caulerpa peltata.

The common bands observed in IR spectrum of all SPS sample were 3425(br, s), 2930(sh), 2367(w), 2341(s), 2159(sh), 1648 (br, s), 1561(sh), 1545(sh), 1425(m), 1381 (sh), 1377(sh), 1256(s), 1044 (s), 926(sh), 848(w), 825(w), 789(s) 730, 750 and 614(brs) cm⁻¹. The band intensities are: [s=strong, w=weak, sh=shoulder, br=broad, m=medium]. The characteristics bands for algal SPS, strong band at ≈ 1250 cm⁻¹ indicate the presence of ester sulphate (S = O stretching). The other absorbance are 825 cm⁻¹ (C-6 sulphate), 848 cm⁻¹ (C-4 sulphate), 1648 and 1545 cm⁻¹ (-CO-NH-), 730, 750 and 1065 cm⁻¹ (C-S linkage).

With different concentrations (100 -2000 μg/ml) of algal SPS products, blood anticoagulant activity was evaluated. All cold and hot water extracts of Caulerpa spp. exhibited potent activity (≈140 IU/mg) than extracts of other species. Cold water extract of Caulerpaceae plants except C. taxifolia showed higher activity than their respective hot water extracts (Table 1). The maximum activity was observed in cold water extract of Caulerpa peltata with heparin units ≈151 IU/mg. In the case of Cladophora fascicularis, Boodlea composita, Chaetomorpha media and C.torta, Valoniopsis pachynema and Bryopsis plumosa, hot water extracts were more active than their respective cold water extracts with moderate activity (60 IU/mg), however, hot water extract of Chaetomorpha torta and B. plumosa had good activity with heparin units 122 and 88 IU/mg respectively. Both cold and hot water extracts of Ulva and Enteromorpha spp. showed feeble anticoagulant activity(≈6.0 IU/mg).

Discussion

Sulphated polysaccharides extracted from green algal species of Indian waters showed promising anticoagulant activity and activity was associated with chemical composition and MW. Total sugars, sulphate, protein and uronic acid contents and MW range distribution of cold water and hot water extracts of sixteen species of Indian marine green algae were
investigated. All these extracts were evaluated for their blood anticoagulant activity taking different concentration. Both cold and hot water extracts of all Caulerpa species possessing different MWs and contain comparatively higher sugar and sulphate contents and lower protein and uronic acid contents exhibited promising 33 anticoagulant activity (≈ 140 IU/mg) which were very much comparable to heparin. Particularly, cold water extract of C. peltata exhibited highest activity with heparin units 151 IU/mg. It was observed that anticoagulants of Caulerpa were located comparatively in high MW fraction. In general, extract with comparatively high content of sugar and sulphate showed considerable activity. Hot water extract of C. fascicularis, B. composita, C. media, C. torta, V. pachynema and B. plumosa exhibited higher activity than those of their respective cold water extracts, and their heparin units were 60.8, 74.2, 47.2, 122.2, 53.5 and 88.9 IU/mg respectively; heparin units of cold water extracts were 45.2, 16.9, 45.9, 63.3, 52.5 and 55.9 IU/mg respectively. It was observed that anticoagulant activity is associated with sugar, sulphate contents and molecular weight range. Extract with low MW and comparatively high uronic acid and protein content showed no activity or they were flaccid, and this has been observed in the extract
of *Ulva* and *Enteromorpha*. Therefore, it can be concluded that higher blood anticoagulant activity is associated with relatively high contents of sugar and sulphate and lower protein and uronic acid contents with high MW range in very complex proportion and this observation is in good agreement with previous reports.\textsuperscript{12,22,24} Though high MW compound having anticoagulant potency is not very common, it is reported for algal anticoagulants.\textsuperscript{7,12,14,25}

Pericival & McDowell\textsuperscript{16} reported that different species have different neutral sugar composition in varying amounts, i.e. the major neutral sugar composition of *Caulerpa* are galactose, xylose, arabinose; in *Cladophora*, galactose, arabinose and xylose; in *Chaetomorpha* arabinose, galactose and xylose in the increasing order of proportion; in *Bryopsis* xylose and in *Ulva* and *Enteromorpha* rhamnose are being major ones. Nature of individual sugar of SPS may have definite role in manifesting anticoagulant activity as they vary in quantity of activity with varying sugar composition. Species of *Caulerpa* comprising galactose and xylose as major sugars and these sugar sulphates may have role in exhibiting promising activity. In moderately active alga, neutral sugar composition is different. For example, arabinose and galactose in *Chaetomorpha*, galactose and arabinose in *Cladophora* and xylose in *Bryopsis*. Shanmugam\textsuperscript{12} and Shanmugam \textit{et al.}\textsuperscript{14} have reported that anticoagulant of *Codium dwarkense* and *C. tomentosum* contained solely arabinose and same was observed in *C. latum* of Japanese waters.\textsuperscript{9} Galactan sulphates possessing potent anticoagulant activity have been isolated from a red alga *Grateloupi* *indica*\textsuperscript{25} and fucoidan sulphates (fucose polymers) are identified as active component from brown algae.\textsuperscript{24} However, further studies are required to locate molecular species and their sugar composition responsible for activity which are found in the promising species of the present investigation.

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