Separation and Determination of α- and β-Galactose from Agar-Type Polysaccharides by Liquid Chromatography

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Abstract

A combination of reversed-phase liquid chromatographic technique and diode-array detection is used for quantitation of the precolumn phenylisocyanate derivatives of monosaccharides. These compounds are stable for at least two months if protected from the light and kept at room temperature. The detection limit is about 5 pmol. The method exhibits good reproducibility for both retention times and chromatographic peak areas. The method also enables a rapid and reproducible determination and separation of anomeric forms of α- and β-galactose from agar-type polysaccharides.

Introduction

Agar is a linear polymer mixture of polygalactans from red algae that has repeating units in alternating sequence of 3-0-(3,6-anhidro-L-galactopyranosyl)-B-D-galactopyranose (1,2). The neutral form of this polysaccharide is agarose.

The main sources of commercial-grade agar and agarose are several species of different genera of marine algae: *Gelidium, Pterocladia*, and *Gracilaria* (1,3). Among them, *Gelidium sesquipedale* is the most important raw material in the obtainment of Spanish agar. One of the most important characteristics of the agar-type polysaccharides is the chemical grade of substitution. This average is expressed as a percentage of the substituted available sites. It depends on the average molecular weight and the molecular weight distribution.

In previous reports, many different analytical techniques have been investigated for the separation and qualitative determination of agar components. IR techniques (4,5), gas–liquid chromatography (6,7), ^{1}HNMR (8,9), and ^{13}C-NMR spectroscopy (2,3,10) have been used for assessing the chemical structure of agar polymers.

In this study, a reversed-phase high-performance liquid chromatographic (HPLC) technique with diode-array detection is described for simultaneous quantitation of the two main anomeric forms in the agar molecule, α- and β-galactose units.

This technique provides a rapid, reproducible, and reliable quantitation of derivatized polysaccharides (11) and was developed to measure sugars at the picomole level in biological samples.

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Experimental

Apparatus

A 1090 HP liquid chromatograph system (Hewlett-Packard, Waldbroon, Germany) that was equipped with a diode-array detector system, a Rheodyne loop injector (20 µL) (Waldbroom, Germany), a 15-cm Supelcosil LC-18 column, and a 2-cm Supelguard LC-18 precolumn (Supelco, Bellefonte, PA) was used for the chromatographic separation of the phenylisocyanate (PIC) derivatives. A Hewlett-Packard system consisting of a workstation plus operation software was used for continuous on-line quantitation of chromatographic peaks. Reagents and chromatographic conditions were described by Dethy et al. (11). The separation of anomers was achieved by optimizing the column temperature (10°C) and carrying out the experiments in a temperature-contolled room. An increase in temperature accelerated the rate of interconversion between the α and β anomers of galactose units. UV wavelength for detection was 240 nm ± 4. The coefficient of variation (CV) was determined as described by Sokal and Rohlf (12).

Analytical procedure

Specimens of the alga *Gelidium sesquipedale* (Clem.) that were collected at the Strait of Gibraltar coast were heated to extract polysaccharides by using the method reported by Santos and Doty (13) and modified by Torres et al. (14). Agar and agarose were provided by Hispanagar S.A. (Burgos, Spain) as additional material for this study.

Polysaccharides (0.015 g) were hydrolyzed with hydrochloric acid (0.01M, 1 mL) for 24 h in a shaker water bath (100°C). The resulting solution was cooled to room temperature, filtered through HVLP 013 Millipore membranes (0.45-µm pore size) (Bedford, MA), and dried in a Speedvac concentrator (Savant Instruments, New York, NY).

PIC derivatives were obtained as described by Dethy et al. (11)

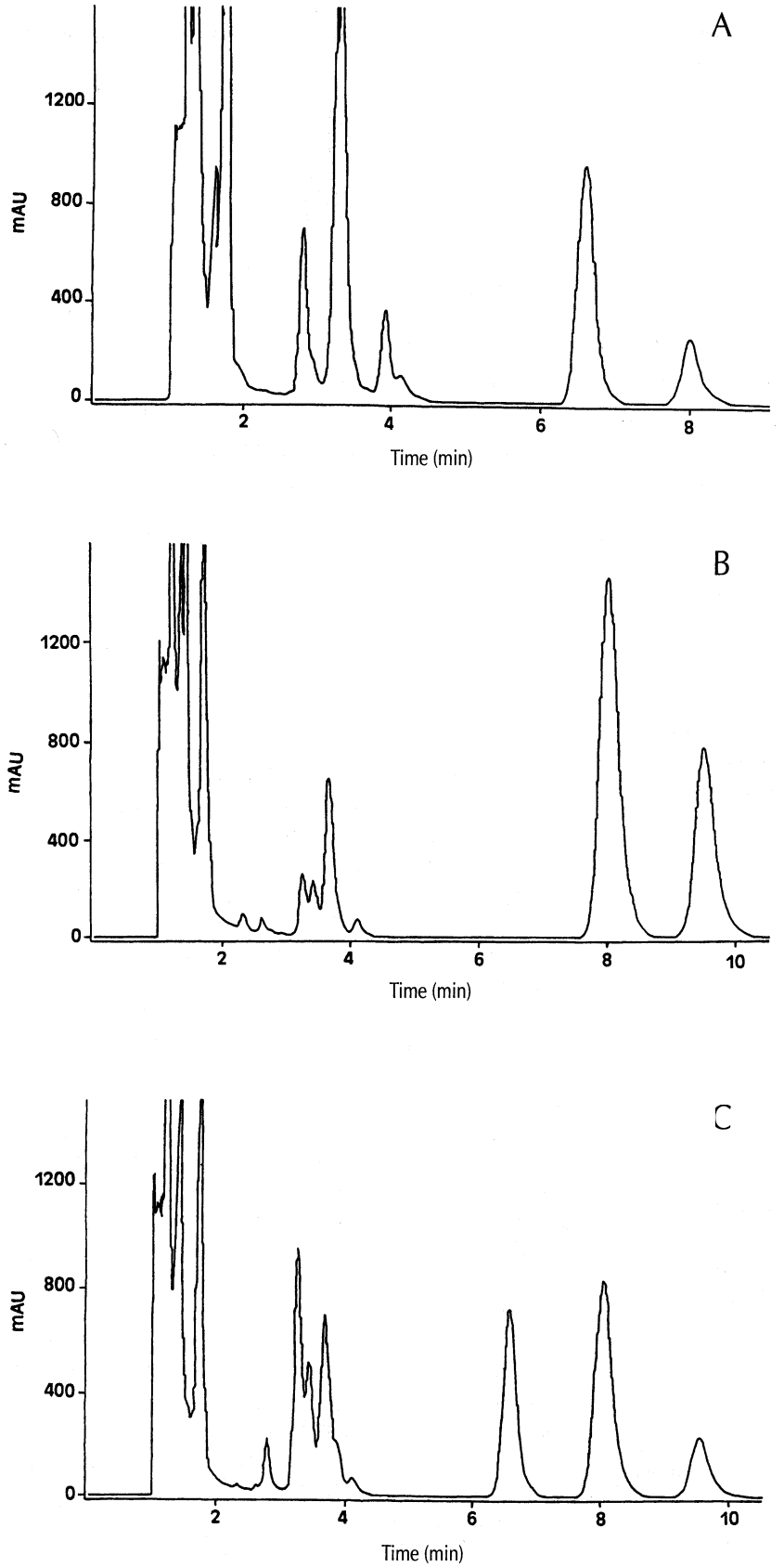

Figure 1. Typical chromatogram (wavelength, 240 nm) of a commercial standard of α-glucose (A), a commercial standard of α-galactose (B), and the mixture of both standards (C); α-Glucose (A,C) elutes at 6 min; β-glucose (A,C) and α-galactose (B,C) elute at 8 min; β-galactose (B,C) elutes at 10 min.

with minor modifications. To obtain derivatives of sugars, the dried samples were dissolved with 200 µL of PIC and 800 µL of pyridine with vigorous vortex mixing in a fume hood protected from the light.

After the incubation period, the mixture was cooled and 0.5 mL of methanol was added to eliminate the excess PIC. To reduce the interferences due to the absorption of the chemicals, the solutions were supplemented with 1.5 mL of pyridine and filtered through GV-Millex 0.13 Millipore membranes (0.22-µm pore size) (15). A mixture of 60% acetonitrile and 40% 0.01% K_2HPO_4 adjusted to pH 7.0 with H_3PO_4 in double-distilled water was used for the separation of the derivatives. The flow rate was 1.6 mL/min. PIC, methanol, and pyridine were of analytical grade (Merck, Darmstadt, Germany); sugars were of biochemical grade (Merck).

Results and Discussion

The aim of our study was to devise a method to separate and quantitate α- and β-galactose units from agar-type polysaccharide. After 4–5 min, background noise is not present in the chromatograms. There are no peaks after this time in a blank derivatization chromatogram (not shown). To have a standard of reference, pure galactose was used and compared with glucose to test the discriminant power of the method. These results were compared with chromatograms of samples that contained both glucose and galactose. A second approach was to study the chromatogram of hydrolyzed and nonhydrolyzed agarose samples. The third experimental material was agar extracted from *Gelidium* plants. Figure 1 shows typical chromatograms of commercial standards of the anomeric forms of α-glucose, α-galactose, and the mixture of both α-glucose and α-galactose. Two peaks were obtained after derivatization of α-glucose and α-galactose. The biggest peak corresponds to the α anomer and the smaller one to the β anomer. One peak (8 min) is coincident on each chromatogram, so three peaks appear when the mixture was assayed. The chromatographic retention time for β-glucose and α-galactose was 8.13 min ± 0.06. The elution times of α-glucose and β-galactose were 6.38 min ± 0.05 and 9.12 min ± 0.13, respectively. Glucose and galactose produced different chromatograms (Figure 1A and 1B).

The reproducibility of the retention times

of the PIC derivatives of tested sugars was calculated from 30 analyses. The CV was less than 5%. The reproducibility of the areas was tested in all sugar derivatives; there was a linear response in the concentration range 20–20,000 pmol in the sample. The calibration curve of response with varying concentrations of commercial standard of α-galactose PIC derivative was determined by duplicate. It was constructed by spiking samples of a hydrolyzate with different amounts of α-galactose prior to derivatization. Then, following derivatization and analysis of the spiked and unspiked samples, the unspiked value for α-galactose was subtracted from each of the spiked samples. The PIC derivatives were stable at room temperature for at least 2 months if protected from the light. The detection limit obtained by this method was about 5 pmol (15).

Figure 2 shows the chromatograms of hydrolyzed and nonhydrolyzed samples of agarose. The same profile of the glucose peak could be seen with agar from *Gelidium* in Figure 3. It is noteworthy that only the two peaks of α and β units of galactose appear, and that both peaks are different, revealing the anomeric ratio between α- and β-galactose (Figures 2 and 3). The chromatograms of hydrolyzed and nonhydrolyzed agar-type polysaccharides of *Gelidium sesquipedale* are not shown.

The ratio of α-galactose to β-galactose in agarose, agar, and agar-type polysaccharides of *Gelidium sesquipedale* extracts was calculated by subtracting the nonhydrolyzed values from the hydrolyzed ones before calculation. The expected value of the ratio should be approximately 1, which is in accordance with the release of units along the chain. Therefore, the ratio obtained for agarose is 1.41, but for the agar of *Gelidium*, it is 2.91, and for the agar-type polysaccharide, it is 4.04.

The determination of anomeric forms was carried out in biological samples of different agar-type compounds: agarose, agar from *Gelidium,* and agar-type polysaccharides of *Gelidium sesquipedale.* The sensitivity of the determination of sugars is very good, as reported by others (11). The chromatograms of PIC derivatives of sugars in biological samples closely matched the standard profiles (Figures 2 and 3).

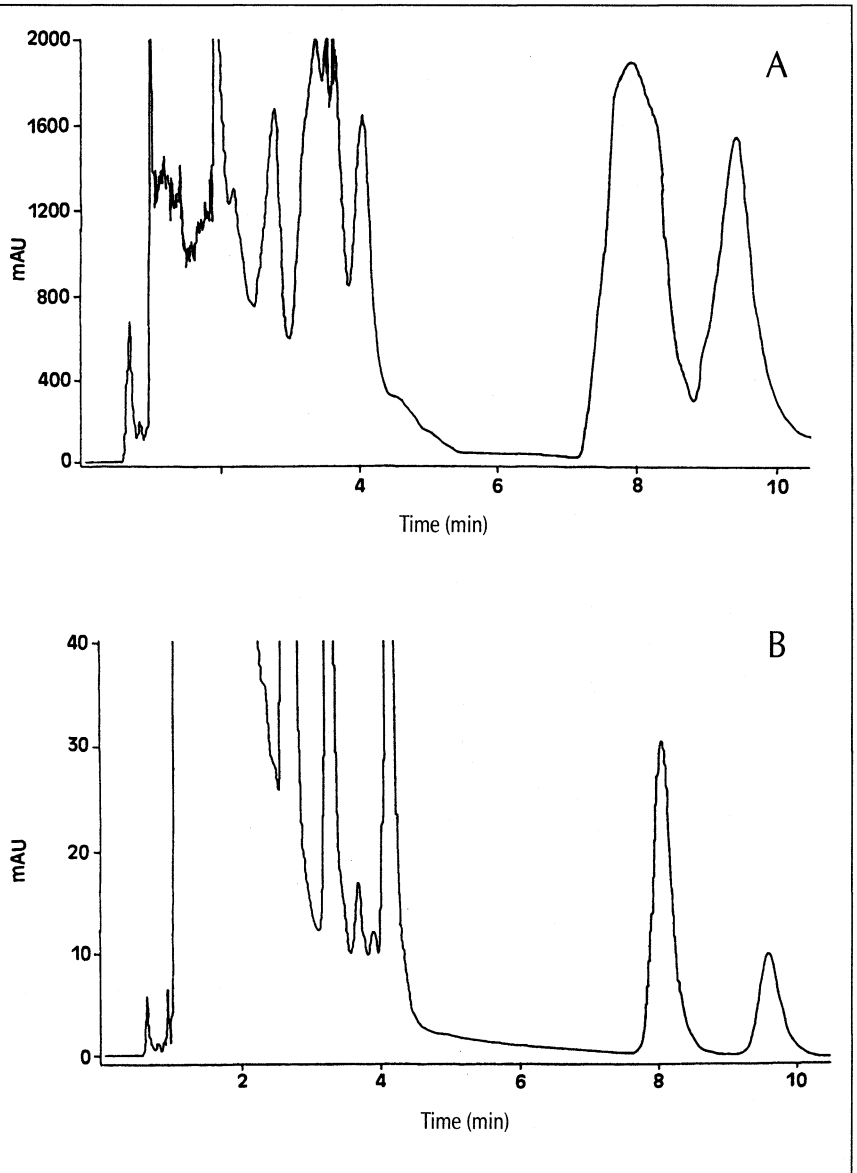

Figure 2. Typical chromatograms (wavelength, 240 nm) of a hydrolyzed (A) and a nonhydrolyzed (B) sample of agarose.

An assay at room temperature increased the previously unadded anomeric form of the analyzed carbohydrate (result not shown). Decreasing the temperature yielded better results. When 10°C was used as standard temperature, the interconversion kinetics looked sufficiently slow so that no significant amount of mutarotation occurred on-column. The α–β ratio we obtained shows the differences due to the average molecular weight and the molecular weight of the polysaccharides assayed. The weight of the molecule of agarose was close to the average value. The larger difference in their length chain justifies the major values in the α–β ratio for the other molecules studied.

Epimeric structures as chrondroitin disaccharides were separated (16). The method that we propose allows the separation of the anomeric forms of assayed monosaccharides in a reliable and rapid manner. Specific rotations, circular dichroism absorption or enzymic susceptibilities of the monosaccharides (oxidation, isomeration, or phosphorylation) are some techniques used to determine the type of configuration of anomeric compounds, but these methods are qualitative, not quantitative. In addition, the latter method is dependent on the availability of enzymes with the desired specificity for the α and β anomers (17). On the other hand, the sensitivity of the refractive index is low and inadequate for the detection of these sugars (11). Therefore, the use of PIC as a derivatizing agent for compounds that containactive hydrogen atoms, such as agars, and the use of diode-array detection are the most useful and simple techniques for determination of anomeric forms of sugars at the picomole level.

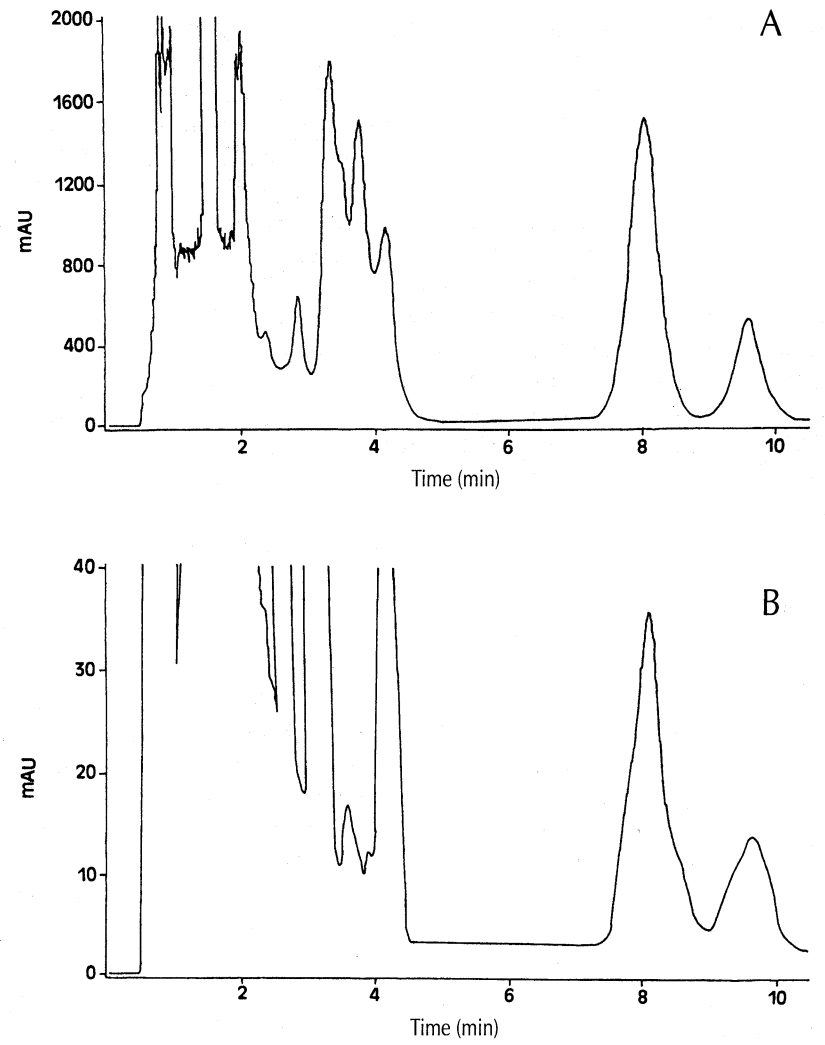

Figure 3. Typical chromatograms (wavelength, 240 nm) of a hydrolyzed (A) and a nonhydrolyzed (B) sample of agar from *Gelidium*.

Conclusion

α- and β-galactose and glucose units were separated and determined by using a routine, rapid, and reliable method that combined reversed-phase HPLC and diode-array detection, quantitating the precolumn PIC derivatives of sugars. Assays were achieved in different biological samples by optimizing the column temperature to 10°C to obtain the α–β ratio. The detection limit for monosaccharides was 5 pmol.

Acknowledgments

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