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Determination of the cyanobacterial osmolyte glucosylglycerol by high-performance liquid chromatography

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Abstract

A combination of reversed-phase chromatographic (RPC) [octadecyl silica (ODS)] and ion-moderated partition chromatographic (IMPC) (Ca^{2+}) stationary phases with water as mobile phase provides separation of the cyanobacterial osmolyte glucosylglycerol (2-O-α-D-glucopyranosylglycerol, GG) from other ubiquitous osmolytes (sucrose, trehalose, glycinebetaine) and major natural carbohydrates, also in the presence of common osmotic stressors (mannitol, sorbitol). The method allows investigations of GG biosynthesis in vitro where glucose and glycerol can be released. The separate use of RPC or IMPC columns is restricted to samples containing no significant amounts of sucrose and glucose, respectively. Amino-bonded silica and acetonitrile–water mixtures provide excellent separation of GG from disaccharides but separation from important hexoses is limited.

1. Introduction

Cyanobacteria are able to compensate hyperosmotic stresses by synthesis of low-molecular-mass organic solutes exhibiting osmotic and protective functions, e.g. trehalose, sucrose, glucosylglycerol and glycinebetaine [1]. The salt-dependent accumulation of GG was first detected in the cyanobacterium *Synechococcus* in 1980 [2] and meanwhile found in numerous freshwater and marine cyanobacteria [1,3–10]. Similar glucosylglycerols were found in some higher plants [11,12].

Detection and quantification of GG were performed by three different techniques: ^{13}C NMR spectroscopy [1,2,4,5,7], gas-liquid chromatography [8,9] and enzymatic determination [3,10]. All methods are time-consuming and, partly, need harmful chemicals or high sample amounts [8,13]. Additionally, the enzymatic estimation of glucose after acid hydrolysis of GG leads to overestimation since other soluble glucose-containing compounds (sucrose, trehalose) may interfere [3]. To overcome many of these disadvantages it seemed promising to introduce HPLC methods for the practical estimation of GG. Recently, the biochemical pathway of GG biosynthesis has been discovered [19]. Also for studies of the regulation of this pathway HPLC should be promoted.

HPLC methods have been already used for the separation of macroalgal organic osmolytes [13,14]. However, research of microalgal salt adaptation includes some special problems, as availability of biomass and the high salt load of cells. Additionally, studies of salt adaptation need a rapid and inexpensive assay technique for the osmolytes which does not suffer from inter-

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ference by salt and addition of biochemical precursors, activators and inhibitors.

Karsten et al. [13] reported on an HPLC method for the determination of macroalgal organic solutes using IMPC columns. Resolution of several polyols, carbohydrates and the most important heteroside of red algae (floridoside) was superior. However, selectivity of IMPC (Ca^{2+}, H^+, Pb^{2+}) is often insufficient for disaccharides [20]. Usually, IMPC columns require periodic regeneration of the ionic phase or extensive employment of guard columns during determinations of samples containing non-compatible salts. Common polystyrene-based columns need more attention to prevent obstruction.

The use of silica columns and addition of small amounts of an aliphatic silica amine modifier in the mobile phase were shown to be valuable tools in the general screening of various macroalgal osmolytes, but common steel columns were unsuitable for long-term use [14]. In addition, the carry-over of salt results in a tailing peak in the region of hexitols and hexoses and may produce difficulties in peak integration.

Resolution of hexoses and hexitols is often problematic. Finally, the mobile phase acetonitrile is hazardous and disadvantageous for the detection of carbohydrates without derivatization.

In this paper, an HPLC method is described which results from the comparison of three standard separation techniques and which is intended for determinations of GG in the presence of common osmolytes and metabolites in living cells as well as in bioassays. The method should be helpful, both in biochemical and ecological research.

2. Experimental

2.1. Apparatus

All chromatographic experiments were performed with a chromatograph consisting of LC-9A pumps, SIL-9A autoinjector (sample loop: 1–50 μl), CTO-6A column oven, DGU-4A solvent degasser and RID-6A refractive index detector (Shimadzu Corp., Kyoto, Japan). Data processing and integrations were performed by the chromatographic software ChromStar Vers. 3.12 (Bruker-Franzen Analytik, Bremen, Germany).

The following columns were used: LiChrosorb/HibarRT ODS (5 μm, 250 × 4 mm I.D.) from Merck (Darmstadt, Germany), HPX-87C (250 × 4 mm I.D.) connected with a corresponding Carbo-C guard column (30 × 4.6 mm I.D.) from Bio-Rad (Richmond, USA), Nucleosil/100 NH_2 column (5 μm, 250 × 3 mm I.D.) with guard column (11 × 3 mm I.D.) of identical material, and Nucleosil/100 ODS columns (5 and 10 μm, 250 × 4 mm I.D.) with corresponding guard columns (11 × 4 mm I.D.) from Machery-Nagel (Oensingen, Germany).

2.2. Reagents

All chromatographic solvents (HPLC grade) used were obtained from J.T. Baker (Deventer, Netherlands). Carbohydrates and inorganic chemicals were purchased from Merck (Darmstadt, Germany). Hexitols, glycinebetaine and the glucose determination kit (510-A) were from Sigma (St. Louis, MO, USA). Polystyrene-based ion-exchange resins (Wofatit KPS-H^+ and SBW-Cl^-) were from VEB Farbenfabrik (Wolfen, Germany). Cellulose (300MN) was obtained from Serva (Heidelberg, Germany). Floridoside was purified from the red alga *Palmaria palmata* by HPLC according to the procedure described in Ref. [13] using the HPX-87C column.

2.3. Extraction, sample preparation and purification of GG

GG was extracted from the axenic unicellular cyanobacterium *Synechocystis* PCC 6803 grown at 20 W/m^2 (continuous light), 29°C and continuous aeration with CO_2-enriched air (5% v/v) in NaCl-enriched (10–40 g/l) basal medium (according to Ref. [15]).

Cells were harvested by centrifugation. A simple treatment of all pellets with 80% ethanol (v/v) results in nearly complete extraction of

low-molecular-mass compounds [8]. All extractions were performed within 4 h at 65°C. The residue of freeze-dried ethanol extracts was redissolved in water (HPLC grade). Usually, GG containing solutions were cleared by membrane filtration (0.45 μm) before injecting 10-μl samples. Further special sample preparations and injection volumes will be described in the text.

Higher amounts of nearly pure GG (~98% w/w) were prepared by use of polystyrene-based cationic and anionic ion-exchange columns and thin-layer chromatography (cellulose, acetonitrile–water, 85:15, v/v). The concentration of purified GG was determined by enzymatic estimation of glucose after acid hydrolysis [3].

3. Results and discussion

3.1. Octadecyl silica phase column

Reversed-phase HPLC enables principally the separation of carbohydrate oligomers [16–18]. According to the molecular structure of GG, its separation from hexoses and disaccharides was assumed.

Samples could be used without desalting because salts rapidly eluted near the dead time. GG showed retention using water or 50 m*M* boric acid as mobile phase. It separated from the monosaccharides glucose and fructose which have lower, but equal retention times, and from the disaccharides trehalose and maltose. Unfortunately, GG nearly coeluted with the disaccharide sucrose which is a widespread osmolyte in cyanobacteria, algae and higher plants (Table 1). Use of various aqueous inorganic buffers at different pH values, variation of temperature, and addition of organic solvents provided no resolution of GG and sucrose (data not shown). Sodium chloride solutions (0.5 and 1 *M*) as mobile phase gave slightly enhanced retention times without remarkably better resolution. Differences between chromatograms of the three ODS stationary phases tested (see Apparatus) were insignificant (data not shown).

With respect to investigations of GG biosynthesis in vitro, precursors were tested. They showed different retention behaviour under standard conditions (Table 1). Glycerol 3-phosphate (G3P) and the common sugar intermediate uridine diphosphate glucose (UDPG) eluted before the dead time. Under the same conditions, adenosine diphosphate glucose (ADPG) eluted near the hexoses and hexitols tested whereas glycerol showed markedly enhanced retention (Table 1). Glycerol could be released during GG biosynthesis experiments in vitro due to hydrolysis of added glycerol phosphate and should be monitored for exact characterization of the GG synthesizing enzyme system [19].

Table 1
Capacity ratios of compounds relevant to osmo-acclimation studies

Substance	k'
UDPG	(−0.20)
G3P	(−0.18)
NaCl	0.03
Sorbitol/mannitol	0.15
ADPG	0.16
Glucose/fructose	0.16
Glycerol	0.21
Trehalose	0.25
Maltose	0.27
Glycinebetaine	0.33
GG	0.42
Sucrose	0.43

Merck ODS column, 250 × 4 mm I.D.; solvent, 0.05 *M* boric acid; flow-rate, 1.0 ml min^{-1} (μ = 0.20 cm s^{-1}); temperature, 30°C.

Due to its limited resolution, RPC (ODS) is only recommended for GG determinations of biochemically well characterized cyanobacterial strains since coelution with various substances cannot be excluded. Nevertheless, simple sample preparation, fast elution of precursors, and use of water as mobile phase make this method attractive. Additionally, RPC allows nearly complete purification of samples with severe salt contamination. Recovery experiments with osmolyte additions (50 μg, injection volume: 50 μl) to 0.85 m*M* NaCl solutions showed only 0.4% of the NaCl peak overlapping with the GG peak (0.8% in the case of glycinebetaine). This method may be helpful when desalting proce-

dures with mixed-bed ion-exchange resins are impracticable (low amounts of GG, estimation of the charged osmolyte glycinebetaine).

3.2. Ion-moderated partition chromatography column

The usefulness of cation-binding stationary phases for determinations of carbohydrates in diverse fields is well documented, e.g. [13,20–25]. In the widely used Ca^{2+}-form, desalting of samples could be ignored. However, high salt contaminations should be eliminated to prevent extensive utilization of guard columns and anionic interferences [13]. The separation of GG from sucrose and fructose was excellent (Table 2). Surprisingly, GG did not show a retention time between those of the glucose containing disaccharides tested and glucose. GG and glucose eluted as a single peak.

Generally, modifications of the separation conditions are limited for polystyrene-based columns. Variation of column temperature and ionic strength of the mobile phase (5–50 m*M* calcium nitrate) or additions of the organic modifier acetonitrile up to 30% (v/v) brought about no resolution of glucose and GG. GG showed slightly lower retention times than glucose without chromatographic significance (data not shown). The macroalgal heteroside floridoside which is structurally related to GG emerged also in the region of glucose. But separation of this heteroside and its corresponding sugar (galactose) seems to involve fewer problems since resolution of galactose and glucose is usually high.

In the case of GG determination, the coelution of GG and glucose prevents a wide application of the Ca^{2+}-moderated stationary phase although osmotic significant production of glucose in vivo has not been documented. But artificial modifications during in vivo and in vitro experiments (heterotrophic growth on glucose, additions of glucose-containing precursors, screening of mutants with defects in C-metabolism) could lead to enhanced concentrations of free glucose. Apart from this restriction and due to the selectivity for a wide range of known osmolytes (disaccharides, polyols, heterosides) [13,20,25], IMPC (Ca^{2+}) is a valuable tool also

Table 2
Relative retention times of selected sugars, polyols and heterosides in comparison to that of sucrose for two Ca^{2+}-moderated partition chromatography phases

Substance	Relative retention times		
	Aminex HPX-87C[a]	Aminex HPX-87C[b]	Polysphere CHCA[c]
Sucrose	1.00	1.00	1.00
Maltose	1.02	1.01	–
Floridoside	–[d]	–	1.23
GG	1.17	–	–
Glucose	1.17	1.21	1.26
Galactose	–	1.37	–
Fructose	1.46	1.52	1.49
Mannitol	1.77	–	1.71
Sorbitol	2.07	–	2.04

Retention times were made comparable by transformation with respect to column dimensions assuming insignificant external dead volumes and similar physical properties of the stationary phases because exact informations from the producers were not available.

[a] 250 × 4.0 mm I.D., (Bio-Rad); solvent, water; flow-rate, 0.25 ml min^{-1}; temperature, 85°C; own measurements.

[b] 300 × 7.8 mm I.D., (Bio-Rad); solvent, water; flow-rate, 0.6 ml min^{-1}; temperature, 85°C; according to the producer [20].

[c] 300 × 6.5 mm I.D., (Merck); solvent, water; flow-rate, 0.5 ml min^{-1}; temperature, 90°C; according to Ref. [13].

[d] not determined or data not available.

to characterize low-molecular-mass substances of cyanobacteria.

3.3. Aminopropyl silica phase column

Chromatography of sugars and polyols by amino-bonded silica as stationary phase and acetonitrile–water mixtures as mobile phase is widely used and characterized just as in situ modification of silica phases with amines [14,22,26–29].

Using amino-bonded silica and acetonitrile–water mixtures of 72.5 up to 87.5% (v/v) acetonitrile, sufficient separations of disaccharides and some important monosaccharides were obtained. Major results were similar to those of the IMPC column: GG showed retention times more comparable to those of hexoses than to the expected intermediate retention between mono- and disaccharides. A mobile phase of 75% (v/v) acetonitrile gave separation from fructose and glucose, but resolutions were insufficient for quantitative determinations (Fig. 1). Higher proportions of acetonitrile improved the selectivity for GG/fructose but, simultaneously, lowered the selectivity for GG/glucose (Fig. 2). Linear detector response was achieved for purified GG over the range tested (1–500 μg, $n = 7$, $r =$ 0,998, peak-height method). However, exact peak integrations of samples containing only a few micrograms of GG were complicated or impossible due to carry over of salt and unknown impurities.

Amino-bonded silica showed instability after injection of several hundred samples simply purified by filtration and not deionized. This led to a rapid decrease of capacity ratios. In Table 3 capacity ratios of a new and an old column (loss of about 40% of column efficiency) are compared. Substantial loss of retention power can be seen, especially for the late eluting disaccharides. Furthermore, peak symmetry became poor ($\tau =$ 1.07 and $\tau = 1.80$ for the new and old column, respectively). Interestingly, capacity ratios of

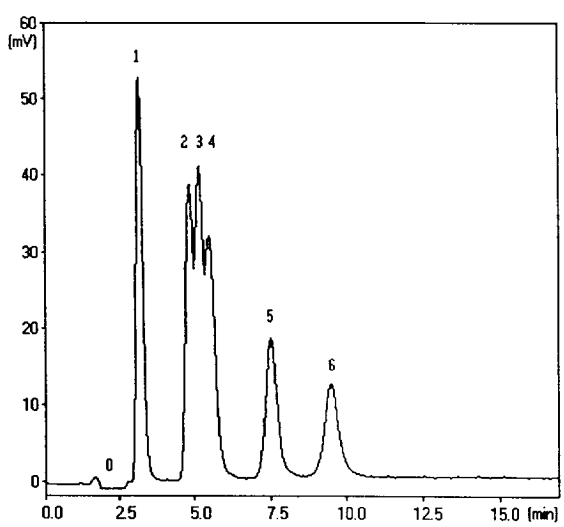

Fig. 1. Chromatogram of standard compounds. Peaks: 0 = injection solvent (water, inverts), 1 = glycerol, 2 = fructose, 3 = GG, 4 = glucose, 5 = sucrose, 6 = trehalose. NH_2 column (new), 250 × 3 mm I.D.; solvent, acetonitrile–water (75:25); flow-rate, 0.8 ml min^{-1} ($\mu = 0.25$ cm s^{-1}); temperature, 30°C.

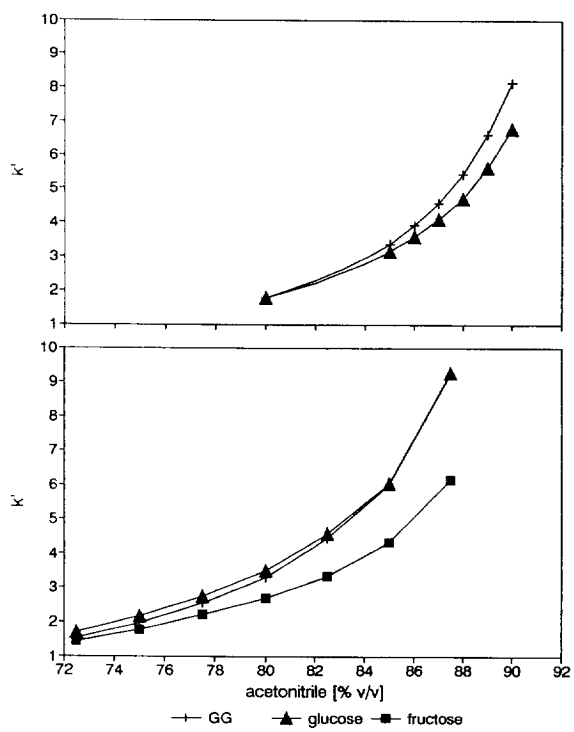

Fig. 2. Dependence of capacity ratios (critical compounds) on acetonitrile content of the mobile phase and column age (upper figure, old column; lower figure, new column). Further analytical conditions as in Table 3.

Table 3
Dependence of capacity ratios of selected compounds on age of amino-bonded silica

Substance	k' (old)[a]	k' (new)
Glycerol	1.35	1.62
Fructose	3.68	6.14
Glucose	4.84	9.26
GG	5.53	9.22
Floridoside	5.87	9.41
Sucrose	11.18	23.75
Trehalose	19.98	45.11

NH_2 column, 250 × 3 mm I.D.; solvent, acetonitrile–water (87.5:12.5); flow-rate, 0.8 ml min^{-1} (μ = 0.25 cm s^{-1}); temperature, 30°C.
[a] The old column suffered from a loss of 7000 theoretical plates per metre (GG, injection volume: 30 μl).

GG became markedly higher compared to glucose (Fig. 2). With high acetonitrile contents (87–90%, v/v) acceptable resolution (R_s = 1.10–1.25) of glucose and GG was obtained. Finally, an acetonitrile–water mixture of 87.5% (v/v) was chosen to reduce separation times of sucrose containing samples to 25 min (analytical conditions as in Table 3). Solvent peaks could be reduced by use of a sample solvent comparable to the mobile phase (usually acetonitrile–water, 80:20, v/v). This injection solvent was a good indicator for sample purity. Highly purified samples containing no salts and charged compounds gave clear solutions. Light scattering was visible in low contaminated samples and high salt contaminated samples provided a biphasic system (acetonitrile in the upper layer and sample and salts in the lower water layer), unsuitable for injection.

Sample cleaning by use of cationic and anionic ion-exchange resins provided excellent baseline stability and prevented further inactivation of the stationary phase. Only a slight loss of retention and selectivity was detected after injection of several hundred samples cleaned by deionization. However, use of controlled degraded amino-bounded silica is not recommended since it is difficult to obtain reproducible degradations. Improvements of the chromatographic system should be directed to in situ modifications of (aminopropyl) silica by amines.

3.4. Combination of octadecyl silica phase column and ion-moderated partition chromatography column

A combination of RPC (ODS) and IMPC (Ca^{2+}) columns was investigated to improve the low selectivity of the IMPC column with respect to GG and glucose. At the same time separation from important polyols should be conserved and water as advantageous mobile phase favoured.

Capacity ratios of carbohydrate oligomers could be controlled by temperature during RPC [16] whereas temperature effects (60–85°C) on the IMPC column were low (data not shown). Temperatures below 55°C were not suitable for IMPC because α- and β-D-glucose seemed to be partly resolved. Separations were specified by resolution (R_s; measurement of peak width at half-height) because compounds showed different peak width at comparable retention times. The IMPC column was set to 60°C and installed behind the ODS column (28°C, ambient).

The most important cyanobacterial disaccharides, GG and some potential metabolites of in vitro studies (glucose, glycerol [19]) separated with acceptable resolutions at the optimal flow-rate of 0.3 ml min^{-1} (Fig. 3, Table 4). Complete separation from common osmotic stressors (mannitol, sorbitol) was also conserved. But resolutions of trehalose and glucose as well as of GG and fructose almost disappeared.

If RPC was also performed at 60°C, resolution between GG and fructose was enhanced mostly at the costs of resolution between GG and glucose just as those between disaccharides (Table 4). This can be helpful especially for samples cleaned by strong ion-exchange resins where breakdown of sucrose and release of fructose may occur.

3.5. Biochemical application

Using the two configurations of combined RPC–IMPC, GG was determined in both salt-

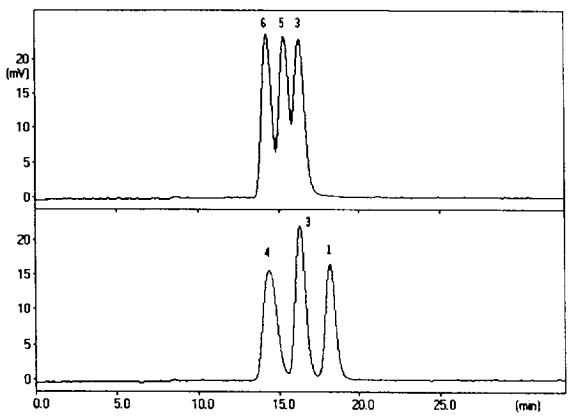

Fig. 3. Chromatograms of standard compounds relevant to in vivo (upper figure) and in vitro (lower figure) studies. Numbers as in Fig. 1. Merck ODS column, 250 × 4 mm I.D.; temperature, (ambient) 28°C; combined with an HPX-87C column, 250 × 4 mm I.D.; temperature, 60°C; solvent, water; flow-rate, 0.3 ml min $^{-1}$; injection volume: 30 μl.

adapted cells and cell-free extracts of *Synechosystis* PCC 6803 (Fig. 4) with high reproducibility (retention times, all compounds tested, $n = 3$, C.V. $< 0.4\%$). GG gave a linear detector response over the range tested (1–500 μg, $n = 6$, $r = 0.9996$, peak-height method) and a detection limit of about 150 ng was obtained (signal-to-noise ratio of 3).

Sucrose showed low significance in *Synechocystis* PCC 6803 and peak integrations were no problem in spite of the limited resolution between GG and this compound. Free fructose was never detected if samples were quickly desalted by ion-exchange resin. Fructose may become a problem for algal strains containing high amounts of sucrose. Careful desalting prevents release of fructose. Unknown substances interfering with GG were not detected. Significant amounts of unknown substances as well as sub-

Table 4
Dependence of chromatographic resolutions during RPC/IMPC on temperature of RPC

Substance	Sucrose	Trehalose	Glycine-betaine	Glycerol	Glucose	Fructose	Mannitol	Sorbitol	Maltose	Proline
GG	0.98	1.74	8.50	1.74	1.52	0.20	2.40	4.76	1.60	9.29
	1.07	1.42	8.58	2.27	0.85	0.79	2.94	5.14	1.17	9.39
Sucrose	x	1.02	9.46	2.76	0.74	1.05	3.34	5.67	0.71	11.19
		0.38	9.67	3.40	0.06	1.80	4.01	6.18	0.20	12.13
Trehalose	x	x	10.38	3.81	0.09	1.93	4.31	6.58	0.24	12.01
			9.94	3.75	0.37	2.14	4.34	6.48	0.14	12.37
Glycine-betaine	x	x	x	7.25	8.64	7.43	5.99	3.38	9.58	2.60
				6.73	8.24	7.41	5.66	3.19	9.06	2.76
Glycerol	x	x	x	x	3.03	1.33	0.83	3.37	3.32	9.29
					2.81	1.33	0.81	3.19	3.29	9.39
Glucose	x	x	x	x	x	1.55	3.50	5.45	0.11	10.21
						1.49	3.36	5.25	0.22	10.44
Fructose	x	x	x	x	x	x	1.95	4.08	1.62	9.20
							2.01	4.14	1.84	9.84
Mannitol	x	x	x	x	x	x	x	2.40	3.82	8.03
								2.30	3.86	8.26
Sorbitol	x	x	x	x	x	x	x	x	5.99	5.61
									5.86	5.79
Maltose	x	x	x	x	x	x	x	x	x	11.21
										11.37

Merck ODS column, 250 × 4 mm I.D.; temperature, (ambient) 28°C (upper values) and 60°C (lower values); combined with an Aminex HPX-87C column, 250 × 4 mm I.D.; temperature, 60°C; solvent, water; flow-rate, 0.3 ml min^{-1}.

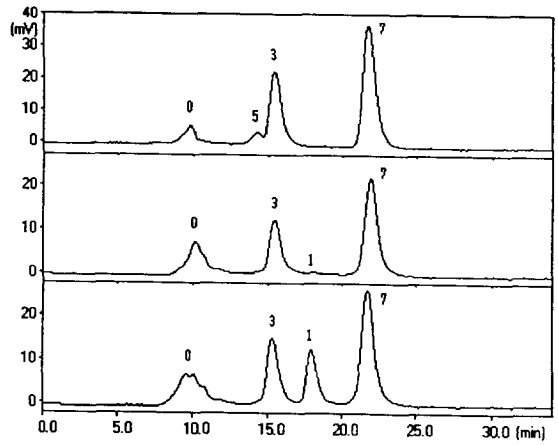

Fig. 4. Elution profiles of GG containing samples cleaned by ion-exchange resin (upper profile, extract of whole cells adapted to 684 m*M* NaCl; middle profile, GG synthesized in vitro by a protein extract according to Ref. [19]; lower profile, as middle profile, sample treated with alkaline phosphatase before desalting). Peaks: 0 = unknown compounds, 7 = sorbitol (internal standard), further numbers as in Fig. 1. Analytical conditions as in Fig. 3.

strates of GG biosyntheses in vitro eluted near the estimated dead time (data not shown).

For samples containing disaccharides and GG, separations can be completed within 18 min whereas in the presence of hexitols, glycinebetaine or proline separation times of up to 32 min are needed. The latter charged compounds can be easily removed by ion-exchange resin. Glutamic acid is one of the most important amino acids accumulated in *Synechocystis* under osmotic stress (unpublished data). Retention time of this compound was controlled by ionic strength of the mobile phase (2–50 m*M* calcium nitrate) and appeared in the region of disaccharides and GG. Glutamic acid was not retained using water as mobile phase. Ionic strength slightly influenced retention times of the other compounds tested (data not shown).

The RPC column provided additional protection of the IMPC column. Although chromatography was stable the RPC column was cleaned after injection of about 150 samples by rinsing with methanol or acetonitrile.

4. Conclusions

Separation of GG by three HPLC methods commonly used for carbohydrate detection is unsatisfactory because its retention behaviour is similar to that of important osmolytes (sucrose) or monosaccharides (glucose, fructose).

Using reversed-phase mechanisms (ODS), capacity ratios are low and more comparable to disaccharides. During chromatography on amino-bonded silica with acetonitrile–water mixtures capacity ratios are similar to common hexoses. Controversy over the mechanisms of that chromatography exists [14]. Interestingly, separation of GG from hexoses was improved after partial degradation of amino-bounded silica. Altogether, amino-bonded silica allows rapid determination of ubiquitous cyanobacterial osmolytes including GG if significant amounts of glucose or fructose are absent. In samples carefully prepared from whole cells without breakdown of sucrose this is mostly the case in contrast to samples where GG is synthesized in vitro. Deionization is necessary to prevent baseline shift and rapid inactivation of the stationary phase. Loss of reducing sugars [22] and the disadvantageous use of acetonitrile has to be taken into account.

Also IMPC (Ca^{2+}) provides insufficient resolution of GG and glucose. However, a combination of RPC and IMPC results in sufficient resolution of major (cyanobacterial) osmolytes, monosaccharides and polyols depending on temperature of RPC. It allows detection of GG synthesized in vivo and in vitro within acceptable time and using water as mobile phase. Desalting is recommended to prevent extensive use of guard columns. This method can be further optimized by variation of column dimensions, particle size and independent temperature setting for both IMPC and RPC and may be applicable for the determination of samples containing charged osmolytes (glycinebetaine), disaccharides and GG in the presence of high amounts of salt by switching the flow of the mobile phase between RPC and IMPC automatically. The combination of RPC and IMPC

may also be helpful for determinations of unusual carbohydrates without testing sets of expensive IMPC columns or doubling chromatographies due to column change, though advantages and common disadvantages of a column combination have to be weighted.

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