Large-scale preparation of α-D-(1→4)-oligogalacturonic acids from pectic acid

Hong-Ni Fan, Mei-Zheng Liu, and Yuan C. Lee

Abstract: An efficient and inexpensive method for large-scale preparation of α-D-(1→4)-oligogalacturonic acids (oligo-GalA), up to DP 5, from pectic acid is described. Pectic acid was digested with a commercially available pectinase to yield a mixture of oligo-GalA, which was effectively separated by a combination of low-pressure – size-exclusion chromatography based on ion-exchange chromatography to obtain pure oligo-GalA of DP 2–5.

Key words: pectic acid, galacturonic acid, galabiose, galatriose, pectinase.

Résumé : On décrit une méthode efficace et peu coûteuse de préparation à grande échelle des acides α-D-(1→4)-oligogalacturoniques (oligo-GalA), jusqu'à 5 DP, à partir de l'acide pectique. L'acide pectique est soumis à une digestion avec une pectinase commercialement disponible pour conduire à un mélange d'oligo-GalA que l'on peut séparer efficacement par une combinaison de chromatographie d'exclusion de taille à basse pression et de chromatographie d'échange ionique pour obtenir les oligo-GalA de DP allant de 2 à 5.

Mots clés : acide pectique, acide galacturonique, galabiose, galatriose, pectinase.

[Traduit par la Rédaction]

Introduction

Pectic acid, derivable from an abundant plant polymer, pectin, is essentially composed of a repeating unit of α-(1→4)-linked D-galacturonic acid. α-D-(1→4)-Oligogalacturonic acids (oligo-GalA) have been shown to be physiologically active elicitors of plant-defense responses to pathogens (1). On the other hand, galabiose (α-Gal-(1→4)-Gal) has the same anomeric and positional linkages as the repeating unit of pectic acid, differing only by the presence of hydroxymethyl group rather than the carboxyl group at C-6. Galabiose is an important structural unit of some glycolipids, such as Gb3 and Gb4, and is the recognition marker for bacterial toxins (e.g., Shiga toxin and Shiga-like toxins) as well as for certain microbial invasions of human tissues, e.g., uropathogenic *Escherichia coli*, which causes hemorrhagic colitis and hemolytic uremic syndrome (2–6). Synthetic glycoconjugates containing the carbohydrate moiety of Gb_3 (globotriose, α-Gal-(1→4)-β-Gal-(1→4)-Glc) proved to be effective biomimetics for detecting and trapping of certain bacteria toxins and viruses (6–8). Galabiose has been prepared from pectic acid by first obtaining (α-GalA-(1→4)-α-GalA)$_n$ oligomers by partial digestion with pectinase followed by chemical reduction of the carboxylic groups (9–11). There are also some reports describing analysis, separation, and preparation of oligo-GalA with different degrees of polymerization (DP) (9–15). These methods, however, either require non-commercial enzymes, an expensive special ion-exchange resin, or preparative HPLC, and are not suitable for economical large-scale preparation of oligo-GalA. We wanted to prepare large amounts of galabiose and oligosaccharides containing galabiose by an inexpensive procedure, for the purpose of preparing high-affinity inhibitors for some toxins (e.g., Shiga toxin, Shiga-like toxins) and for certain pathogenic bacterial adhesion (16).

In this paper, we describe an efficient large-scale preparation of oligo-GalA (DP 2–5) from pectic acid by partial hydrolysis of pectic acid with a commercially available pectinase and a combination of inexpensive low-pressure column-chromatographic separation methods based on size exclusion and ion exchange.

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Dedicated to the memory of Professor Raymond U. Lemieux.

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Materials and methods

Materials

Apple pectic acid was obtained from Sigma-Aldrich-Fluka (Milwaukee, WI). Pectinase from *Aspergillus niger* (EC 3.2.1.15), galacturonic acid (GalA), di- and tri-galacturonic acid ((GalA)$_2$ and (GalA)$_3$, respectively), Dowex-50 W × 8 resin (H^+ form, 200 mesh), Dowex-1 × 4 resin (400 mesh), and Sephadex G-25 were from Sigma (St. Louis, MO). Ultrafiltration membranes (YM3, NMWL 3 000) were from Millipore (Bedford, MA).

General methods

Analysis of GalA-oligomers was carried out with high-performance anion-exchange chromatography, HPAEC (17) using a Bio-LC (Dionex, Sunnyvale, CA) with a CarboPac PA-1 column (4.6 × 250 mm) in combination with a pulsed amperometric detector (PAD-2). The detector sensitivity was set at 1 μA. Potential and time settings of the detector were:

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Fig. 1. Time course of hydrolysis of pectic acid with pectinase examined by HPAEC. The number labels mark the DP of the oligogalacturonic acid for the indicated peaks. The time labels on the right of each curve indicate the lengths of hydrolysis, and GalA and $(GalA)_2$ are standards. Equal amounts of the reaction mixture were injected for each run.

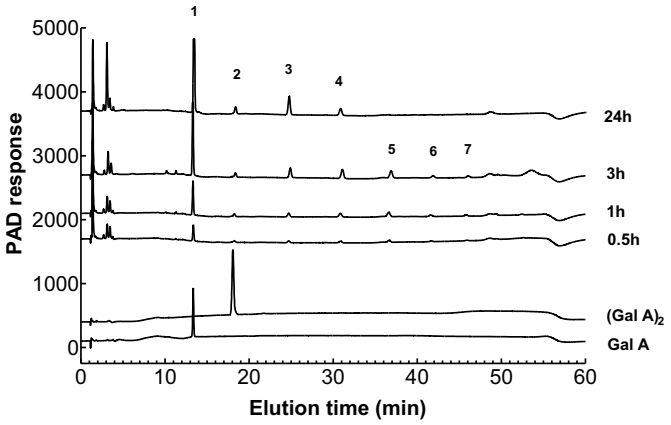


Table 1. Elution gradient (%) of HPAEC for analysis of $(GalA)_n$.

	E2	E3	E4
Time (min)	0.20 M NaOH	Water	0.20 M NaOH/ 1 M NaOAc
0–2	50	48	2
2–9	50	48–30	2–20
9–51	50	30–0	20–50
51–52	50	0–48	50–2
52–62	50	48	2

E_1 = +0.05 V (t_1 = 0.42 s), E_2 = +0.65 V (t_2 = 0.18 s), and E_3 = –0.10 V (t_3 = 0.36 s). Oligo-GalA samples were eluted using the gradients shown in Table 1.

Carbohydrates were determined with a version of the phenol–sulfuric acid method (18). Thin layer chromatography (TLC) was performed on a 0.25-mm layer of silica gel 60 coated on aluminum sheets (E. Merck, Darmstadt, Germany).

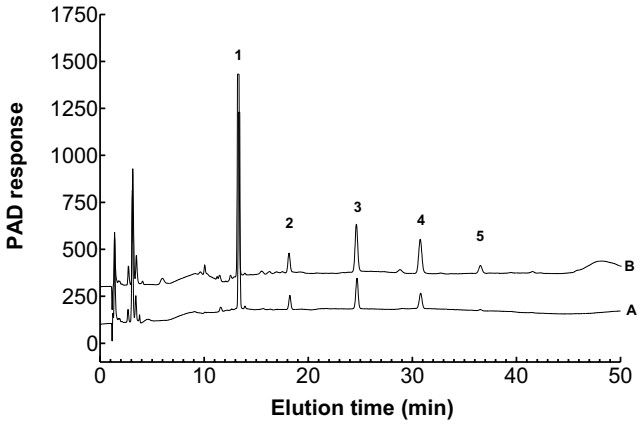
Results and discussion

Optimizing conditions for generation of oligo-GalA with DP 1–5 by pectinase digestion

Mixtures of oligo-GalA with different DPs can be obtained by hydrolysis of pectic acid with autoclaving or enzymatic digestion (9–12), but the enzymatic method was found to be the more efficient to degrade pectic acid. For example, depolymerization of pectic acid or pectin (partially esterified pectic acid) with an endopolygalacturonase (19) produces the desired saturated oligo-GalA, while with pectin lyases or pectate lyases, both saturated and unsaturated oligo-GalAs are produced. Unfortunately, such enzymes are not readily available, and thus we chose to use a commercially available pectinase to generate oligo-GalA from pectic acid.

The optimal conditions for generating oligo-GalA (DP 1–5) mixtures were determined by TLC and HPAEC–PAD analysis of the oligosaccharides released in the process of pectinase digestion. During the digestion with 10 or 20 units of pectinase–pectic acid (1.0 g) in 0.1 M acetate buffer, pH 4.0 (see below) at room temperature for 24 h, portions of the digest were removed after 0.5, 1, 3, and 24 h, immediately frozen in a dry-ice acetone bath, and stored frozen. Before analysis, the samples were thawed and heated at 100°C for 10 min to inactivate the pectinase and the precipitate was removed by microcentrifugation. Digestion with 20 units of pectinase–pectic acid (1.0 g) yielded α-(1→4)-oligo-GalA (DP 1–4) as the main products, in which GalA was predominant (Fig. 1). On the other hand, when 10 units of pectinase–pectic acid (1.0 g) were used under the same conditions, a much lower amount of GalA was produced, and the yields of oligo-GalA (DP 2–5) were higher (Fig. 2).

Fig. 2. Elution profiles of pectic acid digested with different amounts of pectinase by HPAEC. A: pectinase (20 units)/pectic acid (1.0 g); B: pectinase (10 units)/pectic acid (1.0 g). The numbers show the DP of the oligogalacturonic acid in the indicated peaks.



Preparation of oligo-GalA (DP 1–5) mixtures

Pectic acid powder (10.0 g) was suspended in HOAc–NaOAc buffer (each 0.1 M, 500 mL, pH 4.0) and the mixture was incubated at 55°C, sonicated, and stirred until the pectic acid was totally dissolved. The homogeneous but viscous solution was again adjusted to pH 4.0 with 0.1 M NaOAc, and the pectinase (250 μL, ca. 100 units) was added. The solution was gently stirred for 24 h at room temperature; the enzymatic hydrolysis was terminated by heating the mixture at 100°C for 10 min. After cooling, the precipitate and the remaining polymer were removed by ultrafiltration with a stirred cell (200-mL, Millipore, Bedford, MA) using a YM3 membrane. To remove the cations, the filtrate was applied to a column (2.5 × 17.6 cm) of Dowex-50W × 8 resin (H^+ form, 200 mesh) and washed with distilled water (200 mL). The washing was concentrated by rotary evaporation, and the residue was lyophilized to afford 7.68 g of oligo-GalA mixture (76.8% yield).

Size-exclusion chromatography

The oligo-GalA mixture (4.0 g) in HOAc (0.1 M, 20 mL) was applied to a Sephadex G-25 column (5.0 × 218 cm) in 0.1 M HOAc and eluted at a flow rate of 4 mL min^{-1} in 0.1 M HOAc. Fractions (16 mL) were analyzed for carbohydrates by the phenol–H_2SO_4 method (Fig. 3). The

Fig. 3. Elution of oligo-GalA on a Sephadex G-25 column (5.0 × 218 cm). The *y* axis is $A_{480\ nm}$ obtained by the phenol–sulfuric acid method. Approximate positions of the DP of the oligogalacturonic acid are indicated.

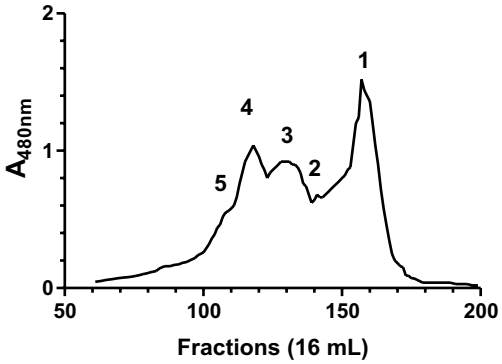
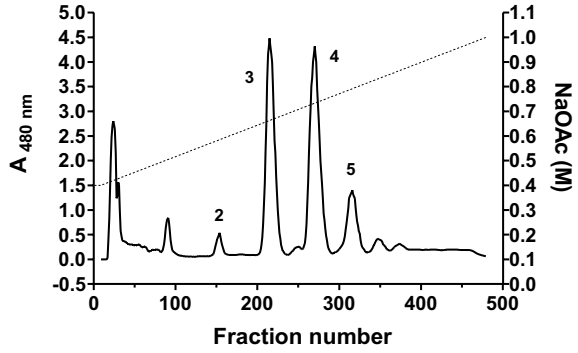


Fig. 4. Separation of oligo-GalA on Dowex-1 × 4 column (2.5 × 46 cm). The column was eluted with a linear gradient generated with 1 L each of 0.4 M and 1.0 M NaOAc (pH 6.0). The numbers indicate the DP of oligo-GalA in the peaks.

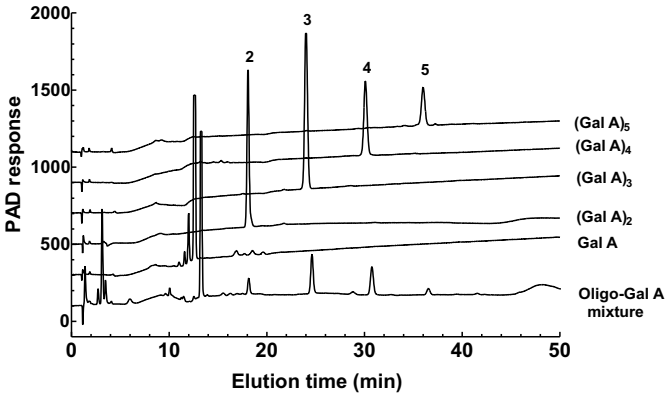


carbohydrate-containing fractions were examined by TLC using silica gel 60 sheets with 1-butanol–formic acid–water (v/v, 4:6:1) as the developing solvent. The R_f values of the oligo-GalA with DPs 1, 2, 3, 4, and 5 were ca. 0.48, 0.35, 0.28, 0.22, and 0.17, respectively. The fractions containing oligo-GalA of DP 2–5 were combined, concentrated by rotary evaporation, and lyophilized to give 2.58 g of product (64.5% yield). Although the Sephadex G-25 column did not fully separate oligo-GalA of DP 2–5, most of the monomer (GalA), which constituted a fairly large proportion of the total digest, could be removed.

Anion-exchange chromatography of oligo-GalA

An example of the anion-exchange chromatography of oligo-GalA is described below. The oligo-GalA mixture (DP 2–5, 1 g) was dissolved in water (10 mL), and the solution was adjusted to pH 12 with 50% aqueous NaOH solution. The mixture was left at room temperature for 1 h and the pH was re-adjusted to 6.0 with 2 M HOAc. The resulting solution was applied to a column (46.5 × 2.5 cm) of Dowex-1 × 4 resin (400 mesh) that had been equilibrated in 0.4 M NaOAc buffer, pH 6.0. The column was washed with 25 mL of the equilibration buffer, and eluted with a linear gradient generated with 1 L each of 0.4 M and 1.0 M NaOAc (pH 6.0). Finally, the column was washed with 1 L of 2.0 M NaOAc (pH 6.0). Fractions were analyzed for carbohydrate

Fig. 5. Identification of each oligo-GalA peak isolated from the Dowex-1 × 4 column (see Fig. 4) with HPAEC.



content by the phenol–H_2SO_4 method; the results are shown in Fig. 4. The carbohydrate-containing fractions were analyzed by HPAEC for identification of DP. The purity of each of the isolated peaks is shown in Fig. 5. The individual oligo-GalA peaks were pooled and passed through a column (17.6 × 2.5 cm) of Dowex-50W × 8 resin (H^+ form, 200 mesh) for decationization; the effluent was concentrated by rotary evaporation, followed by lyophilization to give di-, tri-, tetra-, and pentagalacturonic acid ($(GalA)_2$, $(GalA)_3$, $(GalA)_4$, $(GalA)_5$, respectively), with the yields of 1.8, 18.2, 19.9, and 4.9%, respectively.

The alkaline treatment of the oligo-GalA mixture (pH 12 for 1 h) was to open any lactone rings that might have been formed during the gel filtration procedure. Lactonization under acidic condition is often a problem for separation of anionic oligo/polysaccharides with anion-exchange chromatography (20). The separation of oligo-GalA mixture on Dowex 1, a common anion-exchange resin, was excellent (Fig. 4), separating the oligomers better than those reported previously with sophisticated instrumentation (14, 15). Although the column described above has a loading capacity of ~1.0 g of the oligo-GalA mixture, it can be used repeatedly or can be readily scaled up.

Summary

We present here a simple and efficient preparative method of oliogalacturonic acids, up to DP 5, from an inexpensive source, pectic acid. The advantages of our method are the use of an inexpensive commercial enzyme and low-cost chromatographic procedures to yield pure oligo-GalA of DP 2–5 in good overall yield.

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