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Journal of Chromatography A, 697 (1995) 329–335

JOURNAL OF CHROMATOGRAPHY A

Soluble glycoproteins from sugar cane juice analysed by high-performance liquid chromatography and fluorescence emission

Maria Estrella Legaz[a,*], Mercedes M. Pedrosa[a], R. De Armas[b], Maritza M. Martinez[b], C. Vicente[a]

[a]Department of Plant Physiology, Faculty of Biology, Complutense University, 28040 Madrid, Spain
[b]Department of Plant Physiology, Faculty of Biology, Havana University, Havana, Cuba

Abstract

Soluble polysaccharides have been isolated from sugar cane by size-exclusion chromatography using two successive columns of Sephadex G-10 and G-50. Two main fractions of different molecular mass were separated and their homogeneity was tested by HPLC using a Zorbax GF-450 and a Zorbax GF-250 column in series. Whereas the high-molecular-mass soluble polysaccharide (SP) fraction seems to be composed of two different polymers with retention times of about 16.5 and 24.5 min, the mid-molecular-mass carbohydrate (MMMC) fraction is resolved as only one peak with a retention time of about 2.45 min. This implies that Sephadex G-50 is not able to discriminate between the two categories of macromolecules, as SP eluted from Sephadex G-50 is clearly contaminated by MMMC. Fractionation of clarified juice with 80% (v/v) ethanol separates SP as an insoluble fraction and MMMC as an ethanol-soluble macromolecule. The natural fluorescence of both polymers reveals that they are glycoproteins and, in addition, they can clearly be recognized by the degree of tryptophan exposure to its chemical microenvironment.

1. Introduction

Fructans are polyfructosylsucrose polymers that occur widely as storage carbohydrates in vegetative parts of plants [1]. Three main types of fructans occur, depending on plant species [2]:

(i) inulins, which consist of continuous $(2\rightarrow 1)$-linked β-D-fructofuranosyl units;

(ii) linear fructans of cereal grasses, which are $(2\rightarrow 1)$-linked β-D-fructofuranosyl units;

(iii) highly branched fructans, consisting of both 1- and 6-linked β-D-fructofuranosyl units with a sucrose unit at the end of the chain. These fructans also contain small proportions of atypical linkages.

On the other hand, sugar cane stalks accumulate heterofructans that are composed of both fructose and galactitol [3]. These fructans occur in two main groups of molecular mass forms: mid-molecular-mass carbohydrates (MMMC), which are $[\text{galactitol}_5\text{: fructose}_4]_n$, and high-molecular-mass soluble polysaccharide (SP), which are $[\text{galactitol}_3\text{: fructose}_2]_n$ [3,4]. MMMC occurs in at least two forms of M_r about 871 000 and 380 000 [3]. Both SP and MMMC are hydrolysed by a glycosidase system which produces fructose and galactitol from its substrates [3–5].

Both SP and MMMC seem to be produced from structural polymers occurring in the cell

* Corresponding author.

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SSDI 0021-9673(94)00948-1

wall of parenchymatous cells [4], in contrast to fructans synthesized by C3 grasses [6,7], where fructans are produced when the amount of sucrose exceeds that required for transport and metabolism [8]. The structural sugar cane polymer is partially hydrolysed by a glycosidase to liberate mainly SP to the cytosol, where MMMC is produced later [4].

Fructans of the *Gramineae* are usually separated by using strongly alkaline conditions for their elution from anion-exchange columns and pulsed amperometric detection [9]. Oligosaccharides from partially hydrolysed fructans can be separated by HPLC using a Spherisorb-5-NH_2 column and running it with a mobile phase of acetonitrile–water [10]. Gas–liquid chromatography also allows the separation of several oligosaccharides as trimethylsilyl derivatives or methylated alditol acetates [11]. Separation of heterofructans from sugar cane has been carried out by HPLC using a PWSX GO209 column packed with G5000 PWXL [4].

In this work, both SP and MMMC were analysed by HPLC and fluorescence emission in order to obtain some additional information about their chemical nature.

2. Experimental

2.1. Plant material

Saccharum officinarum L., of variety Jaronu 60-5, field-grown, was used throughout.

2.2. Preparation of soluble polysaccharides

Stalks from 12-month-old plants were mechanically crushed, immediately after cutting, and the raw juice was adjusted to pH 8.0 by adding a saturated solution of ammonium carbonate, followed by filtration through filter-paper. Sodium azide was added to the filtrate to obtain a final concentration of 0.02% (w/v) [12].

This clarified juice was then filtered through a 15 cm × 2.5 cm I.D. column of Sephadex G-10 (Pharmacia, Uppsala, Sweden), pre-equilibrated with a saturated solution of ammonium carbonate containing 0.02% sodium azide. The first 20 × 1.0-ml fractions of eluate were discarded. Fractions 21–39 were collected and filtered through a Sephadex G-50 column (30 cm × 2.5 cm I.D.), pre-equilibrated as above. Fractions 40–70 contained the soluble, high-molecular-mass polysaccharides (SP fraction) whereas the mid-molecular-mass carbohydrates (MMMC fraction) eluted from 71 to 120 ml [12]. Carbohydrates were quantitatively measured in the different fractions by the method of Dubois et al. [13]. Both GLC and HPLC analyses [3] showed the absence of sucrose and monosaccharides in fractions from 40 to 120 ml. Clarified juice and soluble polysaccharides were dialysed overnight, at 4°C, against 0.2 *M* phosphate buffer (pH 6.9) using Visking dialysis tubing 27/32 from Serva (Heidelberg, Germany), which retains more than 90% of cytochrome *c* (M_r 12 400).

Alternatively, raw juice was brought to 5% (w/v) trichloroacetic acid or to 80% (v/v) cold ethanol, filtered through a double cheese-cloth and centrifuged for 30 min at 20 000 *g* at room temperature. Pellets were discarded and supernatants were heated at 60°C for 20 min and subjected twice to the same precipitation procedure with 80% (v/v) ethanol [14]. The last supernatants were evaporated to dryness under reduced pressure. Each residue was dissolved in 2.0 ml of 0.2 *M* sodium phosphate buffer (pH 6.9). Finally, solutions were loaded on to the chromatographic column.

The different samples were also assayed for the Folin phenol reaction according to Lowry et al. [15], using bovine serum albumin as a standard.

2.3. HPLC analysis

Approximately 20 μg of soluble polysaccharides were chromatographed on two columns (both 25 cm × 9.4 mm I.D.) Zorbax GF-450 and Zorbax GF-250 (DuPont–Hichrom, Reading, UK), connected in series, according to Pedrosa and Legaz [16], using 0.2 *M* phosphate buffer (pH 6.9) as the mobile phase at a flow-rate of 1 ml min^{-1}. The dead time was determined as 12.3

min, using Blue Dextran 2000 as a standard. The equipment was a Spectra-Physics (Fremont, CA, USA) SP8800 liquid chromatograph equipped with an SP 4290 computer. Detection was performed at 210 and 275 nm by using a Spectra-Physics SP8490 UV–Vis detector.

2.4. Measurements of absorbance and fluorescence spectra

Absorption spectra were recorded using a Varian (Walnut Creek, CA, USA) DMS 90 dual-beam spectrophotometer. Fluorescence spectra of different polysaccharides were determined by using a Kontron (Milan, Italy) SFM-25 spectrofluorimeter equipped with quartz cuvettes of 1-cm path length. The wavelength of the exciting radiation was either 210 or 275 nm.

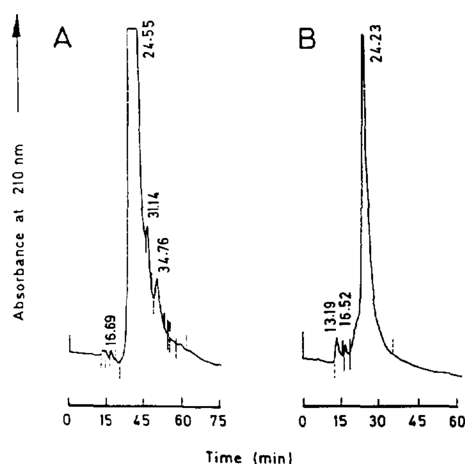

Fig. 1. HPLC elution profiles of (A) filtered raw sugar cane juice and (B) centrifuged and dialysed sugar cane juice. Numbers near the peak represent retention times in minutes.

3. Results

3.1. HPLC separation of water-soluble glycoproteins from sugar cane juice

The conventional procedure for SP and MMMC preparation includes clarification of sugar cane juice by precipitation with ammonium carbonate, filtration through filter-paper and subsequently sequential filtration through successive columns of Sephadex G-10 and G-50. The HPLC traces obtained for clarified sugar cane juice showed a main peak at 24.55 min and secondary peaks at 13.25, 14.42 (traces), 16.69, 31.14 and 34.76 min. This same juice, after dialysis followed by a second centrifugation, produced only three peaks at 13.19, 16.52 and 24.23 min (Fig. 1A and B). As the dialysis membrane retained polymers with molecular masses higher than 10 000, substances eluted at 31.14 and 34.76 min should have molecular masses lower than this value.

SP solution, after dialysis, produced two peaks recorded at 210 nm, with retention times of 16.6 and 23.86 min (Fig. 2A). Only this last peak was also recorded at 275 nm, with a retention time of 23.53 min (Fig. 2B). The ratio of area counts at 210 nm to those at 275 nm was 3.25. However,

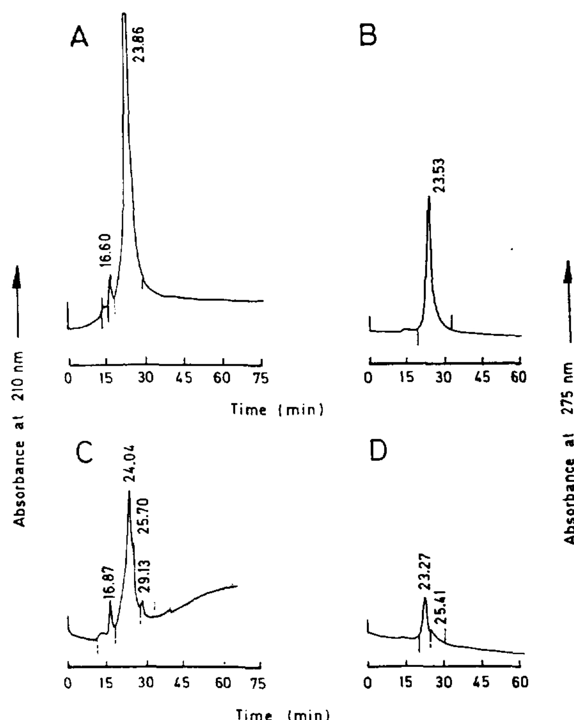

Fig. 2. HPLC elution profiles of (A) and (B) dialysed SP and (C) and (D) SP recently isolated from a Sephadex G-50 column.

SP recently prepared by filtration of sugar cane juice through Sephadex G-10 and G-50 contained two main substances that eluted at 16.87 and 24.04 min, with monitoring of the eluate at 210 nm (Fig. 2C). However, only one peak at 23.27 min was detected at 275 nm (Fig. 2D). The ratio of area counts at 210 nm to that at 275 nm was 4.64. Only one peak in HPLC was detected for MMMC samples filtered through Sephadex or dialysed, with a retention time near 24 min. This substance was detected at 210 and 275 nm, although its absorbance at 210 nm was 3–4 times higher than that observed at 275 nm (Fig. 3).

3.2. *HPLC separation of both ethanol-soluble and ethanol-insoluble glycoproteins from sugar cane juice*

An alternative procedure for isolating soluble polysaccharides from sugar cane juice was attempted in order to separate proteins from the polysaccharide fraction. Precipitates produced after ethanol extraction were dried for later chromatography. In parallel, juices were first precipitated with 5% (w/v) TCA, the precipitate was discarded and the supernatant extracted with cold 80% (v/v) ethanol as described previously. The different precipitates were dried and subsequently analysed. Finally, the last supernatant was dried and chromatographed. Only the peak with a retention time of about 24.5 min appeared in the last supernatant (Fig. 4), similar in chromatographic behaviour to that shown as the sole component of the MMMC fraction. Chromatographic analysis of the pellets redissolved in distilled water indicated that the substance with a retention time of 16.55 min is completely recovered in these pellets, independently of the previous treatment with TCA.

3.3. *Spectral characteristics of soluble glycoproteins from sugar cane juice*

The UV absorption spectra of dialysed SP and MMMC showed an absorption maximum at 274 nm and only that of MMMC a shoulder at 250 nm (Fig. 5A). The absorbance at 210 nm was about three times higher than at 274 nm. The UV absorption spectrum of Sephadex filtered SP showed an absorption maximum at 275 nm whereas that of MMMC showed a main maximum at 271 nm and a secondary maximum at 246 nm (Fig. 5B), very similar to that found for the dialysed carbohydrate. The absorbance at 210 nm was 3.5 times higher than at 271–275 nm.

Both preparations of SP, that only filtered through Sephadex G-50 and that dialysed, showed some similarities in their fluorescence spectra when the wavelength of the excitation

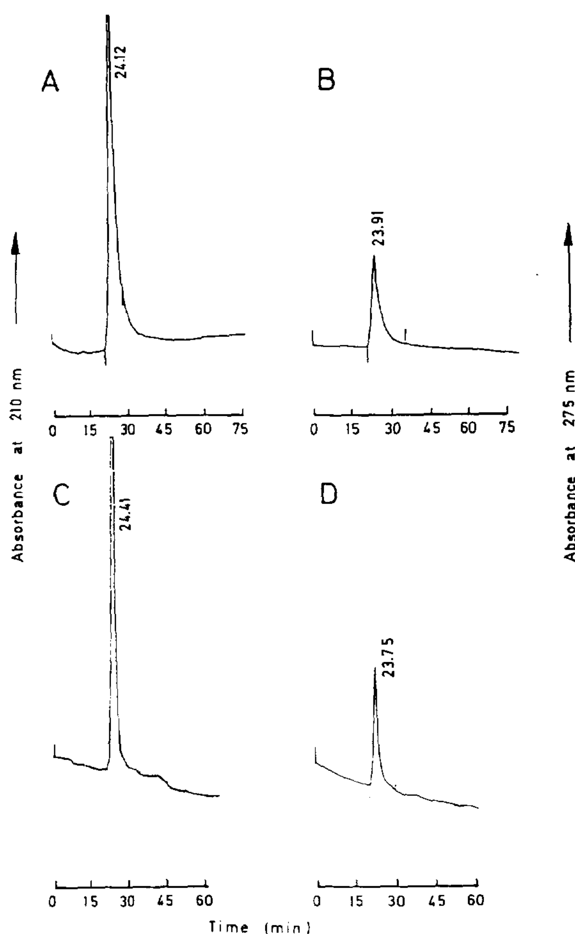

Fig. 3. HPLC elution profiles of (A) and (B) dialysed MMMC and (C) and (D) MMMC recently isolated from a Sephadex G-50 column.

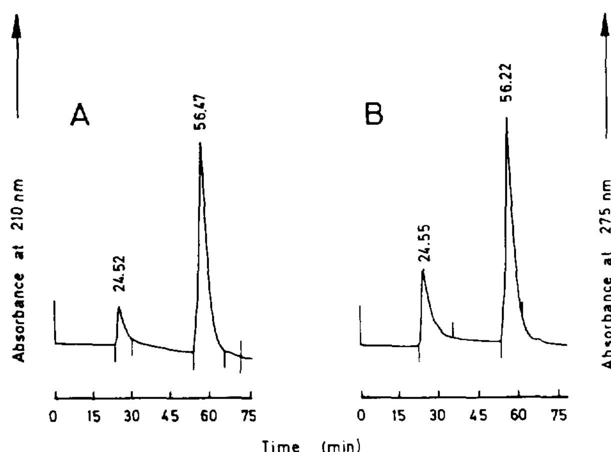

Fig. 4. HPLC elution profiles of the last supernatants after ethanol extraction of raw sugar cane juice monitored at (A) 210 and (B) 275 nm.

radiation was 210 nm. Whereas SP filtered through Sephadex had a net maximum of fluorescence emission at 422 nm, a secondary maximum at 296 nm and a shoulder at 332 nm (Fig. 6A), dialysed SP had two maxima of fluorescence emission, a secondary maximum at 427 nm and another, the main maximum, at 327 nm, and two shoulders at 380 and 302 nm (Fig. 6B). MMMC showed a net maximum of fluorescence emission at 296–297 nm on using exciting radiation of 210 nm, but only that filtered through Sephadex had a secondary maximum at 347 nm (Fig. 6C and D). By exciting samples with radiation of 275 nm, both forms of SP showed only one maximum of fluorescence emission at 334–336 nm (Fig. 7A and B). However, MMMC filtered through Sephadex showed a maximum of emission at 347 nm (Fig. 7C) whereas dialysed MMMC had a secondary maximum at 346 nm and a main maximum at 320 nm (Fig. 7D).

Results for the determination of different the polysaccharides as glycoproteins, according to Lowry et al. [15], showed that SP was composed of equal proportions of the protein and polysaccharide moieties, whereas MMMC was 20% protein and 80% polysaccharide (data not shown).

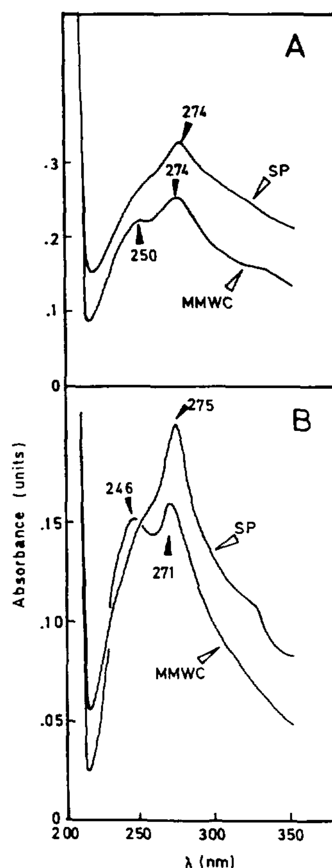

Fig. 5. UV absorption spectra of (A) dialysed SP and MMMC and (B) recently SP and MMMC filtered through Sephadex G-50.

4. Discussion

Martínez et al. [4] were able to find that SP, purified by column chromatography on Sephadex from Cuba 374-72 sugar cane juice, was a heteropolymer with a molecular mass of about 1 735 000, whereas MMMC had a molecular mass of about 870 000. Both purified fractions were contaminated with a dextran with a molecular mass of about 9000. Analyses were carried out by HPLC using a PWSX GO209 column packed with G5000 PWXL. However, purification of both SP and MMMC from Jaronu 60-5 sugar can juice using the procedure described in this work did not completely separate the two

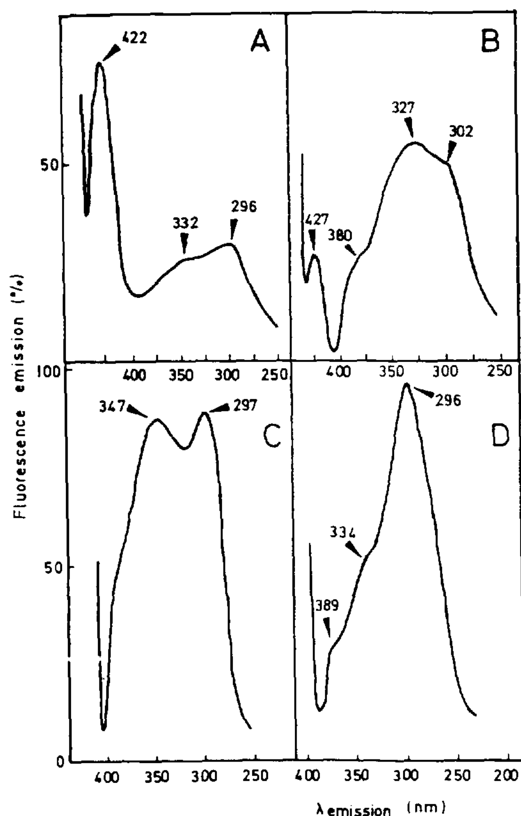

Fig. 6. Fluorescence emission spectra of (A) SP recently filtered through Sephadex G-50, (B) dialysed SP, (C) MMMC recently filtered through Sephadex G-50 and (D) dialysed MMMC, obtained by exciting samples with radiation of 210 nm. Fluorescence emission was recorded from 440 to 250 nm.

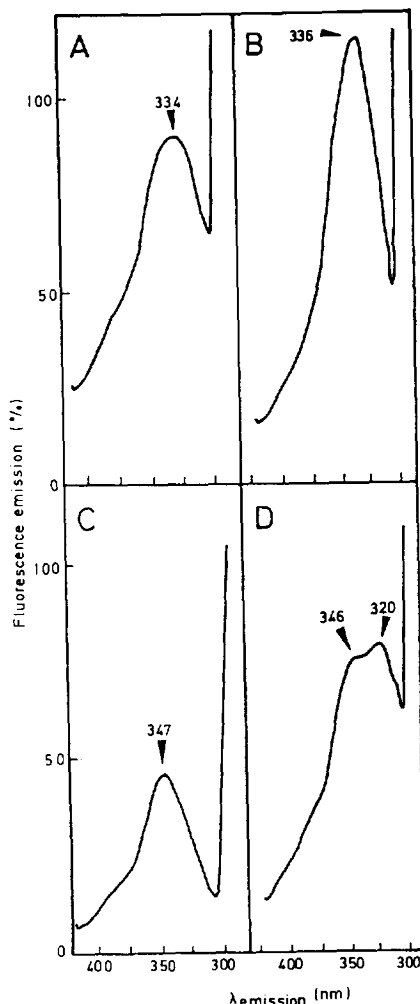

Fig. 7. Fluorescence emission spectra of (A) SP recently filtered through Sephadex G-50, (B) dialysed SP, (C) MMMC recently filtered through Sephadex G-50 and (D) dialysed MMMC, obtained by exciting samples with radiation of 275 nm. Fluorescence emission was recorded from 440 to 280 nm.

forms of heterofructan, as MMMC behaves as a macromolecule purified to homogeneity with a sole peak in HPLC with a retention time of about 24.5 min, and this same peak also appeared in the chromatographic traces of SP analysis in addition to one with a retention time of about 16.5 min (Figs. 2 and 3). The last peak corresponds to a substance largely insoluble in 80% (v/v) ethanol and then the final supernatants after three successive extractions with ethanol do not contain this SP form (Fig. 4). Insolubility in ethanol can be explained as a consequence of the high molecular mass of this polymer [4].

However, some optical properties of these heterofructans reveal that they are not pure polysaccharides but glycoproteins. The fluorescence of both SP and MMMC cannot be explained in terms of polysaccharide excitation and subsequent emission, as this class of macromolecules fluoresces only when they are highly oxidized [17], although the polysaccharide moiety of both SP and MMMC is a heterofructan composed of fructose and galactitol [3] and hence

they are even more reduced than homofructans from grasses [1,8]. However, the possible glycoproteic nature of these soluble molecules could explain several of their fluorescence characteristics. Tryptophan becomes evident in MMMC by the maximum of fluorescence emission at 347–346 nm after exciting the samples with radiation of 275 nm (Fig. 6C and D). However, this maximum displaces towards 334–336 nm for SP (Fig. 6A and B). According to these results, tryptophan seems to be highly exposed in MMMC molecules whereas the amino acid could be placed in a more hydrophobic position in SP, far from the hydrophilic surface of the glycoprotein [18,19]. By exciting the samples with radiation of 210 nm, the same behaviour of fluorescence emission of tryptophan is recorded but, in this instance, tyrosine can also be detected by a net peak of fluorescence at 296–302 nm (Fig. 7).

Acknowledgements

This work was supported by a grant from the Dirección General de Cooperación Internacional (Spain). We are indebted to Miss Raquel Alonso for technical assistance.

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