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H.p.l.c. of 4-nitrophenyl-α-D-malto-oligosaccharides

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ABSTRACT

The distribution of 4-nitrophenyl-α-D-malto-oligosaccharides produced by phosphorolytic synthesis was analyzed by an amine-modified and a C_{18}-bonded silica gel. With both systems high resolution of components up to d.p. 40 can be achieved by gradient elution using acetonitrile–water and water–methanol, respectively. The use of water–acetonitrile in reversed-phase (C_{18}) chromatography allows only a separation up to d.p. 9. The elution behaviour of oligomers in the d.p. range of 1–10 glucosyl residues per molecule eluted by water–methanol is discussed. The observation of rearrangement products of 4-nitrophenyl-α-D-malto-oligomers was successful by normal-phase chromatography.

INTRODUCTION

In order to elucidate detailed structures of amyloses and amylopectin, model substances are required. For this purpose, low molecular weight amyloses (LMWA) with modified terminal groups in a range of d.p. 10–20 glucosyl units per molecule are very useful[1–3]. These compounds can be obtained either by phosphorolytic synthesis[4] or by cyclodextrin-transfer reactions[5,6] with corresponding primers or acceptors. The analysis of the enzymatically synthesized products should be convenient, accurate, and quantitative enough to give reasonable information, within short time, about the oligomer distributions and reaction mechanisms. The separation of LMWA with modified terminal groups on a preparative scale for further investigations, *e.g.*, spectroscopic measurements and single-crystal X-ray diffraction, must deliver substances in a high purity.

Size fractionation on Bio-Gel P-4 has been reported for LMWA by several authors[7–9]. However, the procedure is time consuming and has been shown to lack sufficient resolution for modified LMWA[6]. By comparison, high-performance liquid chromatography (h.p.l.c.) seems to be the method of choice for analysis of oligomer mixtures as well as for the purification of species with definite chain length[6,10].

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In general, three separation modes have been used: (*i*) size fractionation by ion-exchange resins, which either show poor separation for oligosaccharides d.p. >10[11-13] or, although very useful for analytical applications[14-16], are relatively expensive, (*ii*) reversed-phase partition, and (*iii*) normal-phase chromatography using amine-bonded silica gel[17-21]. The latter method has usually been carried out with a water–acetonitrile solvent system and shows satisfactory separation for neutral oligosaccharides and LMWA in d.p. ranges up to 30.

As a detection method for LMWA, differential refractive index has been widely used, but it gives low sensitivity and is susceptible to changes in temperature and solvent composition, which precludes gradient elution. Gradient elution, which requires pre-column modification of the sugars is more convenient and permits a higher resolution. Several chemical conversions have been described as helpful procedures for the analysis of various oligosaccharides[22-24]. The aim of this study is to investigate the separation and retention behaviour of 4-nitrophenyl-α-D-malto-oligomers obtained by phosphorolytic synthesis in a range of d.p. 4–40 on an aminopropyl-modified silica gel, in comparison to reversed-phase partition on an octadecylsilane-derivatized silica gel. The methods are tested in view of their capability for the fractionation of LMWA with modified terminal groups on a preparative scale.

Detection of compounds resulting from hydrolysis of 4-nitrophenyl-α-D-malto-oligomers is herein reported, and their fractionation is demonstrated by the rearrangement products originating from 4-nitrophenyl α-D-maltopentaoside.

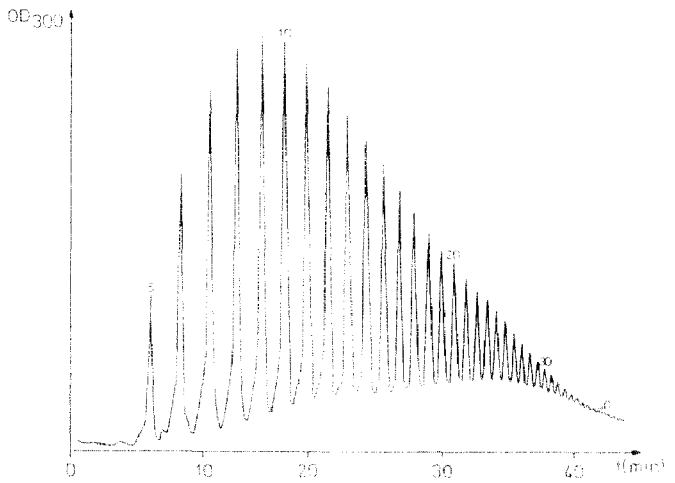

Fig. 1. H.p.l.c. elution profile of 4-nitrophenyl-α-D-malto-oligomers obtained by synthesis with potato phosphorylase[3]. Stationary phase: Hypersil APS-2, 5 μm; column dimensions: 4 × 250 mm; mobile phase: acetonitrile–water, linear gradient 75:25, 60 min; injection: 20 μl; sample concentration 100 mg/ml. The numbers indicate the d.p. of the oligomers.

RESULTS AND DISCUSSION

H.p.l.c. on aminopropyl silica gel. — The oligomer mixtures of LMWA with modified terminal groups produced by syntheses with potato phosphorylase[4] exhibited a surprisingly broad chain-length distribution due to a disproportionation activity superimposing the desired synthetic reaction. This yet unknown phenomenon could be revealed by h.p.l.c. analysis of the 4-nitrophenyl-α-D-malto-oligomers on aminopropyl silica gel[25].

A complete separation of these compounds is achieved within a d.p. range from 5–40 glucosyl residues per molecule even at material with a particle size of 5μm and a column of 4 × 250 mm (Fig. 1). The substances are eluted in sequence of their increasing molecular weight. A baseline separation up to d.p. 17 or even higher occurs on an aminopropyl-modified phase consisting of 3-μm particles and a column of 4 × 300 mm (Fig. 2). The excellent resolution and high capacity of the columns indicates that this method is well suited for a preparative fractionation of LMWA, using modified terminal groups to obtain components with definite chain lengths, in the desired range, in a high degree of purity. One disadvantage, the deterioration of the amino-bonded phases[27,28] can be delayed by adding small amounts of a water-soluble amine such as tetraethylenepentamine to the eluent (0.5 mL/L).

By this method, not only is the high resolution of 4-nitrophenyl-α-D-malto-oligomers attained, but also rearrangement products originating from hydrolysis under alkaline or even neutral conditions in aqueous solution[6,26] can be observed. These can be detected as well-separated peaks in the low molecular weight range d.p. 3–7 and as shoulders of the signals in the range of d.p. 8–20 (Fig. 3). 4-Nitrophenol is eluted

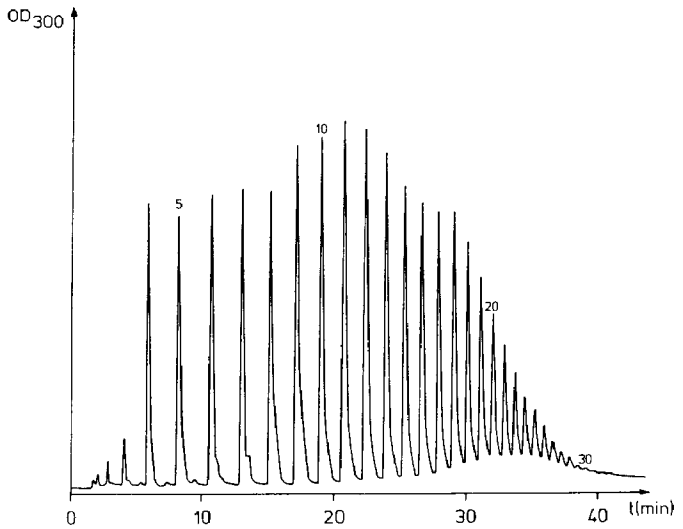

Fig. 2. See Fig. 1. Stationary phase: Hypersil APS-2, 3 μm; column dimensions: 4 × 300 mm.

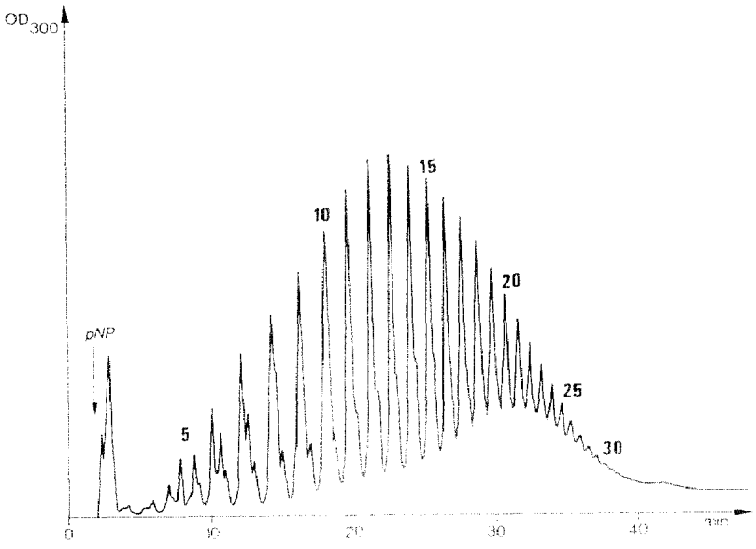

Fig. 3. H.p.i.c. elution profile of a mixture of 4-nitrophenyl-modified LMWA rearrangement products resulting from alkaline hydrolysis. For other conditions, see Fig. 1.

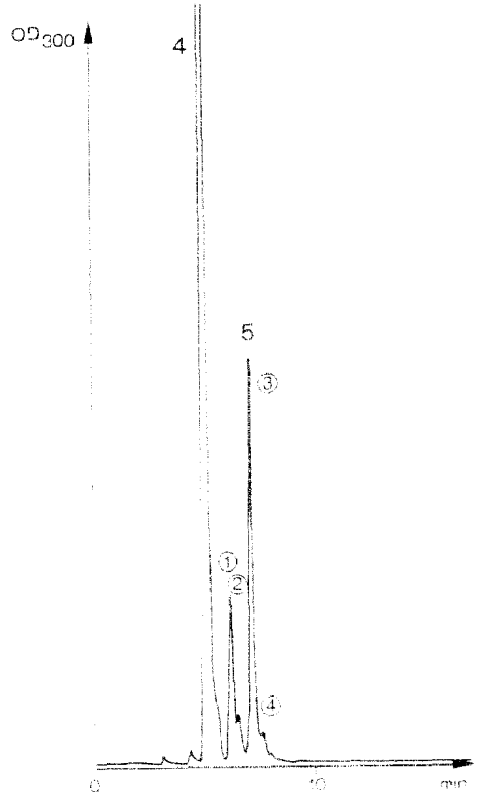

Fig. 4. Rearrangement products of 4-nitrophenyl α-D-maltopentaoside (5, peaks 1–4) in comparison to 4-nitrophenyl α-D-maltotetraoside (4) added as a marker. Other conditions see Fig. 2.

immediately after the void volume of the column, and the 4-nitrophenolate ion elutes in the region of d.p. 2–3.

The elution profile of the reaction products from alkaline hydrolysis of 4-nitrophenyl α-D-maltopentaoside (Fig. 4) reveals three peaks (1, 2, and 4) besides the starting material (3). These are eluted significantly later than the tetrasaccharide which is added as a marker, and these could be all identified as pentasaccharides. Their separation on a large scale, as well as their characterisation by chemical and spectroscopic methods as the corresponding 2-*O*-(4-nitrophenyl)-D-maltopentaose and 3-*O*-(4-nitrophenyl)-D-maltopentaose and their epimers is described[6] and will be published in detail in a future paper.

H.p.l.c. on octadecylsilyl silica gel. — As an alternative for the analysis of LMWA with modified terminal groups with respect to their fractionation on a preparative scale, the separation on C_{18}-modified silica gel was investigated. An advantage of this method would be that desalting of the substances delivered by enzymatic synthesis is not necessary before analysis. In addition, the C_{18}-modified stationary phase has a good durability[27].

At first, the eluent system water–acetonitrile was tested with 4-nitrophenyl-α-D-malto-oligosaccharides d.p. 2–7. Using a linear gradient of 100:80 water–acetonitrile over a period of 60 min, the saccharides are eluted in order of decreasing molecular weight (Fig. 5b), but it must be added that the species of d.p. 6 and 7 are unresolved. For the analysis of products of phosphorolytic synthesis, the method is not suitable because

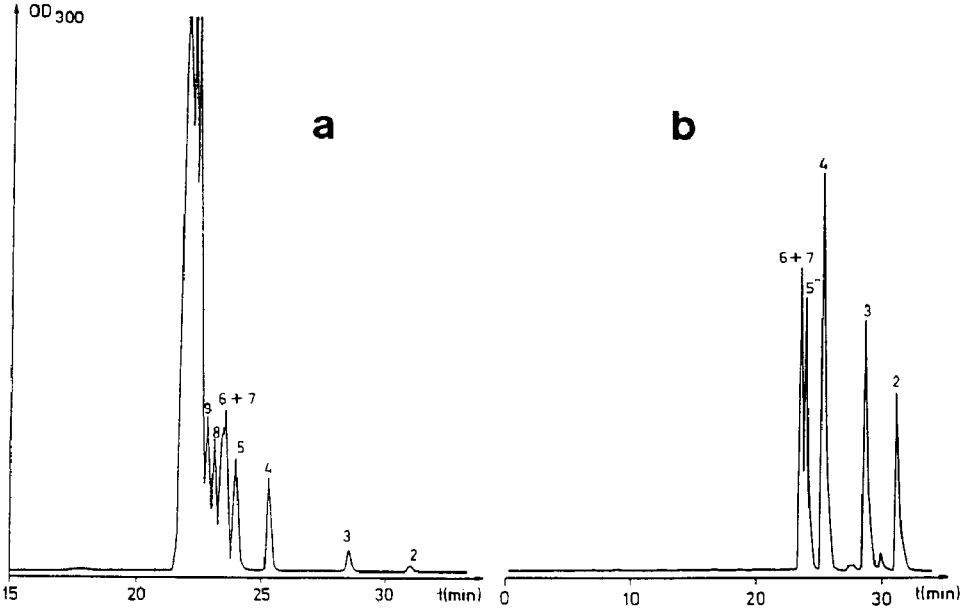

Fig. 5. H.p.l.c. elution profile of (a) 4-nitrophenyl-modified LMWA and (b) 4-nitrophenyl-α-D-malto-oligosaccharides d.p. 2–7 on C_{18}-modified silica gel. Stationary phase: ODS-Hypersil, 3 μm; column dimensions: 4 × 250 mm; mobile phase: water–acetonitrile, linear gradient 100:80, 60 min; injection: 20 μL; sample concentration (a) 20 mg/mL and (b) 5 mg/mL.

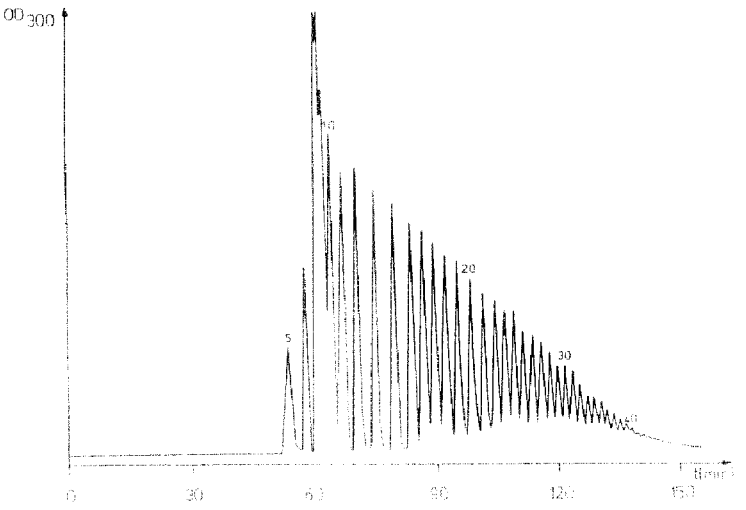

Fig. 6. H.p.l.c. elution profile of 4-nitrophenyl-α-D-malto-oligomers (see Fig. 1) on ODS-Hypersil. Mobile phase: water–methanol, linear gradient 96:90, 180 min. For other conditions see Fig. 5a.

a resolution higher than d.p. 9 cannot be attained (Fig. 5a). When acetonitrile was replaced by methanol, after optimization of the gradient, the 4-nitrophenyl-modified LMWA in a d.p. range of 5–40 could be separated in a manner heretofore not observed (Fig. 6).

However, the elution profiles show some peculiarities. The saccharides in the range of d.p. 7–9 are only incompletely resolved. In the region beyond d.p. 10, the oligomer mixtures are eluted in a good resolution in sequence of their increasing molecular size. This result seems to be in contradiction to a regular elution of homologous substance series with increasing polarity under reversed-phase chromatography[29,30].

A closer investigation of the retention behaviour of the 4-nitrophenyl-α-D-malto-oligosaccharides d.p. 2–8 revealed a "regular" elution of the compounds of d.p. 5–2 according to their decrease in polarity (Fig. 7). The oligomers of d.p. 6–8 are more strongly retarded with increasing molecular weight.

The unexpected reversion of the elution sequence prompted us to investigate the solubility of the saccharides in methanol and water and mixtures of both. The substances of d.p. 2–5 are increasingly more soluble in water due to the decreasing relative influence of the 4-nitrophenyl residue. In contrast, their solubility in methanol decreases in the same direction. Because oligomers with a d.p. > 5, according to their chain length, are also less soluble in water–methanol mixtures (*e.g.*, 96:4, water–methanol v/v), at least a tentative explanation can be advanced for the unusual return of retention for species of d.p. 6–8 range.

Another explanation might be the potential ability of these malto-oligosaccharides and amyloses to form "inclusion complexes" with the alkyl chains of the octadecylsilyl groups attached to the silica gel. This assumption is supported by the fact that

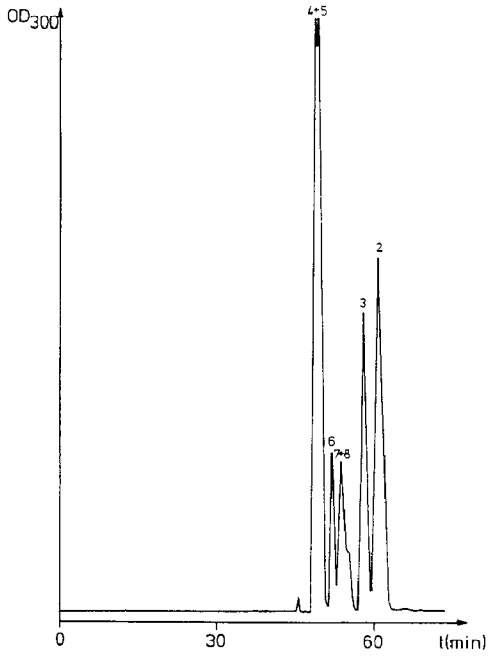

Fig. 7. Retention behaviour of 4-nitrophenyl-α-D-malto-oligosaccharides d.p. 2–8. Conditions as in Fig. 6.

4-nitrophenyl α-D-maltohexaoside was the first, and, until now, the only malto-oligosaccharide which has been crystallized as its polyiodide complex in a reasonable size for single-crystal X-ray diffraction analysis[31]. Furthermore, this would be in good agreement with the structure of V-amylose complexes consisting of helices with six glucose residues per turn[32–34].

The loading capacity of the octadecylsilyl silica gel was surprisingly low. Only 20% of the amounts of substances which are fractionated by aminopropyl silica gel in a good resolution can be injected on an ODS-column of the same dimension to obtain comparable separations. Similar results were observed by Hicks and Sondey[27]. Vrátny *et al.*[35] reported a remarkable decrease of the capacity of C_{18}-modified silica gel when methanol was used as a component of the eluent. However, the method would be satisfactory to separate LMWA with modified terminal groups in a range of d.p. 10 20.

EXPERIMENTAL

H.p.l.c. apparatus, columns, and operating conditions. — A complete system consisting of an autosampler Model A2095, two pumps Model 364, a Dynamic Mixing Chamber, a column oven Model 3, a Variable Wavelength detector 87 and a two-channel recorder 42 (Knauer, Bad Homburg, F.R.G.) was used. Columns (4 × 250 mm, Bischoff, Leonberg, F.R.G. and 4 × 300 mm, Waters Assoc., Milford, MA, U.S.A.) were filled with Hypersil APS-2, 5 μm and 3 μm respectively or ODS-Hypersil, 3 μm

(Shandon, U.K.). A linear gradient of 75:25 acetonitrile–water, developed over a time course of 60 min was used for elution on aminopropyl silica gel (A), a linear gradient of 100:80 water–acetonitrile, 60 min, and 96:90 water–methanol, 180 min, for octadecylsilyl silica gel (B). With flow rates of 1.5 mL/min (A) and 1.0 mL/min (B), pressure rose to 15–25 MPa (A) and 25–28 MPa (B), respectively. Injection volume was 20 μL. The substances were detected at 300 nm.

Solvents. — Acetonitrile (Baker, Deventer, The Netherlands) and methanol (E. Merck, Darmstadt, F.R.G.) were of h.p.l.c. grade. Deionized water was obtained by a Milli-Q filter unit (Millipore, Bedford, MA, U.S.A.). All solvents were degassed and filtered through a 0.45 μm HV filter (Millipore).

Preparation of samples. — LMWA, prepared by phosphorolytic synthesis[3], were carefully desalted on a mixed-bed ion-exchanger (AG 50W-X12, AG 3X-4A, 100 mesh, Bio-Rad Laboratories, Richmond, CA, U.S.A.), lyophilized, and redissolved in water (10–100 mg/mL) for analysis. The products of the alkaline hydrolysis of 4-nitrophenyl α-D-maltopentaoside (Boehringer, Mannheim, F.R.G.), obtained by heating of 100 mg of the saccharide and 60 μL triethanolamine in 250 μL water (pH 9.5), were injected after appropriate dilution without any other treatment.

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