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Note

A simple method for preparation of D-rhamnose

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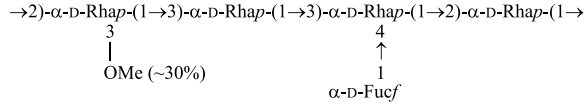
Abstract

A rapid procedure for the preparation of D-rhamnose from bacterial lipopolysaccharide (LPS) has been developed. It involves purification of LPS from *Pseudomonas syringae* pv. *phaseolicola* by phenol extraction and hydrophobic interaction chromatography (HIC), followed by mild hydrolysis and cleavage of the O-antigen into D-fucose and D-rhamnose. The monosaccharides were separated by column chromatography, and D-rhamnose recovered after filtration over Sephadex-LH 20. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: D-Rhamnose; 6-Deoxyhexose; *Pseudomonas syringae* pv. *phaseolicola*; Lipopolysaccharide; Endotoxin; Hydrophobic interaction chromatography; Column chromatography

Rhamnose is a common 6-deoxyhexose. The L-enantiomer occurs in numerous oligo- and polysaccharides of plant and microbial origin.[1] D-Rhamnose, in contrast, is rare in nature and has been identified up till now only in a few bacteria.[2] D-Rhamnose is an important moiety in the O-specific antigen of lipopolysaccharides (LPS) of human and plant pathogenic pseudomonads.[3–5] A loss of the carbohydrate portion in LPS leads to strains with rough colony morphology. Typically, the virulence of rough strains is strongly reduced, their sensitivity towards antibiotics or serum components increased. Given the fact that D-rhamnose is encountered in microorganisms only, but neither in humans, animals and plants, this monosaccharide and the key enzymes for its biosynthesis are promising targets for the development of new antiinfectives. The investigation of biosynthesis and structure of virulence polysaccharides requires access to D-rhamnose in larger quantities as precursor and reference compound. Furthermore, this 6-deoxyhexose is a precursor for the preparation of corresponding nucleotides such as GDP-D-rhamnose by in vitro regeneration systems. As demonstrated for other monosaccharides, they can be used for the synthesis of biologically important glycoconjugates catalyzed by recombinant glycosyltransferases.[6,7] There is, to our knowledge, no commercial supply for D-rhamnose. Chemical syntheses of D-rhamnose have been reported,[8–12] but they are not readily feasible in a typical biochemical or microbiological laboratory. We have, therefore, devised a simple and efficient method for the preparative extraction of reference grade D-rhamnose from bacterial LPS.

The O-specific polysaccharide of LPS from the phytopathogenic *Pseudomonas syringae* pv. *phaseolicola* consists of D-rhamnose and D-fucose in a 4:1 ratio. The repeating unit has the following structure (see Scheme 1):[4,5,13]



Scheme 1.

Strain NCPPB 1321 was grown on a fully synthetic medium with limited glucose supply and harvested upon exhaustion of the carbon source. A crude LPS preparation was obtained by classical hot phenol/water extraction of the cells.[14] Accompanying nucleic acids, proteins and extracellular polysaccharides were removed by preparative HIC over Butylsepharose®.[15] This method permits a rapid one-step purification of

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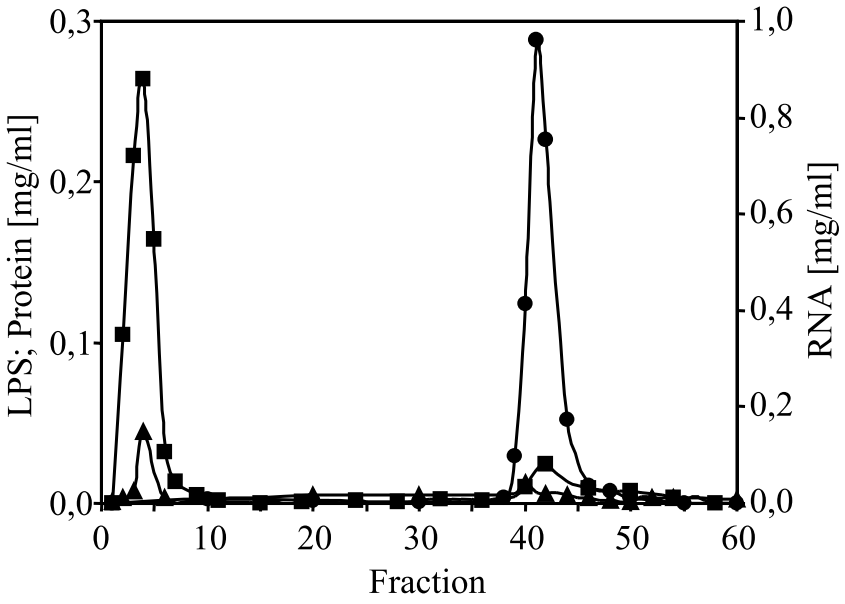

Fig. 1. Elution profile of LPS and contaminants on preparative Butylsepharose column (30 × 3 cm I.D.). Symbols: ●, LPS; ■, RNA; ▲, Protein. For further conditions, see Section 1.

LPS due to the strong interaction of the lipid A moiety with the hydrophobic matrix. A typical elution profile is shown in Fig. 1. The lipid A portion of the LPS was cleaved off by mild hydrolysis in dilute acetic acid and removed by extraction with chloroform. Hydrolysis of the remaining O-antigen was achieved in 4 M TFA. The resulting monosaccharide mixture was separated by rapid column chromatography on silica gel using ethyl acetate–methanol–acetic acid–water (60:15:15:10, v/v) as mobile phase. A baseline separation of D-rhamnose and D-fucose was achieved (see Fig. 2). D-Rhamnose containing fractions were evaporated to dryness and passed through a Sephadex LH-20 column eluted with methanol. Purity and identity were confirmed by thin layer chromatography (TLC), high performance liquid chromatography (HPLC), ^{1}H and ^{13}C NMR, and measurement of optical rotation, in comparison with literature data.[16–18]

The entire procedure, including precultivation was carried out in 2 weeks. The time consuming steps, i.e., dialysis and lyophilization of raw and purified LPS, proceeded unattended. Hydrolysis and chromatographic purifications, in contrast, were comparably fast. Separation on the HIC column took approx 2 h, the purification of D-rhamnose on silica gel and Sephadex columns about 4 h each. The effective working time was an estimated 24 h. Each step of the procedure was scalable. For production of larger quantities of D-rhamnose, growth of bacteria in conventional fermenters can be substituted by high-density cultures which allow 20 to 70-fold higher cell density.[19]

1. Experimental

1.1. General

Methanol and EtOAc were distilled from technical grade solvents. HPLC grade pyrogen-free water was freshly obtained from a Seralpur Delta water purification system. Dialysis was carried out in phenol resistant dialysis tubing (Visking, Serva, Heidelberg, Germany) against demineralized water for at least 48 h at ambient temperature. For TLC analysis, silica gel 60 coated Al-sheets (Merck, Darmstadt, Germany) were used. Monosaccharides were visualized after spraying with vanillin-sulfuric acid reagent and heating for 3 min at 105 °C.[20] ^{1}H and ^{13}C NMR spectra were recorded in D_2O on a Bruker AMX spectrometer operating at

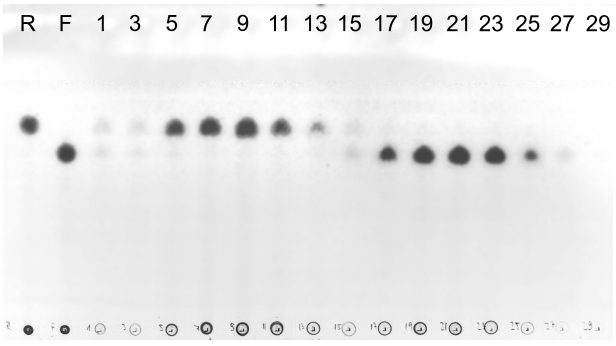

Fig. 2. TLC chromatogram of fractions eluted from silica gel column. Symbols: R and F, reference L-rhamnose and L-fucose, respectively; numbers correspond to the fractions of column chromatography. For TLC conditions, see Section 1.

250.13 and 62.90 MHz, respectively. Optical rotation was measured with a Polartronic-E polarimeter (Schmidt/Haensch, Berlin, Germany).

1.2. Fermentation of *P. syringae* pv. *phaseolicola*

The strain *P. syringae* pv. *phaseolicola* NCPPB 1321 was obtained from Dr K. Rudolph, Institute of Plant Pathology and Plant Protection, University of Göttingen. Preservation was in liquid nitrogen. Cultivation was carried out at 26 °C in phosphate buffered mineral medium pH 7.0 containing (per liter) 5.5 g $Na_2HPO_4 \times H_2O$, 2.6 g KH_2PO_4, 2.0 g NH_4Cl, 1.0 g Na_2SO_4, 0.1 g $MgSO_4$, 10.0 mg $FeSO_4$, 10.0 mg $MnSO_4$.[21] The medium was supplemented with 5 g glycerol (precultures) or glucose (fermentation), respectively. Glucose was filter sterilized and added after the media were autoclaved.

The first preculture was carried out in 500 mL shake flasks (100 mL, 180 rpm, 18 h). The second preculture (2.5 L) was performed in a Braun Biostat B fermenter (Braun, Melsungen, Germany) over 12 h. Main fermentations (25 L) were in a Braun Biostat C-DCU fermenter. Cell density was determined by absorbance measurement at 578 nm, and glucose concn was measured with an commercial kit (Bioquant® glucose, fructose, sucrose, Merck, Darmstadt, Germany). Cells were harvested by centrifugation (10,000*g*, 4 °C, 30 min) in the late log phase when glucose was completely consumed. Loosely bound surface polysaccharides were removed by washing the pellet once with 10 mM Na_2–EDTA in 0.85% NaCl and twice with 10 mM $MgSO_4$ and repeated centrifugation. Cells were lyophilized and stored at −20 °C until use.

1.3. Purification of LPS

Cells were resuspended at 50 mg/mL in water and heated to 68 °C. After addition of preheated 90% aq phenol, extraction of LPS was carried out in a Erlenmeyer flask under intensive stirring at 68 °C for 15 min. The mixture was brought to 4 °C. Phase separation was achieved by centrifugation (6000*g*, 10 min) at 4 °C. The upper, aq phase was carefully removed and submitted to dialysis.

The resulting crude LPS fraction was purified on a Butyl Sepharose 4 Fast Flow (Amersham Biosciences, Freiburg, Germany) column (25 × 3 cm I.D.) at a flow rate of 12 mL/min. The mobile phase consisted of: (A) 0.2 M aq AcONa, pH 4.7; (B) *n*-PrOH. The elution profile (% A) was: 9 min 100%, linear gradient over 90 min to 60%. Aliquots of 50 mg crude LPS in 5 mL buffer were injected, and fractions of 25 mL were collected. LPS containing fractions were identified by a commercial Limulus Amebocyte Lysate (LAL) assay (Kinetic-QCL, BioWhittaker Europe, Verviers, Belgium) and by SDS-PAGE with silver staining according to Tsai and Frasch.[22] LPS containing fractions were combined and concd in vacuo to one tenth of the initial volume, submitted to extensive dialysis and freeze dried to afford a purified LPS fraction.

1.4. Hydrolysis of LPS

LPS was suspended under sonication in 2% aq AcOH (v/v), at a concn of 20 mg/mL. The suspension was sealed in a glass vial and kept at 105 °C for 2 h. Lipid A was removed by extraction (2 ×) with equal volume of $CHCl_3$. The aq phase containing O-antigen was repeatedly evapd under reduced pressure to remove AcOH.

1.5. Hydrolysis of O-antigen

Hydrolysis of O-antigen was achieved in a sealed glass vial with 4 M TFA at 105 °C for 1.5 h. TFA was removed by repeated evaporation under reduced pressure. The aq soln was finally lyophilized.

1.6. Purification of D-rhamnose

The monosaccharide mixture was submitted to open column chromatography on silica gel (95 × 3.2 cm I.D.; 40–63 μm; Macherey & Nagel, Düren Germany). Ethyl acetate–MeOH–AcOH–water (60:15:15:10, v/v) was used as eluent at a flow rate of 1.3 mL/min. Fractions of 13 mL were collected with a SuperFrac (Pharmacia LKB, Uppsala, Sweden) fraction collector. For sample introduction, 1 part of monosaccharide mixture in water and 10 parts of silica gel were mixed, lyophilized and loaded onto the column prior to elution. Rhamnose-containing fractions were detected by phenol–sulfuric acid assay for carbohydrates[23] and by TLC (same eluent as for column chromatography). TLC staining was with vanillin–sulfuric acid reagent.[20] Rhamnose-containing fractions were pooled, evapd to dryness, redissolved in MeOH, and submitted to gel filtration on Sephadex LH20 (50 × 2.5 cm I.D.; Pharmacia, Uppsala, Sweden) at a flow rate of 3.5 mL/min, to afford 40 mg (3.4% of total purified LPS) of D-rhamnose as an amorphous white powder after lyophilization.

Purity was checked by TLC, NMR, and by HPLC on a HP 1100 system (Agilent, Waldbronn, Germany) equipped with a Chrompack Valco Carbohydrates Pb column (7.8 mm × 300 mm). The column was thermostatted at 80 °C, eluted with water at a flow rate of 0.4 mL/min. Detection was at 192 nm.

1.7. D-Rhamnose

$[\alpha]_D^{20}$ −7° (*c* 0.5, H_2O); R_f 0.64 (EtOAc–MeOH–AcOH–water (60:15:15:10, v/v)); ^1H NMR (D_2O): δ_H

4.99 (br s, α H-1), 4.75 (br s, β H-1), 3.81 (unres. m, α H-2), 3.80 (unres. m, β H-2), 3.73 (dd, α H-5), 3.68 (dd, α H-3), 3.48 (m, β H-3), 3.31 (dd, α H-4), 3.20 (unres., β H-4 and β H-5), 1.18 (d, β H_3-6), 1.15 (d, α H_3-6); ^{13}C NMR (D_2O): δ_C 93.9 (α C-1), 93.4 (β C-1), 72.7 (β C-3), 72.1 (α C-4), 72.0 (β C-5), 71.8 (β C-4), 71.3 (β C-2), 70.7 (α C-2), 69.9 (α C-3), 68.2 (α C-5), 16.7 (α C-6), 16.6 (β C-6).

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