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Anticoagulant sulfated polysaccharides: Part I. Synthesis and structure–activity relationships of new pullulan sulfates

S. Alban*, A. Schauerte, G. Franz

Institute of Pharmacy, University of Regensburg, Universitätsstraße 31, 93040 Regensburg, Germany

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Abstract

In order to develop new anticoagulants as potential heparin alternatives, two pullulans with different molecular weight (MW) were used as starting polymers for the partial synthesis of a structurally new class of sulfated polysaccharides. Sulfation of these linear α-1,4-/1,6-glucans was carried out by a method with a SO_3–pyridine complex in DMF, which had been optimized for the modification of β-1,3-glucans. Modifications of this methods resulted in pullulan sulfates with degrees of sulfation (DS) ranging from 0.17 to 1.99 and MW between 15 and 250 kDa. More than 50% of the sulfate groups were bound to the secondary C atoms in positions 2, 3 and 4 of the glucose monomers. The anticoagulant activity of the obtained pullulan sulfates was determined in the coagulation assays prothrombin time (PT), activated partial thromboplastin time (APTT), Heptest® and thrombin time (TT). They represent potent anticoagulants reaching the efficacy of heparin. Their activity not only improves with increasing DS and MW, but also with increasing part of sulfate groups in positions 2, 3 and 4. In addition, their action profile changes in dependence on their individual structure as reflected by the ratio of the TT- to the APTT-activity. The pullulan sulfates specifically interfere with different stages of the coagulation cascade, and these interactions have different requirements on the chemical structure.

Keywords: Sulfated polysaccharides; Pullulan sulfates; Partial synthesis; Anticoagulant activity; Structure–activity relationships

1. Introduction

For more than 60 years, the glycosaminoglycan heparin is the drug of choice in the prevention and treatment of thromboembolic disorders. However, there are some well-documented problems related to its clinical application such as its inefficacy in antithrombin-deficient patients, bleeding complications and heparin-induced thrombocytopenia as severe side effects (Weitz, 1994). Moreover, heparin shows wide variations in its structural parameters and consequently in its physiological activities (Linhardt, Loganathan, al-Hakim, Wang, Walenga, Hoppensteadt et al., 1990). Its isolation from animal materials results in further disadvantages such as the risk of contamination, e.g. with prions inducing BSE. Therefore, an important field of research is the development of alternatives to substitute heparin. One possibility is substances chemically related to heparin, that is, sulfated polysaccharides.

Another reason which, in general, attracts attention to these compounds is the increasing knowledge about their manifold biological activities. Besides their anticoagulant and antithrombotic activities (Alban, 1997), antiatherosclerotic (Engelberg, 1991), antiproliferative (McCaffrey, Falcone, Borth, Brayton & Weksler, 1992), antiadhesive (Ley, Cerrito & Arfors, 1991), antiangiogenetic (Linhardt & Toida, 1997), antimetastatic (Zacharski & Ornstein, 1998), antiinflammatory (Arfors & Ley, 1993; Winkelhake, 1991), complement-inhibiting (Boisson-Vidal, Haroun, Elloouali, Blondin, Fischer, de Agostini et al., 1995) and antiviral effects (DeClercq, 1993) are described. At present, there is also an increasing interest in the question whether and to what extent these actions support the therapeutic benefit of heparin in some cases. For instance, the increased survival time of tumor patients treated with heparin is discussed to be due to its antiproliferative, antiangiogenetic and antimetastatic effects (Zacharski & Ornstein, 1998).

Therefore, sulfated polysaccharides having a similar or even improved action profile are of special interest. They are widespread in nature, they occur as components of the extracellular matrix and on the cell surface of vertebrates, and are produced by marine organisms. However, like heparin, most natural sulfated polysaccharides are complex mixtures of macromolecules showing wide variations in

* Corresponding author. Tel.: +49-941-943-4792; fax: +49-941-943-4762.

E-mail address: susanne.alban@chemie.uni-regensburg.de (S. Alban).

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their structure and their activities, which complicates the development of such polymers into new drugs.

For this reason, we aimed to produce structurally defined sulfated polysaccharides by partial synthesis in order to establish structure–activity relationships as the basis for the development of a heparin alternative. Sulfation of the linear β-1,3-glucan curdlan has been proven to result in potent anticoagulants in vitro, which exhibit antithrombotic activity in vivo comparable to that of heparin (Alban, Jeske, Welzel, Franz & Fareed, 1995). Their activity turned out to depend on several structural parameters such as the degree of sulfation (DS) (Alban, Kraus & Franz, 1992), the molecular weight (MW) (Alban & Franz, 1994a), the sulfation pattern (Alban & Franz, 1994b) and glycosidic branchings (Alban, 1997).

In this study, pullulan was used as starting polymer in order to investigate if similar structure–activity relationships apply to this glucan as found in earlier studies (Alban, 1997). This linear α-1,4-/1,6-glucan is an exopolysaccharide produced by the black yeast *Aureobasidium pullulans*. Pullulans were shown to lack any toxicological activity (Kimoto, Shibuya & Shiobara, 1997), are useful for numerous applications in the food, cosmetic and pharmaceutical industry (Deshpande, Rale & Lynch, 1992), and can be produced in large amounts by fermentation methods, especially using agro-industrial wastes as substrates (Israilides, Scanlon, Smith, Jumel & Harding, 1994).

The sulfation of two representatives of pullulan was carried out with an SO_3–pyridine complex in DMF. By several modifications of the method described by Larm, Larsson, Scholander, Andersson, Holmer and Soderström (1979), the procedure was optimized for the sulfation of the β-1,3-glucan laminarin resulting in reproducible, highly sulfated products without degradation of the polysaccharide (Alban, 1997; Alban et al., 1992). However, as presented in the following, one sulfation method resulting in predictable derivatives of a certain polysaccharide is not easily applicable to another polymer. The anticoagulant activity of the obtained pullulan sulfates was determined in the coagulation assays prothrombin time (PT), activated partial thromboplastin time (APTT), Heptest® and thrombin time (TT) which record influences on different steps of the coagulation cascade.

2. Materials and methods

2.1. Materials

Pullulan NM (PN) with an average MW of 50 kDa as determined by gel permeation chromatography (GPC) (see above) was obtained from the 'Consortium für elektrochemische Industrie, GmbH' (Munich, Germany), and Pullulan 200 (PH) with an average MW of 200 kDa from Wacker-Chemie, GmbH (Burghausen, Germany). According to the methylation analysis performed as described by Harris, Henry, Blakeney and Stone (1984), the ratio of 1,4-glucose to 1,6-glucose was 2.3:1.0 for both, PN and PH. Consequently, they consisted not exclusively of α-1,6-maltotriose units, but contained also small amounts of α-1,6-maltotetraose as reported by Yalpani (1988a).

Unfractionated heparin (UFH) of porcine mucosal origin (147 USP-U/mg), used as reference substance in the coagulation assays, was obtained from Sigma (Deisenhofen, Germany). The specific activities (U/mg) of UFH and pullulan sulfates were determined by means of the 4th International Standard (4th I.St.) of Heparin (1983) (National Institute for Biological Standards and Control, London, UK). Selectively *O*-6-desulfated heparin (deS-UFH) was produced according to Baumann, Scheen, Huppertz and Keller (1998).

Thromborel®S, Pathromtin®, Test-Thrombin (bovine) (30.0 IU/ml), calcium chloride (0.025 mol/l) and diethylbarbiturate–acetate buffer solution (pH 7.6) were purchased from Dade-Behring (Marburg, Germany), and the Heptest® from Laborservice (Augsburg, Germany). All other chemicals used were of analytical grade.

2.2. Preparation of pullulan sulfates

The sulfation of PN and PH was performed by modifying the method established for the sulfation of β-1,3-glucans (Alban et al., 1992) resulting in the pullulan sulfates PN-S, PH-S, PHDe-S, PNNa-S, PHNaSu-S, PHNaRe-S, PN-2S, and PH-2S (Table 1). Both the activation of pullulans and their sulfation were carried out under argon atmosphere.

PN-S and PH-S: For activation, 1.0 g of dried (under reduced pressure in the presence of P_2O_5) PN and PH, respectively, were dissolved (PN) or finely suspended (PH) in 15 ml dry *N*,*N*-dimethylformamide (DMF) followed by the addition of dry pyridine equimolar to the sulfating reagent. The mixtures were heated up to 75°C and stirred for 16 h. Sulfation was carried out by stepwise addition of a 2 M SO_3–pyridine complex solution in DMF over 1 h at 75°C. An excess of 6 mol SO_3–pyridine complex per mole glucose was applied. After stirring for an additional 2 h at 75°C, the reaction was stopped by withdrawing the supernatant and dissolving the insoluble residue in ice-cold 4 N NaOH. The product was twice precipitated with EtOH (99%), washed, and re-dissolved in water. The alkalized solution was exhaustively dialyzed against water (MW-cutoff: 3500 Da) to remove pyridine, salts and potential degradation products. The solution, which turned to a slightly acidic pH during dialysis, was neutralized with NaOH and lyophilized resulting in the sodium salts of the pullulan sulfates PN-S and PH-S.

PHDe-S: For activation, PH was first dissolved in water and lyophilized resulting in a DMF-soluble starting polymer. In order to remove any residual water, the solution of PH in DMF was distilled in vacuo followed by ultrasonic treatment for 16 h. The sulfation reaction was only modified by prolonging the time of SO_3–pyridine complex addition to

Table 1
Reaction parameters of the sulfation (performed with an excess of 6 mol SO_3–pyridine complex per mole glucose) of the pullulans PN (50 kDa) and PN (200 kDa) and structural parameters of the resulting pullulan sulfates

Pullulan sulfate	Reaction parameters			Structural parameters		
	Activation[a]	T (°C)[b]	t (h)[c]	DS	MW (kDa)[d]	SS (%)[e]
PN-S	Ultrasound	75	3	0.17	48	62
PHDe-S	Hydration/drying	75	5	0.47	15[f]	n.d.
PH-S	Ultrasound	75	3	0.47	90	52
PNNa-S	Alkali-complex	95	5	0.52	40	76
PHNaSu-S[g]	Alkali-complex	95	5	0.56	65	61
PN-2S	Re-sulfation of PN-S	75+95	3+5	0.66	90	52
PHNaRe-S[g]	Alkali-complex	95	5	1.80	170	61
PH-2S	Re-sulfation of PH-S	75+95	3+5	1.99	250	58

[a] For details see Section 2.
[b] T denotes temperature during the sulfation reaction.
[c] t denotes reaction time consisting of the time of addition of the SO_3–pyridine complex and 2 h stirring.
[d] MW_{GPC} denotes the apparent average molecular weight as determined by GPC using neutral pullulans as standards.
[e] SS (%) is the percentage of sulfate groups bound to C2, C3 and C4 on the total sulfate groups.
[f] Excessive degradation of PhDe-S occurred during lyophilization.
[g] PHNaSu-S and PHNaRe-S were obtained by fractionating isolation after the sulfation reaction.

3 h. The purification procedure was the same as described above apart from the lyophilization, which was performed without neutralization of the solution after dialysis.

PNNa-S, PHNaSu-S, PHNaRe-S: The first step of activation was the formation of alkali–pullulan complexes by dissolving PN and PH, respectively, in 1 N NaOH at 60°C, stirring for 2 h at room temperature (RT) and lyophilization. Then, the resulting Na–PN and Na–PH were suspended in DMF, pyridine was added, and the mixture was stirred for 16 h at the reaction temperature, which was increased to 95°C. PNNa-S was obtained as described for PN-S and PN-H. For the isolation of PHNaSu-S and PHNaRe-S, the insoluble pyridinium salt of the product was first suspended in 0.1 N NaOH, and the mixture was poured into EtOH (99%). The precipitate was re-suspended in 0.1 N NaOH and centrifuged. The supernatant was removed, precipitated in EtOH (99%), dissolved in 0.1 N NaOH and exhaustively dialyzed against water. Lyophilization of the neutralized solution resulted in PHNaSu-S. The insoluble residue was dissolved in 0.5 N NaOH. By precipitation in EtOH (99%), dissolving in 0.5 N NaOH, dialysis, neutralization and lyophilization, PHNaRe-S was obtained.

PN-2S and PH-2S: The sulfated pullulans PN-S and PH-S were subjected to a second sulfation. After suspending in DMF and stirring overnight at RT, they were sulfated and purified as described for PNNa-S resulting in the products PN-2S and PH-2S.

2.3. Determination of the degree of sulfation

The pullulan sulfates were hydrolyzed with 2 M trifluoroacetic acid (TFA) by autoclaving for 60 min at 121°C. After dilution with water and devolatilization by lyophilization, the sulfate ions were quantified by ion chromatography on a high-performance liquid chromatography (HPLC) system (Waters Corp., Milford, MA, USA) consisting of a HPLC pump 501, an autosampler WISP 712, a Waters 434 conductivity detector and an IC-Pak anion (4.6 mm × 50 mm) column. The eluent consisted of 20 ml borate–gluconate concentrate (16 g sodium gluconate, 18 g boric acid, 25 g sodium tetraborate decahydrate, 250 ml glycerol, and water ad 1000 ml), 20 ml *n*-butyl alcohol, 120 ml acetonitrile, and water ad 1000 ml. The flow rate was 1.0 ml/min. Sodium sulfate dilutions (1.5–25.0 μg SO_4^{2-}/50 μl) were used for calibration. The hydrolysis and ion chromatography of each pullulan sulfate was performed in triplicate. The calculated DS corresponded with the DS obtained by the methylation analysis.

2.4. Determination of the molecular weight

The MW is presented as the hydrodynamic volume as determined by GPC on a fast-performance liquid chromatography (FPLC) system consisting of a P-500 pump, a Valve V7 injection system (Amersham Pharmacia Biotech, Uppsala, Sweden), a RI detector ERC 7512 (Erma, Brussels, Belgium) and a Sephacryl™ S-500 HR 16/600 (MW fractionation range: 40–20 000 kDa) or a Superose™ 12 HR 10/30 (MW fractionation range: 1–300 kDa), respectively (Amersham Pharmacia Biotech, Uppsala, Sweden). The eluent was 0.1 M sodium chloride containing 0.05% sodium azide at a flow rate of 45 or 30 ml/h, respectively. The pullulan sulfates were dissolved in the eluent (1.0 mg/ml). The columns were calibrated using neutral pullulans of defined MW ranging from 5.8 to 853 kDa (Polymer Laboratories: Separation Science Division, Shropshire, UK) as standards.

2.5. Methylation analysis

The sulfation pattern of the pullulan sulfates and the

percentage of the potential 16 different glucose monomers on the respective pullulan sulfates was evaluated by a modified methylation procedure (Alban & Franz, 1994b) based on that of Harris et al. (1984). The resulting methylated, ethylated alditol acetates were analyzed by combined gas–liquid chromatography–mass spectrometry (GLC/MS) providing qualitative and quantitative information about the polysaccharide structure and the distribution of the sulfate groups. The methylation analysis of each pullulan sulfate was performed in triplicate.

2.6. Coagulation assays

The coagulation assays were carried out using citrated human platelet poor plasma (PPP) pooled from at least eight healthy volunteers. PPP was obtained according to the guidelines for preparing citrated plasma for hemostaseological analyses (Witt, Beeser & Müller-Berghaus, 1995) and stored at −70°C until use. The PPP was supplemented with dilution series of the pullulan sulfates and UFH in 0.9% sodium chloride resulting in final concentrations ranging between 0.1 and 100 μg/ml. The clotting times were recorded in seconds with a Kugel coagulometer KC 10 (Amelung, Lemgo, Germany). The specific anticoagulant activities (U/mg) of the pullulan sulfates and UFH in the different assays were evaluated by means of a standard curve with the 4th I.St..

The following reagents were used: Thromborel®S for the PT, Pathromtin® for the APTT, Test-Thrombin (bovine) (3.0 IU/ml) in diethylbarbiturate–acetate buffer solution (pH 7.6) for the TT, and Heptest® for the determination of the anti-factor Xa activity. The assays were carried out according to the respective instructions of the manufacturers.

All assays were performed in duplicate and repeated at least three times on different days ($n = 6$). In general, the standard deviations were less than 3% of the mean.

3. Results

3.1. Sulfation of pullulans

Two highly purified, water-soluble pullulans with an identical ratio of 1,4- to 1,6-glycosidic bonds, but differing in their respective MW were used as starting polymers for the sulfation. Whereas PN with an average MW of 50 kDa was soluble in DMF, PH with an average MW of 200 kDa was insoluble, but could be obtained as a well-dispersed suspension. After activation of the pullulan solution/suspension by exposing to ultrasound, the sulfation with SO_3–pyridine complex resulted in the low-sulfated products PN-S and PH-S. Though PN was soluble in DMF, the DS of the PN-S (0.17) was lower than that of PH-S (0.48) (Table 1). In order to investigate whether sulfation in a homogenous system might increase the DS, a modified hydration/drying method based on the principle described by Guiseley (1978) was applied for the activation of PH. Since the precipitation of PH, dissolved in water, with various hydrophilic organic solvents led to water- and DMF-insoluble gum, it was dehydrated by lyophilization followed by vacuum distillation with anhydrous DMF to remove residual water. After this treatment, PH was also soluble in DMF. However, despite the special activation procedure and prolongation of the sulfation time up to 5 h, the DS of the obtained PHDe-S was as low as 0.48 (Table 1). Further, the pullulans were activated by formation of an alkali-complex as reported by Yalpani (1985), which had been shown to be a suitable activation method for the sulfation of the β-1,3-glucan curdlan (Alban, 1993). In addition, the temperature of the sulfation reaction was increased from 75 to 95°C with a sulfation time of 5 h as in the case of PHDe-S. As a consequence, the sulfated products PNNa-S, PHNaSu-S, and PHNaRe-S had higher DS values (Table 1). Again the PN-based product was lower sulfated than the two fractions derived from PH. The latter were isolated utilizing the different solubility of the pyridinium salt of the product mixture in 0.1 N NaOH: PHNaSu-S with a DS of 0.56 was soluble in 0.1 N NaOH, whereas PHNaRe-S with a DS of 1.80 required 0.5 N NaOH. Based on the assumption that sulfation disaggregates highly ordered polysaccharide structures similar to the various activation procedures, the low-sulfated products PN-S and PH-S were re-sulfated for 5 h at 95°C without any further activation. In this way, the highest DS for PN and PH were achieved, which were increased by a factor of 4 compared to PN-S and PH-S (Table 1). However, just like the other two PH derivatives, PH-2S with a DS of 1.90 was higher sulfated than the corresponding PN-based product PN-2S with a DS of 0.66.

3.2. Molecular weight of pullulan sulfates

Besides the DS, a second important parameter for the assessment of a sulfation method is the resulting MW, since degradation of the polysaccharides during sulfation is a common problem (Engelskirchen, 1987; Whistler & Spencer, 1961). For lack of suitable sulfated polysaccharides with defined MW, neutral narrow MW distribution pullulans were used as standards for the MW determination by GPC. Consequently, the MW measured as hydrodynamic volumes only represent relative values being higher than the real MG due to the high degree of hydration of negatively charged sulfate groups. The produced pullulan sulfates showed symmetric GPC elution profiles. In order to estimate the extent of degradation, the theoretical MW was calculated on the basis of the sodium salts of the pullulan sulfates with known DS and compared with the apparent MW as determined by GPC (Table 1). In the case of the sulfated PH derivatives, a more pronounced degradation took place compared to the products based on PN. As obvious from PNNa-S, PHNaSu-S, and PHNaRe-S, activation by alkali-complex formation, the increased reaction temperature (95°C) and the prolonged reaction time (5 h)

Table 2
Percentage of non-substituted (AGU-N), mono- (AGU-S), di- (AGU-2S) and tri-sulfated glucose units (AGU-3S) on the composition of the pullulan sulfates as determined by methylation analysis

Pullulan sulfate	AGU-N (%)	AGU-S (%)	AGU-2S (%)	AGU-3S (%)	DS_{Meth}[a]
PN-S	86.9	11.0	0.9	1.0	0.16
PHDe-S	57.0	40.0	2.0	1.0	0.47
PH-S	64.5	27.9	6.3	1.3	0.44
PNNa-S	65.4	16.6	14.3	3.7	0.56
PHNaSu-S	64.7	19.3	13.6	2.4	0.54
PN-2S	49.6	37.7	10.7	2.0	0.65
PHNaRe-S	3.8	40.0	43.3	12.9	1.65
PH-2S	1.2	37.8	42.1	18.9	1.79

[a] Degree of sulfation calculated as AGU-S + 2 × AGU-2S + 3 × AGU-3S/total AGU.

favored degradation. However, the re-sulfation of already sulfated polymers for 5 h at 95°C did not induce further degradation as shown by the MW of PH-2S and PN-2S. The pretreatment of pullulan by the hydration/drying method did not induce any degradation, because the MW of PNDe-S amounted to 230 kDa before dialysis and lyophilization. In this case, the lyophilization of the product, containing sulfate groups in the free acid form, is responsible for the strong degradation to as low as 15 kDa.

3.3. Methylation analysis of pullulan sulfates

The methylation analysis of the pullulan sulfates should provide information about the distribution of the sulfate groups and thus on the homogeneity and the selectivity of the sulfation. The DS of the pullulan sulfates as calculated from the methylation analysis amounted to 90–108% compared to the DS as found by ion chromatography (Table 2). There were no significant differences in the homogeneity of sulfation between the products based on the dissolved PN and the suspended PH. All polymers consisted of mono-, di- as well as tri-sulfated glucose units besides non-substituted glucose moieties (Table 2). Their respective percentage varied corresponding to the DS, i.e. in the low-sulfated products the content of non-sulfated monomers was the highest and in the high-sulfated that of the di-sulfated ones. PNDeS showed the most homogenous sulfation with only low amounts of di- and tri-sulfated glucose units. In general, most of the sulfate groups were linked to the primary OH group on the C6 of the glucose. The relative distribution of the sulfate groups on the four potential positions was C6/C2/C3/C4 = 1.00:0.66:0.46:0.19 (mean values without PN-S and PNNa-S) (Table 3). Sulfation of the different OH groups occurred in the order C6 > C2 > C3 > C4 independently of the original starting pullulan, the derivatization procedure and the DS. However, two exceptions were obvious: PN-S with the lowest DS and PNNa-S. In PN-S, the relative amount of sulfate groups in position 4 (30.5%) exceeded those in position 2 (20.3%) and 3 (11.3%). However, the percentage of sulfate groups linked to secondary C atoms (SS) on the total sulfate groups was similar to that of the other pullulan sulfates ranging between 52 and 61%. In PNNa-S, the content of 76.1% SS was significantly higher, and the sulfation of position 6 amounted to only 23.9%.

3.4. Anticoagulant activity of pullulan sulfates

The anticoagulant activity of the pullulan sulfates was investigated by the classical coagulation assays APTT, PT, Heptest®, and TT using UFH as reference compound (Weißbach, 1990). The specific activities (U/mg) in the various assays were determined by means of the 4th I.St.. Depending on the individual structural characteristics, the pullulan sulfates turned out to represent potent anticoagulants (Table 4). PDNaRe-S with a DS of 1.80 and an MW of 170 kDa was as active as heparin in the TT with an APTT-activity being half as high compared to that of heparin. Even PN-S with a DS of only 0.17 and an MW of 48 kDa had still an activity of 7 APPT-U/mg and 12 TT-U/mg. In contrast to this, deS-UFH with a DS of 0.6 was completely inactive. In general, the highest activity was found in the TT, followed by that in the APTT with the exception of the short-chain PHDe-S exhibiting higher APTT- than TT-activity. In the

Table 3
Distribution of the sulfate groups of the pullulan sulfates on the various positions of the glucose unit

Pullulan sulfate	$-SO_4^-$ (%) bound to				SS (%)[a]
	C2	C3	C4	C6	
PN-S	20.3	11.3	30.5	37.9	62.1
PHDe-S	n.d.	n.d.	n.d.	n.d.	n.d.
PH-S	23.4	17.1	11.0	48.4	51.6
PNNa-S	31.1	28.0	17.0	23.9	76.1
PHNaSu-S	28.5	21.6	10.5	39.3	60.7
PN-2S	23.5	15.1	13.8	47.6	52.4
PHNaRe-S	34.9	21.8	4.1	39.2	60.8
PH-2S	31.5	24.8	2.0	41.7	58.3

[a] SS (%) = C2–SO_4^- +C3–SO_4^- + C4–SO_4^- (%) = percentage of sulfate groups bound to secondary C-atoms.

Table 4
Anticoagulant activity of the pullulan sulfates in relation to the reference UFH

Pullulan sulfate	% of the anticoagulant activity of UFH				Ratio
	APTT[a]	PT[b]	Heptest®[a]	TT[a]	TT/APTT[c]
PN-S	4.8	4.9	0.7	8.3	0.36
PHDe-S	4.8	2.0	1.1	1.7	1.71
PH-S	9.0	7.3	1.4	13.8	1.54
PNNa-S	11.7	22.9	2.8	35.9	3.06
PHNaSu-S	14.5	7.3	2.8	22.1	1.52
PN-2S	23.4	19.6	2.1	29.0	1.24
PHNaRe-S	53.1	20.0	4.8	100.0	1.88
PH-2S	52.4	24.9	4.8	81.4	1.55

[a] Anticoagulant activity of UFH and pullulan sulfates determined by means of the 4th I.St.. The anticoagulant activity of UFH amounted to 147 U/mg in each assay.
[b] Anticoagulant activity of UFH and pullulan sulfates determined on the basis of the DC (μg/ml), i.e. the concentration for doubling the baseline coagulation time. DC of UFH amounted to 17.4 μg/ml.
[c] Ratio of the anticoagulant activity of the pullulan sulfates in the TT to that in the APTT. The TT/APTT-ratio of UFH was 1.00.

Heptest®, the clotting assay for the anti-factor Xa activity, the effects of the pullulan sulfates were relatively weak and reached at most 4.8% of the heparin activity. The PT-activity amounted up to 25% of that of heparin, but it has to be considered that heparin itself exhibits only moderate activity in this assay (Hemker & Beguin, 1989).

In order to evaluate possible structure–activity relationships, the anticoagulant activities of respective pairs of pullulan sulfates only differing in one structural parameter were compared: PN-2S with a DS of 0.66 has a more than twice as high APTT- and TT-activity than PH-S with a DS of 0.47 indicating that the anticoagulant activity improves with increasing DS (Fig. 1). Moreover, as shown in Table 4, where the pullulan sulfates are arranged by increasing DS, the APTT-activity clearly increases in the same order regardless of the MW and the percentage of SS. Above a DS of 1.80 no further increase of the APTT-activity was observed. The identical APTT-activities of PN-S and

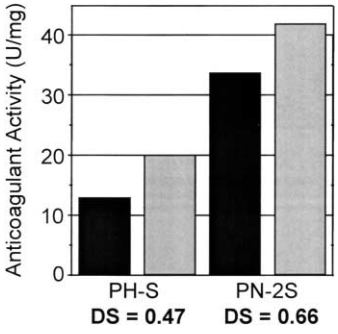

Fig. 1. Degree of sulfation dependence of the anticoagulant activity in the APTT (■) and TT (▒) (U/mg) as demonstrated by two pullulan sulfates only differing in their DS: PH-S and PN-2S with a MW of 90 kDa and 52% SS.

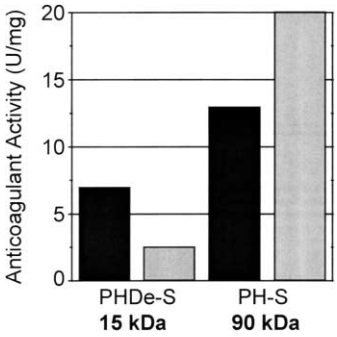

Fig. 2. Molecular weight (MW) dependence of the anticoagulant activity in the APTT (■) and TT (▒) (U/mg) as demonstrated by two pullulan sulfates PHDe-S and PH-S with a DS of 0.47 considerably differing in their MW.

PHDe-S demonstrate that besides the DS, the MW also influences the activity, since the higher-sulfated PHDe-S has a considerably lower MW than PN-S. In the APTT, the higher DS of PHDe-S compensates its lower MW. However, in the TT, it is eight times less active than PN-S, so that in this assay the MW is more important than the DS and a certain minimum chain length is essential for a marked effect in the TT. This is obvious from the ratio of the TT- to the APTT-activity, which is only lower than 1.0 in the case of the short-chain PHDe-S. The MW dependence of the anticoagulant activity becomes more evident in view of the higher APTT-and TT-activities of PH-S compared to those of PHDe-S, which has the same DS and percentage of SS, but a lower MW (Fig. 2). Comparing PHNaRe-S and PH-2S, the activity improves up to a maximum DS and MW. Further increase in both DS and MW does not promote the anticoagulant effect, but even rather reduces it in the TT. PNNa-S with its extraordinary high percentage of SS presents a special action profile. Whereas its APTT-activity is 20% lower than that of PHNaSu-S according to its lower DS and MW, its TT-activity is more than 60% higher resulting in a remarkably high ratio of the TT- to the APTT-activity of 3.1 (Fig. 3). This indicates that at least for the

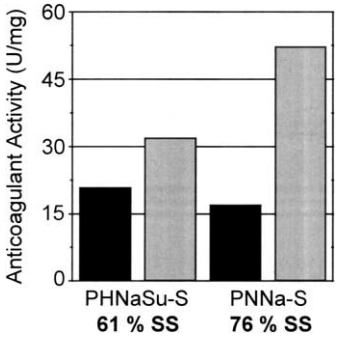

Fig. 3. Sulfation pattern dependence of the anticoagulant activity in the APTT (■) and TT (▒) (U/mg)as demonstrated by the pullulan sulfates PHNaSu-S (DS = 0.56, MW = 65 kDa) and PNNa-S (DS = 0.52, MW = 40 kDa), which also differ in their percentage of SS.

TT-activity, the arrangement of the sulfate groups within the glucose moieties also plays an important role.

4. Discussion

In this study, for the first time two representatives of the α-1,4/1,6-glucan pullulan were used as starting polymers to produce sulfated polysaccharides with anticoagulant activities. For the sulfation of β-1,3-glucans, a method with SO_3–pyridine in DMF was developed reproducibly resulting in highly sulfated compounds without degradation (Alban, 1997). However, applying this procedure to the pullulans, only low-sulfated, more or less degraded products (DS = 0.17 and 0.48) were obtained. Consequently, a defined sulfation method established for a certain polysaccharide cannot be transferred easily to another. The reason for the less-efficient sulfation of the pullulans might be their amount of primary OH groups, which is reduced by about 30% due to their α-1,6-glycosidic bonds between the maltotriose units. The OH group bound to the primary C atom turned out to have ten-fold higher accessibility to sulfation compared to those bound to secondary C atoms under the used reaction conditions (Alban et al., 1995). This observation corresponds to the report of low-sulfated dextran and alginic acid derivatives, which have only few or no free OH groups bound to the primary C atom, respectively (Whistler & Spencer, 1961).

Moreover, the comparison of the DS and the extent of degradation between corresponding PH and PN derivatives suggests that apart from the basic polysaccharide structure also physicochemical properties of the starting material influence the resulting product: the soluble, shorter-chain PN generally resulted in lower-sulfated, less-degraded products than the insoluble, longer-chain PH. In contrast to Lang, Masci, Dentini, Crescenzi, Cooke, Gidley et al. (1992), sulfation in a homogeneous system did not favor the efficacy of substitution. This might be due to the fact that the insoluble PH was well dispersed in DMF and activated prior to the sulfation. Further, it has to be considered also that initially dissolved polysaccharides become insoluble as soon as they are sulfated. Accordingly, as shown by the methylation analysis, the homogeneity of substitution in homogeneous systems was similar to that in heterogeneous ones. The results of this study support the hypothesis that the activation of the polysaccharide prior to the sulfation is crucial for a high and homogenous substitution (Yalpani, 1988b).

Pretreatment with DMF is assumed to activate polysaccharides to some extent by association of the highly polar DMF with their polar OH-group and to prevent their degradation (Schweiger, 1972). But in the case of the pullulans, the sulfation was insufficient and especially PH was degraded. As an alternative, the hydration/drying method according to Guiseley (1978) was applied to PH. This procedure avoids excessive drying of the polysaccharide, which was shown to deactivate polysaccharides. Although PH became soluble in DMF and in addition, the reaction time was prolonged, no increase in DS was found. This corresponds to the lower DS received with the soluble PN and to the observation that about 80% of the possible substitution occurs within the first 60 min (Guiseley, 1978). However, since during the lyophilization considerable degradation occurred, it cannot be excluded that the product was also partly desulfated.

The activation by alkali-complex formation turned out to be more successful with regard to the DS. Also the increase of the reaction temperature may contribute to the higher DS. However, the extent of degradation increased, so that in the case of the PH derivative two different fractions were obtained. It may be assumed that the higher reaction temperature was responsible for degradation (Nagasawa, Tohira, Inoue & Tanoura, 1971), but the re-sulfation of PH-S and PN-S under the same conditions did not result in degradation to a similar extent. The latter method resulted in the highest DS and the highest MW.

Consequently, by two-fold sulfation it is possible to overcome the low reactivity of pullulan. For the first sulfation, mild, non-degrading reaction conditions can be chosen. The homogeneity of the product can be increased by using stepwise solution of the insoluble pyridinium salt of the product. If products with low MW are desired, degradation during the lyophilization by omitting neutralization after dialysis represents a comfortable method. All these findings demonstrate that not only the sulfation procedure, but also the pretreatment of the polysaccharide and the purification of the product are crucial steps for the resulting sulfated polysaccharide. However, as shown by the four-fold repetition of the same procedure (Alban, 1993), identical products can be obtained if all conditions influencing the outcome are exactly maintained.

In order to receive detailed information about the structure of the pullulan sulfates, methylation analysis was performed. Since all derivatives contained non-substituted up to tri-sulfated glucose moieties, the substitution pattern was not very homogenous. Only the activation by the hydration/drying method resulted in a more regular substitution. This suggests that in this way the intra- and intermolecular hydrogen bonds are most effectively destroyed.

In view of the distribution of the sulfate groups on the various C-atoms of the glucose monomers, it turned out that the sulfation pattern of the pullulan derivatives considerably differed from that of the β-1,3-glucan sulfates produced by the same method (Alban & Franz, 1994b). In β-1,3-glucans, the OH-group in position 6 is about 10 times more accessible to sulfation than the OH-groups in positions 2 and 4. In contrast to this, the pullulan sulfates are mainly C6-substituted as well, but the preference of position 6 is less marked. After the OH group on C6, the OH group on C2, which has the highest degree of acidity, is most favored followed by that on C3. The low sulfation of position 4 can be attributed to the fact that about 70% of these OH groups

are involved in glycosidic bonds. Analogously, the decreased C6-substitution may be explained by the part of 30% 1,6-glycosidic bonds in pullulan. These results suggest that the sulfation pattern is determined not only by the reaction conditions, but also by the basic polysaccharide structure. Further experiments using different sulfation methods have to be carried out in order to evaluate whether the sulfation pattern of pullulans can be modified.

The modifications of the sulfation method used in this study resulted in pullulan sulfates covering a wide DS- and MW range. In the coagulation assays, they proved to represent potent anticoagulants reaching the activity of heparin. Their activity improves with increasing DS and MW as found for other sulfated polysaccharides like fucoidans (Nagumo & Nagumo, 1991; Nardella, Chaubet, Boisson-Vidal, Blondin, Durand & Jozefonvicz, 1996), sulfated dextran derivatives (de Raucourt, Mauray, Chaubet, Maiga-Revel, Jozefowicz & Fischer, 1998), dermatan sulfates (Ferrari, Marchesini & Maggi, 1994), glycosaminoglycans from sea cucumber (Suzuki, Kitazato, Takamatsu & Saito, 1991), and β-1,3-glucan sulfates (Alban et al., 1992; Alban & Franz, 1994a, 1994b). Already pullulan sulfates with DS as low as 0.17 show a basic anticoagulant activity, whereas e.g. β-1,3-glucan sulfates (Alban, 1997) or dextran sulfates (Nagasawa, Harada, Hayashi & Misawa, 1972) of similar MW require higher DS for any anticoagulant effect. In addition, the distribution of the sulfate groups on the various positions of the glucose markedly influences the activity and also the action profile. The increased TT-activity of PNNa-S, which has a higher content of sulfate groups bound to secondary C atoms than the other derivatives, correlates with the finding that β-1,3-glucan sulfates selectively sulfated in positions 2 and 4 are more active than those mainly sulfated in position 6 (Alban et al., 1994b). Corresponding results were reported recently for fucoidans and dermatan sulfates (Mulloy, Mourao & Gray, 2000; Pereira, Mulloy & Mourao, 1999). It is concluded that not only the chain length and the charge density but also the three-dimensional structure and flexibility of a sulfated polysaccharide influence its interactions with the coagulation proteins.

The action profile of the pullulan sulfates differs from that of heparin. Whereas UFH had the same specific activity in APTT, TT and Heptest® as determined by means of the 4th I.St., the pullulan sulfates showed the best effect in the TT, followed by that in the APTT and were only moderately active in the Heptest®. Since the various coagulation assays record interactions with different stages of the coagulation, they provide basic information about the mode of action of anticoagulants (Weißbach, 1990). The Heptest®, which was especially developed for heparin, measures the inhibition of factor Xa. The PT determines an interference with the extrinsic and the APTT with the intrinsic coagulation process. The TT is representative of the last coagulation step, the thrombin-mediated fibrin formation. Heparin mainly inhibits blood coagulation by catalyzing the factor Xa and thrombin neutralization by the endogeneous coagulation inhibitor antithrombin (Hirsh, Warkentin, Raschke, Granger, Ohman & Dalen, 1998). Owing to the non-significant effect of the pullulan sulfates in the Heptest®, these sulfated polysaccharides do inhibit factor Xa neither directly nor mediated by antithrombin. In addition, this has been proven by an amidolytic anti-factor Xa assay with chromogenic substrates (data not shown). The slight activity of the pullulan sulfates in the Heptest® has to be attributed to the insufficient specificity of the Heptest® measuring not exclusively the anti-factor Xa activity, but also the anti-thrombin activity to some extent (Bara, Mardiguian & Samama, 1990). Owing to the pronounced TT-activity of the pullulan sulfates, an important point of interference with the coagulation cascade is the thrombin-mediated fibrin formation. Provided that they have high MW and DS, they can be as active as heparin. The evaluated structure–activity relationships suggest that a certain minimum chain length is essential for this mechanism of action. In contrast to this, the efficacy in the APTT is not dependent on the MW in the same manner, because short-chain pullulan sulfates also are relatively active. Therefore, the anticoagulant effect of pullulan sulfates is suggested to be not limited on the interference with the last step of the coagulation cascade. Further investigations using amidolytic assays with chromogenic substrates showed that they strongly inhibit the intrinsic factor Xa generation (data not shown). In this way, they reduce the thrombin generation, the key process during the development of thrombosis (Hemker & Beguin, 1989).

In summary, the anticoagulant activity of the new pullulan sulfates changes not only quantitatively but also qualitatively in dependence on their individual structure as reflected by the ratio of the TT- to the APTT-activity. Consequently, they do not inhibit the coagulation non-specifically due to their negative charge, but they specifically interfere with several stages of the coagulation cascade, and these interactions have different requirements on the chemical structure. Accordingly, by controlled partial synthetic approach, pullulan sulfates with specific action profiles can be obtained. In addition, the pullulan sulfates turned out to strongly inhibit the activation of the complement system (manuscript in preparation). Therefore, they represent promising candidates for the development of new antithrombotics or of biocompatible, athrombogenic artificial surfaces for use during extracorporeal circulation, which is combined with severe activation of both coagulation and complement systems resulting in severe complications (Mollnes, 1998).

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