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Journal of Chromatography A, 814 (1998) 105–110

JOURNAL OF CHROMATOGRAPHY A

Separation of the branch chain hexose, hamamelose, by high-performance liquid chromatography, and amperometric detection of hamamelose and related compounds

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Received 12 January 1998; received in revised form 4 May 1998; accepted 4 May 1998

Abstract

The identification and purification of the branch chain hexose hamamelose (2-hydroxymethyl-D-ribose) from plant leaves is described. The procedure includes metabolite extraction in acid and a series of isocratic HPLC separations. Amperometric detection of hamamelose is shown to be quantitative, having a detection limit below 1 nmol. Similar detection of the related compound, 2-carboxy-D-arabinitol, and its monophosphate ester, yields unusually small peaks.

Keywords: Plant materials; Sugars; Hamamelose; Carboxyarabinitol; Rubisco; Enzymes

1. Introduction

Interest in the sugar hamamelose has recently been revived because of its relationship to a regulator of the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco, EC 4.1.1.39) [1,2]. Rubisco is responsible for the stable assimilation of atmospheric CO_2 into organic carbon by plants and its catalytic activity is modulated in response to changes in its immediate environment. The activity of Rubisco is decreased at night by a tight-binding, phosphorylated, branch chain sugar acid, 2-carboxy-D-arabinitol 1-phosphate (CA1P) [3]. Structurally related compounds may also regulate Rubisco activity during the day [4–6].

Hamamelose (2-hydroxymethyl-D-ribose) is a branch chain hexose found in leaves of most (if not all) plant species [7] which has the same carbon skeleton as both CA1P and its immediate precursor, 2-carboxyarabinitol (CA) [6]. Owing to the structural similarity between hamamelose, CA and CA1P, it is likely that all three are related metabolically [1,2]. Hamamelose, CA and CA1P are apparently unique to plants. Little is known about the metabolic or physiological function of hamamelose apart from its likely role in the regulation of Rubisco (as a precursor of CA1P) and as a possible intermediate in the synthesis of the compatible solute hamamelitol [8].

Previous identification of hamamelose in leaves [7] was dependent on the prior incorporation of ^{14}C (from $^{14}CO_2$) into hamamelose, followed by metabolite extraction and identification, by means of paper chromatography and autoradiography. With the ad-

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0021-9673/98/$19.00
PII: S0021-9673(98)00409-9

vent of new high-performance liquid chromatography (HPLC) strategies, it is possible to identify and resolve hamamelose from leaf material without the need for radioisotopes. Such an approach is described here, incorporating anion-exchange and ion-mediated partitioning HPLC in conjunction with amperometric detection. In each case, sample separation has been achieved by isocratic elution, and so should be possible using a very simple (e.g., single pump) HPLC system. Pulsed amperometric detection (PAD) of hamamelose, CA and CA1P is shown to be quantitative, although the signal intensities were considerably lower than for straight chain sugars and sugar acids.

2. Experimental

2.1. Plant material

French bean (*Phaseolus vulgaris* cv. Tendergreen) was grown in a glasshouse with supplementary lighting to ensure a photoperiod of 16 h, with a minimum photon flux density (PFD) of approximately 200 μmol m^{-2} s^{-1}. Leaves were harvested between 14 and 21 days after sowing.

2.2. Metabolite extraction

Frozen leaf material (10 g fresh mass, FM) was ground to a fine powder in liquid nitrogen and mixed rapidly with 8 ml of ice-cold 3.5% (v/v) aqueous trifluoroacetic acid, using a pestle and mortar. After 30 min at 0°C the extract was clarified by centrifugation (15 800 *g* for 5 min at 4°C) and the clear supernatant applied to a column containing 10 g of Mega Bond Elut C_{18} material (Phenomenex UK Ltd.) and washed through with 10 ml water. The resulting colourless solution was evaporated to dryness in vacuo over NaOH pellets and anhydrous $CaCl_2$. The residue was dissolved in water and passed through a column containing 20 ml of Dowex-50 (H^+) cation-exchange resin. The pH of the eluate was adjusted to 5–6 by addition of Dowex-1 (CO_3^{2-}). The ion-exchange resin was then removed by filtration, and the sample evaporated to dryness, as above. The sample was redissolved in 1 ml water immediately before HPLC fractionation.

2.3. HPLC equipment

We used a Bio-LC chromatography system (Dionex, UK) incorporating an eluent degas module (EDM-2), a gradient pump (GPM-1), a PAD system (PAD-2), a post-column reagent pump (RP-1) and an analogue-to-digital converter, for Figs. 1 and 2. For Fig. 3, a Dionex DX500 chromatography system was used, comprising an autosampler (AS3500), a gradient pump (GP40) and an electrochemical detection system (ED40) with one of two measuring cells (ED40 or PAD-2 cell, part Nos. 44108 and 36276, respectively) both with a gold working electrode and silver–silver chloride reference electrode, but with different electrode areas and active cell volumes. Both cells were used for pulsed amperometry, measuring the current caused by sample oxidation at the electrode surface, set at electrode potential, E_1. Thereafter, the electrode was brought to potentials E_2 (hyperpolarisation) then E_3 (depolarisation), which served to remove oxidation products from the electrode surface, preparing the electrode for the next measurement. The potential sequence applied using the PAD system (Figs. 1 and 2) was: E_1=50 mV (240 ms duration); E_2=650 mV (180 ms); and E_3=−100 mV (360 ms); and the oxidation current was sampled from 40 to 57 ms after the initiation of E_1. The potential sequence used with both cells in conjunction with the ED40 detector (Fig. 3) was: E_1=50 mV (410 ms duration); E_2=750 mV (200 ms); and E_3=−150 mV (390 ms); integrating the oxidation current from 200 to 400 ms after the initiation of E_1. Both potential sequences were developed for carbohydrate detection by the manufacturer (Dionex).

2.4. HPLC identification/purification

Hamamelose purification from the acid stable, neutral metabolites was achieved after three successive HPLC treatments (Table 1). These were:

(1) Anion-exchange HPLC. Each sample application contained material from 1 g FM of leaf material. Fractions corresponding to the peak at 16 min (Fig. 1A) were treated with Dowex-50 (H^+) (0.06 ml resin per ml) to remove the NaOH, then dried in vacuo over anhydrous $CaCl_2$.

(2) Ion-moderated partitioning HPLC. Samples

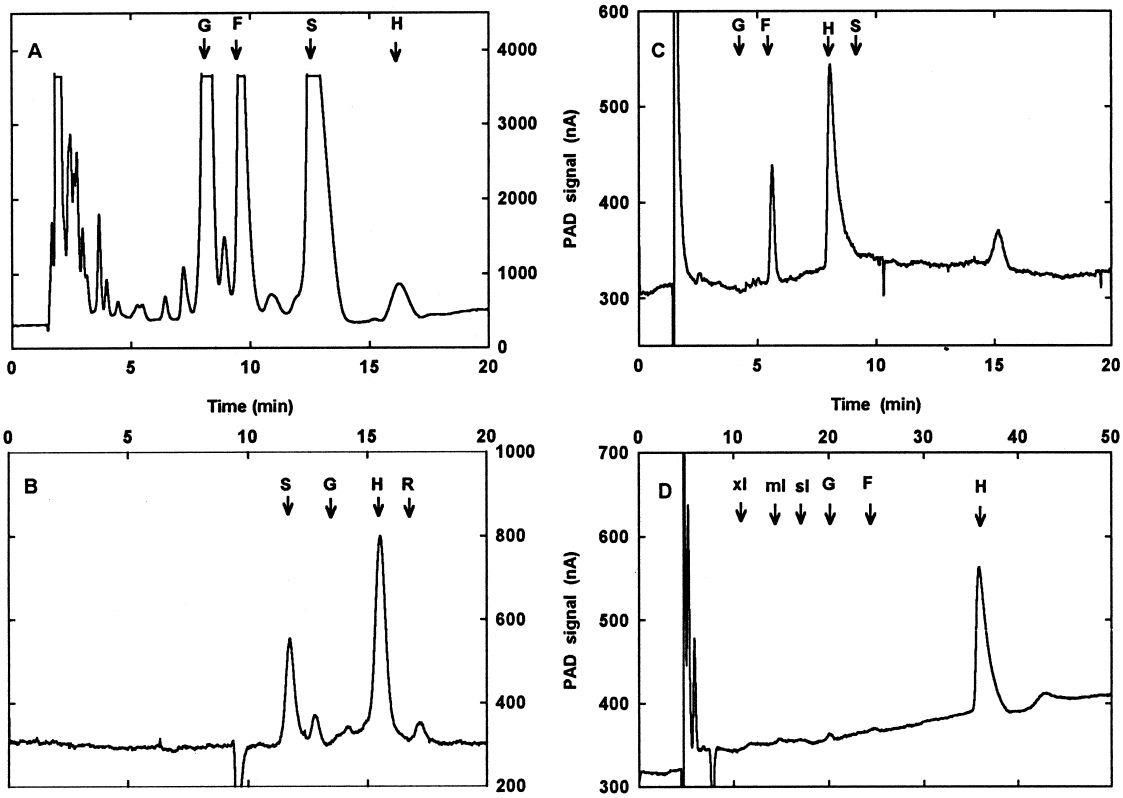

Fig. 1. Sequential purification of hamamelose by HPLC. (A) Initial separation of neutral leaf extract by anion-exchange chromatography. Isocratic elution using 40 m*M* NaOH. (B) Further purification of hamamelose peak from A, by ion-moderated partition chromatography. (C) Final purification of hamamelose from B, by anion-exchange chromatography. Isocratic elution using 100 m*M* NaOH. (D) Confirmation of purity of hamamelose from C, by anion-exchange chromatography using a CarboPac MA1 analytical column (Dionex, UK) with isocratic elution using 0.48 *M* NaOH at a flow-rate of 0.4 ml min^{-1}. In all cases, peak detection was by means of pulsed amperometry. The retention times of purified components, determined immediately before sample application, are shown above each chromatogram. Abbreviations: F=fructose; G=glucose; H=hamamelose; ml=manitol; R=ribose; S=sucrose; sl=sorbitol; xl=xylitol. For further details, see Section 2.

from (1) were rehydrated in up to 0.5 ml water. In order to visualise the peaks by amperometric detection, post-column addition of 200 m*M* NaOH at 0.4 ml min^{-1} was necessary. However, since the retention time of hamamelose is very consistent, after establishing its retention time using a small amount of the extract from (1) above, we could then proceed without the need for detector or post-column addition. As before, each sample application contained material from 1 g FM of leaf material. Fractions corresponding to the peak at 15.5 min (Fig. 1B) were treated with Dowex-1 (CO_3^{2-}) (0.06 ml resin per ml) to remove SO_4^{2-} ions and neutralise. The combined sample was then dried, as before.

(3) Anion-exchange HPLC, similar to (1) above, but using 100 m*M* NaOH throughout (Fig. 1C). Material from up to 10 g FM could be applied in a single application. Fractions were collected at 1 min intervals and those corresponding to the peak at 8.5 min (Fig. 1C) were treated with Dowex-50 (H^+) (0.15 ml resin per ml) to remove the NaOH, then dried in vacuo over anhydrous $CaCl_2$.

2.5. Quantification of purified components

This was achieved by measurement of mass (glucose, glucose 6-phosphate, gluconic acid, 6-phosphogluconate and hamamelose), by titration of the lactone (CA), or by determination of organically bound phosphate (CA1P) [9].

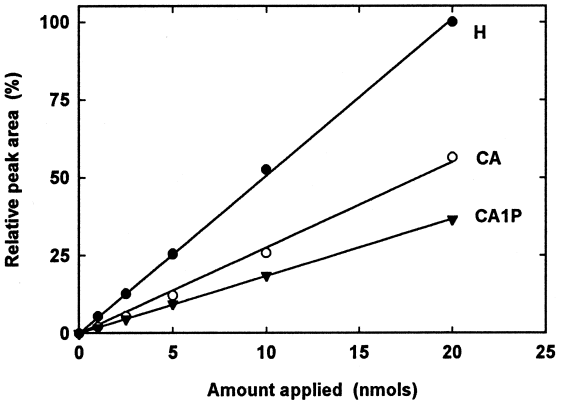

Fig. 2. Dependence of signal from amperometric (PAD) detector on the amount of hamamelose (●), CA (○) and CA1P (▼). Fifty μl of a solution containing the stated amounts of hamamelose, CA and CA1P was loaded onto a CarboPac PA1 analytical column, pre-equilibrated with 100 m*M* NaOH, 50 m*M* sodium acetate. Sample resolution was effected using a linear gradient (50–750 m*M*) of sodium acetate, developed from 5 to 20 min after sample application (with 100 m*M* NaOH throughout). The flow-rate was 1 ml min^{-1}.

2.6. Other materials

CA and CA1P were synthesised as described previously [10] from ribulose 1,5-bisphosphate and potassium cyanide. For use as a chromatographic standard, hamamelose was isolated from the bark of witch hazel, as described previously [11]. [U-^{14}C]-Hamamelose was synthesised according to the method of Yanagihara et al. [12]. Dowex resins (AG 50W-X8 and AG 1-X8) were supplied by Bio-Rad Labs., UK. All other chemicals were of analytical grade, and used without further purification.

3. Results and discussion

3.1. Identification and purification of hamamelose

Authentic hamamelose, from hamamelitannin, was found to be resolved from mixtures of commonly occurring sugars by a variety of HPLC procedures, which were subsequently found to be suitable for the purification of free hamamelose from neutral leaf extracts. The first was anion-exchange HPLC with 40 m*M* NaOH as eluent. Such separations are possible in the presence of NaOH, since the hydroxyl

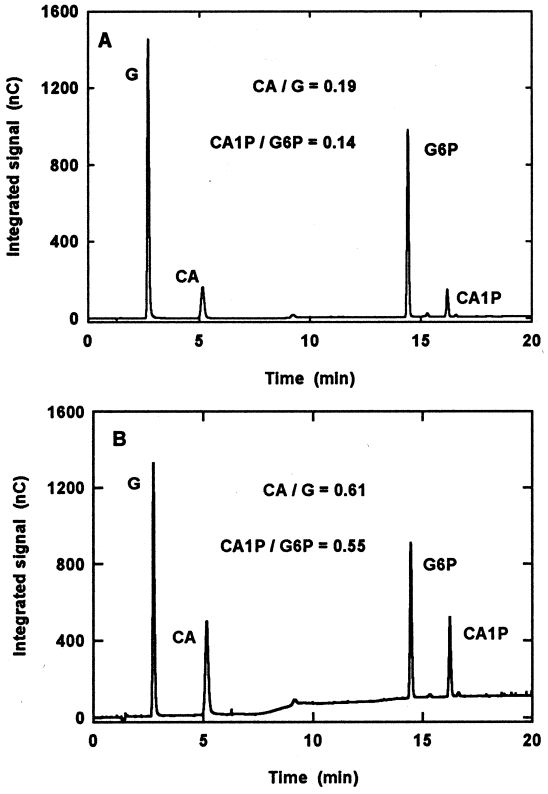

Fig. 3. Comparison of the response of different amperometric detector cells to glucose, glucose 6-phosphate, CA and CA1P. Ten nmol each of glucose (G), glucose 6-phosphate (G6P), CA and CA1P were loaded onto a CarboPac PA1 analytical column (plus guard), in a volume of 50 μl and resolved using a gradient of sodium acetate, as described in Fig. 2. Peak detection was by integrated amperometry using alternative electrochemical cells. (A) Standard ED40 cell; (B) experimental PAD cell.

groups of sugars become deprotonated ($pK_a \approx 12$) rendering them anionic. Hamamelose (H, retention time 16.0 min; Fig. 1A) was resolved from glucose (G), fructose (F) and sucrose (S) and a variety of minor components. The greater retention time of hamamelose than of any other hexose (glucose, fructose, galactose, mannose) or pentose (ribose, xylose) or even sucrose, suggest either that hamamelose is more anionic under the prevailing conditions, or that its conformation facilitates adsorption to the ion-exchange matrix.

The resulting hamamelose fraction was further resolved by means of ion-moderated partition HPLC, using 5 m*M* H_2SO_4 as eluent (Fig. 1B). Under these

Table 1
Columns and conditions used in hamamelose purification from acid stable, neutral metabolites

Step	1 (Fig. 1A)	2 (Fig. 1B)	3 (Fig. 1C)
Column	Carbopac PA1 Analytical (250×4 mm)	Aminex HPX-87H (300×7.8 mm)	Carbopac PA1 Analytical (250×4 mm)
Guard column	Carbopac PA1 Guard (50×4 mm)	Mico-Gurad CationH Cartridge	Carbopac PA1 Guard (50×4 mm)
Supplier	Dionex, UK	Bio-Rad Labs., UK	Dionex, UK
Eluent	40 m*M* NaOH	5 m*M* H_2SO_4	100 m*M* NaOH
Flow-rate	1.0 ml min^{-1}	0.4 ml min^{-1}	1.0 ml min^{-1}
Sample volume	100 μl	100 μl	100 μl
Fraction size	1.0 ml	0.2 ml	1.0 ml
Column regeneration	200 m*M* NaOH	None	None

conditions, the retention time of hamamelose was 15.5 min. These two HPLC procedures exploit different properties of hamamelose and so their combined use provides a useful tool in the separation of this sugar from complex mixtures.

Surprisingly, the resulting material contained traces of other compounds, one having a retention time similar to fructose (Fig. 1C). Interestingly, ion-moderated partition chromatography is unable to resolve fructose and hamamelose and so any fructose remaining after the first round of HPLC (or released thereafter by hydrolysis of other contaminants) would not have been removed in the second. However, the hamamelose fraction resulting from an additional round of anion-exchange HPLC was pure (Fig. 1D), to the extent that no other oxidizable material was detected. Thin-layer chromatographic analysis of the purified sugar, using silica gel and a mobile phase of ethyl acetate–65% (v/v) aqueous isopropanol followed by staining for reducing sugars using *p*-anisidine, confirmed the presence of a single substance with the same R_F (0.29) as hamamelose (not shown).

Additional support for the correct identification of hamamelose in each chromatogram was obtained by a separate purification, in which the leaf homogenate (in trifluoroacetic acid) was spiked with a small amount of high specific radioactivity [U-^{14}C]-hamamelose (6.67 kBq; 10.2 GBq $mmol^{-1}$). Fractions collected during each HPLC separation were subsequently found to contain radioactivity exclusively in the peaks with retention times corresponding to those for hamamelose. The recovery of radioactivity (as a percentage of the total) after each step in the purification – after Dowex treatment of the relevant fractions – gives a good indication of the losses at each stage, and were: 60% for the initial extraction/selection of neutral components; 56% for the first, 27% for the second and 20% for the third (final) HPLC treatment. See Fig. 1A–C.

3.2. Amperometric detection of hamamelose, CA and CA1P

Using PAD, the presence of hamamelose, CA or CA1P in the detector cell gives rise to sample oxidation in direct proportion to the amount of material present. A graph showing the dependence of current generated (integrated peak signal) on quantity applied shows a linear dependence from 1 to 20 nmol (Fig. 2). The signal intensity for hamamelose is less than that obtained for other hexoses (e.g., about 60% of the signal using D-glucose). CA and CA1P gave even smaller signals – about 50% and 35%, respectively, of the corresponding signal for glucose (Figs. 2 and 3B). The lower signal for CA1P than for CA must be due to the replacement of a primary alcohol for a phosphate group, as they are identical

in all other respects. An analogous difference in signal intensity has been observed between gluconic acid (an isomer of CA) and 6-phosphogluconate (an isomer of CA1P).

Thus, the introduction of a branch chain structure or a phosphate group into otherwise identical molecules, reduces the detector sensitivity, presumably by altering the oxidation characteristics of such compounds.

Such signal attenuation is particularly pronounced using the latest ED40 electrochemical cell whose properties (Fig. 3A) were compared with those of the more familiar PAD cell, configured (by Dionex) for use with the current ED40 detector (Fig. 3B). The signals from both cells were generated and measured in an identical fashion. While both measuring cells give very similar peak areas for both glucose (G) and glucose 6-phosphate (G6P), the ED40 electrochemical cell gave a 3- to 4-fold lower signal for CA and CA1P than the PAD cell (compare Fig. 3A with Fig. 3B). However, in terms of absolute sensitivity, the ED40 cell was found to provide a higher signal/noise ratio than the PAD cell for any of the sugars detected, being about 2-fold higher for CA and CA1P and 10-fold higher for straight chain hexoses and hexose monophosphates. This was due to a 10-fold reduction in baseline noise.

We are currently investigating whether the electrode potential profile can be further optimized for these unusual compounds and whether the differential sensitivity of the two amperometric cells can be used to identify branch chain carbohydrates.

Acknowledgements

We would like to thank Berk (Materials for Industry) Ltd. (UK) for the generous gift of witch hazel bark. IACR receives grant-aided support from the Biotechnology and Biological Research Council of the United Kingdom.

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