Synthesis of silica-bound amylose by phosphorolytic elongation of immobilised maltoheptaosyl hydrazides

Hans-Georg Breitinger*

Institut für Organische Chemie und Makromolekulare Chemie II, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1, D-40225 Düsseldorf, Germany

Accepted 2 July 2002

Abstract—Maltoheptaoside-alkoxysilane anchor molecules were synthesised by fusing aliphatic ω-Si(OEt)₃ hydrazide linkers with maltoheptaose. After immobilisation of the primers on porous silica, support-bound amylose was synthesised by phosphorolytic synthesis. The hydrazone linkage as a pre-formed cleavage site allowed removal and subsequent characterisation of immobilised amylose, which showed a broad molecular weight distribution. Under HPLC conditions, amylose assumed a non-helical conformation, making surface interactions and not complexation the primary separation mechanism. © 2002 Elsevier Science Ltd. All rights reserved.

1. Introduction

Amylose is a linear polysaccharide composed of α-1–4 linked glucose moieties, which, together with the highly branched amylopectin forms the polysaccharide component of starch. Native starch, derivatives of amylose and cellulose, as well as cyclodextrins have been used for chromatographic enantioseparation. Amylose can adopt a helical conformation which is able to form inclusion complexes with various guest compounds. Similar complexation mechanisms are known for cyclodextrins, which have long been used for chiral recognition and encapsulation of compounds. Carbanate derivatives of polysaccharides, adsorbed on or covalently linked to solid supports are frequently used for chiral separation. Silica-bound amylose carbamate was prepared by enzymatic synthesis followed by immobilisation and derivatisation. However, inclusion complexes of amylose have not yet been exploited for chromatography. Here, synthesis and immobilisation of oligosaccharide primers on porous silica, followed by solid-phase enzymatic chain elongation, is reported. The synthetic route ensured that silica-bound amylose was attached to the support via its reducing end, allowing for maximal conformational flexibility of the immobilised polysaccharide chain. Immobilised amylose could be removed via breaking of a pre-formed cleavage site, allowing analysis of the synthetic process. Properties of the new phases in high-pressure liquid chromatography (HPLC) were tested.

2. Results and discussion

Bifunctional maltooligosaccharide-silane linkers were synthesised from ω-unsaturated carboxylic acids by conversion to the corresponding hydrazides (Fig. 1). The synthetic route ensured that silica-bound amylose was attached to the support via its reducing end, allowing for maximal conformational flexibility of the immobilised polysaccharide chain. Immobilised amylose could be removed via breaking of a pre-formed cleavage site, allowing analysis of the synthetic process. Properties of the new phases in high-pressure liquid chromatography (HPLC) were tested.

Keywords: immobilised amylose; phosphorolytic synthesis; maltooligosaccharides; silica gel; silane linkers; hydrazides.

* Present address: Institut für Biochemie, Emil-Fischer-Zentrum, Friedrich-Alexander-Universität Erlangen-Nürnberg, Fahrstraße 17, D-91054 Erlangen, Germany. Tel.: +49-(0)9131-852-6206; fax: +49-(0)9131-852-2485; e-mail: hgb@biochem.uni-erlangen.de

0040-4039/02/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PH: S0040-4039(02)01293-5
2.1. Solid-phase amylose synthesis

Enzymatic synthesis of amylose using potato phosphorylase (EC 2.4.1.1) and glucose-1-phosphate allows generation of amylose polymers with a narrow molecular weight distribution,28,29 a method which can also be used for the generation of amylose-containing copolymers by heterogeneous phosphorylolytic synthesis.30,31 Similar to anionic polymerisation, a desired molecular weight of the polymer can be defined via the ratio of starter to monomer.32 Since the enzyme requires a maltooligosaccharide starter of at least 4 glucose units in length (Glc4), immobilised maltoheptaose was indeed an efficient starter for polymer synthesis. Enzymatic chain elongation of immobilised maltoheptaose primers afforded silica-bound amylose (Fig. 3A).32

Variation of reaction time and amount of enzyme used for synthesis gave an optimal duration of enzymatic synthesis of 15 min, using 0.29 units33 of potato phosphorylase per µmol of immobilised maltoheptaose starter. The total amount of immobilised polysaccharide after phosphorolytic synthesis was ca. 6%, irrespective of the spacer length (Fig. 3B).34 The relative increase in amylose content of the modified silica support was higher for the -(CH2)11- spacer, indicating that although fewer immobilised primers were present on the surface, these were better accessible for enzymatic synthesis. Variation of the ratio of monomer (Glc-1-P) to starter (immobilised maltoheptaose) revealed a saturation behaviour, reaching a maximum at a theoretical degree of polymerisation of ca. 100 for -(CH2)6-, and ca. 250 for the -(CH2)11- spacer (Fig. 3B). This is consistent with a more efficient amylose synthesis from maltoheptaose molecules that are bound to the support via the longer spacer. The saturation behaviour also indicates that an equilibrium of amylose synthesis and removal from the support by a disproportionation reaction of the enzyme32 was being reached, regardless of the amount of available monomer.

2.2. Characterisation of immobilised amylose

The hydrazide linker had been introduced as a preformed cleavage site to monitor the progress of solid phase amylose synthesis. Under the conditions of synthesis, coupling and deprotection, the hydrazide bond was stable, but synthesised amylose could be cleaved from the support by shaking the material in 0.2 M citrate buffer at pH 4.0 at 60°C for 4 h (Fig. 3A). The supernatant was directly tested by iodine complexation and HPLC (Waters Ultrahydrogel 250, 0.2 M citrate buffer pH 6.0, 0.5 ml/min, room temperature). Synthetic amylose standards10,35 were used for calibration. Solid-phase synthesised amylose was characterised by a broad molecular weight distribution (Fig. 3C). Reduced accessibility of immobilised primers, leading to variations in the rate of chain elongation on different sites on the support, and beginning disproportionation may account for the observed chain length distribution. High molecular weight of support-generated amylose, and the presence of unreacted primer indicated that only few of the immobilised primer molecules were utilised by the enzyme.
2.3. Chromatography

Amylose-modified silica was suspended in water/0.2% I2/KI solution added to induce formation of the helical amylose–iodine inclusion complex prior to column packing. Water/methanol (95:5, v/v) was used as medium for packing and HPLC. Under these conditions, complex formation ability of amylose was fully preserved, while swelling of the material during column packing was minimised. The observed differences in the elution sequence for complexands of starch and cyclodextrin between base material and amylose-modified silica indicated that bound amylose dominated the separation process (Table 1). In the case of D- and L-menthol, enantioseparation was observed. The chiral separation factor of \( a = 1.04 \), however, was not sufficient for baseline separation. Note also that known complexands of amylose were not retarded as strongly as expected. Apparently, no inclusion complexes were formed between analytes and immobilised amylose. When amylose phases were removed from the columns and probed with iodine solution, the typical blue colour only became visible after 1–2 min, compared to instantaneous staining with materials that had not been used in HPLC.

Thus, the conditions of HPLC column packing and use (>25 bar) induced a conformational change of the amylose, abolishing the low-density, helical V-conformation. Retardation, therefore, was most likely due to interactions on the carbohydrate surface and not complex formation between analyte and amylose helices. Amylose-grafted chromatography materials should thus be useful in low and medium pressure chromatographic applications, where helical inclusion should be the predominant separation mechanism.

Table 1. Chromatographic properties of modified silica phases

<table>
<thead>
<tr>
<th>Compound</th>
<th>Column material</th>
<th>Amylose (wt%)</th>
<th>Theor. plates</th>
<th>Capacity factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fenchone</td>
<td>S 250 (base mat.)</td>
<td>6.2</td>
<td>2700</td>
<td>1.73</td>
</tr>
<tr>
<td>D-Menthol</td>
<td>S 250-(CH₂)₁₁amylose</td>
<td>1.94</td>
<td>2100</td>
<td>2.10</td>
</tr>
<tr>
<td>L-Menthol</td>
<td></td>
<td>1.94</td>
<td></td>
<td>2.18</td>
</tr>
<tr>
<td>2-Hexanone</td>
<td></td>
<td>1.51</td>
<td></td>
<td>1.39</td>
</tr>
<tr>
<td>Phenol</td>
<td></td>
<td>1.02</td>
<td></td>
<td>1.09</td>
</tr>
</tbody>
</table>

Thus, the conditions of HPLC column packing and use (>25 bar) induced a conformational change of the amylose, abolishing the low-density, helical V-conformation. Retardation, therefore, was most likely due to interactions on the carbohydrate surface and not complex formation between analyte and amylose helices. Amylose-grafted chromatography materials should thus be useful in low and medium pressure chromatographic applications, where helical inclusion should be the predominant separation mechanism.

Acknowledgements

Supported by the Federal Ministry of Research and Technology, program ‘Renewable Resources’ and Eridania-Begin-Say. Support and helpful discussions by Professor Dr. G. Wulff are gratefully acknowledged.

References

22. Maltoheptaose 4: 125 g of β-cyclodextrin (Avebe, Krefeld, Germany) were heated to reflux for 2 h in 500 ml of 0.01 N HCl. After neutralisation, 1 ml 1 M phosphate buffer pH 7.0 was added and the solution stored at 4°C overnight. Unreacted β-cyclodextrin was removed as p-xylene complex. Repeated precipitation from water/ethanol and precipitation with acetone gave 4, Rf 0.19 (n-butanol/methanol/water 4:3:3).

23. 5 mmol of maltoheptaose, 4, were dissolved in 40 ml of dry pyridine; 15 mmol of o-alkenyl hydrazide were added and the mixture kept at 60°C for 48 h. Solvent was removed and the residual washed with ethyl acetate until pyridine-free. d-Maltoheptaosyl-10-undecenoyl hydrazide 5: Yield 94%, mp 158–200°C (dec.). Anal. calc. for C32H59N4O4: H2O: C, 46.60; H, 7.09; N, 2.12. Found: C, 46.77; H, 7.34; N, 2.25%.

24. Per-o-trimethylsilyl-d-maltoheptaosyl-10-undecenoyl hydrazide: Yield 90%, mp 80–83°C, 1H NMR (CDCl3): δ 4.93–5.32 (m, 9H), 3.19–4.21 (m, broad, 42H), 2.1 (2, 2H), 0.14 (s, Si-CH3). Anal. calc. for C122H277N2O36Si23: C, 48.93; H, 9.32; N, 0.94. Found: C, 48.62; H, 9.54; N, 0.93%.


26. Per-o-trimethylsilyl-d-maltoheptaosyl-ω-(trioxsylyl)-n-undecenoyl hydrazide 8: mp 80–83°C, 1H NMR (CDCl3): δ 4.95–5.23 (m, 9H), 3.26–4.18 (m, broad, 42H), 2.1 (2, 2H), 0.14 (s, Si-CH3). Anal. calc. for C117H267N2O36Si23: C, 47.45; H, 9.27; N, 0.97. Found: C, 46.76; H, 9.26; N, 1.05%.

27. Silica 250 K (Amicon, Hamburg, Germany) was neutralised with HNO3. 1 μmol of anchor per m2 silica surface was added and the suspension gently shaken at 60°C for 72 h. TMS was removed by repeatedly shaking...
modified silica in 10 ml/g of 0.5 % acetic acid in methanol/water (1:1) for 1 h.


32. Pfannemüller, B.; Burchard, W. *Makromol. Chem.* 1969, 121, 1. 1 g of maltolheptaose-modified silica was suspended in 6 ml of 0.2 M citrate buffer at pH 6.0, 15 ml of Glc-1-P solution and 10 units of phosphorylase were added. The mixture was shaken at 40°C for 15 min, washed with water, ethanol, acetone, and dried.


36. HPLC columns (0.4×25 cm) were packed using a Knauer pneumatic dual piston pump (H. Knauer GmbH, Berlin, Germany), and Knauer Vertex cartridges. The HPLC setup consisted of a Waters 6000 pump, Latek 7125 Rheodyne injector, Waters R401 refractory index detector (Waters GmbH, Eschborn, Germany), and a Hewlett-Packard 3390 A recorder/integrator (Hewlett-Packard, Ratingen, Germany); flow rate 0.5 ml/min, pressure 25 bar.