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Chemoenzymatic synthesis of 2-chloro-4-nitrophenyl β-maltoheptaoside acceptor-products using glycogen phosphorylase b

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Abstract

In the present work, we aimed at developing a chemoenzymatic procedure for the synthesis of β-maltooligosaccharide glycosides. The primer in the enzymatic reaction was 2-chloro-4-nitrophenyl β-maltoheptaoside (G_7-CNP), synthesised from β-cyclodextrin using a convenient chemical method. CNP-maltooligosaccharides of longer chain length, in the range of DP 8–11, were obtained by a transglycosylation reaction using α-D-glucopyranosyl-phosphate (G-1-P) as a donor. Detailed enzymological studies revealed that the conversion of G_7-CNP catalysed by rabbit skeletal muscle glycogen phosphorylase b (EC 2.4.1.1) could be controlled by acarbose and was highly dependent on the conditions of transglycosylation. More than 90% conversion of G_7-CNP was achieved through a 10:1 donor–acceptor ratio. Tranglycosylation at 37 °C for 30 min with 10 U enzyme resulted in $G_{8\rightarrow12}$-CNP oligomers in the ratio of 22.8, 26.6, 23.2, 16.5, and 6.8%, respectively. The reaction pattern was investigated using an HPLC system. The preparative scale isolation of $G_{8\rightarrow11}$-CNP glycosides was achieved on a semipreparative HPLC column. The productivity of the synthesis was improved by yields up to 70–75%. The structures of the oligomers were confirmed by their chromatographic behaviours and MALDI-TOF MS data.

Keywords: Maltooligosaccharide series; Transglycosylation; Glycogen phosphorylase b; Chain elongation

1. Introduction

The widening interest in well-defined maltooligosaccharides and their glycosides has stimulated research for new and efficient syntheses of these compounds. The homologous maltooligosaccharide substrates are of current interest because of their importance in the investigation of the binding sites and the actions of different depolymerising enzymes. In these studies, well defined, high purity, structurally well characterised substrates are preferred.[1–3] Recently, a chemoenzymatic procedure was developed in our laboratory for the synthesis of CNP-maltooligosides of shorter chain length in preparative scale[4] and these compounds were used as model substrates for the elucidation of the action of human salivary amylase.[5]

In the present work, we aimed at developing a procedure for the preparation of CNP-maltooligosaccharides of longer chain length, in the range of DP 8–11. These maltooligomer substrates are indispensable tools in the inves-

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tigation of the binding sites and the actions of α-amylases having a longer binding area than that of human α-amylases.

Unfortunately, there is no efficient chemical method for carbohydrate chemists to form glycosidic linkages stereospecifically, or to generate higher-molecular weight oligosaccharide glycosides with chromogenic aglycons. Therefore, we decided to focus on the enzymatic synthesis of these compounds.

In the enzymatic conversion, G_7-CNP was used as a starting material which was synthesised from β-cyclodextrin by a convenient and efficient chemical method.[6]

2. Results and discussion

The chemical synthesis to introduce a glycosyl moiety having an α-(1→4) linkage to the non-reducing end of maltooligosaccharides is an exciting and difficult challenge. There is no real efficient method available for a carbohydrate chemist because of the great number of steps necessary for such synthesis and due to the low yield. We therefore decided to focus on enzymatic synthesis to gain a series of CNP β-maltooligosaccharides from DP 8 to 12.

There are two groups of enzymes, glycoside hydrolases[7–9] and glycosyl transferases,[7,8] which can be applied for the synthesis of oligosaccharides. The physiological function of glycoside hydrolases (e.g., α-glucosidases, α-amylases) is to cleave glycosidic bonds; however, under certain experimental conditions, they can catalyse the stereospecific formation of glycosidic linkages. Glycosyl transferases are biocatalysts responsible for the synthesis of glycosidic linkages in vivo. They form regio- or stereospecific linkages between the activated donor and an acceptor in very good yields. The activating group at the anomeric centre of the donor is either a nucleoside diphosphate in the case of the Leloir transferases or a simple sugar-1-P in the case of the non-Leloir glycosyl transferases, for example, phosphorylases.[10,11]

In our previous work,[4] glycogen phosphorylase b was used as a favourable system for oligosaccharide degradation and the distribution of products could be controlled to some extent by reaction conditions. However, enzymatic phosphorolysis is a reversible process and, therefore, it can be used also in the synthesis of oligo- or polysaccharides.

Two important examples are the syntheses of sucrose and trehalose, catalysed by sucrose phosphorylase[12] and trehalose phosphorylase,[12] respectively. Moreover, potato phosphorylase was used to synthesise maltose oligomers,[13–15] and a family of linear, as well as star- and comb-shaped polymers.[16] Artificial primers with functional groups can also be used to prepare tailor-made polysaccharides which readily conjugate to proteins or other compounds.[15] It is worth noting that there are several quite recent reports on the use of phosphorylases in the synthesis of oligosaccharides, e.g., in the production of trehalose from starch,[17] the synthesis of three branched trisaccharides,[18] cellodextrins[19] and Gal-β-(1→3)GlcNAc derivatives.[20]

We thought that glycogen phosphorylase b, the most intensively studied α-glucan phosphorylase,[21] should be the enzyme of choice, again to meet our particular needs. In the experiments on CNP-oligosaccharide syntheses G_7-CNP was used as a primer and conditions were adjusted in a way that only oligosaccharides with DP 8–12 were produced. It is worth noting that the preparation of CNP β-maltooligomers via chain elongation by glycogen phosphorylase b has not been reported in preparative scale yet.

Effect of acarbose on product pattern.—An interesting feature of phosphorylase is that the inorganic phosphate liberated from G-1-P can be recycled in a degradation reaction. It is, therefore, of interest as a possible agent for controlling the removal of phosphate to drive the formation of oligosaccharides. Initially, we thought to control the distribution of products by acarbose. Acarbose is used as a potent inhibitor for the degradative pathway of the α-amylase reaction where it competes with the starch substrate.[22] Its inhibitory activity for phosphorylase is also described in the degradation reaction.[23]

To ensure that acarbose does not function as a substrate when tested as an inhibitor in the chain elongation and degradation direction, the substrate activity of acarbose was

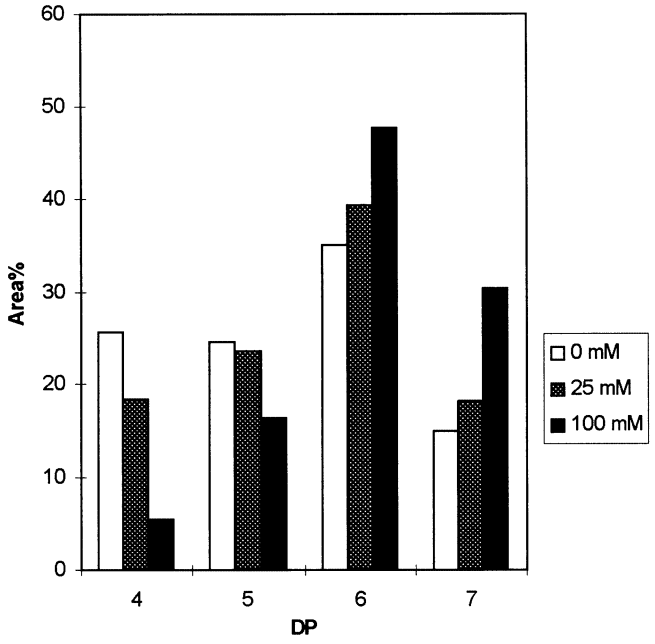

Fig. 1. Distributions of phosphorolysis products at different acarbose concentrations. Reaction conditions: 5 mM CNP-G_7, 100 mM phosphate buffer (pH 6.8), 10 °C, 10 min.

first studied. No substrate activity could be detected for acarbose either in the direction of degradation or elongation using conditions similar to those in normal experiments.

In the first set of experiments, the presence of acarbose was studied in the direction of phosphorolytic cleavage of G_7-CNP. As shown in Fig. 1, acarbose inhibits the conversion of G_7-CNP into shorter oligomers and changes product distribution significantly.

It should be mentioned that phosphorolysis is a multistep reaction, the formed products are substrates as well, and are degraded further, which makes the results more difficult to interpret. Increasing concentrations of acarbose (25 and 100 mM) result in a higher amount of longer oligomers (G_7-CNP, G_6-CNP) but decrease the amount of the shortened ones (G_5-CNP, G_4-CNP).

In the next set of experiments, the effect of 100 mM acarbose on the phosphorolytic synthesis was studied in the case of the same donor and acceptor concentration; 5 mM G-1-P and 5 mM G_7-CNP, respectively.

As shown in Fig. 2, acarbose promotes oligosaccharide synthesis which has not been published so far. The distribution of products shifted towards the formation of longer oligomers (CNP-$G_{9\rightarrow12}$), while the ratio of the shorter oligomers (CNP-$G_{6\rightarrow5}$) has not changed or decreased (CNP-$G_{8\rightarrow7}$). In the presence of acarbose, the reaction conditions are favourable for phosphorylase to convert CNP-G_7 into longer oligosaccharides, so that the final degree of polymerisation (DP) can be regulated to some extent simply by the addition of acarbose. The effect of acarbose on the

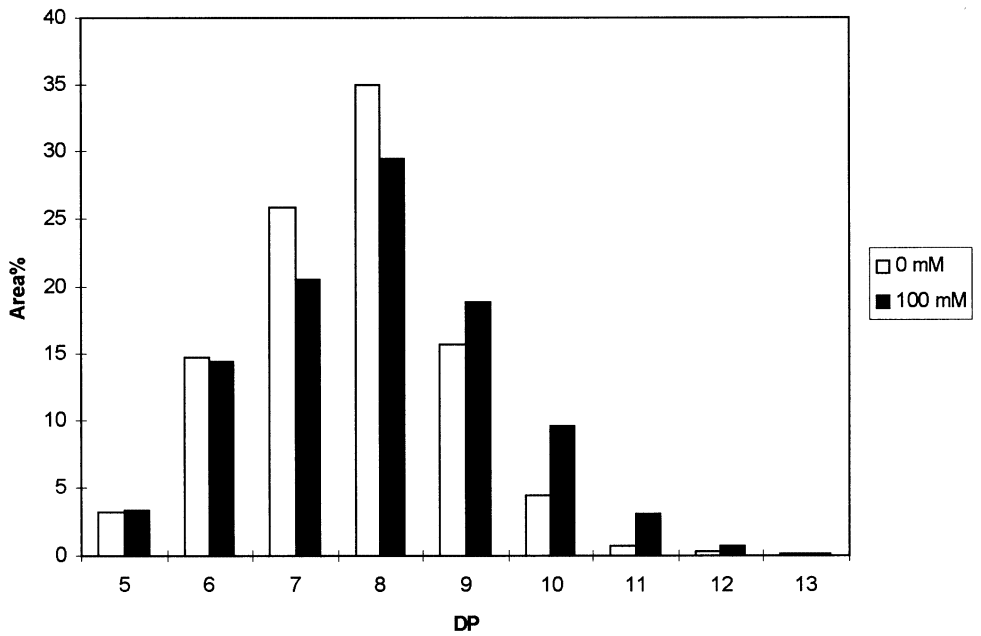

Fig. 2. Effect of acarbose on the distribution of products in a phosphorylase catalysed reaction. Reaction conditions: 5 mM CNP-G_7, 5 mM G-1-P, 25 mM glycerophosphate buffer (pH 6.8), 37 °C, 30 min.

product pattern should be interesting from an enzymological point of view, although, acarbose is a weak inhibitor when its affinity for phosphorylase is compared with that for amylases.[22] High acarbose concentrations are obviously disadvantageous for the separation of products, therefore the effectiveness of the enzymatic conversion should be optimised for experimental conditions.

Effect of temperature on conversion.—To shift the enzymatic reaction towards the formation of CNP-$G_{8\rightarrow12}$ oligomers, all chain elongation were conducted with 5 mM of CNP-G_7 as the acceptor and 50 mM of G-1-P as the donor. The concentration of phosphorylase was not changed (1000 U/mL). Distributions of products after an incubation period of 30 min at different temperatures (10, 15, 37 °C) are shown in Fig. 3.

With increasing temperature, a continuous increase in the degree of polymerisation was found. At 10 °C the reaction mixture contained CNP-maltooligosides in the range of DP 7–11. Conditions of transglycosylation at 10 °C were probably the best to obtain high quantities of CNP-G_8 glycoside. At 15 °C the final reaction mixture was equally enriched in CNP-$G_{8\rightarrow10}$ glycosides.

The conversion of CNP-G_7 increased with rising temperatures and reached almost 100% at 37 °C. Incubation at 37 °C resulted in oligomers in the range of DP 8–22.

Time course of tranglycosylation at 15 °C.—A summary of the results is given in Table 1. The chain elongation of CNP-G_7 by phosphorylase at 15 °C for 15 min resulted in 82.6% conversion with the octamer glycoside as the main product (47.4%). Incubation for 30 min was more favourable for the formation of the desirable oligomers in a ratio of 22, 26, 23, 15% for the octamer, nanomer, decamer, and undecamer glycosides, respectively. When the enzymatic reaction was performed over a longer period of time (45 min), the conversion of CNP-G_7 was shifted towards the formation of longer glycosides (CNP-$G_{10\rightarrow14}$).

In conclusion, the elongation of CNP-G_7, using G-1-P as a donor, in a ratio of 1:10, respectively, in 25 mM glycerophosphate buffer (pH 7.0) for 30 min at 15 °C can be highly recommended as an alternative way for obtaining longer oligosaccharides.

Effect of enzyme concentration.—High enzyme concentrations (1000 U/mL) are obviously disadvantageous from an economical point of view, therefore, reaction conditions should be optimised for enzyme concentrations, as well. Results show (Table 2) that the efficiency of the conversion of CNP-G_7 and the distribution of oligomers at 15 °C with 10 U phosphorylase or at 37 °C with 1 U enzyme is almost identical.

Structural characterisation of the CNP-G_7 acceptor products.—Structural parameters of the elongated and separated compounds were established by mass spectrometric data, chromatographic behaviours and the hydrolysis test using glucoamylase (GA), (EC 3.2.1.3). As shown in Fig. 3, the applied HPLC method allowed the separation of products with a DP ranging between 3 and 22. The homologous maltooligosides were eluted in an order of increasing DP. The mass spectrum (Fig. 4) showed molecular masses of the elongated oligomers $[M + Na]^+$ were 1331.6, 1493.7, 1655.8, 1817.9, 1980.2 and 2142.8, corresponding to the oligosaccharides in the range of DP 7–12.

To identify the position and type of the formed glycosidic linkages, retention times of oligomers were analysed. Retention times were determined on the amine column using simple isocratic elution with acetonitrile–water. As it can be seen in Fig. 5, there was a strong relationship between DPs of oligomers and their retention times (R_t). A logarithmic plot of retention time versus DP of CNP-oligosaccharides gave a straight line suggesting the existence of one family of homologous molecules. Oligosaccharides (DP 3–11) were produced in the same reaction catalysed by glycogen phosphorylase, starting from CNP-G_7 as a primer. Consequently, the glycosidic linkages in the case of oligomers shorter than CNP-G_7 should be α-(1→4) linkages. Since the values of the log R_t for the longer products are on the same line, they suggest the formation of α-(1→4) linkages in the direction of synthesis also.

Linear plots of log R_t versus DP have already been reported[24,25] but this is the first evidence on homologous products of CNP-maltooligosides (DP 3–11) synthesised by glycogen phosphorylase. Results are consis-

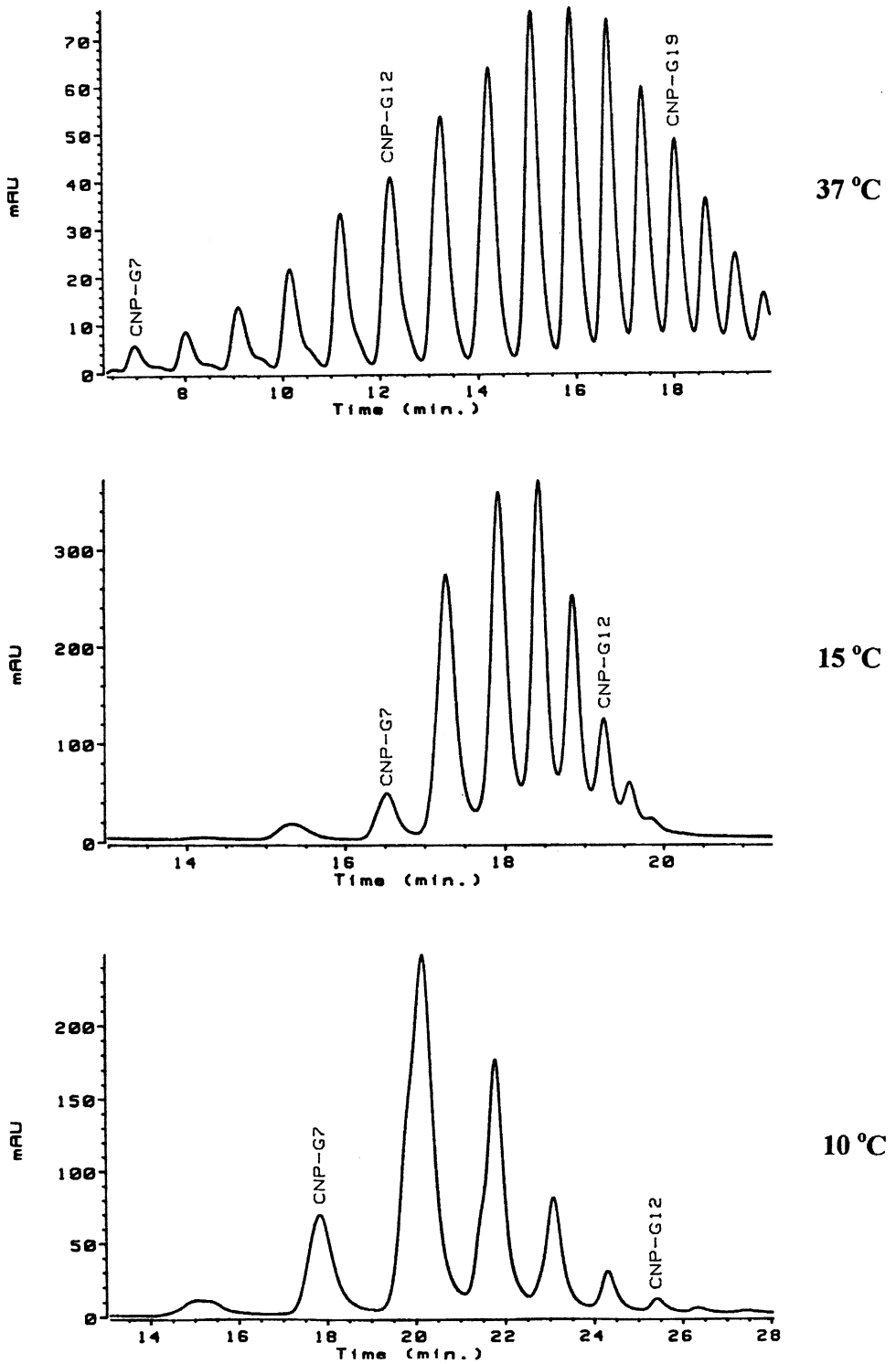

Fig. 3. HPLC profiles of the elongated CNP-oligomers at different temperatures. For the reaction conditions, see Section 3.

tent with those of the hydrolysis test using GA. Since GA is an exo-type enzyme and hydrolyses α-(1→4) glucosidic bonds, glucose residues are liberated one by one from the nonreducing end. When CNP-G_9 or CNP-G_{10} and GA were incubated, substrates were hydrolysed to give glucose and 2-chloro-4-nitrophenyl β-glucopyranoside. Results of the hydrolysis test on the elongated substrates demonstrated convincingly that the transfer of the glucose residues occurred at the nonreducing end by α-(1→4) linkages.

3. Experimental

Materials.—Buffer chemicals and other reagents (reagent grade) were obtained from Sigma–Aldrich Co. and MeCN (gradient grade) was purchased from Tisons. Purified water was obtained from a laboratory purification system equipped with both ion-exchange and carbon filters (Millipore, Bedford, MA, USA). G_7-CNP was synthesised from β-CD as previously described in Ref. 6. Glycogen phosphorylase b was isolated from

Table 1
Distribution of reaction products at 15 °C as a function of time [a]

Reaction time (min)	DP of CNP-glycosides (%) [b]									
	6	7	8	9	10	11	12	13	14	15
15	2.0	15.4	47.4	23.3	8.2	2.7	1.0			
30	2.0	3.8	21.8	25.5	23.3	15.0	6.6	2.0		
45	2.1	2.9	10.0	18.3	21.5	20.0	13.7	7.7	3.8	

[a] Reaction conditions: 5 mM CNP-G_7, 50 mM G-1-P, 25 mM glycerophosphate buffer (pH 6.8), 10 μL of 1000 U/mL enzyme.
[b] Area % measured by HPLC.

Table 2
Distribution of reaction products after 30 min as a function of temperature and enzyme concentration [a]

Temperature (°C)	Enzyme (U)	DP of CNP-glycosides (%) [b]									
		6	7	8	9	10	11	12	13	14	15
15	100	2.0	3.8	21.8	25.5	23.3	15.0	6.6	2.0		
37	10		0.8	22.8	26.6	23.2	16.5	6.8	2.4	0.7	0.2

[a] Reaction conditions: 5 mM CNP-G_7, 50 mM G-1-P, in 1 mL of 25 mM glycerophosphate buffer.
[b] Area % measured by HPLC.

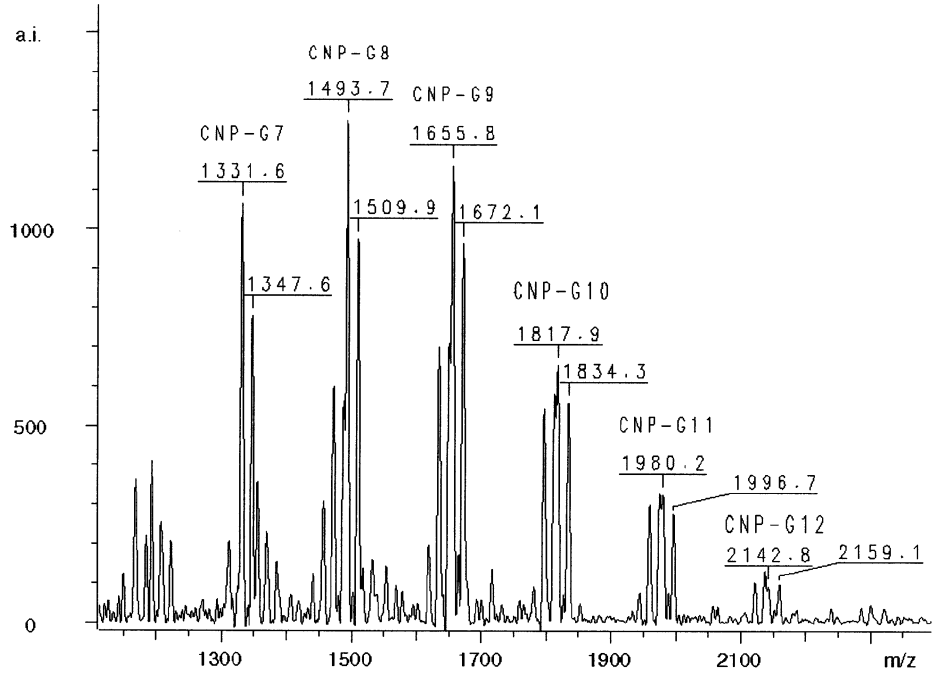

Fig. 4. MALDI-TOF MS of CNP oligomers.

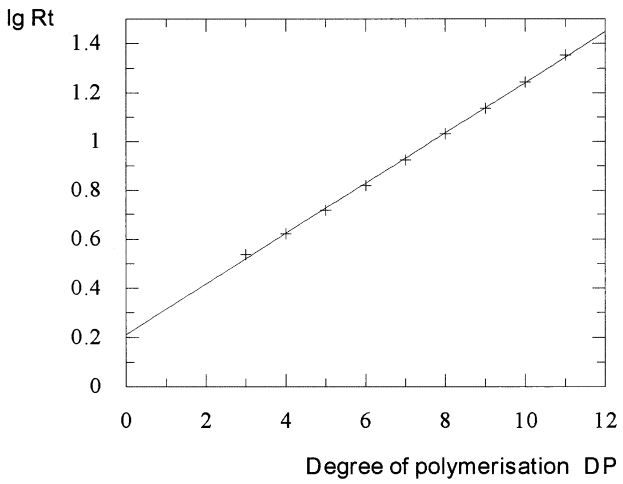

Fig. 5. Retention time analysis of CNP-oligomers obtained in phosphorylase reaction.

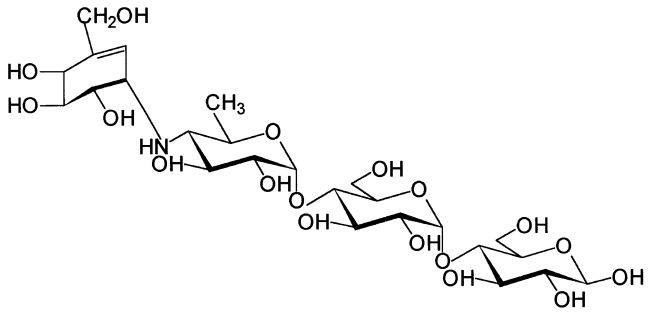

Fig. 6. Structure of acarbose.

rabbit skeletal muscle. A glycerol suspension of crystallised enzyme was a gift from Professor V. Dombrádi (activity: 1000 U/mL, protein content: 62 mg/mL). Acarbose (pseudotetrasaccharide) was extracted from Glucobay tablet (Bayer) and its purity was confirmed by NMR and MALDI-TOF MS data. Fig. 6 shows the structure of acarbose.

General procedure for synthesis of elongated CNP-oligomers.—Glycogen phosphorylase b (1000 U/mL, 10 μL) was added to a solution of G_7-CNP (6.5 mg, 5 mM) and G-1-P (16.8 mg, 50 mM) in glycerophosphate buffer (25 mM, pH 6.8, 1 mL) containing 10 mM β-mercaptoethanol, 2 mM EDTA and 4 mM AMP. The mixture was incubated at different temperatures for different periods of time and enzymatic conversion was stopped by boiling for 1 min. After cooling, the precipitated enzyme was removed by filtration through a Millipore 0.2-μm filter. Samples (20 μL) were injected on the HPLC column. Reproducible values were obtained at four experiments and the mean values are given in Tables 1 and 2.

Chromatographic analysis.—For HPLC, a Hewlett–Packard 1090 series II liquid chromatograph equipped with a diode array detector, automatic sampler, and ChemStation was used. The samples were separated on a LiChrospher 100 NH_2 5 μm column (250 × 4 mm) by a linear gradient from 80 to 40% CH_3CN with water at a flow rate of 1 mL/min. Gradient time was 20 min. The effluent was monitored for the CNP group at 302 nm. Quantitative results are given on the basis of peak area percentage. Fig. 3 shows the separation of the oligomers.

Product separation and quantitation.—A typical experiment was performed on a scale of 65 mg G_7-CNP. Phosphorylase b (1000 U/mL, 100 μL) was added to a solution of G_7-CNP (65 mg, 5 mM) and G-1-P (168 mg, 50 mM) in glycerophosphate buffer (25 mM, pH 6.8, 10 mL) containing (16 mg, 4 mM) AMP as an allosteric activator. The reaction mixture was incubated at 15 °C for 30 min and stopped by boiling for 5 min. After cooling, the precipitated enzyme (6.2 mg) was removed by filtration through a Millipore 0.2-μm filter. The filtrate was lyophilised, and the reduced volume (1.5 mL) was applied to a Toyopearl HW-50F (30–60 μm, 900 × 15mm) column. Purified distilled water or methanol–water mixture were used as mobile phases at a flow rate with 1.5 mL/min. Although partial separation was observed only, AMP, unreacted G-1-P and inorganic phosphate were liberated. The purified and desalted mixture was lyophilised again, and the reduced volume was separated on a semi-preparative column (Hypersil NH_2, 250 × 10 mm, 5 μm) by isocratic elution using 60:40 MeCN–water. Fig. 7 shows the HPLC profile of the semipreparative separation. Repeated injections of the sample gave pure compounds; 22 mg CNP-G_8, 20 mg CNP-G_9, 14 mg CNP-G_{10} and 7 mg CNP-G_{11} and resulted in 70–75% yield for the required oligomers. The yields obtained with the enzyme system were superior to those obtained with chemical synthesis.

Mass spectrometry.—MALDI-TOF MS analyses of the compounds were performed in positive-ion mode using a Bruker Biflex

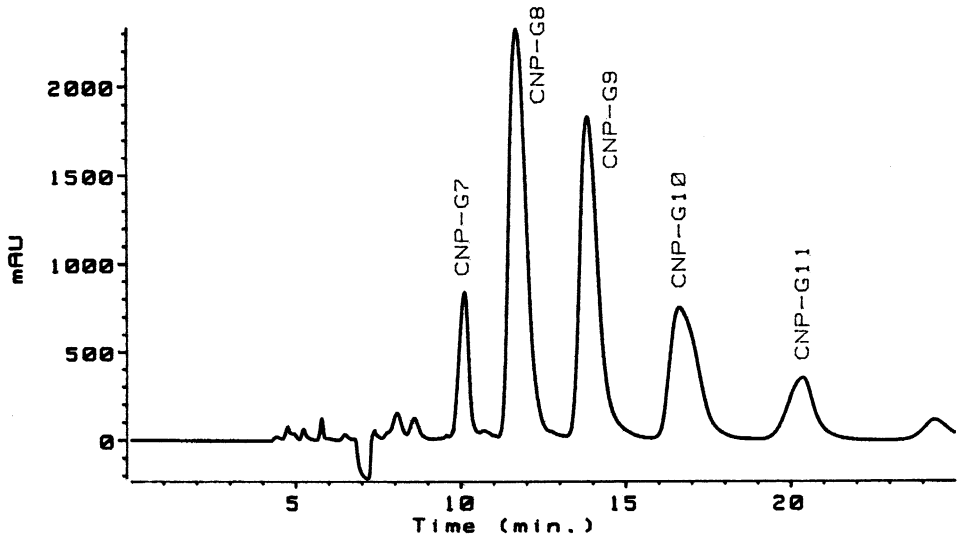

Fig. 7. Separation of reaction products by semipreparative HPLC. For HPLC conditions, see Section 3.

MALDI-TOF mass spectrometer equipped with delayed-ion extraction. Desorption/ionisation of the sample molecules was effected with a 337 nm nitrogen laser with a pulse width of 3 ns. Spectra from multiple (at least 100) laser shots were summarised using 19 kV accelerating and 20 kV reflectron voltage. External calibration was applied using the $[M + Na]^+$ peaks of maltooligosaccharides DP 3–7 and maltoheptaose peracetate, m/z: 527.15, 689.21, 851.26, 1013.31, 1175.36 and 2142.84, respectively. The spectrum was performed in 2,4,6-trihydroxiacetophenon (THAP) matrix by mixing 10 μL of satd matrix solution with 10 μL of sample dissolved in water, then 0.5 μL was applied to the sample target and it was allowed to dry at rt. The identification of compounds was done on the basis of the mass of $[M + Na]^+$ peaks.

Acknowledgements

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