ELSEVIER

PII: S0144-8617(96)00171-3

Carbohydrate Polymers 32 (1997) 89–93

0144-8617/97/$17.00 + .00

New investigations on heparin-like derivatized dextrans: CMDBS, synergistic role of benzylamide and sulfate substituents in anticoagulant activity

O. Maiga-Revel, F. Chaubet* & J. Jozefonvicz

Laboratoire de Recherches sur les Macromolécules, CNRS URA 502 Institut Galilée, Université Paris Nord, Av. J. B. Clement, 93430 Villetaneuse, France

(Received 16 April 1996; revised version received 18 October 1996; accepted 19 November 1996)

Dextran derivatives: CMDSu (carboxymethyldextran sulfate) and CMDBS (carboxymethyldextran benzylamide sulfonate/sulfate) show heparin-like properties. More specifically, their anticoagulant activity is closely dependent on their sulfate content. Functionalization of dextran follows a statistical substitution of hydroxyl groups of the α-(1-6)-D-glucosyl units with different chemical groups. CMDSu was obtained by carboxymethylation and sulfation, while CMDBS was prepared using four distinct reactions: carboxymethylation, benzylamidation and sulfonation of aromatic ring with concomitant sulfation of free hydroxyl groups. The proportion of sulfates was estimated following desulfation by solvent action. CMDBS exhibit higher anticoagulant activity than CMDSu for a given sulfate composition. This phenomenon was probably due to some steric role of the B units, the hydrophobic interactions from aromatic rings inducing a polymer conformation promoting a better interaction with blood proteins. © 1997 Elsevier Science Ltd

INTRODUCTION

The importance of heparin as an anticoagulant agent has stimulated, for over 20 years, a great interest in the preparation of sulfated forms of many diverse polysaccharides exhibiting heparin-like properties. Widely studied examples are glycosaminoglycans (Bourin and Lindahl, 1993), natural or semi-synthetic bioactive polysaccharides such as pentosan polysulfate (Fischer *et al.*, 1982), derivatized dextrans (Mauzac *et al.*, 1984; Jozefonvicz and Jozefowicz, 1994) and fucan extracted from seaweeds (Boisson-Vidal *et al.*, 1995). These polymers interact with biological macromolecules such as proteins and nucleic acids through selected binding sites. The specificity of these sites depends upon short saccharide sequences along the polymer backbone (Krentsel *et al.*, 1996). Such sequences could act as active sites as is the case for the pentasaccharide responsible for the anticoagulant activity of heparin (Choay *et al.*, 1983). Among the polysaccharides studied for their biological properties, the derivatized dextrans called CMDBS (Fig. 1) exhibit some heparin-like properties such as anticoagulant activity (Mauzac and Jozefonvicz, 1984), anticomplementary activity (Crepon *et al.*, 1987) and modulation of vascular cell proliferation (Letourneur *et al.*, 1993a; Logeart *et al.*, 1993).

Their structure was previously described as a statistical substitution of dextran with three different groups: carboxymethyl (CM), CM as the benzylamide (B), the latter being sulfonated on the aromatic ring by chlorosulfonic acid (S) (Chaubet *et al.*, 1995). These functionalized dextrans exert their anticoagulant activity mainly through a heparin-like mechanism, requiring the presence of AT as well as HCII and they also directly inhibit thrombin (Fischer *et al.*, 1985; Maaroufi *et al.*, 1993). The anticoagulant activity of CMDBS was demonstrated to be dependent on the molecular weight, the CM and S contents, and on the relative proportions of these groups (Mauzac and Jozefonvicz, 1984; Chaubet *et al.*, 1995). Neither carboxymethyl dextran (CMD) nor carboxymethyldextran benzylamide (CMDB) exhibited anticoagulant activity.

In this paper we focus on the formation of sulfate groups taking place during the reaction of CMDB with

*To whom correspondence should be addressed.

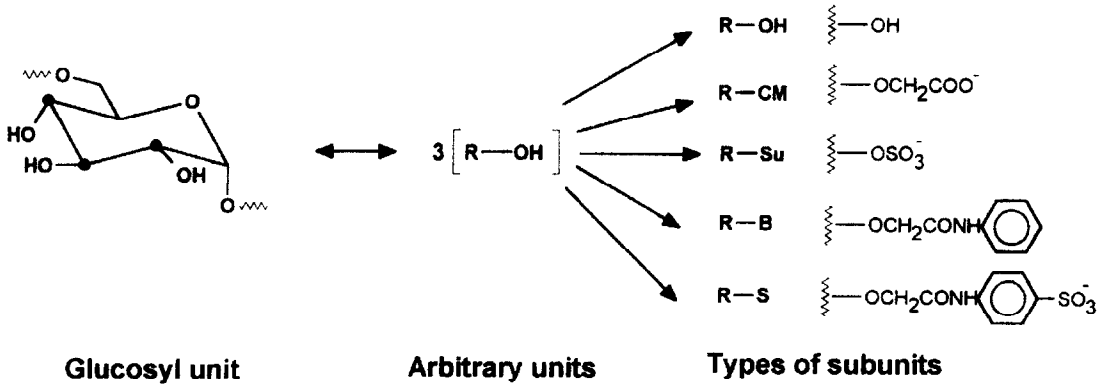

Fig. 1. Schematic structure of CMDBS. The use of subunits built from one-third of a starting arbitrary glucosyl unit can be conveniently used to describe the compounds in terms of average hydroxyl degree of substitution. Derivatized dextrans are considered as copolymers of these subunits (Chaubet *et al.*, 1995; Maiga-Revel *et al.*, 1995).

chlorosulfonic acid (Maiga-Revel *et al.*, 1995). The bioactive samples were specifically desulfated in order to determine the relative proportions of sulfate and sulfonate. We present preliminary results on the importance of both groups on anticoagulant activity. In addition, our data suggest that benzylamide groups are implied in the anticoagulant activity of CMDBS.

EXPERIMENTAL

Materials

Dextran T40 from *Leuconostoc mesenteroides* was purchased from Pharmacia (batch 13541, $M_w = 37.200$, $M_n = 22.800$). All chemical reagents were purchased as analytical grade from Carlo Erba, Fluka, Merck and Sigma. Dichloromethane was distilled on P_2O_5, and methanol and dimethylsulfoxide were dried over 4 Å molecular sieves.

Syntheses of dextran derivatives

The syntheses of CMDBS precusors (CMD and CMDB) were performed as previously described (Chaubet *et al.*, 1995). In brief: carboxymethylations of dextran were performed with monochloroacetic acid in alkaline medium at 60°C for 1 h. Three successive reactions were necessary to obtain CMD with a degree of substitution (d.s.) ranging from 0.7 to 1.1. Then CMDB with d.s. (B) from 0.2 to 0.6 were obtained quantitatively by coupling benzylamine to carboxylic groups of CMD using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoleine as coupling agent in water–ethanol (1:1, v/v) mixture at room temperature.

Sulfonation and sulfation

The compositions of all the samples are gathered in Table 1. Eight sulfur containing dextran derivatives were prepared, four CMDBS (CMDBS 1–4) and four CMDSu (CMDSu 1–4) as follows: 2 g of the dried precursor was dispersed in dry dichloromethane. The suspension was stirred for 20 min under a stream of argon, then chlorosulfonic acid diluted in a small volume of CH_2Cl_2 was quickly added. After 1 h of reaction at room temperature the suspension was filtered and washed successively with CH_2Cl_2, CH_2Cl_2/dioxane mixture and dioxane. Finally the crude acidic sample was dissolved in water, maintained at pH 9 for 1 h before neutralization and lyophilization. CMDBS1, CMDBS2, CMDSu1 and CMDSu2 were prepared using 0.1 M of chlorosulfonic acid. Ratios of [HSO_3Cl]/[B units] for CMDB and [HSO_3Cl]/[free OH] for CMD varied from 0.8 to 3 (see Table 1). All compounds were obtained in yields ranging from 60 to 90%.

Desulfation

An aqueous solution of samples: CMDBS or CMDSu (250 mg in 10 ml) was shaken slowly with 3 ml of Amberlite IR 120 H^+ cation exchange resin. After 2 h, the acidic solution was filtered, neutralized with pyridine and evaporated to dryness under reduced pressure. The residue was treated with dry methanol (2×10 ml) which was evaporated under reduced pressure before dispersion into 25 ml of a dimethylsulfoxide/methanol/pyridine mixture (90:9:1), and heated at 90°C under stirring for 48–72 h. The reaction was stopped by addition of water (25 ml), the pH was then adjusted to 9 with 1 M NaOH and the mixture was stirred for 1 h. The desulfated product was purified by chromatography on Sephadex G15 followed by dialysis under pressure in a cell equiped with a 1000D cut-off membrane (Amicon). The purification was followed by conductimetry, 160 to 180 mg of the desulfated samples were obtained.

Analytical methods

Analyses were performed on dried samples purified using an ultrafiltration device (Filtron, Cut-off

Table 1. Characterization of derivatized dextrans

Compoound	Conditions of synthesis		Degree of substitution of subunits					
	C[a]	R[b]	CM	B	S	Su	M_p±3000 (g/mol)	AAc[d]±0.2 (IU/mg)
CMDBS1	0.10 M	0.8	0.76	0.41	≤0.01	0.08	130,000	2.2
D1[c]	—	—	0.76	0.41	≤0.01	0	61,000	0.2
CMDBS2	0.10 M	1.0	0.77	0.41	≤0.01	0.14	93,000	2.7
D2[c]	—	—	0.77	0.41	≤0.01	0	57,000	0.2
CMDBS3	0.15 M	3.0	1.10	0.24	≤0.01	0.18	90,000	4.2
D3[c]	—	—	1.10	0.24	≤0.01	0	66,000	0.2
CMDBS4	0.15 M	3.0	1.07	0.66	≤0.01	0.76	80,000	5.8
D4[c]	—	—	1.07	0.66	≤0.01	0	40,000	0.1
CMDSu1	0.10 M	1.0	1.05	—	—	0.06	81,000	1.3
CMDSu2	0.10 M	1.0	1.35	—	—	0.36	67,000	3.1
CMDSu3	0.15 M	1.0	1.00	—	—	0.42	57,000	3.1
CMDSu4	0.15 M	1.0	1.09	—	—	0.72	55,000	3.5
D(%)	—	—	2	2	10	8	—	—

[a]C: [$ClSO_3H$].
[b]R: [$ClSO_3H$]/[B units] for CMDBS, [$ClSO_3H$]/[free OH] for CMDSu.
[c] Desulfated products.
[d] Specific anticoagulant activity.
SD: Standard deviation.

membrane: 5000D) followed by lyophilization. The carboxymethyl content was determined on dried aliquots (15–25 mg) by acidimetric titration in water/acetone mixture (1:1, v/v) acidified with HNO_3 (10%). The proportion of B, S and sulfate (Su) groups was assessed from N and S elemental analysis performed by CNRS (Gif/Yvette).

The chromatographic molecular weight of dextran derivatives samples was determined by high-performance steric exclusion chromatography in 0.15 M NaCl, 0.05 M NaH_2PO_4, solution buffered at pH 7, using two columns assembled in series (respectively Licrospher Si 300 diol by Merck-Clevenot and a Hema Sec Bio 40 by Alltech) and a 510 model pump (Merck) with a 7125 Rheodyne injection valve with 100 µl loop. The effluent was monitored with a high pressure differential refractometer (by Jobin-Yvon). The flow rate was 0.5 ml/min. The columns were calibrated with standard polysaccharides of narrow molecular weight pullulans (853,000–5800 g/mol, Polymer Laboratories, Interchim); dextran (1500 g/mol), melezitose (522 g/mol), sucrose (342 g/mol) and glucose (180 g/mol). The peak-molecular weight (M_p) was determined with Chromstar software (Bruker, Merck-Clevenot).

FTIR spectra

Spectra were measured on KBr pellets (150 mg of KBr and 1 mg of sample). Data were analysed using the Perkin-Elmer software (IRDM).

Coagulation assays

Anticoagulant activity of each sample was obtained by measuring the activated partial thromboplastin time (APTT) using the APTT kit (Organon Teknika). Owen Koller buffer (controls) 100 µl, or heparin dilution (anticoagulant standard), or sample dilution, 100 µl of human platelet-poor plasma and 100 µl of APTT test reagent were incubated for 3 min at 37°C. The clotting time was measured after the addition of 25 mM $CaCl_2$ solution (100 µl). Heparin (H410, anticoagulant specific activity of 170 IU/mg) was diluted at different concentrations in buffer (from 0 to 1 µg/ml). A linear curve was established by determining the log of the clotting time for each concentration of heparin standard. The sample was also diluted in buffer (from 0 to 50 µg/ml) and a linear curve was established (log clotting time for each concentration tested). The anticoagulant activity of each dextran derivative was calculated (IU/mg) with respect to heparin standard.

RESULTS AND DISCUSSION

Syntheses and characterization

Four CMDBS were prepared (CMDBS 1–4) with different sulfur contents and an aliquot of each was desulfated to determine the sulfate to sulfonate ratio (compounds D1–D4 are the corresponding desulfated samples). Similarly, four CMDSu were prepared (CMDSu 1–4) from CMD with variable sulfate contents. The compositions of CMDBS and CMDSu, and the conditions of preparation are summarized in Table 1. The sulfate contents were established after desulfation of products in the pyridinum salt form according to the method of Nagasawa *et al.* (1977. Under these conditions the aryl–sulfonate linkage is stable (Suter, 1944). Results are presented in Table 2.

Table 2. Desulfation of CMDBS and CMDSu

		CMDBS72 h, 100°C				CMDSu48 h, 100°C	
		1	2	3	4	3	4
Sulfur % (g/100 g)	before	1.23	1.66	2.04	5.82	3.20	6.12
	after	0.20	0.10	0.18	0.12	0	0

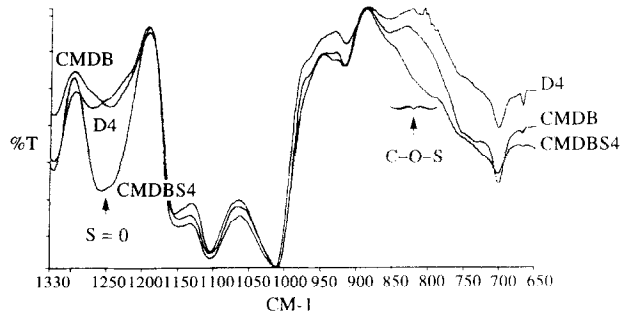

Fig. 2. FTIR spectra of CMDBS4, its desulfated form D4 and the starting CMDB in the 650–1330 cm^{-1} region.

CMDBS were completely desulfated within 72 h, however in the case of CMDSu, all sulfate groups were removed within 48 h. Thus the remaining sulfur could be attributed to the sulfonate groups. Fig. 2 presents FTIR spectra of CMDBS4, its desulfated form (D4) and the carboxymethyldextran benzylamide precurcor in the 650–1330 cm^{-1} region. After desulfation, the SO stretching vibration at 1255 cm^{-1} and the C—O—S bending vibrations between 820 and 870 cm^{-1} (Orr, 1954) decreasingly leading to a pattern of D4 spectrum similar to that of CMDB. We concluded that sulfation mainly occurred during treatment of CMDB with chlorosufonic acid, whatever the conditions of preparation. Preliminary results indicated that with other solvents and at higher temperatures, it was possible to increase the contents of sulfonate groups (work in progress).

Anticoagulant property of the products

The anticoagulant activity of CMDBS was previously described as closely dependent on three main parameters: the CM content which should be higher than 0.4 (Mauzac and Jozefonvicz, 1984), the molecular weight which should be greater than 40 000 g/mol (Crepon *et al.*, 1987), and finally the amount of sulfur containing groups (sulfate and sulfonate groups) promoting an enhancement of the clotting time (Mauzac and Jozefonvicz, 1984). The specific anticoagulant activities of all compounds we have prepared are listed in Table 1. Both CMDBS and CMDSu exhibited a significant anticoagulant activity proportional to their sulfur content although it was fairly low compared to the activity of heparin. However, the CMDBS samples lost

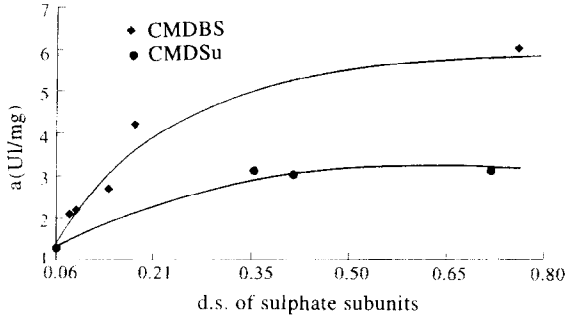

Fig. 3. Specific anticoagulant activity of CMDBS and the corresponding CMDSu vs the degree of substitution of sulfate containing subunits.

the greatest part of their activity after complete desulfation. The very low remaining sulfonate content contributed weakly to the activity. Fig. 3 shows the plots of the specific anticoagulant activity from Table 1 samples vs d.s. of sulfate containing subunits. We observed that CMDBS exhibited a higher activity than CMDSu for the same sulfate content. We can explain this observation by considering a steric role of B units. The spatial arrangement of sulfate along a polymer backbone being important for anticoagulant activity, B units could induce conformations promoting a better interaction with blood proteins. Investigations are now in progress in our laboratories concerning this particular aspect.

ACKNOWLEDGEMENTS

This work was supported by the Centre National de la Recherche Scientifique (CNRS), France.

REFERENCES

Boisson-Vidal, C., Haroun, F., Ellouali, M., Blondin, C., Fischer, A. M., de Agostini, A. & Jozefonvicz, J. (1995). Biological activities of polysaccharides from marine algae. *Drug of the Future*, **20**, 1237–1249.

Bourin, M. C. & Lindahl, U. (1993). Glycosaminoglycans and the regulation of blood coagulation. *Biochemical Journal*, **289**, 313–330.

Chaubet, F., Champion, J., Maiga, O., Mauray, S. & Jozefonvicz, J. (1995). Synthesis and structure–anticoagulant property relationships of functionalized dextrans. *CMBDS Carbohydrate Polymers*, **28**, 145–152.

Choay, J., Petitou, M., Lormeau, J. C., Sinay, P., Casu, B. & Gatti, G. (1983). Structure–anticoagulant relationships in heparin: a synthetic pentasaccharide with high affinity for antithrombin III and eluciting high anti-factor Xa activity. *Biochemical and Biophysical Research Communications*, **116**, 492–499.

Crepon, B., Maillet, F., Kazatchkine, M. D. & Jozefonvicz, J. (1987). Molecular weight dependency of the anticomplementary and anticoagulant activities of special substituted dextrans. *Biomaterials*, **8**, 248–253.

Fischer, A. M., Barrowcliffe, T. W. & Thomas, D. P. (1982). A

comparison of pentosan polysulfate (SP54) and heparin. I: Mechanism of action on blood coagulation. *Thrombosis and Haemostasis*, **47**, 104–108.

Fischer, A. M., Mauzac, M., Tapon-Bretaudière, J. & Jozefonvicz, J. (1985). Anticoagulant activity of dextran derivatives. Part II: mechanism of thrombin inactivation. *Biomaterials*, **6**, 198–202.

Jozefonvicz, J. & Jozefowicz, M. (1994). Blood contacting polymers, in *Polymeric Biomaterials*, ed. S. Dumitriu, pp. 349–371. Marcel Dekker, New York.

Krentsel, L., Chaubet, F., Rebrov, A., Champion, L., Ermakov, I., Bittoun, P. Fermandjian, S., Litmanovich, A., Platé, N. & Jozefonvicz, J. (1996). Anticoagulant activity of functionalized dextrans. Structure analyses of carboxymethylated dextran and first Monte Carlo simulations. *Carbohydrate Polymers* (in press).

Letourneur, D., Champion, J., Slaoui, F. & Jozefonvicz, J. (1993). *In vitro* stimulation of human endothelial cells by derivatives of dextrans. *In Vitro Cellular and Developmental Biology*, **29A**, 67–72.

Logeart, D., Avramoglou, T. and Jozefonvicz, J. (1993). Inhibition effect of functional dextrans on rat arterial smooth muscle cell growth *in vitro*. *Colloids and Surfaces B: Biointerfaces*, 1–8.

Maaroufi, M. R., Tapon-Bretaudière, J., Jozejonvicz, J. and Fischer, A. M. (1993). Native and artificial sulfated polysaccharides: effect on thrombin inhibition by anti-thrombin III and heparin cofactor II. XIVth Congress of the International Society on Thrombosis and Haemotosis, New York, USA, 4–9 July.

Maiga-Revel, O., Clairbois, A. S., Champion, J., Chaubet F. and Jozefonvicz, J. (1995). Structure–anticoagulant property relationships of dextran derivatives: CMDBS. VIIIth European Carbohydrate Symposium, Abstract no 53, Seville, Spain, 2–7 July.

Mauzac, M. & Jozefonvicz, J. (1984). Anticoagulant activity of dextran derivatives. Part I: synthesis and characterization. *Biomaterials*, **5**, 301–304.

Nagasawa, K., Inoue, Y. & Kamata, T. (1977). Solvolytic desulfation of glycosaminoglycan sulfates with dimethyl sulfoxyde containing water or methanol. *Carbohydrate Research*, **58**, 47–55.

Orr, S. F. D. (1954). Infra-red spectroscopic studies of some polysassarides. *Biochimica et Biophysica Acta*, **14**, 173–181.

Suter C. M. (1944). Tetracovalent sulfur compounds, in *The Organic Chemistry Of Sulfur*, pp. 387-413. Wiley, New York.