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A novel process for preparation of (1→3)-β-D-glucan sulphate by a heterogeneous reaction and its structural elucidation

Ya-Jun Wang[a,b], Shan-Jing Yao[a,*], Yi-Xin Guan[a], Tian-Xing Wu[c], J.F. Kennedy[d,e]

[a]*Department of Chemical and Biochemical Engineering, Zhejiang University, Hangzhou 310027, People's Republic of China*
[b]*Institute of Bioengineering, State Key Laboratory Breeding Base of Green Chemistry Synthesis Technology, Zhejiang University of Technology, Hangzhou 310014, People's Republic of China*
[c]*Department of Chemistry, Zhejiang University, Hangzhou 310027, People's Republic of China*
[d]*Birmingham Carbohydrate and Protein Technology Group, School of Chemical Sciences, The University of Birmingham, Birmingham BI5 2TT, UK*
[e]*Chembiotech Laboratories, University of Birmingham Research Park, Vincent Drive, Birmingham BI5 2SQ, UK*

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Abstract

(1→3)-β-D-Glucans have received increasing attentions with respect to their biological functions. Previously, a method combining cellular induced autolysis with subsequent oxidation by sodium hypochlorite to prepare (1→3)-β-D-glucan from *Saccharomyces cerevisiae* was developed. However, the poor aqueous solubility of the resultant polysaccharide was a huge hindrance to its application especially in pharmaceuticals. Herein, a novel glucan sulphation process, also a heterogeneous reaction system, composed of sulfuric acid and *n*-propanol with a molar ratio of 1.4, is proposed to improve polysaccharide polarity and its aqueous solubility. After the heterogeneous sulphation at −6 °C for 90 min, (1→3)-β-D-glucan sulphate yield was about 37.4% (w/w), the degree of sulphation 0.36, and its empirical formula was $(C_6H_{10}O_5)_{25}\cdot 9SO_3\cdot 7H_2O$ according to elemental analysis. In addition, sulphation made the polysaccharide morphologically loosened according to observation under scanning electron microscopy. The presence of characteristic absorptions at 1246 and 813 cm^{-1} in the FTIR spectrum, as well as the ^1H NMR spectrum demonstrated that the material was a sulphate ester of glucan.

Keywords: (1→3)-β-D-Glucan sulphate; Sulphation; Heterogeneous reaction; Infrared spectrum; ^1H NMR

1. Introduction

(1→3)-β-D-Glucans, (1→3)-β-linked linear and branched glucose homopolymers, as a kind of constitutive fungi cell wall material, belong to the class of drugs known as biological response modifiers (BRMs). Extensive studies have demonstrated that (1→3)-β-D-glucans exhibit considerable immunomodulatory activity by binding specific macrophage receptors and activating macrophages, resulting in antitumor, antibacterial, and wound healing activities (Müller et al., 2000; Ohno, Miura, Miura, Adachi, & Yadomae, 2001; Patchen et al., 1998; Šandula, Kogan, Kacuráková, & Machova, 1999; Tsiapali et al., 2001). Moreover, (1→3)-β-D-glucans exert other biological activities potentially exploitable in therapy as a clinical immunostimulant (Liang et al.,1998).

In our previous work (Wang, Yao, & Wu, 2003), a novel method for preparing (1→3)-β-D-glucan from *Saccharomyces cerevisiae* with a significant increased yield was developed, which was based on the combination of cellular induced autolysis and subsequent oxidation of the autolyzed cell by sodium hypochlorite. However, the aqueous solubility of the glucan was as poor as that of glucans prepared by other processes, meaning that it was less suitable for pharmaceutical application. The abundance of –OH groups in glucan facilitates hydrogen bonds formation, causing the natural glucan to exist as a compact triple stranded helix, which in turn contributes to poor glucan aqueous solubility. As solubility of polymers always depends on their degree of polymerization, degree of

* Corresponding author. Tel.: +86 571 8795 1982; fax: +86 571 8795 1015.
E-mail address: yaosj@che.zju.edu.cn (S.-J. Yao).

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branching and chemical derivation and so forth. Glucan depolymerization by acidic or alkali hydrolysis, enzymatic degradation, supersonic treatment, as well as sulphation and phosphation (Machová, Kogan, Šoltès, Kacuráková, & Šandula, 1999; Williams et al., 1992) has been developed to enhance glucan solubility in aqueous solution. Among them, sulphation is a preferred method due to the positive impacts on their biological functions.

Previously, a homogeneous process through partially sulphating β-D-glucan in the presence of dimethyl sulfoxide and urea was developed (Williams et al., 1992). However, the sulphated glucan contained a significant amount of nitrogen (3.23% mol/mol), which might have been derived from the urea employed as the chaotropic agent. Besides this, the used reaction solution after sulphation by the homogeneous process would be an environmental waste load problem. Bohlman, Schneider, Andresen, and Buchholz (2002) and Yao (2000) have conducted a heterogeneous reaction process for the sulphation of cellulose by employing the mixture of sulfuric acid and *n*-propanol. Cellulose sulphate was prepared with good quality and stability by this process.

In this work, one goal is to develop a novel method based on a heterogeneous reaction process similar to the process of cellulose sulphation, in which β-D-glucan, prepared from yeast cell cultivation, could be sulphated with a reaction solution of sulfuric acid and *n*-propanol. Because the substrate glucan, product glucan sulphate are not dissolved by the reaction solution, the outstanding advantages of this heterogeneous reaction process for glucan sulphation are easy separation of target product from reaction solution and recycling of the reaction solution. It is especially of benefit to a large-scale production. This work also sets out to exemplify the use of elemental analysis, electron microscopy, infrared spectroscopy and ^{1}H NMR techniques to detect the structural characteristics of (1→3)-β-D-glucan sulphate prepared by the new heterogeneous process.

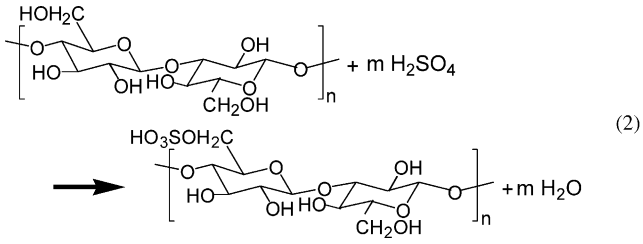
2. Description of heterogeneous reaction process

2.1. Reaction mechanism of (1→3)-β-D-glucan sulphation by a heterogeneous process

According to the reaction mechanism of cellulose sulphation (Yao, 2000), the reaction solution is formed from sulfuric acid and *n*-propanol at follows

$$\alpha H_2SO_4 + \beta CH_3CH_2CH_2OH \rightarrow \beta CH_3CH_2CH_2OSO_3H + (\alpha - \beta)H_2SO_4 + \beta H_2O \quad (1)$$

In this reaction, the sulfuric acid is in excess to *n*-propanol with a molar ratio of sulfuric acid to *n*-propanol 1.4. (1→3)-β-D-glucan sulphation is carried out between the solid (1→3)-β-D-glucan and excess sulfuric acid in the reaction solution as the following expression,



(2)

The extent of glucan sulphation varies with the concentration of sulfuric acid in the reaction solution. In addition, the esterification (1) and *n*-propanol have vital roles in controlling the concentration of sulfuric acid because too high a concentration of sulfuric acid will bring about adverse reactions, such as hydrolysis, carbonization and so on.

2.2. Heterogeneous process for (1→3)-β-D-glucan sulphate preparation

The heterogeneous process for the preparation of (1→3)-β-D-glucan sulphate, in which a mixture of sulfuric acid and *n*-propanol is applied as the reaction solution, is presented in Fig. 1. Reaction solution is prepared at −10 °C. In addition to little amount of H_2O in the chemical reagents at level of about 3%, the reaction solution contains three principal components: sulphuric acid, *n*-propanol and *n*-propanol sulphate. The glucan sulphation was performed at −6 °C for 90 min. Because the glucan and its sulphate are both insoluble in the reaction solution, a simple solid–liquid separation operation can realize the isolation of substrate and product from the reaction solution after sulphation, and the used reaction solution can be regenerated after a slight adjustment of sulphuric acid content. Further, the target product, glucan sulphate, can be easily separated from glucan by resuspending their mixture in water and subsequent centrifugation due to the water solubility of glucan sulphate and the aqueous insolublility of glucan. The resultant supernatant is combined and lyophilized.

3. Materials and methods

3.1. Microorganism and cell culture

The strain of nonpathogen *S. cerevisiae* FL1 was utilized in all the experiments. Yeast was streaked on solid YEPD agar plates (20 g L^{-1} glucose, 20 g L^{-1} peptone, 10 g L^{-1} yeast extract and 18 g L^{-1} agar), grown at 28 °C for 2 days, and then maintained at 4 °C (Wang et al., 2003).

The *S. cerevisiae* FL1 preinocula were prepared in liquid YEPD media with no agar, starting from plates. One colony was transferred into a 50 mL Erlenmeyer flask containing 15 mL YEPD medium and incubated for 8 h at 30 °C with agitation of 220 r min^{-1}. Then 10 mL preinocula was transferred into a 250 mL Erlenmeyer flask with 50 mL YEPD medium in aseptic manner, and further incubated for

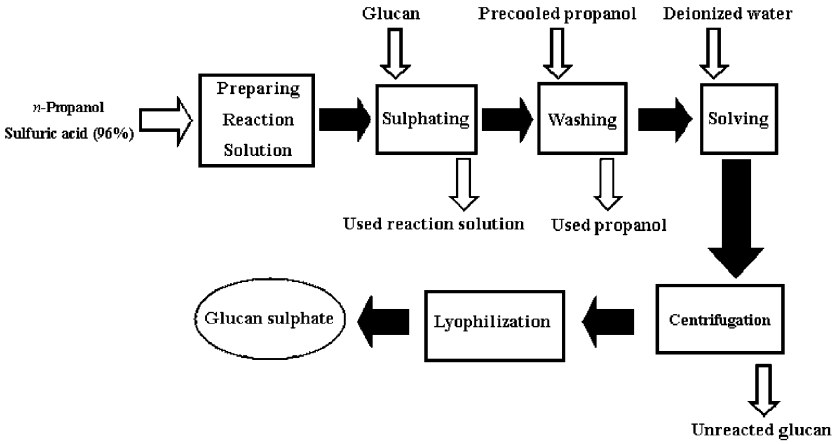


Fig. 1. Heterogeneous reaction for (1→3)-β-D-glucan sulphate preparation: process scheme.

6 h at 30 °C. Cultivations were carried out in a 3.7 L fermenter (Bioengineering, Switzerland) at 30 °C, agitation speed at 400 r min^{-1}, and an air flow rate of 1 vvm. The working volume of the fermenter was set at 2 L. 40 mL inocula were transferred into the growth medium containing 25 g L^{-1} yeast extract, 115.5 g L^{-1} sucrose, 10 g L^{-1} ammonium sulphate, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 g L^{-1} KH_2PO_4, 0.1 g L^{-1} NaCl, 0.1 g L^{-1} $CaCl_2 \cdot 2H_2O$. After culture for 36 h, *S. cerevisiae* FL1 cells at the stationary phase were collected by centrifugation at 8900 *g* for 10 min, followed by a triple washing in physiological saline solution.

3.2. Chemicals

Peptone and yeast extract were purchased from Oxoid (UK). Agar was supplied by Shanghai Chemical Reagent Corporation (China). Dimethyl-d_6 sulfoxide was obtained from Cambridge Isotope Laboratory Inc (UK). All other chemical reagents were from commercial market and of analytical grade.

3.3. (1→3)-β-D-Glucan isolation

(1→3)-β-D-Glucan was prepared using a two-step treatment. During the first stage, yeast induced atuolysis was conducted by exposing *S. cerevisiae* FL1 slurry to 1.5% (v/v) ethyl acetate aqueous solution (pH5.5) at 52 °C for 36 h with agitation at about 140 r min^{-1} to avoid cell precipitation. Autolyzed cells were collected by centrifugation and further oxidized under alkali condition in the second stage. Autolyzed cells (20 g) were suspended in 0.1 mol L^{-1} sodium hydroxide solution (2.0 L) with an appropriate amount of sodium hypochlorite at 0.8% level of final concentration of available chlorine. The oxidation continued for 5 h at room temperature, after which the insoluble fraction was harvested by centrifugation at 13,900 *g*, and washed extensively with deionized water. The resulting (1→3)-β-D-glucan was obtained by lyophilization.

3.4. (1→3)-β-D-Glucan sulphation procedure

In the first step of two steps, sulfuric acid (125 mL) was slowly dropped with gentle agitation into a flask containing 125 mL *n*-propanol at −10 °C to make a reaction mixture. Then, (1→3)-β-D-glucan (10 g) was suspended into the reaction mixture. Sulphation was performed at −6 °C for 90 min. After the reaction, the sediment was harvested by centrifugation at 27,200 *g* for 12 min at −10 °C, followed by a triple washing with 150 mL *n*-propanol at −10 °C. Then, the sediment was dissolved in deionized water, and centrifuged to discard the unreacted (1→3)-β-D-glucan. The supernatant was collected and lyophilized.

3.5. Elemental analysis

Elemental analysis of C, H, O, N, and S were carried out with a Carlo Erba 1110 (Italy) instrument. According to the results of elemental analysis, the degree of sulphation (***DS***) is defined as the following expression (3).

$$\mathrm{DS} = \frac{72s}{32c} \tag{3}$$

Where *s* refers to the mass ratio of S element in the product glucan sulphate, and *c* the mass ratio of C element in the product glucan sulphate. Hence, by the definition, ***DS*** denotes the number of sulphate group per glucose residue.

3.6. Electron microscope detection

Transmission electron microscopy (TEM) was employed to examine yeast structural alterations after autolysis. Cells were fixed in the pentamethylene aldehyde solution at 4 °C overnight. After rinsing extensively with pH 5.2 phosphate buffer, they were embedded in epoxy resin. Cells were dehydrated gradually with a series of ethanol aqueous solution from 10 to 100% (v/v) after washing with the above buffer. Dehydrated cells were sliced into thin sections and stained with uranyl acetate and lead citrate

(Martýnez-Rodrýguez, Polo, & Carrascosa, 2001). All preparations were examined using a TEM JEM-1200 EX instrument (JEOL, Japan).

Glucan and its derivate were checked with a scanning electron microscope (SEM). Samples were sprinkled onto a double-sided tape, sputter coated with gold, then examined under a SEM JSM-T20 instrument (JEOL, Japan).

3.7. Infrared spectroscopy detection

Samples were pressed into KBr disks with an approximate weight ratio of 1:50. The resulting KBr disks were analyzed using a Fourier transform infrared spectrometer Impact 400D (Nicolet, Japan). In order to obtain more exact band positions, Fourier self-deconvolution was applied.

3.8. ^{1}H NMR spectroscopy detection

The ^{1}H NMR spectra of the polysaccharide and its derivate were obtained at FT-NMR spectrometer DXM 500 (Bruker, Switzerland) equipped with a 5-mm multinuclear inverse probe. Samples were dissolved in dimethyl-d_6 sulfoxide at the concentration of about 20 mg mL^{-1}. All spectra were carried out at 25 and 80 °C, respectively. The scanning number was 16. Chemical shifts (δ) are given in parts per million downfield from tetramethylsilane (TMS), which was used as an internal standard.

4. Results and discussion

4.1. (1→3)-β-D-Glucan preparation and its sulphation

S. cerevisiae FL1 at the stationary phase, grown for 48 h, was selected as the starting material. Cells are ovoid, elongate, and within the cytoplasm there is a large vacuole containing some spherical bodies, located mainly on the edges of the vacuole (Fig. 2A). After exposure to 1.5% (v/v) ethyl acetate aqueous solution (pH 5.5) for 36 h at 52 °C, the autolyzed cells display many wrinkles or folds on the walls without significant variations in cell volume, volumetric reduction in vacuoles, and the distinct separation of cell wall from cytoplasm as well (Fig. 2B). Moreover, notable structural and morphological alterations take place on *S. cerevisiae* FL1 with no observable variations in the cellular wall after induced autolysis for 36 h, compared with that of the starting reference cells (Fig. 2). It demonstrates that induced autolysis is helpful to discard unwanted intracellular substances and nearly keeps cell wall intact.

Further, dilute sodium hypochlorite oxidation under alkali environment was applied to oxidize the autolyzed cells for preparing (1→3)-β-D-glucan. The yield of (1→3)-β-D-glucan is much improved compared with that of the traditional method. However, its aqueous solubility is still poor. To solve this problem, sulphation by a heterogeneous reaction process was proposed to enhance

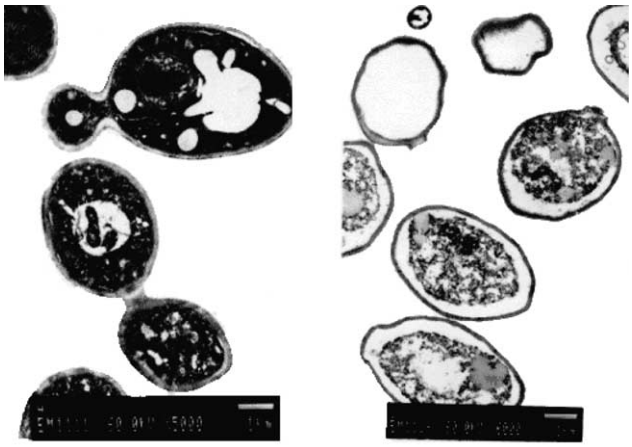


Fig. 2. Morphological and structural alterations of *S. cerevisiae* FL1 after induced autolysis in 1.5% (v/v) ethyl acetate solution (pH 5.5) at 52 °C: A, prior to autolysis; B, after autolysis.

glucan polarity and solubility accordingly. The resulting preparation is white, completely aqueous soluble, which indicates that the heterogeneous process is practicable and efficient to elevate glucan solubility. The product yield of sulphation, which is defined as the ratio of the amount of glucan sulphate formed to that of original glucan, is, however, 37.4% (w/w).

4.2. SEM observation

The original water-insoluble (1→3)-β-D-glucan is compact and sheet-like (Fig. 3A), which may be due to the existence of abundant hydrogen bond and mild treatment. Glucan sulphate prepared by the heterogeneous sulphation process becomes morphologically loosened, fibre-like by observation under scanning electron microscopy (Fig. 3B), indicating partial hydrogen bonds are broken and molecular polarity is increased on sulphation.

4.3. Elemental analysis

To obtain the general formula of the (1→3)-β-D-glucan sulphate and its degree of sulphation, elemental analysis was performed. Elemental analysis of lyophilized glucan sulphate reveals a chemical composition (w/w) of 36.3% C, 6.1% H, 51.1% O, 5.8% S, 0.7% N. According to elemental analysis, the degree of sulphation of (1→3)-β-D-glucan sulphate is therefore 0.36 and its empirical formula is $(C_6H_{10}O_5)_{25}\cdot 9SO_3\cdot 7H_2O$, suggesting that, on average, 36 sulphate groups are substituted on every 100 glucose subunits along the polysaccharide. In addition, the amount of nitrogen existing in the glucan sulphate prepared by the heterogeneous process was negligible compared with 3.23% (mol/mol) nitrogen in product by the homogeneous sulphation process in the presence of dimethyl sulfoxide and urea (Williams et al., 1992).

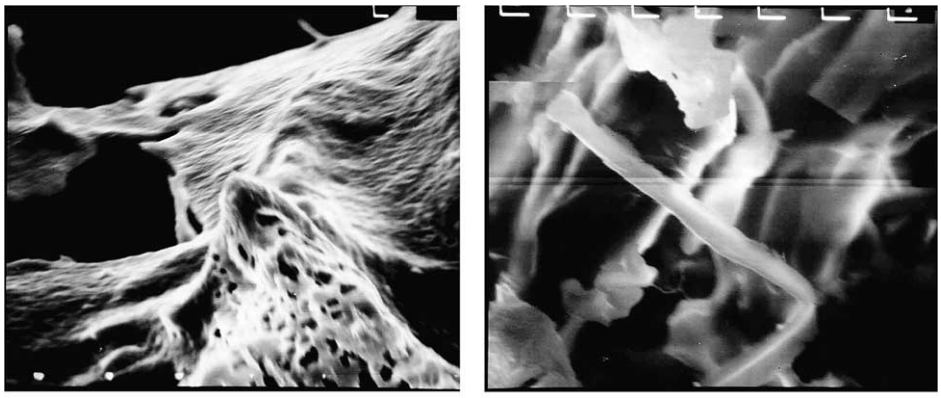


Fig. 3. Observation of (1→3)-β-D-glucan prior to and after sulphation under a SEM: A, original glucan (×1000); B, glucan sulphate (×1500).

4.4. Infrared spectra

Mid-infrared spectroscopy was employed to determine the structural characteristic of the polysaccharide sulphate. In the functional group region of both infrared spectra, there are significant absorptions at 3413, 2922 and 1748 cm^{-1} (Fig. 4), which corresponds to the stretching absorption bands of poly –OH, C–H and C=O, respectively. The C–O vibrations ranging at 1200–950 cm^{-1} as well as in the 'anomeric region' at 950–750 cm^{-1} distinguish β-D-glucan from α-D-glucan spectra (Mathlouthi & Koenig 1986). In the case of our tested samples, there is no absorption at 841 cm^{-1}, suggesting the absence of the α-D-glucopyranosyl residues; the presence of characteristic absorption bands are at 1157, 1074, 1041 and 891 cm^{-1} (Fig. 4), which demonstrate that D-glucosidic linkage configuration is β type prior to and after sulphation. In the FTIR spectrum of sulphated glucan, the presence of characteristic absorptions at 1246 and 813 cm^{-1} is notable (Fig. 4A), which corresponds to S=O stretching and C–O–S vibration as the characteristic bands of the sulphate ester of hexose are at 1240 cm^{-1}, 810 cm^{-1} respectively.

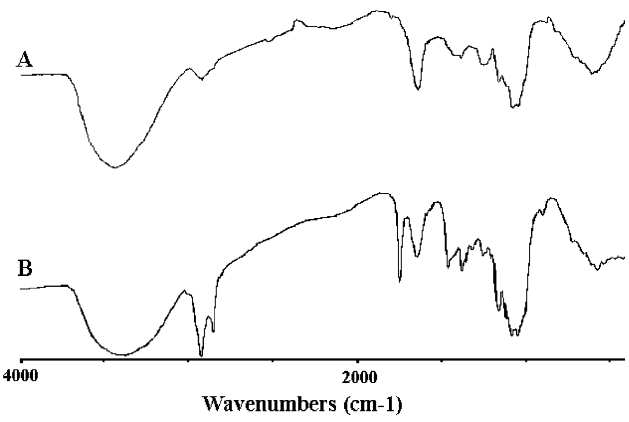


Fig. 4. Comparison of (1→3)-β-D-glucan IR spectra prior to and after sulphation: A, glucan sulphate; B, original glucan.

4.5. ^1H NMR studies of the polysaccharides

^1H NMR spectroscopies of the original glucan (Fig. 5) and sulphated glucan (Fig. 6) were conducted at 25, 80 °C using DMSO-*d6* as solvent. ^1H NMR spectra clearly show that the resolutions are temperature dependent. Higher temperature causes a higher degree of resolution. High molecular mass of the polysaccharides makes signals overlapped and broader and hence elevating temperature reduces the line width. In addition, chemical exchange effect on line width broadening also correlates with temperature. The contribution to exchange broadening decreases at higher temperatures since the chemical exchange rate increases.

Comparison of the 'anomeric region' of ^1H NMR spectra with that of previously reported indicates that they are of the similar pattern. The chemical shifts values support the

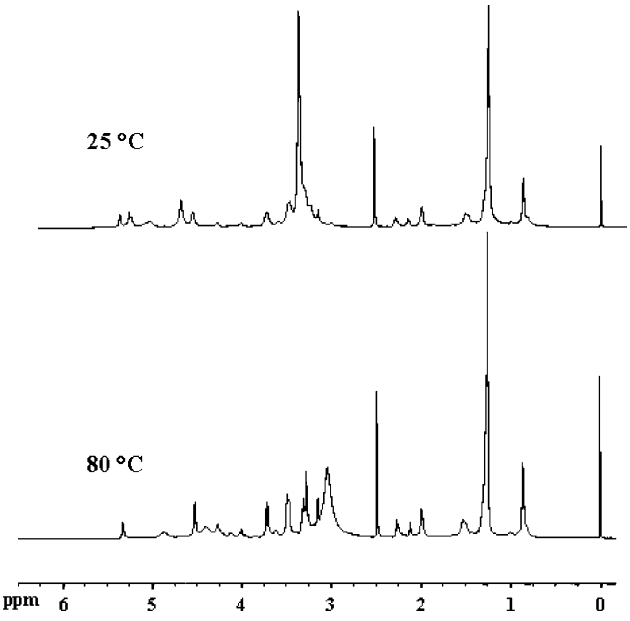


Fig. 5. ^1H NMR spectra of (1→3)-β-D-glucan in Me_2SO-d_6 at 25 and 80 °C.

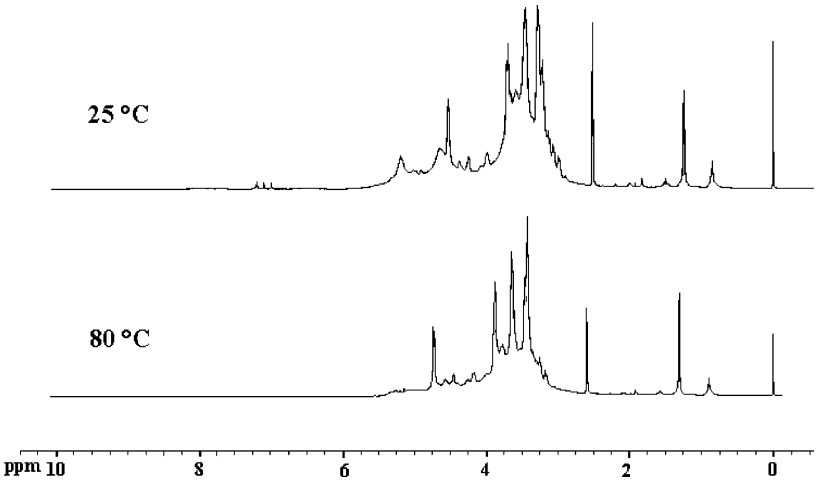


Fig. 6. ^{1}H NMR spectra of (1→3)-β-D-glucan sulphate in Me_2SO-d_6 at 25 and 80 °C.

resonance having β-configuration. From the IR spectrum and ^{1}H NMR spectra, we can draw a conclusion that the preparation is exclusively composed of (1→3)-β-D-linkages. Comparison of the 'anomeric region' of ^{1}H NMR spectrum of sulphated glucan (Fig. 6) with those of the original glucan in Me_2SO-d_6 (Fig. 5) clearly shows that a strong signal is lost in the ^{1}H NMR spectrum of sulphated glucan, that is, a corresponding hydrogen is substituted. Sulphation is responsible for the alteration in ^{1}H NMR spectra.

5. Conclusions

The yield of (1→3)-β-D-glucan sulphate by a heterogeneous process was approximately 37.4% (w/w), and it is relatively low compared to the yield by the homogeneous process (Williams et al., 1992). In the homogeneous process, the presence of polar solvent dimethyl sulfoxide and the chaotropic agent urea made glucan soluble in the above mixture. Therefore, sulphated glucan and original glucan were precipitated together after homogeneous sulphation, which overestimated the yield of soluble glucan sulphate. On the contrary, in the hetergeneous process, the lower yield is attributed to short reaction duration and acidic hydrolysis by sulfuric acid under the severe reaction conditions. The moisture in the glucan accelerates the hydrolysis. However, high yield may be obtained by prolonging the duration, and adjusting the molar ratio of sulfuric acid and *n*-propanol. In addition, heterogeneous sulphation makes glucan structurally loosened.

The combination of NMR with other analysis techniques to gain structural or functional information is employed in this work. ^{1}H NMR data showed conclusively that the D-glucosidic linkages were still in the β-anomeric configuration and that hydrogen atoms in glucan were partially substituted. Moreover, the presence of absorptions at 1246, 813 cm^{-1} in the IR spectrum of the glucan sulphate demonstrated that the preparation was the sulphate ester of hexose. Elemental analysis of glucan sulphate revealed its degree of sulphation to be 0.36 and its empirical formula was $(C_6H_{10}O_5)_{25}\cdot 9SO_3\cdot 7H_2O$, suggesting that, on average, 36 sulphate groups were substituted on every 100 glucose subunits along the polysaccharide. In addition, the amount of nitrogen existing in the glucan sulphate prepared by a heterogeneous process was negligible compared with that of the homogeneous sulphation process in the presence of dimethyl sulfoxide and urea. In addition to easy separation, the outstanding advantage of this heterogeneous process is ecologically more viable. All in all, it is practical to prepare (1→3)-β-D-glucan sulphate by the heterogeneous process.

Acknowledgements

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