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Microwave-assisted desulfation of sulfated polysaccharides

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Abstract

Several sulfated polysaccharides were desulfated by heating their pyridinium salts dissolved in dimethyl sulfoxide in a microwave oven. The procedure was applied to different products like a λ-carrageenan, a partially cyclized μ/ν-carrageenan, an agar-like product like corallinan, a fucoidan and a chondroitin sulfate. When their pyridinium salts were generated in carefully chosen conditions, the desulfation proceeds smoothly after 1 min of heating, leading to a removal of 60–93% of the original sulfate present. Some depolymerization was found to occur, but its effect is moderate (yields of the recovered products are 64–89%), even for polysaccharides with labile bonds. An *in situ* methylation procedure was coupled with the microwave-aided desulfation method in order to facilitate the retrieval of structural data. Both spectroscopical and chemical studies showed that the integrity of the polysaccharides was not affected by this procedure (besides the loss of sulfate and the above-mentioned depolymerization).

Keywords: Carrageenan; Agaran; Fucoidan; Chondroitin; Microwave irradiation; Desulfation

1. Introduction

The determination of the covalent structure of polysaccharides is usually a complex task, which requires addressing several issues as their composition, linkage and sequence (Aspinall, 1982). Nowadays, spectroscopical methods, especially ^{13}C NMR spectroscopy have provided an increasing aid in answering those questions, particularly those related to linkage analysis. However, the structures of many polysaccharides are too complex to be determined by spectral data alone, and thus chemical methods are needed (Usov, 1998). Among them, methylation analysis is an old but widespread method for the linkage analysis of polysaccharides. It usually involves the complete etherification of free hydroxyl groups, so that a subsequent hydrolysis and identification of methylated monosaccharides allows to recognize the hydroxyl groups that were involved in glycosidic linkages (Aspinall, 1982). The problem arises when a polysaccharide also carries an alkali-stable group like most of the sulfate hemiester groups. In such circumstances, methylation analysis leads to ambiguities, as both the position of linkage and the position of sulfation will remain free after methylation. Several important sulfated polysaccharides are known to occur naturally, as the red seaweed galactans (agarans and carrageenans), many of them with industrial importance (Usov, 1998), brown seaweed fucoidans, some with significant biological activities (Ponce, Pujol, Damonte, Flores, & Stortz, 2003) and several important mammalian glycosaminoglycans like chondroitin, heparin, keratan and dermatan sulfates (Fransson, 1985). Their structural determination has been a matter of study for many years.

If a smooth procedure can be applied for desulfating the polysaccharide without affecting other points of the polysaccharide structure, then a subsequent methylation analysis can unequivocally determine the linkage positions and thus, the sulfate hemiester positions can be inferred by difference with the results of methylation of the original polysaccharide. Methanolic hydrogen chloride was the first

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desulfation agent ever used (Kantor & Schubert, 1957). This reagent worked nicely with a compound containing strong glycosidic linkages like chondroitin sulfate, but produced depolymerization in others like λ-carrageenan (Dolan & Rees, 1965), making it unfeasible for galactans containing labile 3,6-anhydrogalactosidic bonds. Several other methods were developed, most of them related with a solvolytic desulfation with DMSO alone or mixed with other solvents (Usov, Adamyants, Miroshnikova, Shaposhnikova, & Kochetkov, 1971; Usov, Miroshnikova, & Kochetkov, 1972; Nagasawa, Inoue, & Kamata, 1977; Furneaux & Stevenson, 1990), the use of pyromellitic acid (Miller & Blunt, 1998), sodium dithionite in alkaline medium (Miller & Blunt, 2002), and chlorotrimethylsilane (Kolender & Matulewicz, 2004). Some of these methods led to incomplete desulfations, depolymerizations and a sharply different behavior with distinct polysaccharides (e.g. Cases, Stortz, & Cerezo, 1994).

In recent years an increasing number of papers reported the use of microwave irradiation to speed up chemical reactions (Gabriel, Gabriel, Grant, Halstead, & Mingos, 1998; Lidström, Tierney, Wathey, & Westman, 2001; Stadler, Pichler, Horeis, & Kappe, 2002), some of them applied to polysaccharides (Kunlan et al., 2001; Singh, Tiwari, Tripathi, & Malviya, 2003; Zhou et al., 2004; Uy, Easteal, Farid, Keam, & Conner, 2005; Navarro & Stortz, 2005; Zhou, Yao, & Wang, 2006). It was shown that the heating mechanisms are different in conventional and microwave ovens (Gabriel et al., 1998; Lidström et al., 2001). In the present work we report the applications of microwave-assisted solvolytic desulfation (with a domestic microwave oven) of different sulfated polysaccharides, with good yields and high desulfation rates, and the follow-up by an external methylation analysis in a one-pot reaction, which sharply simplifies the structural analysis of sulfated polysaccharides.

2. Materials and methods

2.1. Microwave oven

A Sharp domestic microwave oven (model R353EA, 1200 W, operating at 2450 MHz) was used for all the experiments. Closed-vessel reactions were carried out in home-made threaded Teflon 50 ml tubes with screw caps, manufactured from 42 mm (tube) and 52 mm (cap) rods, with tube walls 5 mm thick. All the reactions were carried out at full microwave power, using the desired steps of microwave heating (10 s) followed by opening the vessel and cooling shortly (50–60 °C) in an ice-bath to relieve the pressure. With the current procedure, risks of accidents are minimized (Navarro & Stortz, 2005).

2.2. Polysaccharide materials

The cystocarpic and tetrasporic carrageenans from *Iridaea undulosa* (=*Sarcothalia crispata*) were obtained as described elsewhere (Stortz & Cerezo, 1993), though the λ-fraction from the tetrasporic material was isolated from a new batch, originated in seaweeds collected in 2003 in Cueva de Los Leones, Puerto Deseado (Santa Cruz Province). The corallinan was retrieved from the seaweed *Corallina officinalis* (Cases, Stortz, & Cerezo, 1992; Cases et al., 1994; Navarro & Stortz, 2002) using the procedure of Usov, Bilan, and Klochkova (1995). The fucoidan fractions from *Adenocystis utricularis* were obtained as reported elsewhere (Ponce et al., 2003). Chondroitin sulfate (70% type A, remainder type C) was purchased from Sigma (St. Louis, USA).

2.3. General methods

Sulfate was determined by the turbidimetric method of Dodgson and Price (1962), and also by ion chromatography (Kolender & Matulewicz, 2004) for low-sulfate content samples. Molecular weights (uncorrected for the presence of non-carbohydrate components) were calculated from the reducing power, which was determined by the method of Park and Johnson (1949). The methylated polysaccharides were hydrolyzed with 2 M TFA, 90 min at 120 °C, and the partially methylated monosaccharides were derivatized to the alditol acetates, which were analyzed by GLC and characterized by GLC-MS, using for either of them the conditions reported by Ponce et al. (2003). When 3,6-anhydrogalactose was present, a reductive hydrolysis of the methylated polysaccharide was carried out (Stevenson & Furneaux, 1991).

2.4. Pyridinium salts of the polysaccharides

Three different methods were used in order to generate the pyridinium salts. In method A, the polysaccharide samples were dissolved in water, passed through a cation-exchange column of Amberlite IR 120 (H^+), eluted with water and the eluate collected over 20% pyridine (Usov et al., 1972). After dialysis, the salts were recovered by lyophilization. In method B, water solutions of the polysaccharides were passed through a cation-exchange column of Amberlite IR 120 previously equilibrated with pyridine hydrochloride and washed thoroughly with water. The polysaccharide was eluted with water and recovered by lyophilization. Method C was carried out as stated for B, but drops of pyridine were immediately added to the eluate until the pH became neutral, and then lyophilized. The triethylammonium salts were generated by a method equivalent to B, using triethylamine hydrochloride instead of the pyridinium salt.

2.5. General procedure for desulfation and for desulfation–methylation

Twenty to 50 mg of the pyridinium salts of the polysaccharides were dissolved or suspended in 10 ml of dry DMSO (containing 2% dry pyridine) and treated for the desired time in the microwave as stated in 2.1. The reaction

mixture was allowed to cool in an ice-bath and then water was added, dialyzed against tap water and then distilled water (molecular weight cut-off 3500), and lyophilized. For the *in situ* methylation an aliquot of the microwave-treated product (ca. 2 ml) was stirred with 10 mg of powdered NaOH. After 30 min, methyl iodide (1 ml) was added under an ice bath, and the reaction was allowed to proceed at room temperature for 30 more min (Ciucanu & Kerek, 1984). The methylated polysaccharide was recovered by dialysis (molecular weight cut-off 3500) and lyophilization.

3. Results and discussion

Three different red seaweed galactans with known structural features were used: (a) the cystocarpic carrageenans from *I. undulosa* (fraction C, Stortz & Cerezo, 1993), consisting of partly cyclized μ/ν-carrageenans (Stortz, Bacon, Cherniak, & Cerezo, 1994) which contain all of the β-galactose units sulfated on C-4; (b) the purified λ-carrageenan obtained from *I. undulosa* (fraction T_1, Stortz & Cerezo, 1993; Stortz et al., 1994) which carries the β-galactose units sulfated on C-2, and (c) a crude xylogalactan from *C. officinalis* (Cases et al., 1992, 1994; Navarro & Stortz, 2002).

Previous experiments of solvolytic desulfation of polysaccharides showed that a pyridinium salt is needed in order to attain high desulfation rates, which do not occur with sodium salts (Usov et al., 1972). The traditional method of obtaining the pyridinium salt, by neutralization of the acid form of the polysaccharide followed by dialysis (Method A in our nomenclature, see Section 2) showed a considerable degree of depolymerization after heating in DMSO (Usov et al., 1972). However, we observed that this degradation also occurs before heating started indicating that the salt is labile. The degradation could have been originated after passing to the acid form and before neutralizing, or by the pyridinium salt itself. Another possibility comes from an incomplete conversion to the pyridinium salt, leaving some sulfate groups in the acidic form. Such prospect is supported by the work of Usov et al. (1972), who showed that a second solvolytic desulfation did not reduce the residual sulfate content unless the polysaccharide is reconverted to the pyridinium salt. In any case, we have decided to try the conversion to the pyridinium salt (Method B) by passage through a cationic column in the pyridinium form (Stortz & Cerezo, 1993). In such way, the polysaccharide should not be in the H^+ form for any time, and the isolation is faster as no dialysis is required.

The pH of a solution of the pyridinium salt of the μ/ν-carrageenan (obtained by method B) is 3.9. Even if this value is higher than that of the H^+ form (2.95), it is low enough as to expect some acid degradation, as observed previously (Usov et al., 1972; Kolender & Matulewicz, 2004). The degradation indeed occurs: the original average molecular weight of the polysaccharide (54,000) diminishes to 10,900 for the isolated pyridinium salt (method B). When drops of pyridine are added to the eluate, in order to obtain a neutral solution which is afterwards lyophilized, the molecular weight has an intermediate value: 23,100. Neutral conditions have already been used to obtain a pyridinium salt (Falshaw & Furneaux, 1994).

The result of submitting several different polysaccharides to microwave-assisted desulfation is shown on Table 1. For the partially cyclized μ-/ν-carrageenan it was shown that working for 1 min gives better desulfation rates than working for 30 s, without compromising seriously the

Table 1
Microwave-assisted desulfation of several sulfated polysaccharides

	Salt	Method	Original		Reaction time (min)	Desulfated		Reaction yield[a]	Desulfation percentage
			Sulfate (% SO_3Na)	MW (kDa)		Sulfate (% SO_3Na)	MW (kDa)		
C[b]	Py	A	27.9	54	1	8.0	1.9	65	71
C[b]	Py	B	27.9	54	0.5	15.7	4.7	62	44
C[b]	Py	B	27.9	54	1	8.4	2.1	73	70
C[b]	Py	B	27.9	54	1.5	ND	ND	12	ND
C[b]	Py	C	27.9	54	1	10.0	5.6	83	64
λ-Carrageenan	Py	B	45.3	600	1	6.4	5.3	35	86
λ-Carrageenan	Py	C	45.3	600	1	5.4	6.3	77	88
λ-Carrageenan	Et_3N	B	45.3	600	1	33.7	ND	31	26
Corallinan[c]	Py	B	12.0	31.6	1	1.3	11.4	81	89
Corallinan[c]	Et_3N	B	12.0	31.6	1	7.8	ND	91	35
Corallinan[c]	Py	C	12.0	31.6	1	4.6	13.4	89	62
EA1[d]	Py	C	32.0	19.0	1	2.2	8.0	ND	93
EA2-5[d]	Py	C	6.0	8.0	1	2.4	10.8	64	60
EW1-20[d]	Py	C	24.0	⩾100	1	1.6	26.4	74	93
Chondroitin S	Py	C	24.4	40.2	0.5	10.3	13.7	99	43
Chondroitin S	Py	C	24.4	40.2	1	4.3	11.3	74	83

[a] Reaction yield (recovery), after correcting for the loss of sulfate.
[b] Partly cyclized μ/ν-carrageenan (Stortz & Cerezo, 1993).
[c] Crude corallinan (Cases et al., 1992; Navarro & Stortz, 2002).
[d] Fucoidan fraction (Ponce et al., 2003).

molecular weight. On the other hand, longer reaction times lead to a substantial degradation, which renders the product unusable. The addition of pyridine right after conversion (Method C) leads to an even better yields and lesser depolymerization (higher molecular weights), with similar desulfation rates (Table 1). Methods A and B gave rise to similar results (Table 1). As Method B is simpler and faster, Method A was not used in further studies.

Furthermore, the desulfated product can be submitted to an *in situ* methylation in order to differentiate the sulfation positions from those compromised in a glycosidic bond. Table 2 shows the results of submitting the partially cyclized μ/ν-carrageenan and some of its desulfated counterparts to classical (triethylammonium salt) and *in situ* (pyridinium salt) methylation procedures. The Table indicates clearly that both methylation procedures give rise to similar results, indicating the known features of carrageenans of the κ-family (Stortz, 2005): similar amounts of 3-linked and 4-linked galactose units, being the formers mostly sulfated on O-4, and the latters mainly as 3,6-anhydrogalactose units, partly sulfated on O-2. The results of the microwave desulfation confirm those structural details. The partial desulfation experiment indicated that the desulfation of the 3,6-anhydrogalactose units proceeds faster than that of the galactose unit. The presence of small amounts of 2-*O*-methylgalactose in the methylation of the original polysaccharide (absent in desulfated products) and the moderate increase in 2,3,6-tri-*O*-methylgalactose in the desulfated products may be an indication of the presence of 4-linked 3-sulfated (or 3,6-disulfated) units. Sulfation on O-3 was only rarely found to occur on carrageenans (Errea & Matulewicz, 2003; Matsuhiro et al., 2005), but more commonly in agar-like polysaccharides (Kolender & Matulewicz, 2002; Estevez, Ciancia, & Cerezo, 2004). However, the present results can also be attributed to a partial undermethylation (on O-3) occurring for the original polysaccharide given the steric and electrostatic hindrance produce by the negatively charged sulfate groups. On the desulfated products, the fractions can be totally methylated as this hindrance has disappeared.

The desulfation results were similar for the heavily sulfated λ-carrageenan: the microwave-assisted desulfation carried out in standard conditions using the salt generated by the C method yielded a product with higher molecular weight, similar sulfate content and considerable higher yield than that generated by the B method. On the other hand, when the pyridinium salt was replaced by the triethylammonium one, only little sulfate was removed, and the overall yield was poor. Methylation analysis of the desulfated products yielded similar amounts of 2,3,6- and 2,4,6-tri-*O*-methylgalactose, and minor proportions of 4,6-di-*O*-methylgalactose, indicating the expected substitution pattern (Stortz, 2005). The presence of the dimethylated sugar is indicative that the 2-sulfate group on 3-linked β-D-galactose units is more reluctant to desulfation than sulfate groups on O-2 and O-6 of 4-linked α-galactose units.

Corallinan is a red seaweed galactan with low proportions of sulfate (Cases et al., 1992, 1994), for which conventional solvolytic desulfation worked deficiently (Cases et al., 1994). On the other hand, microwave solvolytic desulfation of its pyridinium salt (Table 1) gave excellent yields of products only slightly degraded, and with a sulfate loss of above 50%. The triethylammonium salt also gave a good yield, but the product has lost much less sulfate. In this case, the product obtained by method B gave rise to a larger desulfation without compromising seriously the yield and molecular weight. Probably the desulfation of the products obtained by method B combine solvolytic and acid mechanisms. For the corallinan, where there are no labile 3,6-anhydrogalactosidic linkages, this results in a better reaction.

The reaction was applied also to other polysaccharides. One that is difficult to desulfate is fucoidan, leading to low desulfation rates or low yields (Chizhov et al., 1999; Ponce et al., 2003). With the present method, desulfation proceeds to almost completion with only slight degradation and acceptable yields, whereas the *in situ* methylation gave similar results to those after a three-step methylation (Ponce et al., 2003). The method also works for polysaccharides that are easily desulfated by other procedures, like chondroitin sulfate. In 1 min, the reaction proceeds smoothly, with good yields and only moderate degradation. ^{13}C NMR spectra of the original and desulfated chondroitin samples (Fig. 1) were assigned by comparison with the work of Mucci, Schenetti, and Volpi (2000). The original

Table 2
Methylation analysis (mol/100 mol)[a] of a partly cyclized μ/ν-carrageenan and some desulfated fractions

Methylated sugar	Original polysaccharide	Desulfated polysaccharide			
Pyridinium salt		Method A	Method B	Method C	Method C
MW time	–	1 min	1 min	0.5 min	1 min
Methylation		Isolated[b]	*in situ*	*in situ*	*in situ*
2,4,6-Tri-*O*-Me-Gal	9	44	49	32	46
2,3,6-Tri-*O*-Me-Gal	4	6	8	6	10
2,6-Di-*O*-Me-Gal	44	14	9	29	12
2-*O*-Me-Gal	5	–	–	–	–
2-*O*-Me-3,6-AnGal	30	36	34	33	32
3,6-AnGal	8	–	–	–	–

[a] Only the partly methylated sugars with proportions higher than 4% are shown in the Table.
[b] Methylated after isolation (by dialysis and lyophilization) of the desulfated polysaccharide.

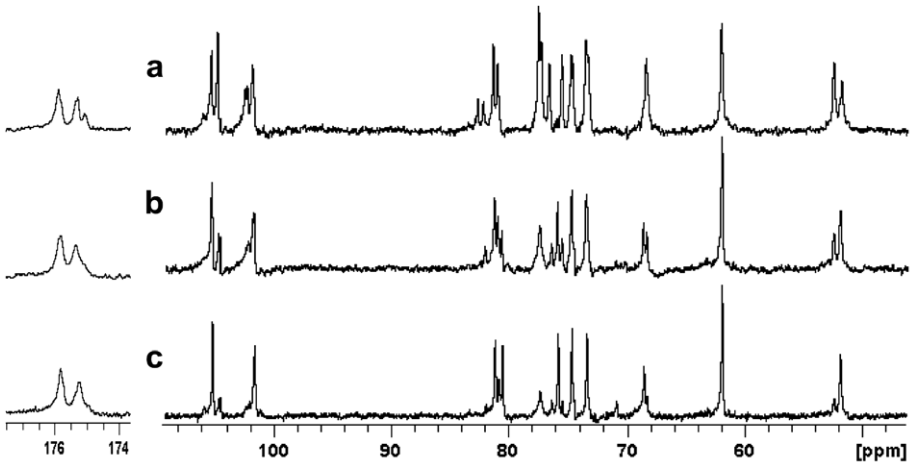

Fig. 1. 125 MHz-^{13}C NMR spectra of (a) chondroitin sulfate, (b) microwave-desulfated (30 s) chondroitin sulfate, and (c) microwave-desulfated (1 min) chondroitin sulfate, in the regions 46–110 ppm and 174–177 ppm.

sample, having both 4- and 6-sulfated units (ca. 6:4, according to the spectrum) has lost a large proportion of both sulfate groups in the 30 s reaction, and an even larger one after 1 min, leaving traces of both sulfate groups. The spectra points out the integrity of the desulfated products (Fig. 1), as indicated by the absence of spurious peaks or reducing terminals.

The present results show that an usually complicated procedure like desulfation can be aided efficiently by microwave heating in just one minute. Although some depolymerization was found to occur, its effect is moderate, even for polysaccharides with labile bonds. The desulfated polysaccharides can be the subject of spectroscopical or further chemical studies. In this sense, the possibility of joining this procedure with an *in situ* methylation opens up the scope even more. The structural determination of sulfated polysaccharides by chemical methods can thus be simplified, giving rise to results in just a few days.

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