ELSEVIER

Carbohydrate Research 289 (1996) 201–208

CARBOHYDRATE RESEARCH

Note

Anticoagulant low molecular weight fucans produced by radical process and ion exchange chromatography of high molecular weight fucans extracted from the brown seaweed *Ascophyllum nodosum*

Alain Nardella [a], Frederic Chaubet [a,*], Catherine Boisson-Vidal [a], Catherine Blondin [a], Patrick Durand [b], Jacqueline Jozefonvicz [a]

[a] *Laboratoire de Recherches sur les Macromolécules, CNRS URA 502, IFREMER, URM 2, Institut Galilée, Université de Paris-Nord, Av. J.B. Clément, F-93430 Villetaneuse, France*

[b] *Laboratoire de Biochimie et Molécules Marines, IFREMER, B.P. 1049, Rue de l'Ile d'Yeu, F-44037 Nantes, France*

Received 5 January 1996; accepted in revised form 12 April 1996

Keywords: Fucan; Seaweed; Anticoagulant activity; Radical process degradation; Ion exchange chromatography

Sulfated polysaccharides extracted from marine algae represent a source of marine compounds with potential applications in medicine. Mention of the use of seaweed products is found in traditional Chinese herbal medicine as early as the sixteenth century [1,2]. Among these, fucans isolated from the cell walls of marine brown algae [3,4] and echinoderms (sea cucumbers and sea urchins) are found [5]. Their anticoagulant activity is low as compared to heparin [6,7], yet they have numerous other biological properties; they are antithrombotic [8], antiinflammatory [9], antiviral [10] and antiangiogenic [11]. Moreover, they can modulate cell adhesion [12], growth factor release [13], clinically relevant events such as tumor metastasis [14,15], and block sperm–egg binding in various species [16,17]. All these activities confer to these polysaccharides potential applications in human and veterinary health care, while taking advantage of the absence of potential risk of contamination by animal viruses. As by-products of alginate

* Corresponding author.

PII S0008-6215(96)00110-3

preparation in the food and cosmetic industries, they also represent a cheap source of molecules of biological interest.

The structural analysis of fucans extracted from the brown seaweed *Ascophyllum nodosum* (*An*) has received little attention from chemists [18–20], while their multipurpose potential has attracted general interest. The sulfated polysaccharides were identified mostly as 4-sulfated 2- or 3-linked α-L-fucopyranans with branched heterosaccharides, such as D-xylose, D-glucuronic acid, D-mannose and D-galactose, and sulfate. In addition, several structurally different polysaccharide components are found [5,21]. This structural heterogeneity is an impediment for a precise characterization using nuclear magnetic resonance spectroscopy [5] or any other spectral technique. Furthermore, secondary structure and structure–activity relationships remain to be established for this class of compounds. They can bind to a large number of proteins as a consequence of their ionic behaviour. Until now, their affinity for most proteins appears to be determined mainly by their negative charge, molecular weight and degree of sulfation rather than by any specific carbohydrate structure [15,22–24] as formally described for heparin [25]. Low molecular weight fucans (LMWF, M_p = 11,000–40,000 g/mol) have been previously produced in low yields by acidic hydrolysis followed by low pressure size exclusion chromatography, starting from crude acidic extracts from various brown seaweeds [4,26]. These fractions exhibited anticoagulant activity similar to heparin [6]. To formally demonstrate the existence of a specific carbohydrate structure responsible for the anticoagulant activity of fucans, it is of interest to prepare and purify in good yields LMWF exhibiting anticoagulant activity, i.e. containing the supposed active site.

In this report, we focus on the relationships between the structure and the anticoagulant activity of LMWF expressed as activated partial thromboplastin time (APTT) activity. LMWF were prepared by radical process degradation from high molecular weight fucans (HMWF) extracted from *An*. This method was used in high yields and good reproducibility by Volpi et al. in the case of heparin and dermatan sulfate [27,28].

Table 1
Characterization of fucan fractions [a]

Fraction	Yield (%)	L-Fucose (g/100 g)	D-Glucuronic acid (g/100 g)	$-SO_3Na$ (g/100 g)	Mp (g/100 g)	Polydispersity (Mw/Mn)	APTT (IU/mg)
F1	—	31.3	5.7	26.1	556,000	2.2	9.1
F2	—	35.8	11.6	18.4	516,000 [b]	na	12.1
DF1	47	36.4	2.6	29.7	8,300	1.5	8.2
DF2	50	32.2	6.5	30.1	7,800	1.7	7.3
F21	30	23.1	23.5	14.2	156,000	2.1	2.2
F22	40	43.2	1.7	35.3	600,000 [b]	na	25.3
DF21	13	22.5	17.7	20.2	6,000	1.7	0.2
DF22	50	42.7	1.3	35.0	13,000	1.8	7.6

[a] Chemical analyses had a SD of 5–15% and the activity assay of 10–20%. M_p were given ±3000 g/mol.
[b] Shoulder at 100,000 g/mol.

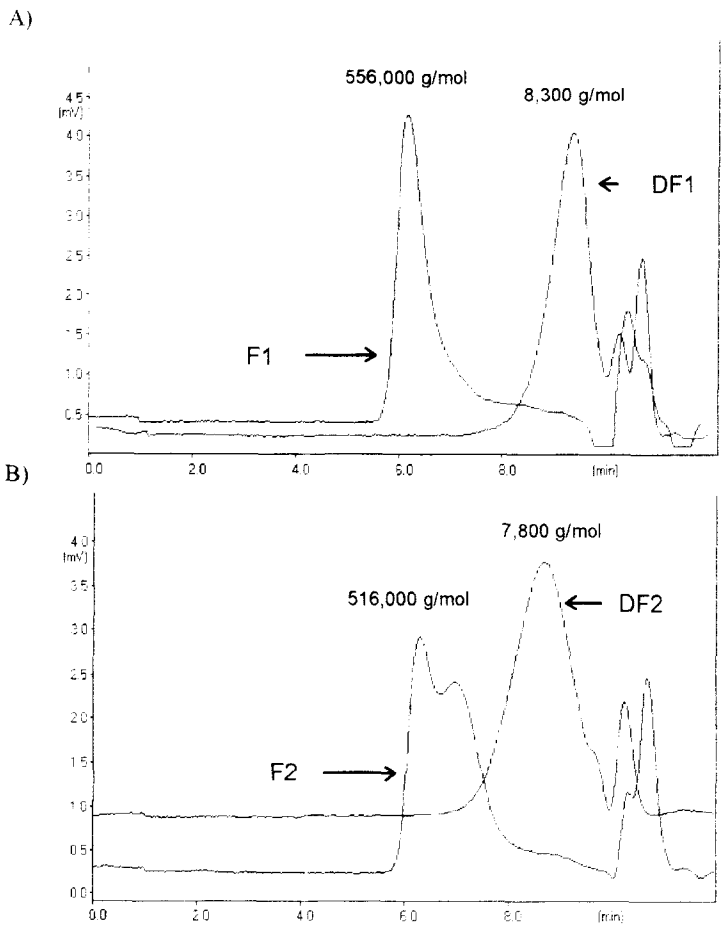


Fig. 1. HPSEC chromatograms of fractions F1 and DF1 (A) and F2 and DF2 (B).

Ion-exchange chromatography, subsequently performed on LMWF, enabled the isolation of an anticoagulant fucan fraction.

The radical process degradation proceeds through the formation of free radicals from the hydrogen peroxyde–cupric redox system [29]. At neutral pH, these species are very reactive and degrade the polysaccharide backbone. Two HMWF, extracted from *An* as previously described [4], were degraded: F1 (M_p = 556,000 g/mol) and F2 (M_p = 100,000 and 516,000 g/mol). Compositions and APTT activities of all fractions are presented in Table 1. Fractions F1 and F2 were degraded at 60 °C in a temperature controlled reactor with addition of hydrogen peroxyde performed continuously for 5 h. High performance steric exclusion chromatograms of LMWF DF1 (M_p = 8300 g/mol) and DF2 (M_p = 7800 g/mol) are presented in Fig. 1A and B. In the case of F1, samples were monitored during the degradation process and analyzed with HPSEC. After 30 min, a 66,000 g/mol fucan fraction peak with a 19,000 g/mol shoulder was obtained.

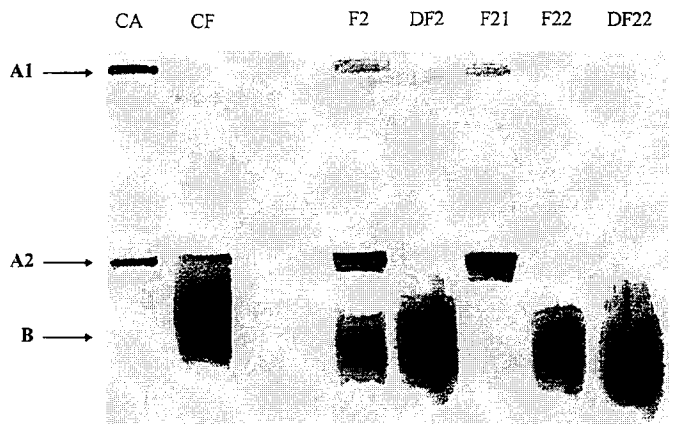
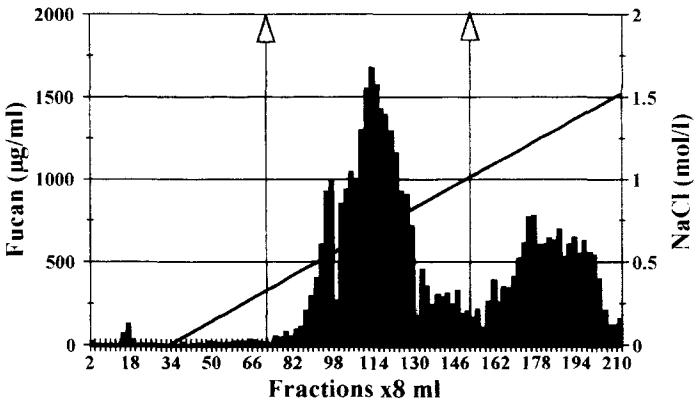


Fig. 2. Electrophoreses on cellulose acetate membrane of commercial ascophyllan (CA), commercial fucoidan (CF), HMWF F2, LMWF DF2 and fraction obtained by anion exchange chromatography of F2 (F21 and F22) and DF2 (DF22).

Between 1 h and 5 h, the molecular weight decreased to give DF1 in a yield of 47%. The degradation process was similar for F2 (data not shown), leading to DF2 in similar yields. DF1 and DF2 presented a more narrow molecular weight distribution as compared to the parent fractions. However, the charged groups composition of DF1 and DF2 was different from that of the starting compounds. Electrophoresis on cellulose acetate membrane was performed with F2 and DF2 and compared qualitatively with commercial fucoidan (CF) rich in sulfated L-fucose and commercial ascophyllan (CA) rich in uronic acid (Fig. 2). The latter presented two narrow bands and CF migrated as a large one. We have observed that F2 migrated as a mixture of CA and CF. In the case of DF2, the radical process degraded mainly the ascophyllan-like species.

Low pressure ion-exchange chromatography experiments were then performed on F2 and DF2 in order to study the role of the different species in the APTT activity of fucan. In Fig. 3A and B are displayed the ion exchange chromatograms of F2 and DF2 fractions which respectively led to four fractions: two HMWF F21 (M_p = 156,000 g/mol) and F22 (M_p = 600,000 g/mol) and two LMWF DF21 (M_p = 6000 g/mol) and DF22 (M_p = 13,000 g/mol). Fractions F21 and DF21, collected with a gradient of 0 to 0.75 M sodium chloride, showed a high amount of D-glucuronic acid (17 to 23 g/100 g) as compared to F2 (11.6 g/100 g) and a decrease in the L-fucose and sulfate contents. Their APTT activities (2.2 and 0.2 IU/mg) were much lower as compared to F2 (12.1 IU/mg). In contrast, F22 and DF22, eluted above 0.75 M sodium chloride were enriched with sulfate (35 g/100 g) and L-fucose (43 g/100 g) while their D-glucuronic acid content was fivefold lower than that of the parent compounds. The APTT activity of F22 (25.3 IU/mg) was found twice that of F2. We attributed the lowest APTT activities of DF2 and DF22 (7.3 and 7.6 IU/mg), compared to that of F2, to their low molecular weight. Electrophoresis performed on cellulose acetate membrane with F21, F22, and DF22 suggest that F21 can be mainly assigned as ascophyllan-like species (A1 and A2 on Fig. 2 and F22 and DF22 as fucoidan-like species (B on Fig. 2).

A) F2



B) DF2

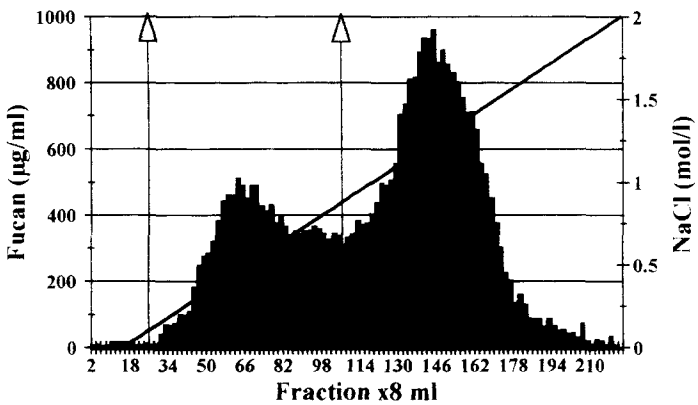


Fig. 3. Low pressure anion exchange chromatograms of fractions F2 (A) and DF2 (B).

These results indicate that fucan extracted from *An* could be a mixture of polysaccharides with composition close to ascophyllan and fucoidan and that a fucoidan-like fraction only is responsible for the APTT activity of these compounds. Radical process degradation followed by anion exchange low pressure chromatography is an improved methodology, as compared to acidic degradation followed by size exclusion chromatography, in order to obtain active LMWF in an overall yield of 25% from the starting material. This may lead to fruitful developments in anticoagulant drug production from seaweeds with high yields and good reproducibility.

1. Experimental

Common chemicals were purchased in analytical grade from Carlo Erba, Fluka and E. Merck and used without further purification. Heparin H410 used for APTT activity was obtained from Choay-Sanofi. HMWF F1 and F2 were obtained from *Ascophyllum nodosum* collected by IFREMER (Nantes) as previously described [4]. All characterizations were performed on fucans dried overnight at 40 °C under vacuum.

Preparation of low molecular weight fucans (LMWF).—Fucan (1.0 g) and copper acetate monohydrate (0.08 g, 0.40 mmol) were dissolved in water (20 mL) at 60 °C in a temperature controlled reactor and the pH was maintained at 7.5 by automatic addition of 2 M NaOH. A 9% (v/v) hydrogen peroxide solution was added with a peristaltic pump at a flow rate of 12 mL/h. The reaction was stopped after 5 h and Chelex 100 chelating resin (Biorad) was added to remove copper from the medium. After neutralization with 0.1 M NaOH, the solution was desalinated under stirring against bidistilled water in a diafiltration cell (Filtron) using a 1000 g/mol cut-off membrane and freeze-dried to yield LMWF.

Ion-exchange chromatography of LMWF.—F2 or DF2 (0.5 g) was fractionated by anion-exchange chromatography on a column (2.6 cm × 40 cm) of DEAE Sepharose CL6B (Pharmacia) with conductimetric monitoring. Elution was performed at 1.6 mL/min with water for 4 h, then with a linear NaCl gradient (0–2 M) to elute ionic fractions. Two fractions were collected according to their fucan concentration, as determined by the phenol–sulfuric acid method as described by Dubois et al. [30], then concentrated, desalinated as described above and finally freeze dried.

Molecular weight determinations.—The molecular weights were determined by high-performance steric exclusion chromatography (HPSEC) in 0.15 M NaCl, 0.05 M NaH_2PO_4 at pH 7.0, using a Licrospher Si 300 diol column (Merck-Clevenot) and a Hema Sec Bio 40 column (Alltech) connected in series. The columns were calibrated with standard polysaccharides of narrow molecular weight distribution (pullulans: 853,000–5800 g/mol, Polymer Laboratories, Interchim; dextran: 1 500 g/mol; melezitose: 522 g/mol, Fluka; sucrose: 342 g/mol; glucose: 180 g/mol, Sigma). Number-average ($\overline{M}_n$), weight-average ($\overline{M}_w$), peak-molecular weight (M_p) and polydispersity ($I = \overline{M}_w/\overline{M}_n$) were determined using the Chromstar software (Bruker, Merck-Clevenot).

Fucose.—Fucose content was determined using the cysteine–sulfuric acid method as described by Dische [31].

D-*Glucuronic acid.*—D-Glucuronic acid content was determined using the modified *m*-hydroxydiphenyl-sulfuric acid method of Filisetti and Carpitta [32] with D-glucuronic acid as standard (Sigma).

Sulfate.—Sulfate content was determined from elemental analysis of sulfur performed at the Service de Microanalyse du CNRS (Gif/Yvette).

Electrophoresis.—It was performed on a cellulose acetate membrane (4.7 × 15 cm^2, Sartorius) which was soaked in 0.1 M zinc acetate (pH 6.6). A fucan solution (2 μL, 10 mg/mL) was applied to the membrane at 3 cm from the cathode end. Electrophoresis was run for 1 h at 200 V (Helena). After the electrophoretic run, the membrane was immersed immediately in a staining solution (0.05 M magnesium chloride; 0.025 M sodium acetate buffer pH 5.8 in 50% v/v EtOH–water) containing 0.2% (w/v) of

alcian blue, and left at room temperature for 30 min. The membrane was then washed three times with 0.05 M magnesium chloride; 0.025 M sodium acetate buffer pH 5.8 in 1:1 EtOH–water. Finally, the membrane was immersed for 10 to 15 s in 25% (v/v) acetic acid and MeOH. Commercial ascophyllan (CA) and fucoidan (CF) (Sigma) were used as standards.

APTT activity.—The activity of each fucan sample was obtained using the APTT kit (Organon) of either buffer Owen Koller (controls, 100 μL), heparin (H410, Institut Choay, Sanofi, 170 IU/mg) solution (0–1 μg/mL), or fucan dilution (0 to 50 μg/mL), 100 μL of platelet poor plasma (PPP) and 100 μL of APTT test reagent were incubated for 3 min at 37 °C. The clotting time was measured after the addition of 100 μL of 25 mM $CaCl_2$ solution. The anticoagulant activity of each fucan was calculated as previously described [6].

Acknowledgements

This work was supported by the Centre National de la Recherche Scientifique (CNRS) and by the Institut Français de Recherche pour l'Exploitation de la Mer (IFREMER). The assistance of Dr. Stephane La Barre is gratefully acknowledged.

References

[1] C. Boisson-Vidal, S. Colliec, A.M. Fischer, J. Tapon-Bretaudiere, C. Sternberg, P. Durand, and J. Jozefonvicz, *Drugs Fut.*, 16 (1991) 539–545.
[2] D.S. McLellan and K.M. Jurd, *Blood Coagul. Fibrinol.*, 3 (1992) 69–77.
[3] G.F. Springer, H.A. Wurzel, G.M. Mc Neal, N.J. Ansell, and M.F. Doughty, *Proc. Soc. Exp. Biol. Med.*, 94 (1957) 404–409.
[4] V. Grauffel, B. Kloareg, S. Mabeau, P. Durand, and J. Jozefonvicz, *Biomaterials*, 10 (1989) 363–368.
[5] B. Mulloy, A.-C. Ribeiro, A.-P. Alves, R.P. Vieira, and P.A.S. Mourão, *J. Biol. Chem.*, 269 (1994) 22113–22123.
[6] S. Colliec, A.M. Fischer, J. Tapon-Bretaudiere, C. Boisson-Vidal, P. Durand, and J. Jozefonvicz, *Thromb. Res.*, 64 (1991) 143–154.
[7] T. Nishino and T. Nagumo, *Carbohydr. Res.*, 214 (1991) 193–197.
[8] S. Mauray, C. Sternberg, J. Theveniaux, J. Millet, C. Sinquin, J. Tapon-Bretaudiere, and A.M. Fischer, *Thromb. Haemostas.*, 74 (1995) 1280–1285.
[9] C. Blondin, E. Fischer, C. Boisson-Vidal, M. Kazatchkine, and J. Jozefonvicz, *Mol. Immunol.*, 31 (1994) 245–253.
[10] M. Baba, D. Schols, R. Pauwells, H. Nakashima, and E. De Clercq, *J. AIDS*, 3 (1990) 493–502.
[11] R. Hanenberger and A.M. Jakobson, *Glycoconjugate J.*, 8 (1991) 350–353.
[12] C.G. Glabe, T. Yednock, and S.D. Rosen, *J. Cell Sci.*, 61 (1983) 475–490.
[13] D.A. Belford, I.A. Hendry, and C.R. Parish, *J. Cell. Physiol.*, 157 (1993) 184–189.
[14] D.R. Coombe, C.R. Parish, I.A. Ramshaw, and J.M. Snowden, *Int. J. Cancer*, 39 (1987) 82–90.
[15] M. Ellouali, C. Boisson-Vidal, P. Durand, and J. Jozefonvicz, *Anticancer Res.*, 13 (1993) 2011–2020.
[16] M.C. Mahony, S. Oehninger, G.F. Clark, A.A. Acosta, and G.D. Hodgen, *Contraception*, 44 (1991) 657–665.
[17] M.C. Mahony, G.F. Clark, S. Oehninger, A.A. Acosta, and G.D. Hodgen, *Contraception*, 48 (1993) 277–289.

[18] E. Percival, in R.H. Mc Dowell (Ed.), *Chemistry and enzymology of marine algal polysaccharides*, Academic Press, London, 1967, pp 157–164.
[19] B. Kloareg and R.S. Quatrano, *Oceanogr. Mar. Biol. Ann. Rev.*, 26 (1988) 259–315.
[20] T. Nishino, C. Nishioka, H. Ura, and T. Nagumo, *Carbohydr. Res.*, 255 (1994) 213–224.
[21] M.S. Patankar, S. Oehninger, T. Barnett, R.L. Williams, and G.F. Clark, *J. Biol. Chem.*, 268 (1991) 21770–21776.
[22] S. Soeda, Y. Ohmagari, H. Shimeno, and A. Nagamatsu, *Thromb. Res.*, 72 (1993) 247–256.
[23] D.B. Volkin, P.K. Tsai, J.M. Dabora, J.O. Gress, C.J. Burke, R.J. Linhardt, and C.R. Middaugh, *Arch. Biochem. Biophys.*, 300 (1993) 30–41.
[24] T. Nishino, Y. Aizu, and T. Nagumo, *Agric. Biol. Chem.*, 55 (1991) 791–796.
[25] J. Choay, *Ann. NY Acad. Sci.*, 556 (1989) 61–74.
[26] S. Colliec, C. Boisson-Vidal, and J. Jozefonvicz, *Phytochemistry*, 35 (1994) 697–700.
[27] G. Volpi, G. Mascellani, and P. Bianchini, *Anal. Biochem.*, 200 (1992) 100–107.
[28] G. Volpi, *J. Chromatogr.*, 622 (1993) 13–20.
[29] F.H. Haber and J. Weiss, *Proc. R. Soc. London*, 147 (1934) 332–351.
[30] M. Dubois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, and F. Smith, *Anal. Chem.*, 28 (1956) 350–356.
[31] Z. Dische, *Methods Biochem. Anal.*, 2 (1955) 313–358.
[32] T.M.C.C. Filisetti-Cozzi and N.C. Carpitta, *Anal. Biochem.*, 197 (1991) 157–162.