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Study of a novel labelled scleroglucan macromolecule

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Abstract

A fast and rapid chemical reaction is presented here for labelling the polysaccharide scleroglucan with iodine to simplify its detection. The conditions proposed for the chemical reaction (temperature, concentrations of reactants and pH) were selected so as to substitute only a few of the OH groups in the polymer. The quantification of the labelled macromolecule is possible by radioactivity or Total Reflection X-ray Fluorescence techniques, depending on the iodine isotope used. Both techniques were adjusted for their use in labelled (with ^{127}I or ^{131}I) scleroglucan solutions.

A comparison of the molecular weight distribution and the viscosity between solutions of the labelled product and the original one is performed and no major differences were found.
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1. Introduction

The aim of this work was to label the polysaccharide scleroglucan by a fast and simple chemical reaction, to simplify its detection.

Scleroglucan is secreted by the fungus *Sclerotium Rolfsii*. This polymer is an exopolysaccharide chosen because of its usefulness in Enhanced Oil Recovery, food and pharmaceutical application (Sandford, 1979). The chemical structure (Fig. 1) has been characterized as a linear chain of linked β-(1,3)-D-glucopyranosyl units, with a single linked β-(1,6)-D-glucopyranosyl unit to every third unit of the main chain. Scleroglucan dissolves in water as a rod-like triple helix. Its average total length is ∼1 μm, its diameter is approximately 2 nm and it has a persistence length of 180 nm. This neutral glucan has a mean molar mass 4×10^6 Da (Biver, 1986). Its solutions, even at low concentrations, attain a high viscosity and there is a tendency to form ordered phases of the liquid crystal type (Deslandes, Marchessault, & Sarko, 1980; Yanaki, Norisuye, & Teramoto, 1984).

The purpose of this work was to label a macromolecule for its subsequent uses to study the displacement through non-transparent porous media, mainly for in Enhanced Oil Recovery applications (Chertcoff, 2000; Grattoni, Rosen, Chertcoff, & Bidner, 1987; Obernauer et al., 1994; Rosen, Chertcoff, & Calvo, 1989; Temprano & Vartuli, 1996).

Classical polysaccharide detection techniques are complex and laborious, they have very high detection limits, low sensitivity and sampling is generally required (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). This has been an impediment to make fast and accurate experiences in both dynamic and static conditions. Labelling with salts to detect the fluid by conductimetry has been used for years in spite of the problem caused by differences in the mobility of the small ions and the large macromolecules (D'Onofrio, 2000; Vartuli, Temprano, Chertcoff, & Rosen, 1991). Fluorescent tagging by reductive amination of the aldehyde end group (Roger et al., 2002) is not appropriate for these polymers because of their long chains. The substoichiometric addition of fluorophores and UV chromophores to the hydroxyl

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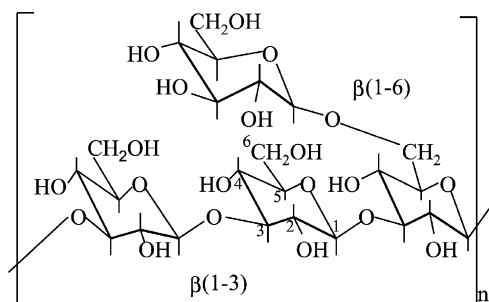E-mail address: sboeyke@fi.uba.ar (S.P. Boeykens).

Fig. 1. Structure of the scleroglucan monomer unit.

Fig. 2. Reaction scheme for preparation of labelled scleroglucan.

groups has been reported (DeAngelis, 2000). The radioisotope detection, a direct no-sampling technique, has been used to study structures of compounds, reaction mechanisms and as a tool in analytical chemistry, though the major application has been in biochemical research (Ugalde et al., 1973).

We have previously proposed the applicability of Total Reflection X-ray Fluorescence analysis (TXRF) for polymer systems (Vázquez, Boeykens, & Temprano, 1998; Vázquez, Custo, & Boeykens, 2001). That technique is a variation of energy-dispersive X-ray Fluorescence. A different optical arrangement makes the primary beam strike the sample at a glancing angle of less than 0.1°. Because of this grazing incidence, the beam is totally reflected (Wobrauschek & Kregsamer, 1989). TXRF is used for chemical micro-, ultramicro- and trace analyses because of its high detection power and sensitivity. Matrix effects cannot build up within the thin layer of the sample. By this technique, only elements of atomic number higher than Si are detectable (Klockenkämper, 1997).

The introduction of iodine atoms into the macromolecule makes the detection possible by any of the above-mentioned two methods, depending on the iodine isotope used. The goal was to label the macromolecule without altering its physicochemical properties (especially its transport properties); this means the substitution of only a few OH groups per macromolecule. Tests were made by conductimetry, ^{13}C NMR and TXRF analysis using the isotope ^{127}I.

The physical properties of the labelled product and the original macromolecule were compared. Molecular weight distribution and solubility of the polymer and rheological behaviour of their solutions were tested and no major differences were found. The labelled polymer is useful for the study of the behaviour of the scleroglucan in adsorption, dispersion and diffusion experiences (D'Angelo, Chertcoff, & Rosen, 2001; Obernauer et al., 1997).

1.1. The chemical reaction

The tracers most used to label organic compounds are isotopic carbon and hydrogen. Other important isotopes are those of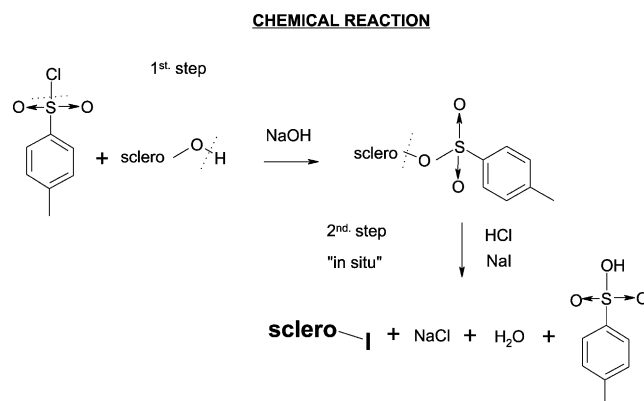 nitrogen, oxygen and halogens. The method for incorporating an isotope within an organic compound largely depends on the nature and availability of the tracer. The introduction of the radioactive material should be at the latest possible stage of a multi-step synthesis, avoiding handling as much as possible, and the recovery of the isotope from unwanted side-products or unused starting material should be made without unnecessary dilution (Arnstein, 1962).

The reaction for labelling the polymer should be fast and simple. The atoms should be covalently linked to assure that the detected signal correspond to the macromolecule displacement. The reaction should not promote polymer degradation.

In this work, an iodation in two steps is proposed to label the scleroglucan, following a procedure previously reported for α-cyclodextrin (Iwakura et al., 1975). The first step or 'activation' consists of the introduction of a very good leaving base (p-toluensulfonyl or p-tosyl) which is displaced by the iodide in the second step, in situ (Fig. 2).

2. Materials and methods

2.1. Reagents

Scleroglucan (Actigum CS11) was provided by Sanofi Bio Industries (France). To obtain the homogeneous dispersions a measured mass of the solid was vigorously shaken during 24 h in high-purity water using a magnetic stirrer. The polymeric systems were purified following a protocol especially tested and described in a previous study (Boeykens, Temprano, & Vázquez, 2002). The membranes used for dialysis were supplied by A.H. Thomas Co., (No. 4465-A2).

p-Toluenesulfonyl chloride (tosyl chloride) was provided by Aldrich (T3595.5), sodium hydroxide and hydrochloric acid by Merck, and sodium iodide u.s.p. (granular) by Mallinckrodt. The Na^{131}I radioactive samples were provided by CNEA with an activity concentration between 70 and 250 C l^{-1}.

Standard solutions of S and I for TXRF calibration were Tritisol Merck (1000 $\mu g\ ml^{-1}$).

2.2. X-ray analysis

TXRF technique was adjusted for the analysis of scleroglucan labelled solutions (using [127]I) and used for test the evolution of the chemical reaction.

X-ray analysis was carried out at the X-Lab in CNEA, using a total reflection system composed of an X-ray spectrometer, an X-ray tube excitation system, a total reflection module and spectrum acquisition and quantitation software (Wobrauschek & Kregsamer, 1989). The X-ray spectrometer consisted of an 80 mm^2 Si(Li) detector with 166 eV of full width half-maximun for 5.9 keV, a 0.008 mm thick Be window, an Ortec 672 fast spectroscopy amplifier and an analogical digital converter Nucleus PCA2. Excitation conditions were 40 kV and 30 mA in all cases.

The total reflection module designed at the Atominstitut der Östereichischen Universitäten, fitted with a cut-off-filtered radiation from a fine focus diffraction molybdenum anode X-ray tube was employed (Van Espen & He 1989).

For spectral data analysis, the AXIL program was employed. The QXAS software package from the International Atomic Energy Agency, was employed for quantification data.

For these experiences, a set of calibration standard solutions was made, containing S, and I covering a range between 5 and 50 $\mu g\ l^{-1}$. Co was chosen as internal standard owing to its non-interfering properties. The system sensitivity required 0.5 $\mu g\ l^{-1}$ Co content. The fluorescent intensity of the Kα or Lα lines was measured for each element. The acquisition time for each spectrum was 200 s.

For measurements, an aliquot of 10 μl of standard solution or sample was pipetted onto a quartz glass sample carrier and allowed to dry to a thin film under an infrared lamp. Four replicates of each sample were analysed.

Intensity ratio between element i and internal standard (Co) was obtained by

$$\frac{I_i}{I_{Co}} = \frac{s_i}{s_{Co}} \frac{C_i}{C_{Co}} \qquad (1)$$

where I_i is the line intensity (counts s^{-1}) of the element i in the sample; C_i is its concentration (ng ml^{-1}); I_{Co} is the line intensity of the internal standard (Co) in the sample; C_{Co} is its concentration; s_i is the system sensitivity (counts ml $ng^{-1}s^{-1}$) for the element i; s_{Co} is the system sensitivity for the internal standard (Co).

Relative quantities (R_i) (ng^{-1} ml) of the element i for this system are defined by

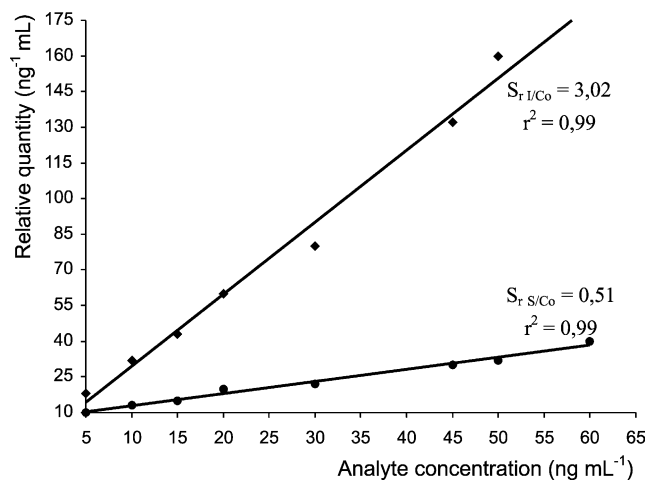$$R_i = \frac{I_i}{I_{Co}} C_{Co} \qquad (2)$$

Fig. 3. TXRF calibration curves for I and S. The relative intensities (S_r) and the correlation coefficients (r^2) are shown beside the curves.

S_{ri}, the no-dimensional relative sensitivity of the system for the element i, is defined by

$$S_{ri} = \frac{s_i}{s_{Co}} \qquad (3)$$

that results

$$R_i = S_{ri}C_i \qquad (4)$$

Fig. 3 shows the relative quantity (R_i) versus the analyte concentration (C_i) in the samples. The slope of these curves, relative sensitivity (S_{ri}), and the regression coefficient (r^2) are also shown.

The detection limits (DL) were calculated (Miller & Miller, 1988) by measuring the element intensities in low concentration solutions (as near to the background as possible).

$$DL_i = 3\frac{C_i}{l_i}\sqrt{2B_g} \qquad (5)$$

where DL_i is the minimum concentration (ng ml^{-1}), that can be detected for the element i; and B_g is the background intensity (counts s^{-1}).

2.3. Radioactivity detection

A radioactivity technique was adjusted for the use of scleroglucan labelled with ^{131}I solutions and used to control the evolution of the labelling reaction.

The radioactivity experiences were carried out at the Radioisotopes Laboratory of the GMP in FIUBA, using a 2M2/2 EG&G Ortec with a 905-3 NaI (Tl) Crystal detector and 276 Photomultiplier tube base and 575 Amplifier assembly, with a TRUMP-PCI_2K Buffercard multichannel and the Maestro-32 MCA emulation software.

A radioisotope gamma (γ) emittor was chosen because γ-rays will pass through the container's walls and also
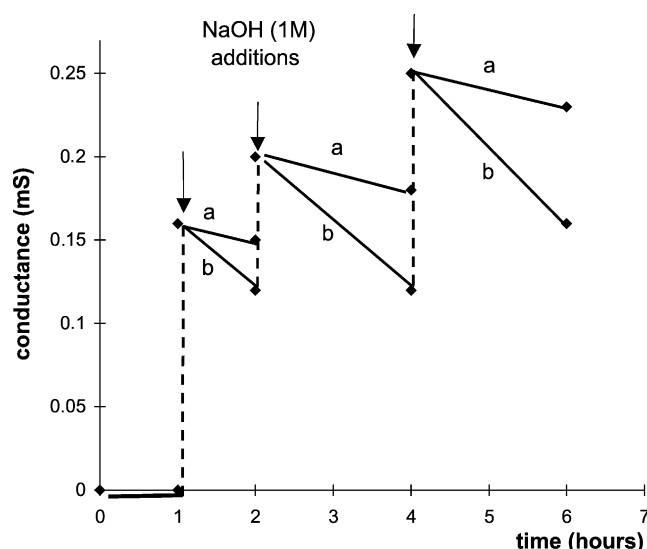
Fig. 4. Conductance evolution during the first step of the labelling chemical reaction at two different temperatures: (a) 25 °C and (b) 60 °C. Arrows mark the addition of equal volumes of 1 M NaOH.

the porous medium during a flux experience. Then, it is possible to study the behaviour of the aqueous dispersion in each section of the porous bed.

Iodine was chosen because the radioactive isotope ^{131}I has a half-life of 8.02 days, which is long enough to make a two or three day experiment and to recover the material used in a reasonable time. The main disadvantage of its short half-life is the impossibility of the storage of the labelled product; it should be used immediately. (Curzio, Mangosio, & Mitta, 1972). Its gamma energy (0.364 MeV) allows reasonable security (Faires & Parks, 1958). The specific radioactivity obtainable is 10,000 C g^{-1} I^{-1}.

It has been shown by Haigh (1954) that scintillation counting is an efficient instrument for determining the activity of liquid samples containing ^{131}I. The real counting rate is obtained by subtracting the background count (measured when no radioactive source is present) from the total observed counting rate of the sample. (Arnstein, 1962).

The relative method was used to determine the activity of the samples (Duggan, 1984). It consists of the introduction of a sample of known activities accumulated for a convenient period of live time to determine the peak sum ($\sum_1$), then, another sample is measured with the same period of live time and determined its peak sum ($\sum_2$). The $\sum_b$ is determined by the peak sum of the background for the same period of live time. The activity of the sample is calculated by

$$\frac{activity1}{activity2} = \frac{\sum_1 - \sum_b}{\sum_2 - \sum_b} \quad (6)$$

when appropriate experimental data was corrected for the radioactive decay of the sample.

2.4. ^{13}C NMR measurements

The covalent binding and location of the iodide atom into the scleroglucan macromolecule were investigated by ^{13}C NMR.

These experiment were carried out in the Laboratoire de Recherche sur les Polymères (LRP) of the Commision Nationale de la Recherche Scientifique (CNRS) at Thiais, France. Solution NMR spectra were obtained using a Bruker AM 300 spectrometer operating at 75 MHz for carbon resonance. A sample of 3 cm^3 (5% polymer in deuterated-DMSO) was placed in a cylindrical cell of 20×1 cm. Chemical shift were calibrated via the ethyl benzene signal in chloroform.

The solvent DMSO is necessary to obtain resolved ^{13}C NMR spectra. In this solvent scleroglucan adopts a single chain random coil conformation thus, the mobility of the chain segments permits a decrease in the proton–carbon dipolar interaction.

2.5. Viscosimetry

Viscosity measurement was used to compare native scleroglucan and the labelled product.

Viscosity measurements were carried out at the Fluids, Authomatique et Systèmes Thermiques Laboratory (FAST) of the CNRS in Orsay, France, with a Contraves LowShear 30 Rheometer for low viscosity and shear rates systems and

Table 1
Physical properties of the product obtained from the reaction in different conditions

Conditions	n Viscosity exponent	M_w[a] weight-average molecular weight (Da)	M_w[b] weight-average molecular weight (Da)	M_w/M_n[a] molecular distribution (polydispersity)	$[\eta]$ Intrinsic viscosity (cm^3/g)
A (pH 1; $T = 50$ °C; 6 h)	0.53	3.2×10^6	3.1×10^6	2.50	100
B (pH 2; $T = 50$ °C; 6 h)	0.60	3.6×10^6	3.7×10^6	2.20	105
C (pH 3; $T = 70$ °C; 12 h)	0.64	4.0×10^6	4.0×10^6	1.60	115
D (pH 4; $T = 80$ °C; 12h)	0.65	4.1×10^6	4.2×10^6	1.50	117
E (pH 3; $T = 80$ °C; 6h)	0.72	4.2×10^6	4.1×10^6	1.23	120

[a] GPC measurements.
[b] Viscosity measurements.

Table 2
Optimal selected conditions for the chemical reaction

	First step	Second step
Scleroglucan concentration	0.2%	0.2%
pH	12	3
Temperature	50 °C	80 °C
Time	24 h	6 h
Purification	Dialysis (6000 Da)	Dialysis (6000 Da)

a Cienctech Stresstech Rheometer for the more viscous dispersions and gels.

The solutions of these macromolecules are non-Newtonian fluids. Their flow curves, at a constant temperature, are not linear. This means, the viscosity is not constant but depends on the shear rate in the fluid. Eq. (7) shows the relation used to characterize these fluids

$$\eta = k\dot{\gamma}^{n-1} \tag{7}$$

where η is the viscosity, $\dot{\gamma}$ is the shear rate, k and n are constants for a particular fluid, k is a measure of the consistency of the fluid and n is a measure of the degree of non-Newtonian behaviour (Wilkinson, 1960). For scleroglucan solutions at 25 °C, the relationship between n and the concentration (c in % m/m) of scleroglucan in the solution (Boeykens, 2003) is:

$$n = 0.87(1 - e^{-c/0.07}) \tag{8}$$

The intrinsic viscosity $[\eta]$ is a characteristic of the polymer and the length and shape of its chains. It is can be defined by:

$$[\eta] = \lim_{c \to 0} \left(\frac{1}{c} \ln \frac{\eta}{\eta_0} \right) \tag{9}$$

where η is the viscosity of the solution and η_0 is the viscosity of the solvent. For $[\eta]$ determinations, measurements on $0.05-0.5\%$ scleroglucan solutions were carried out.

The intrinsic viscosity is related to molecular weight by the Mark–Houwink equation (Hiemenz and Rajagopalan, 1997):

$$[\eta] = kM^a \tag{10}$$

where M is the mass of the polymer molecule, k is a constant determined by measuring M for several homologues with different mass of the same polymer and a is the Mark–Houwink exponent related to the shape of the molecules. When $a = l$ M corresponds to a weight-average molecular weight.

Eq. (10) (by the assumption of the same values for k and a) was used for a comparison between the weight-average molecular weight of the labelled product and the original scleroglucan.
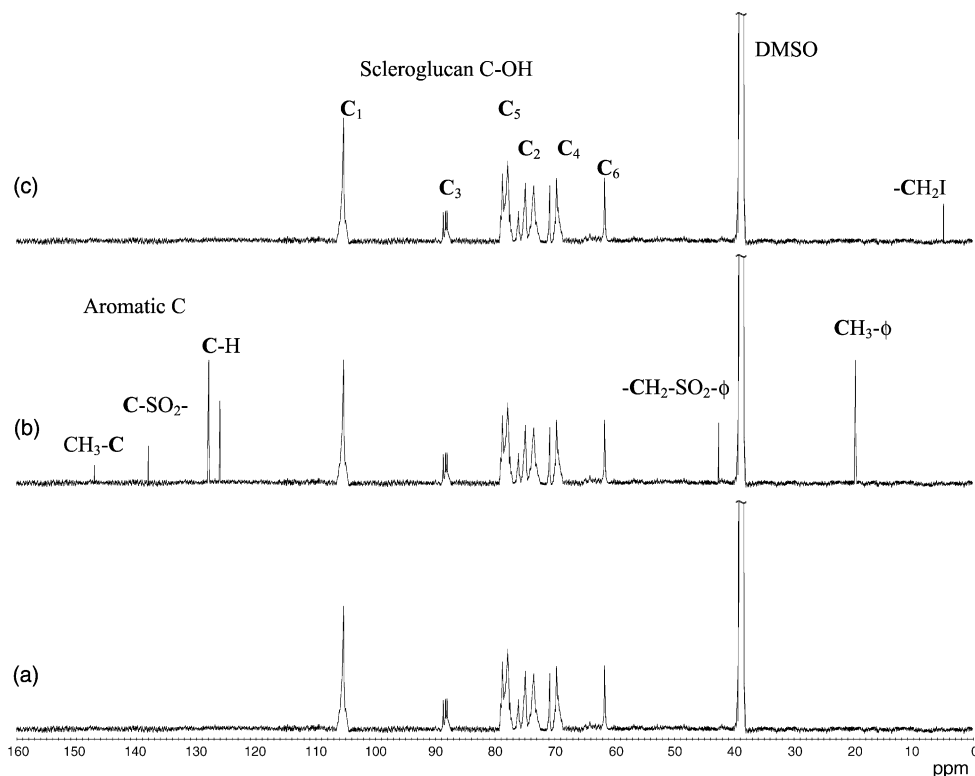

Fig. 5. ^{13}C NMR spectra of (a) scleroglucan, (b) *p*-tosyl ester and (c) iodine labelled scleroglucan.

2.6. Gel permeation chromatography

GPC was also used to determine of polymer degradation occurred during the chemical reaction and to test differences between scleroglucan and the labelled product.

The measurements were carried out on Sepharose CL-4B (Pharmacia Fine Chemicals) using an AC-16 Pharmacia Biotech column eluted with 0.01 M NaOH at a flow rate of 0.5 ml/min at 25 °C. The relative concentration of scleroglucan in the eluent was monitored using a differential refractometer. The column was calibrated with standard dextrans (from *L. mesenteroides* B-512, Sigma).

3. Results and discussion

3.1. Test for the chemical reaction performance

The chemical reaction was tested both in acid and basic media due to the wide pH stability of the triple helix from pH 3 to 12. (Bluhm, Deslandes, & Marchessault, 1982).

In the first step, an esterification, a basic medium is required. The slow dissolution of the *p*-tosyl chloride occurs while the reaction proceeds and the liberated hydrochloric acid is neutralized by the addition of NaOH. The reaction was followed by conductance and pH measurements at the LaQuiSiHe, FIUBA. Scleroglucan solutions have a very low conductance ($\approx 10^{-6}$ mS). The addition of an electrolyte produces a drastic increment in this property. The advance of the chemical reaction can be followed by the decrease in the concentration of the electrolyte reactant, via the conductance. This method does not need any previous calibration study.

Fig. 4 shows the recorded study of the reaction advance at two different temperatures. pH was controlled to be between 7 and 11. During the first hour, evolution was not observed in the water–scleroglucan–*p*-tosyl chloride system. The steps in the conductance are due to the additions of NaOH, until pH 11. While the reaction produces H$^+$, the conductance drops because of neutralization. After 1 h, at pH 7, the conductance was caused by the produced NaCl and a new addition of NaOH was made. An increase in the temperature produces an increase in the velocity of any reaction, including the polymer degradation. The optimal temperature, time and pH (Table 2) were determined, taking into account the limits imposed by the polymer degradation and the destruction of the triple helix that could lead to some very different products.

Before the second step, and only for testing, the first product (intermediate) was purified by dialysis using 6000 D pore size membranes. The selection of the membranes was made taking into account blocking and the time for purification (with 14,000 D pore size, blocking occurs in 24 h, but purification takes much longer) (Boeykens et al., 2002).

In the second step, the OH$^-$ competes preferentially (hydrolysis of the ester) with the iodide for the displacement of the tosyl-attached group and the performance of the reaction drops. Acid medium favours displacement.

The labelled scleroglucan was filtered and its purification by dialysis was tested by TXRF as previously described (Boeykens et al., 2002).

The time, temperature and pH of the reaction were selected so that the molecular weight distribution and the viscosity of the product were as similar as possible to the original scleroglucan.
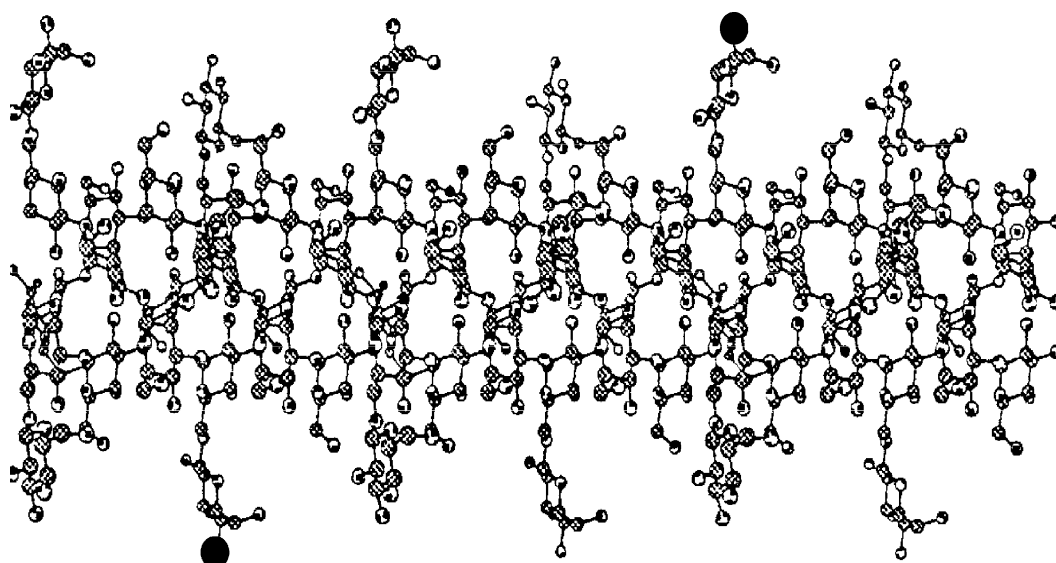

Fig. 6. Stereo view of the triplex of iodine labelled scleroglucan. C and O atoms are grey and white circles, respectively. The two iodine atoms corresponding to this portion of the macromolecule are indicated with black bigger circles. H atoms are omitted.
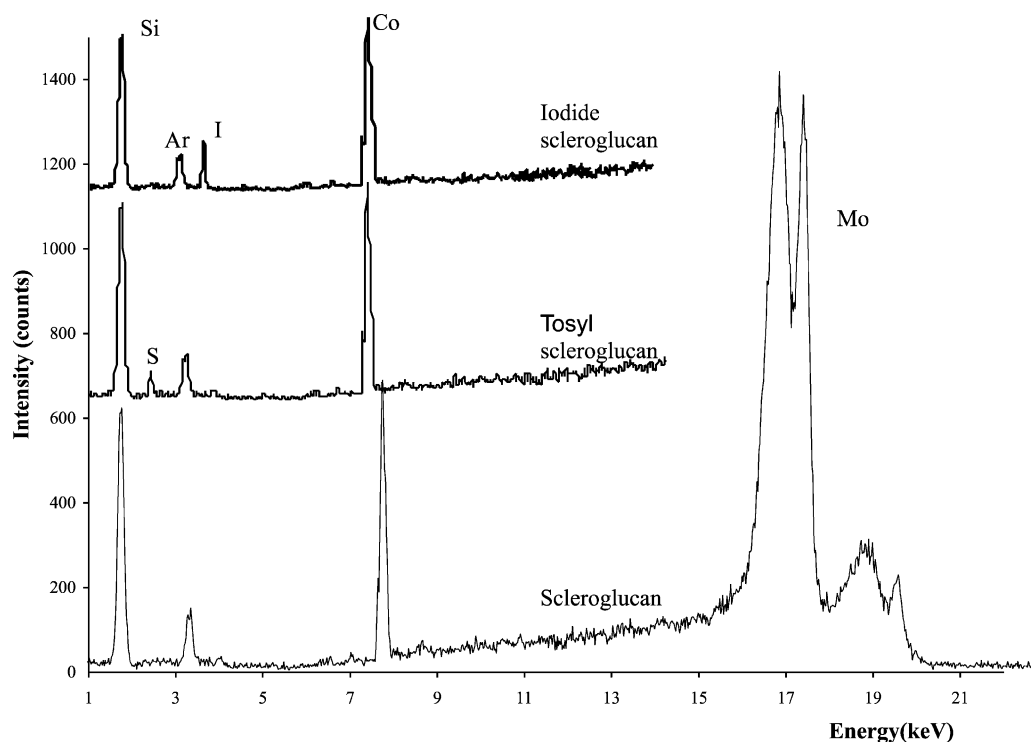
Fig. 7. TXRF spectra from the three macromolecules. Kα and Lα lines from S and I atoms are indicated.

As indication of the influence of these variables on the final product, the properties of the products obtained by five different conditions for the second step of the reaction are shown in Table 1.

The optimum conditions for reaction are shown in Table 2.

In Fig. 5, NMR spectra from native scleroglucan intermediate *p*-tosyl ester and iodide labelled product are shown. Peaks were identified for scleroglucan (Bardet, Rousseau, & Vincendon, 1993), the tosyl group and the iodine group, respectively.

On the bases of this spectra and considering the NMR and X-ray Diffraction studies on oriented fibers and the conformational structure of scleroglucan triple helix (Bluhm et al., 1982; Saito et al., 1987), we conclude that the substitution was placed at the OH group of the C_6, at the pendent β(1-6) linked glucose groups that protrude outside the triple helix. A three dimensional view of the labelled product is shown in Fig. 6.

Quantitative TXRF analysis showed that one *p*-tosyl group was attached to each monomer unit of the polysaccharide in the first step of the reaction. In the second step, a 60% of the introduced tosyl groups were substituted by iodide and the rest of them were hydrolysed. In Fig. 7 the TXRF spectra from the three macromolecules are shown. The fluorescence energy characteristic provided by S and I atoms permitted their identification.

The radioactive performance was measured. A comparison between the activity of the original $Na^{131}I$ and the lost activity during dialysis purification showed that the ^{131}I isotopes were effectively attached to the macromolecule. This concludes in a 15% radioactive performance for the reaction.

3.2. Comparison between scleroglucan and the labelled product

Rheological measurements were done in order to determine the change in the macromolecule behaviour caused by the chemical reaction. In Fig. 8, the logarithmic plots of viscosity (η) and shear rate ($\dot{\gamma}$)
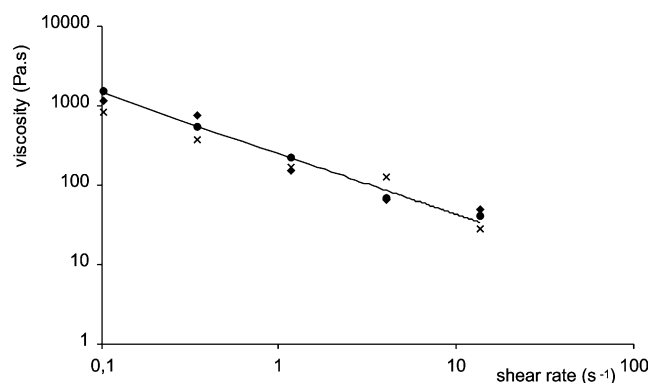

Fig. 8. Viscosity (Pa s) vs shear rate (s^{-1}) plots for (●) scleroglucan, (◆) iodine labelled scleroglucan and (×) *p*-tosyl ester derivative. The three different macromolecules fit in the same equation $\eta = 240\dot{\gamma}^{n-1}$, with $n = 0.72$ for the first two and $n = 0.70$ for the *p*-tosyl ester.

Table 3
Comparative physical properties of 0.2% scleroglucan and derivatives solutions

	n Viscosity exponent	M_w[a] weight-average molecular weight (Da)	M_w[b] weight-average molecular weight (Da)	M_w/M_n[a] molecular distribution (polidispersity)	$[\eta]$ Intrinsic viscosity (cm^3/g)	Water solubility	DMSO solubility
Scleroglucan	0.72	4.0×10^6	3.9×10^6	1.20	113	Very	Very
Tosyl scleroglucan	0.70	5.1×10^6	5.3×10^6	1.35	140	Difficult	Very
Iodide scleroglucan	0.72	4.2×10^6	4.1×10^6	1.23	120	Very	Very

[a] GPC mesurements.
[b] Viscosity measurements.

Table 4
Comparative characteristics of TXRF and radioactivity analysis

	Sample volume	Relative sensitivity	Sample destruction	Detection limits[a] (%)	Accuracy (%)
TXRF	10 µl	0.5–3 (to Co)	Yes	0.01	<1
Radioactivity	No sampling	7 (to ^{131}I)	No	0.05	<1

[a] For labelled scleroglucan solutions.

measured at 25 °C on samples of the same concentration of scleroglucan and iodide derivatives show similar exponents ($n = 0.72$ for 0.2% of macromolecule concentration). The *p*-tosyl ester showed slightly smaller exponent ($n = 0.70$).

The aproximate weight-average molecular weights of the samples listed in Table 3 were obtained using the Mark–Houwink equation. The value of $k = 4 \times 10^{-4}$ (Eq. (10)) according to previous results (Fariña, Siñeriz, Molina, & Perotti, 2001) was used for calculations. The intrinsic viscosity determined for scleroglucan triple helix solutions was $[\eta] = 113$ cm^3/g. These values allow the corresponding Mark–Houwink exponent value for these systems to be estimated as: $a = 0.8$.

A second value to estimate the weight-average molecular weights of the samples was obtained by the exclusion profile by gel permeation chromatography. The polymers showed narrow molecular weight distributions and the data according to the calibration curve with standards dextrans were listed in Table 3. The estimated M_w values were in agreement with the results of intrinsic viscosity determinations.

There were no significant differences between the scleroglucan and the labelled iodide macromolecule in the measured properties. Rather different was the case of *p*-tosyl ester due to the weight and number of the *p*-toluensulfonic groups. Hydrolysis of these groups is very important in the second step of this method, then the final labelled macromolecule is much more similar to the original that the intermediate one.

3.3. Comparison between the techniques for detection

Comparative characteristics belonging to the employed techniques are showed in Table 4. Their detection limits and accuracy are very similar. Both techniques allowed detection and quantification of scleroglucan solution in concentrations from 10^{-2}%.

Even though the *p*-tosyl ester can be detected by TXRF, the reaction should proceed to the second step to avoid difficulties originated by the small differences in the behaviour of the macromolecule.

4. Conclusions

The iodide labelled scleroglucan had a molecular structure with two iodine atoms for each five monomer units. It make a difference of 5% in weight but the transport behaviour was very similar to the original scleroglucan. The use of this labelled molecule makes the dynamic experiences in non-transparent porous media possible.

The proposed techniques for detection are complementary. Their usefulness depends on the condition of the experiment.

We consider the chosen procedure to labelled scleroglucan safely and successfully.

Acknowledgements

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