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Analysis of nucleotide sugars from cell lysates by ion-pair solid-phase extraction and reversed-phase high-performance liquid chromatography

Jarkko Räbinä[1]*, Minna Mäki[1], Emma M. Savilahti[1], Nina Järvinen[1], Leena Penttilä[1] and Risto Renkonen[1,2]

[1] *Biomedicum Helsinki, Rational Drug Development and Department of Bacteriology and Immunology, P.O. Box 63, FIN-00014 University of Helsinki, Finland,* [2] *HUCH Laboratory Diagnostics, Helsinki University Central Hospital, Helsinki, Finland*

Analysis of nucleotide sugar metabolism is essential in studying glycosylation in cells. Here we describe practical methods for both extraction of nucleotide sugars from cell lysates and for their analytical separation. Solid-phase extraction cartridges containing graphitized carbon can be used for the purification of nucleotide sugars by using triethylammonium acetate buffer as a ion-pairing reagent for decreasing retention. After that they are separated by high-performance liquid chromatography using a C18 reversed-phase column and the same ion-pairing reagent for increasing retention. These new sample preparation and analysis methods enable good separation of structurally similar sugar nucleotides, compatibility with rapid evaporative concentration, and possibility to automation. Monitoring the production of GDP-deoxyhexoses in genetically engineered yeast and native bacterial cells are described here as specific applications.

Keywords: **nucleotide sugar, solid-phase extraction, reversed-phase HPLC, deoxyhexose**

Abbreviations: **GDP-KDM, GDP-4-keto-6-deoxy-D-mannose; GMER, GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase/4-reductase; GMD, GDP-D-mannose dehydratase; HPLC, high-performance liquid chromatography; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; RMD, GDP-4-keto-6-deoxy-D-mannose-4-reductase; SPE, solid-phase extraction; TEAA, triethylammonium acetate; UDP-HexNAc, UDP-N-acetylhexosamine.**

Introduction

The biosynthesis of glycans involves specific glycosyltransferases utilizing nucleotide sugars as activated donors. Current research in glycosylation is focused on the differences between glycosyltransferase activities between cells. However, precise information about cellular content of nucleotide sugars is also needed, because the regulation of glycosylation probably includes the availability of nucleotide sugars. Furthermore, nucleotide sugars have also received increased interest as starting materials in glycosyltransferase catalyzed oligosaccharide synthesis. Biocatalytic synthesis is a very attractive way to produce oligosaccharides for use in glycobiology research and medicinal applications [1]. Thus, analysis of nucleotide sugars is essential both in their synthetic production and in studying glycosylation machinery of cells.

*To whom correspondence should be addressed: Jarkko Räbinä, Finnish Red Cross, Blood Transfusion Service, Kivihaantie 7, 00310 Helsinki, Finland. Tel.: +358-9-5801 470; Fax: +358-9-5801 429; E-mail: jarkko.rabina@bts.redcross.fi

In sample preparation of nucleotide sugars, for example deproteinization with boiling [2], perchlorate [3], and organic solvents [4–7] have been used as methods. In this work, we have studied solid phase extraction (SPE) in nucleotide sugar sample preparation to prevent many problems associated with previously used techniques. SPE is an increasingly useful sample preparation technique. It is easy, rapid to perform, and readily automated. Recently, standardized graphitized carbon packings have become available [8]. Activated carbon has been used as a preparative purification method for nucleotide sugars for decades in the form of charcoal [9]. SPE columns containing graphitized carbon have been used for clean-up of oligosaccharides from salts, detergents, and proteins [10]. We found out that nucleotide sugars adsorb to carbon columns so tightly, that recoveries were low with organic solvents. However, we noticed that nucleotide sugars could be recovered quantitatively by adding a ion pairing reagent to elution solvent. Triethylammoniumacetate (TEAA) buffer, which can be removed by evaporation under vacuum, was used as a ion-pairing reagent. If nucleotide peaks need to be avoided in HPLC chromatograms, samples can be treated with alkaline phosphatase and purified

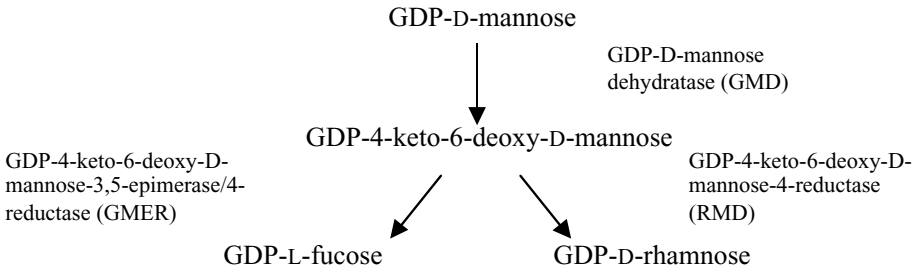

Figure 1. Biosynthesis of GDP-L-fucose and GDP-D-rhamnose from GDP-D-mannose by the *de novo* pathway.

with DEAE anion exchange colums using volatile NH_4HCO_3, which also can be removed by evaporation prior to HPLC analysis. However, for most nucleotide sugars we have tested, simple one-step purification with carbon SPE columns prior to HPLC analysis is enough.

In HPLC of nucleotide sugars CarboPac PA-1 anion-exchange column [4,7] and reversed-phase columns have been used. In reversed-phase HPLC isocratic KH_2PO4 [3] or ion-pairing reagents, such as tetra-, octa-, and hexadecyltrimethylammonium bromide in the presence of borate [11] and tetrabutylammonium salts [2,12], have been used in mobile phases. We show here that ion-pair reversed-phase chromatography with volatile TEAA buffer, which is a well-established technique used for separation of nucleotides [13], works well also in nucleotide sugar analysis. We have evaluated here the separation with 10 nucleotide sugars, which were eluted with acetonitrile gradient.

We have constructed yeast strains that express enzymes producing GDP-L-fucose and GDP-D-rhamnose from GDP-D-mannose (Figure 1) [14,15]. The methods described here were used for monitoring the production of these GDP-deoxyhexoses and also for their preparative purification. A good resolution of GDP-sugars was achieved. We also utilized the newly developed methods to analyse the enzyme activities synthesizing GDP-D-rhamnose in the human pathogen *Pseudomonas aeruginosa* that has D-rhamnose in its LPS.

Materials and methods

Materials

CMP-N-acetyl-D-neuraminic acid, UDP-D-galactose, UDP-D-glucose, UDP-N-acetyl-D-galactosamine, and UDP-N-acetyl-D-glucosamine were from Sigma (St. Louis, MO). GDP-L-fucose, GDP-D-mannose, dTDP-D-glucose, NADPH, and $NADP^+$ were from Calbiochem (San Diego, CA). GDP-D-rhamnose and dTDP-L-rhamnose were generous gifts from M.Sc. Bernd Kneidinger and Dr. Paul Messner (Universität für Bodenkultur Wien, Wien, Austria). Envi-Carb SPE-columns (250 mg) containing graphitized nonporous carbon were from Supelco Inc., Bellafonte, PA. DEAE-Sepharose Fast Flow was from Amersham Pharmacia Biotech (Uppsala, Sweden). TEAA buffer was from Fluka (Buchs, Switzerland) and acetonitrile was from J.T. Baker (Phillipsburg, NJ).

Construction of S. cerevisiae *strains expressing GMD, RMD, and GMER*

Construction of *S. cerevisiae* strains expressing *H. pylori* GDP-D-mannose dehydratase (GMD) [14], *P. aeruginosa* GDP-4-keto-6-deoxy-D-mannose-4-reductase (RMD) [15], or *H. pylori* GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase/4-reductase (GMER) [14] is described in cited references.

Preparation of yeast cell lysates

The recombinant and the vector control strains were grown overnight at 30°C in 25 ml of SD-LEU dropout media. Cells were collected and resuspended in 25 ml of galactose containing SG-LEU dropout induction media and grown for 24 h at 30°C. After induction, 1×10^9 cells were spun down. Cells were resuspended in 500 μl of lysis buffer containing 50 mM MOPS-NaOH (pH 7.5), 1% Triton X-100, and 10% glycerol. Glass beads (1/3 volume) were added and cells were lysed by vortexing for 1 h at 4°C. Lysates were centrifuged at 20,000× g for 10 min and the supernatants were collected for further assays. Protein concentrations were determined by the BCA protein assay (Pierce, Rockford IL). The yeast lysates with or without GMD were incubated in the presence of 500 μM GDP-D-mannose at 37°C for 1 h. Lysates with RMD or GMER were mixed with the lysate containing GMD and all lysates were further incubated for 1 h at 37°C with 1000 μM NADPH and 5 mM $MgCl_2$, after which the samples were subjected to purification with Envi-Carb colums.

Preparation of Pseudomonas aeruginosa *lysates*

P. aeruginosa ATCC 27853 was grown on King's broth agar overnight at 37°C. The bacteria cells were harvested and washed 3 times with 1 ml of PBS. The cells were then resuspended in 600 μl of PBS prior to probe sonication (Soniprep 150, MSE Scientific Instruments, Sussex, UK) for 3 × 15 s bursts on ice. After sonication, the cell debris was removed by centrifugation at 6,000× g for 10 min at 4°C and the supernatants were collected. Half of the cell lysate was kept at −20°C before purification and half was supplemented with 5 mM $MgCl_2$, 200 μM GDP-D-mannose, 200 μM $NADP^+$, and 200 μM NADPH, and incubated for 1 hour at 37°C.

Extraction of nucleotide sugars

Prior to use, Envi-Carb carbon colums were conditioned with 80%(v/v) acetonitrile in 0.1%(v/v) trifluoroacetic acid (3 ml) followed by 2 ml of water. The samples were diluted to volume of 1 ml with 10 mM NH_4HCO_3 and applied to Envi-Carb colums. To remove salts, detergents, and other unwanted materials, the packings were washed with 2 ml of water, 2 ml of 25%(v/v) acetonitrile, and 2 ml of 50 mM TEAA buffer (pH 7). Nucleotide sugars were then eluted with 25%(v/v) acetonitrile containing 50 mM TEAA buffer (pH 7). Recoveries of nucleotide sugars were examined by extraction of mixture of pure nucleotide sugars and extraction of GDP-L-fucose added to *S. cerevisiae* vector control lysate, which does not contain endogenous GDP-L-fucose.

In some experiments, samples were treated with alkaline phosphatase, which removes the phosphate groups from nucleotides but leaves the nucleoside diphosphate sugars intact. After purification with Envi-Carb columns, drying in a vacuum centrifuge, and redissolving in water, samples were incubated for 30 min at 37°C with 50 U of alkaline phosphatase (Finnzymes, Espoo, Finland). After treatment with phosphatase, the reaction mixtures were diluted to volume of 1 ml with 10 mM NH_4HCO_3 and applied on columns of DEAE-Sepharose Fast Flow (1 ml of Sepharose packed in Bond Elut column, Varian, Harbor City, CA). After washing with 5 ml of 10 mM NH_4HCO_3, the nucleotide sugars were eluted with 250 mM NH_4HCO_3. First, 750 μl was discarded and 1500 μl was then collected. NH_4HCO_3 salt was removed by drying and redissolving in H_2O several times, and in 20 mM TEAA buffer once, before analysing nucleotide sugars with HPLC.

Ion-pair reversed-phase HPLC

A Waters (Milford, MA) HPLC system consisting of a model 626 pump, a 600S controller, and a 2487 UV-detector was used. Buffers were made from high purity water (USF Elga, Bucks, England) and filtrated before use. Nucleotide sugars were analyzed by ion-pair reversed-phase HPLC on a Discovery C18 column (0.46 × 25 cm; Supelco) at a flow rate of 1 ml/min. Isocratic 20 mM TEAA buffer (pH 6.0) were used for 15 min, then linear gradient of 0–2% acetonitrile in 20 mM TEAA buffer over 20 min was used. The column was then washed with 4% acetonitrile in 20 mM TEAA buffer for 7 min. The effluent was monitored with a UV detector at 254 nm.

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was performed with a Biflex mass spectrometer (Bruker Daltonics, Germany). Analysis was performed in the negative-ion delayed-extraction mode, using 2,4,6-trihydroxyacetonephenone (THAP, Fluka Chemica) as a matrix [16]. External calibration was performed with THAP matrix dimer and sialyl Lewis X β-methylglycoside (Toronto Research Chemicals, Canada).

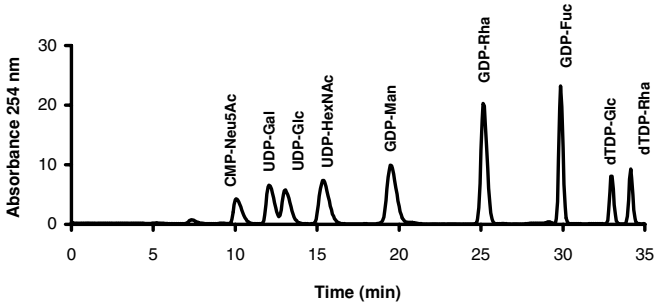

Figure 2. Ion-pair reversed-phase HPLC of nucleotide sugar standard mixture containing 500 pmol of each nucleotide sugar. The HPLC run was performed as described under Materials and Methods.

Results

Separation of nucleotide sugars by ion-pair reversed-phase HPLC

The potential of reversed-phase HPLC with TEAA buffer as a ion-pairing reagent was evaluated with 10 nucleotide sugars (Figure 2). Negatively charged nucleotide sugars bound the ion-pairing reagent and were retained on the column. They were eluted with a gradient of increasing acetonitrile concentration. Of the 10 nucleotide sugar standards tested, 8 were easily resolved, but UDP-N-acetyl-D-galactosamine and UDP-N-acetyl-D-glucosamine had the same retention time. Thus, they are referred to as UDP-HexNAc in the chromatogram. In separation of deoxyhexose nucleotides the method works very well. Both GDP- and dTDP-deoxyhexoses are resolved from the nucleotide hexoses that are their starting materials in the biosynthesis reactions. Injections of 5 to 500 pmol of nucleotide sugar showed a linear relationship between the amounts injected and peak areas monitored by UV absorbance.

Adsorption of nucleotide sugars to cartridges containing graphitized carbon

Nucleotide sugar standards were quantitatively adsorbed to carbon SPE colums and they were retained in columns during washes, as confirmed by HPLC runs of wash fractions (not shown). Salts were washed off with water, and the colums were washed also with 25% acetonitrile and with 50 mM TEAA buffer to remove unwanted, weakly retained materials. Both ion-pair reagent and organic solvent were required in elution of nucleotide sugars. The recovery with pure nucleotide sugars was almost complete, in the range of 93–100% (Table 1). The capacity of carbon colums was tested with internal standard by adding known amount of GDP-L-fucose to *S. cerevisiae* lysate (Table 2.). The recovery of GDP-L-fucose from cell lysate was almost 100%, as with pure nucleotide sugars. As quantitated

Table 1. Recovery of nucleotide sugars after sample preparation with carbon SPE columns. Standard mixtures of nucleotide sugars were subjected to purification protocol with Envi-Carb columns and quantitated then by HPLC. The amounts of nucleotide sugars were calculated from peak areas by reference to standard samples. The mean of three independent experiments is presented

Nucleotide sugar	*Recovery (%)*
CMP-D-Neu5Ac	96
UDP-D-Glc	93
UDP-D-GlcNAc	94
GDP-D-Man	99
GDP-L-Fuc	100
dTDP-L-Rha	98

Table 2. Capacity of Envi-Carb SPE columns (250 mg). Known amounts of GDP-L-fucose was added to *S. cerevisiae* lysate. After purification with Envi-Carb columns, GDP-L-fucose was quantitated by HPLC

Cell lysate (mg protein)	*Recovery (%)*
0.125	98
0.25	93
0.5	98
1	92
2	80
4	68

by protein content, about 1 mg of yeast cell lysate could be loaded into one 250 mg column without remarkable loss in recovery.

Extraction of nucleotide sugars from transgenic yeast with graphitized carbon cartridges

The stable recombinant *S. cerevisiae* strains expressing bacterial enzymes were used to convert GDP-D-mannose to either GDP-D-rhamnose or GDP-L-fucose. Peaks from cell extracts incubated with GDP-D-mannose and purified with carbon SPE were identified by comparison of retention times to runs of standard mixtures of nucleotide sugars. In vector control a GDP-D-mannose peak with no other GDP-sugar peaks was seen (Figure 3A). On the contrary, the strain expressing GMD had a smaller GDP-D-mannose peak and two novel peaks at 23.4 and 28.8 min (Figure 3B). We think that the latter peak is GDP-4-keto-6-deoxy-D-mannose (GDP-KDM), because this peak is seen also in bacterial and animal cell lysates containing GMD activity (not shown). However, we had no standard available for this intermediate product. We tried to confirm its mass with MALDI-TOF MS, but we could not analyse it confidently, possibly because 4-keto-6-deoxysugars are known to be labile. The presence of GDP-4-keto-6-deoxy mannose in reaction mixture was studied also by chemical reduction with $NaBH_4$. As

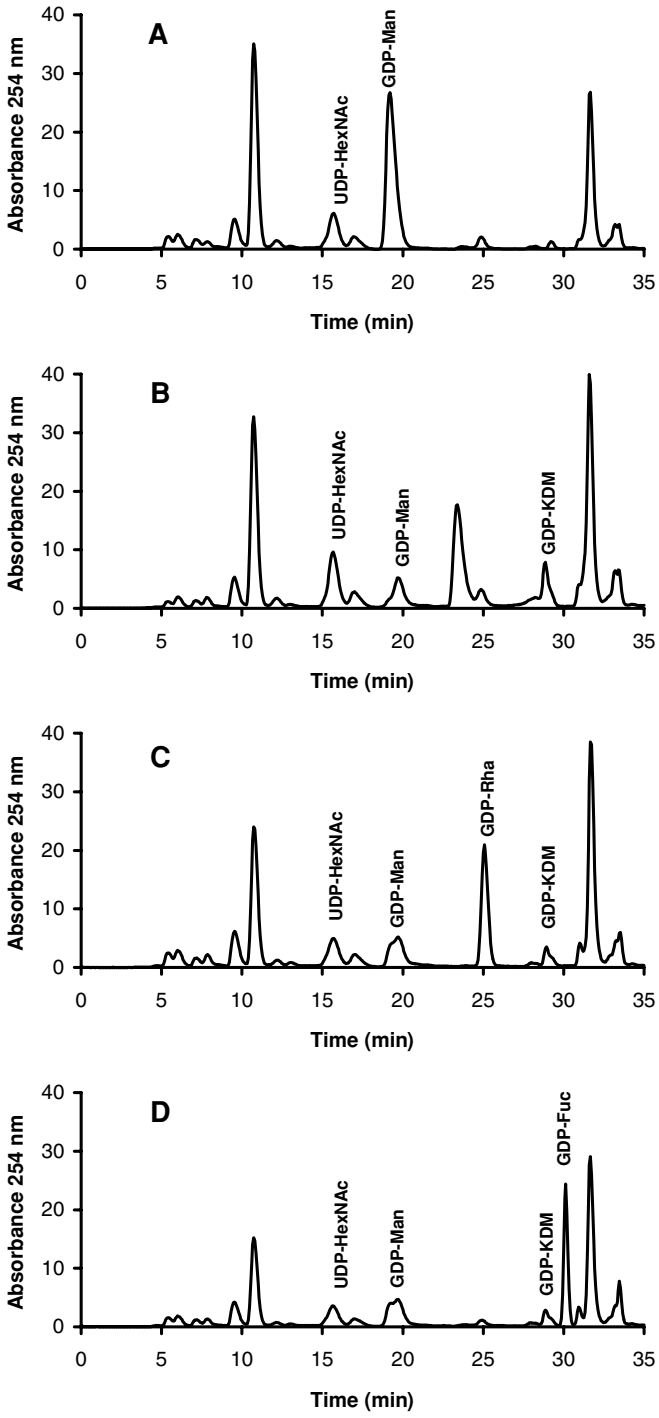

Figure 3. Typical HPLC elution profiles of yeast extracts purified with graphitized carbon. GDP-D-mannose was incubated with lysates of yeast strains transformed with (A) vector control; (B) GMD; (C) GMD and RMD, (D) GMD and GMER.

expected, two new peaks were detected with HPLC (not shown). The minor peak comigrated with GDP-D-rhamnose standard and the retention time of the major peak was near the 4-keto intermediate product. This latter product is probably GDP-6-deoxy-D-talose, but we were not able to prove this because of

Table 3. MALDI-TOF MS of compounds purified with HPLC from *S. cerevisiae* extracts. ND = not detected

HPLC peak (min)	*m/z*	*m/z (calc.)*	*Compound*
10.8	ND	?	?
15.3	605.9	606.1	UDP-HexNAc
19.1	603.8	604.1	GDP-Man
25.0	587.9	588.1	GDP-Rha
28.9	ND	586.1	GDP-KDM?
30.2	588.0	588.1	GDP-Fuc

the lack of a standard for this nucleotide sugar. The putative GDP-4-keto-6-deoxy-D-mannose peak was quite small in HPLC compared to GDP-D-mannose peak in vector control, suggesting that part of this labile intermediate product was degraded already during purification. In samples from strains expressing RMD or GMER, peaks of GDP-D-rhamnose (Figure 3C) or GDP-L-fucose (Figure 3D) were seen, respectively.

In addition to idenfication by comparison of retention times to standards, some peaks were also collected and checked with MALDI-TOF MS, which gave expected masses of endogenous UDP-N-acetylhexosamine of yeast, GDP-hexose, and GDP-deoxyhexose (Table 3). The compound eluting at 10.8 min did not give any peak in the mass area of nucleotide sugars. No attempts were made to identify other unknown peaks, but it is very likely that they are mostly nucleotides and other common metabolites of yeast. Most of these unknown peaks disappear after treatment with alkaline phosphatase (Figure 4), which degrades nucleotides but leaves the nucleotide sugars intact. Most nucleotide sugar peaks seem to be quite clearly resolved already before alkaline phosphatase reaction, but this further purification removes some small impurity peaks that partly fuse with UDP-HexNAc and GDP-Man peaks.

Analysis of GDP-deoxyhexoses in Pseudomonas aeruginosa

The enzyme activities synthesizing GDP-deoxyhexoses in the human pathogen *Pseudomonas aeruginosa* were analysed with the protocol tested with nucleotide sugar standards and yeast lysates. No significant GDP-sugar peaks were seen in the chromatogram (Figure 5A). To boost up the synthesis of GDP-deoxyhexoses, GDP-D-mannose was added to the cell lysate, resulting synthesis of GDP-D-rhamnose in cell lysate (Figure 5B). To analyse more accurately the nucleotide sugars, the same samples were further purified by treatment with alkaline phosphatase to degrade nucleotides, and purification with DEAE anion exchange colums. After this procedure, only GDP-sugar peaks and some minor peaks were left in the chromatograms (Figure 5C and D), which again suggests that the unknown peaks seen after graphite SPE purification were mostly nucleotides.

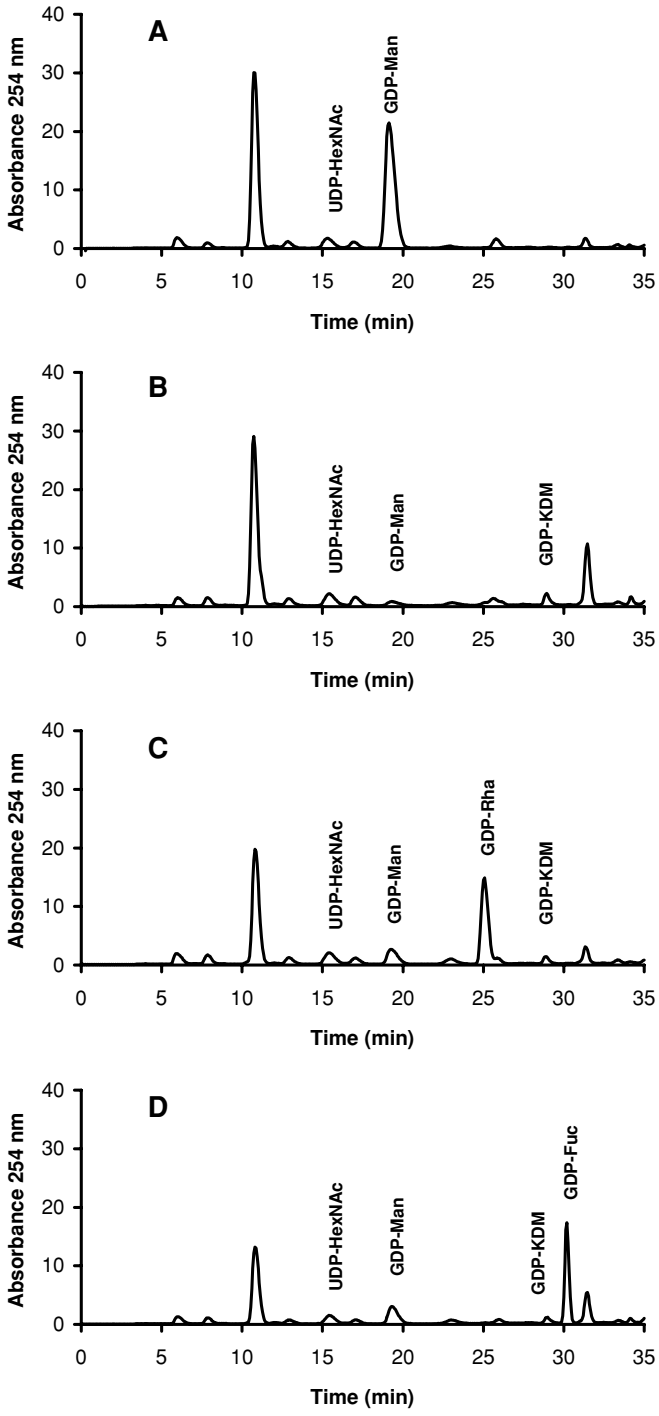

Figure 4. Typical HPLC elution profiles of the same yeast extracts as in Figure 3 that were further purified with alkaline phosphatase treatment and anion exchange columns to remove nucleotide peaks from HPLC chromatograms. (A) vector control; (B) GMD; (C) GMD and RMD, (D) GMD and GMER.

Discussion

The yeast *S. cerevisiae* is an ideal host to express the enzymes that synthesize GDP-deoxyhexoses from GDP-D-mannose as

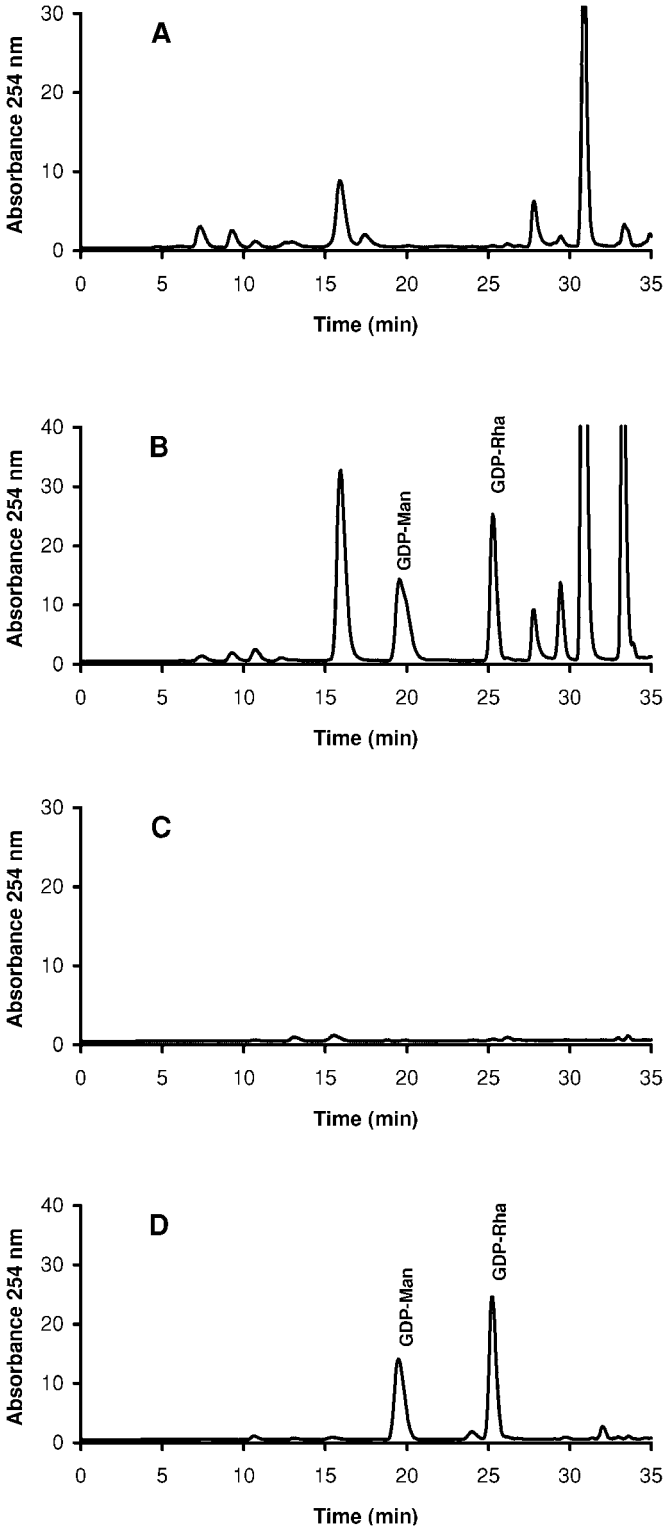

Figure 5. HPLC elution profiles of *Pseudomonas aeruginosa* extracts. Bacterial cell lysates as such (A) or after incubation with GDP-D-mannose at 37°C (B) were purified with graphitized carbon. To eliminate nucleotide peaks in HPLC chromatograms, part of the same samples were treated with alkaline phosphatase and purified with anion exchange columns (C and D, respectively).

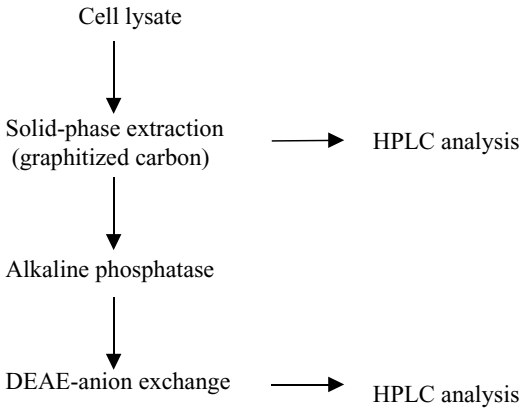

Figure 6. Sample preparation protocol of cell extracts for separation of nucleotide sugars. Salts, proteins, and detergents are removed by graphitized carbon cartridges. If removal of nucleotides is not required the HPLC run can be performed after sample purification with graphitized carbon. If necessary, nucleotides can be removed by treatment with alkaline phosphatase and DEAE anion exchange columns.

it is not known to have any deoxyhexose metabolism of its own. We have already earlier shown that the enzymes producing GDP-L-fucose can be expressed in yeast in functionally active form [17]. In this work, both GDP-L-fucose and GDP-D-rhamnose were produced in yeast. The newly developed SPE and HPLC methods in sample processing and nucleotide sugar separation make easier the analysis and production of nucleotide sugars, which are valuable reagents in functional studies of glycosyltransferases. In analysing or isolating GDP-deoxyhexoses from yeast cell lysates, single purification step with graphitized carbon column prior to HPLC is enough, because there are no overlapping peaks in this area. For more precise nucleotide sugar profile analysis, nucleotides can be removed by treatment with alkaline phosphatase and purification with anion exchange columns (Figure 6), as shown with yeast and *P. aeruginosa* lysates.

The surfaces of all microbes contain glycoconjugates that can function as virulence determinants in the case of pathogens [18]. In this work, the enzymatic machinery producing GDP-D-rhamnose in the human pathogen *Pseudomonas aeruginosa* was detected. It has been observed from the chronic cystic fibrosis infection that the respiratory *P. aeruginosa* isolates express mainly A-band LPS, which contains D-rhamnose [19]. In searching new bacterial enzymes producing rare nucleotide sugars two complementary methods can be used: (1) searches based on sequence homology to previously known enzymes and (2) analysis of nucleotide sugars in cell extracts. Limited amount of nucleotide sugar standards is commercially available, but characterization of unknown nucleotide sugar peaks with mass spectrometry and nuclear magnetic resonance spectroscopy is straigtforward after removing TEAA buffer and acetonitrile by evaporation under vacuum. Enzymatic synthesis and isolation of building blocks required for bacterial glycosylation

will help to resolve the biological roles of the glycoconjugates of pathogenic bacteria.

SPE as a sample clean-up method allows automation and upscaling in sample volumes. Ion-pair SPE is a less frequently applied technique [20], but it offers many advantages over more conventional SPE and other sample preparation techniques. The use of volatile TEAA buffer eliminates the need for further desalting and either HPLC or mass analysis can be performed simply after evaporation under vacuum. Interestingly, it seems that the ion pairing reagent works in opposite ways in carbon SPE and in reversed-phase HPLC by either decreasing or increasing retention, respectively.

Methods for the analysis of nucleotide sugar profiles of different cells will be very useful in analysing the regulation and abnormalities in glycosylation. One example of many possible applications is characterization of nucleotide sugar metabolism in gene knock-out cell lines lacking specific enzymes synthesizing nucleotide sugars. Most probably also nucleotide profiles of different cells could be easily analysed with graphitized carbon SPE and ion-pair reversed-phase HPLC, though we were interested only in nucleotide sugars in this study.

In summary, our results indicate that SPE with graphitized carbon and reversed-phase HPLC are efficient and convenient methods for both analytical and preparative separation of nucleotide sugars.

Acknowledgments

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