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Chemoenzymatic preparation of 2-chloro-4-nitrophenyl β-maltooligosaccharide glycosides using glycogen phosphorylase b

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Abstract

In the present work, we aimed to develop a new 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), which was synthesised from β-cyclodextrin (β-CD) using a very convenient chemical method [E. Farkas, L. Jánossy, J. Harangi, L. Kandra, A. Lipták, *Carbohydr. Res.*, 303 (1997) 407–415]. Shorter chain length CNP-maltooligosaccharides in the range of dp 3–6 were prepared using rabbit skeletal muscle glycogen phosphorylase b (EC 2.4.1.1). Detailed enzymological investigations revealed that the conversion of G_7-CNP was highly dependent on the conditions of phosphorolysis. A 100% conversion of G_7-CNP was achieved during 10 min in 1 M phosphate buffer (pH 6.8) at 30 °C with the tetramer glycoside (77%) as the main product. Phosphorolysis at 10 °C for 10 min resulted in 89% conversion and G_4-, G_5-, and G_6-CNP oligomers were detected in the ratio of 29:26:34%, respectively. The reaction pattern was investigated using an HPLC system. The preparative scale isolation of $G_{3\rightarrow 6}$-CNP glycosides was achieved by size-exclusion column chromatography (SEC) on Toyopearl HW-40 matrix. The productivity of the synthesis was improved by yields of up to 70–75%. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: Maltooligosaccharide series; Glycogen phosphorylase b; Size-exclusion column chromatography

1. Introduction

The widening interest in defined maltooligosaccharides and their glycosides has stimulated research for new efficient syntheses of these compounds. The new synthetic routes may provide us with chemically modified oligosaccharides that possess more desirable physical and biological properties than those of their natural counterparts. In addition, the homologous maltooligosaccharide substrates are indispensable tools in the investigation of the binding site and the action of different depolymerising enzymes. In these studies, well-defined, high-purity, low-molecular-weight substrates are preferred [1–3]. More recently, CNP β-maltooligosides were used in our laboratory [3] as model substrates for the elucidation of the action of porcine pancreatic α-amylase. In addition, maltooligosaccharides having four to six glucose units turned out to be the best substrates for human α-amylases [4]. Unfortunately, there is no efficient chemical method available for carbohydrate chemists to synthesise low-molecular-weight CNP maltooligosides and, therefore, we decided to focus on the enzymatic synthesis of these compounds. The enzymatic procedures are advantageous alternatives to classical

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chemical methods since, in many cases, enzymes operate under the mildest reaction conditions (water, pH 6–8, room temperature) and possess a high specificity for the structures they recognise.

In the present work, chemical synthesis was supplemented with an enzymatic step to gain a series of CNP β-maltooligosides from dp 3 to 6. In the enzymatic conversion, G_7-CNP was used as a starting material. G_7-CNP was synthesised on a preparative scale from β-CD by a very convenient and efficient chemical method as described by Farkas et al. [5]. Although the same chemical procedure could also be used for the preparation of shorter chain length CNP-oligomers, a prolonged acetolysis of β-CD had to be introduced in this case. Following that, the peracetylated maltotriose, -tetraose, -pentaose, and -hexaose were separated, converted into their α-acetobromo derivatives, and then transformed into 2-chloro-4-nitrophenyl β-glycosides. The peracetylated maltooligosaccharides were purified and deacetylated, which led to CNP glycosides only in rather moderate yields. In the present paper, we describe the effective enzymatic conversion of G_7-CNP into shorter chain length oligomers as a promising alternative to their multistep chemical syntheses.

2. Results and discussion

There are two possibilities for the enzymatic cleavage of glycosidic bonds: (i) hydrolysis by α-glycosidases or α-amylases and (ii) phosphorolysis by phosphorylases. The physiological function of these enzymes is to cleave glycosidic bonds; however, under certain experimental conditions, they are able to catalyse the stereospecific formation of glycosidic linkages. Each group of enzymes has certain advantages and disadvantages for cleavage. In reactions catalysed by amylases, neither G_5- nor G_6-CNP was formed [3] and α-glucosidases split maltooligosides up to G-CNP. Therefore, we thought that glycogen phophorylase b, the most extensively studied α-glucan phophorylase [6], should be the enzyme of choice to meet our particular needs. Interestingly, the preparation of CNP oligomers via chain shortening by glycogen phosphorylase b has not yet been reported. The enzymatic phosphorolysis is a highly reversible process and, therefore, it can also be used in the synthesis of oligo- or polysaccharides. Two important examples are the syntheses of sucrose and trehalose, catalysed by sucrose phosphorylase [7] and trehalose phosphorylase [7], respectively. Moreover, potato phosphorylase was used to synthesise maltose oligomers [8–10], and a family of linear as well as star- and comb-shaped polymers [11]. Artificial primers with functional groups can also be used to prepare tailor-made polysaccharides which are ready to be conjugated to proteins or to other compounds [10]. It is worth noting that there are several more recent reports on the use of phosphorylases in the synthesis of oligosaccharides, e.g., in the production of trehalose from starch [12], the synthesis of three branched trisaccharides [13], and the phosphorolytic synthesis of cellodextrins [14].

Glycogen phosphorylase (EC 2.4.1.1).—The enzyme catalyses the first step in the breakdown of glycogen:

$$(\mathrm{Glc})_n + \mathrm{P_i} \leftrightarrows (\mathrm{Glc})_{n-1} + \text{Glc-1-P}$$

where $(\mathrm{Glc})_n$ is an α-(1→4)-linked glucosyl polymer of n residues, Glc-1-P is α-D-glucose-1-phosphate and $\mathrm{P_i}$ is inorganic phosphate. Glycogen phosphorylase is present in two forms in skeletal muscle. The inactive phosphorylase b, which can be observed mainly in resting muscle, can be activated by increasing the level of AMP or by phosphorylation to phosphorylase a. In our experiments, phosphorylase b was activated by the addition of AMP. Both the crystal structure of glycogen phosphorylase b [15] and the structure of its binding sites have been elucidated [16]. It has been demonstrated that, in addition to glycogen, α-(1→4) oligosaccharides also bind strongly to the glycogen binding site [17].

Our results showed that glycogen phosphorylase b effectively splits CNP β-maltoheptaose and the phosphorolysis of G_7-CNP is probably the most practical method for the preparation of shorter chain length homologues. Nevertheless, the effectiveness of the enzymatic degradation depended on some experimental conditions, including:

- the availability of an enzyme preparation which had a satisfactorily high phosphorolytic activity and which was free of any hydrolytic activity,
- the optimisation of reaction conditions to convert G_7-CNP into the shorter oligomers,
- the suitable control of the reaction to give a final reaction mixture equally enriched in $G_{3\rightarrow 6}$-CNP glycosides,
- the analysis and separation of the reaction products, and
- the preparative scale isolation of each glycoside.

The glycogen phosphorylase b preparation used in our experiments fulfilled the first requirement quite well because the enzyme possessed no α-amylase or any other hydrolytic activity. In the absence of inorganic phosphate no cleavage of α-(1 → 4) glycosidic linkages was observed.

Time course of phosphorolysis at 30 °C.— As shown in Table 1, the enzymatic phosphorolysis of G_7-CNP at 30 °C for 10 min resulted in a 100% conversion with the tetramer glycoside as the main product (77%). When the phosphorolysis was performed over a longer period of time (5 h), the reaction mixture also contained substantial quantities of the trimer glycoside with a ratio of 51.3:41.4% for G_3- and G_4-CNP oligomers, respectively. The HPLC elution profiles clearly indicated that the reaction never resulted in oligomers with higher dp than that of the primer (G_7-CNP). The preferred formation of maltotetraoside could well be explained by the earlier observation that this phosphorylase splits glycogen chains up to four residues away from the branch points [18]. However, the formation of G_3-CNP indicated that in the shorter substrates CNP moiety might 'mimic' a glucose residue similar to that found in the case of porcine pancreatic α-amylase [3]. Our results

Table 1
Distribution of reaction products at 30 °C as a function of time[a]

Reaction time (min)	Phosphorolytic products of G_7-CNP (%)[b]					Total conversion (%)
	G_3-CNP	G_4-CNP	G_5-CNP	G_6-CNP	G_7-CNP	
5	1.3	73.7	15	10	0	100
10	2	**77**	12	9	0	100
60	14	66.2	11.5	8.2	0	100
180	33.8	48.9	11.2	6.0	0	100
300	**51.3**	**41.4**	4.4	2.9	0	100

[a] Reaction conditions: 5 mM G_7-CNP, 1 M phosphate buffer (pH 6.8), 10 U mL^{-1} enzyme. Bold type printed numbers are explained in the text.

[b] Area % measured by HPLC.

Table 2
Phosphorolysis products of G_7-CNP at 0 °C as a function of time[a]

Reaction time (min)	Phosphorolytic products of G_7-CNP (%)[b]					Total conversion (%)
	G_3-CNP	G_4-CNP	G_5-CNP	G_6-CNP	G_7-CNP	
10	0	3	8	45	44	56
15	0	9	16	49	25	75
28	0	**23**	**24**	**39**	14	86
42	0	42	23	28	7	93
55	0	48.5	23	22	6	93.5

[a] Reaction conditions: 5 mM G_7-CNP, 1 M phosphate buffer (pH 6.8), 10 U mL^{-1} enzyme. Bold type printed numbers are explained in the text.

[b] Area % measured by HPLC.

Table 3
Distribution of reaction products at temperatures from 0 to 34 °C[a]

Temperature of reaction (°C)	Phosphorolytic products of G_7-CNP (%)[b]					Total conversion (%)
	G_3-CNP	G_4-CNP	G_5-CNP	G_6-CNP	G_7-CNP	
0		3	7.6	44.9	44.5	55.5
5		12	22.4	46.8	18.3	81.7
10		**29**	**26.1**	**33.7**	11.1	88.9
15		68	19.3	11	2.2	97.3
20		76	14	10		100
30	2	77	12	9		100
34	2	89	7	2		100

[a] Reaction conditions: 5 mM G_7-CNP, 1 M phosphate buffer (pH 6.8), 10 min, 10 U mL^{-1} enzyme. Bold type printed numbers are explained in the text.

[b] Area % measured by HPLC.

Table 4
Distribution of reaction products at 10 °C, as a function of P_i concentration[a]

Concentration of P_i (mM)	Phosphorolytic products of G_7-CNP (%)[b]					Total conversion (%)
	G_4-CNP	G_5-CNP	G_6-CNP	G_7-CNP	G_8-CNP	
5	0	0	12.5	76.9	10.6	23.1
40	6.2	15.5	39.6	32.0	6.7	68.0
100	**30.9**	**26.2**	**28.8**	11.5	2.6	**88.5**
500	28.0	26.6	33.5	10.2	1.7	**89.8**
1000	29.0	26.1	33.7	11.1		**88.9**

[a] Reaction conditions: 5 mM G_7-CNP, 10 min, 10 U mL^{-1} enzyme. Bold type printed numbers are explained in the text.

[b] Area % measured by HPLC.

suggest that the conditions of phosphorolysis we used are probably the most efficient to obtain high quantities of G_4- and G_3-CNP oligomers.

Effect of low temperature (0 °C) on the pattern of phosphorolysis.—In order to shift the enzymatic reaction towards the formation of G_5- and G_6-CNP oligomers, phosphorolysis was carried out at 0 °C with a shorter reaction time. As presented in Table 2, the reaction proceeded at a much lower rate at 0 °C but the conversion of G_7- to G_6-CNP improved substantially (49% conversion was achieved after 15 min). An incubation time of 28 min resulted in an overall conversion of G_7-CNP of 86% in the ratio of 23:24:39% for the G_4-, G_5-, and G_6-CNP oligomers, respectively. After incubating G_7-CNP for 55 min with the enzyme, a 93% conversion was achieved with the tetramer glycoside (48.5%) as the main product. As before, under these conditions, no chain elongation could be detected.

Effect of the temperature on the conversion.—The temperature range which was covered in our investigations was between 0 and 34 °C. Distributions of products at different temperatures after a 10 min incubation period are shown in Table 3. The conversion increased with increasing temperatures and reached 100% at 15 °C. Usually a 5 °C decrease in the temperature resulted in a 7–9% decrease in the reaction rate. On the other hand, between 0 and 5 °C the reaction rate decreased by 26.2%. This change could be explained with the formation of inactive tetramers at low temperatures [19]. To avoid the oligomerisation of phosphorylase at low temperature, enzymatic conversions should not be performed below 10 °C for preparation of oligomers. In conclusion, the phosphorolytic cleavage of G_7-CNP with glycogen phosphorylase b in 1 M phosphate buffer (pH 6.8) for 10 min at 10 °C can be highly recommended as an alternative way for obtaining shorter maltooligomers.

Effect of inorganic phosphate concentration.—An interesting feature of the enzyme system used is that Glc-1-P liberated by glycogen phosphorylase can be recycled in a chain elongation reaction when the concentration of inorganic phosphate is low. Since we used a high P_i concentration (1 M) in comparison to the liberated Glc-1-P (max 20 mM), the reaction proceeded without any chain elongation. However, high salt concentrations are obviously disadvantageous for the separation of the products. Therefore, the system also had to be optimised for inorganic phosphate concentration. Phosphorolysis was also performed at lower inorganic phosphate concentrations keeping in mind our aims: conversion of G_7-CNP into shorter oligomers with an acceptable yield, prevention of chain elongation, expansion of the procedure onto a preparative scale, and isolation of desalted oligomers in satisfactory quantities. As shown in Table 4, the efficiency of the conversions of G_7-CNP at 10 °C in 100, 500, and 1000 mM inorganic phosphate buffers was almost identical (85–90%) with chain elongation neglected.

Separation of the oligosaccharides on a preparative scale.—The chromatographic method presented here for the separation of the $G_{3\rightarrow7}$-CNP homologues was based on size exclusion chromatography (SEC) using Toyopearl HW-40F chromatographic matrix. This matrix enabled the separation of products with a dp ranging from 1 to 7, but did not allow separation of the longer oligomers. The homologues were eluted in order of decreasing dp. Although the fractionation range of the matrix was between 10^2 and 10^4 for amino acids and globular proteins, we found that the upper molecular-weight limit for carbohydrates that have expanded molecular structures was considerably lower. This chromatographic matrix was very advantageous because the separation and the desalting of the maltooligomers could be performed in one step. The phosphate ions were eluted first from the column.

Since glycogen phosphorylase is an exotype enzyme, the Glc-1-P residues were released sequentially from the non-reducing end of the primer. Thereby, the utilisation of starting material can drop to 50–70% depending on the product ratio. However, this is still considered to be a noteworthy result since the chemical synthesis of these oligomers requires a multistep synthesis in moderate yields.

These results demonstrate convincingly the potential of glycogen phosphorylase b for the enzymatic synthesis of maltooligosaccharide glycosides in aqueous phase.

3. Experimental

Materials.—Buffer chemicals and other reagents (reagent grade) were obtained from Sigma-Aldrich Co. and acetonitrile (gradient grade) was purchased from Fisons. 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 [5]. Glycogen phosphorylase b was isolated from rabbit skeletal muscle. A glycerol suspension of crystallised enzyme was a gift from Professor V. Dombradi (activity: 1000 U mL^{-1}; protein content: 62 mg mL^{-1}).

General procedure for synthesis of CNP-oligomers.—Glycogen phosphorylase b (1000 U mL^{-1}, 10 μL) was added to a solution of G_7-CNP (6.5 mg, 5 mM) in phosphate buffer (1 M, 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 reaction was stopped by boiling for 1 min. After cooling, 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 incubation times and the mean values are given in Tables 1–4.

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 70 to 50%

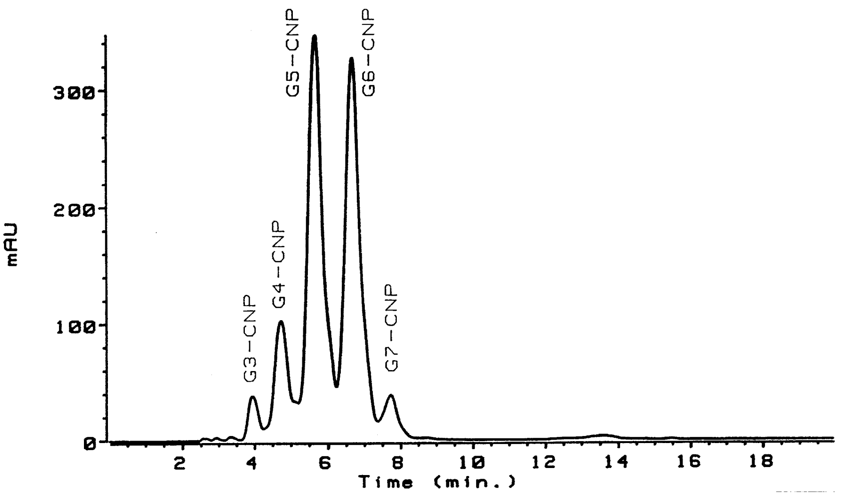

Fig. 1. HPLC profile of phosphorolysis products. Reaction conditions: 5 mM G_7-CNP, 40 mM phosphate buffer (pH 6.8), 0 °C, 50 min. For the HPLC conditions, see Section 3.

CH_3CN with water at a flow rate of 1 mL min^{-1}. Gradient time was 20 min. Effluent was monitored for CNP group at 302 nm and the reaction products were identified by using relevant standards. Quantitative results are given on the basis of peak area %. Fig. 1 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^{-1}, 100 μL) was added to a solution of G_7-CNP (65 mg, 5 mM) in phosphate buffer (100 mM, pH 6.8, 10 mL). Reaction mixture was incubated at 10 °C for 10 min and stopped by boiling for 5 min. After cooling, 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-40F (30–60 μm, 180 × 15 mm) column. Purified distilled water was used as the mobile phase at a flow rate with 1.5 mL min^{-1}. Analysis of fractions was achieved on TLC. TLC was performed on Kieselgel $60F_{254}$ (E. Merck) in 9:1 acetone–water. Compounds were detected under UV light or by charring with 50% H_2SO_4 in ethanol, and pure fractions were combined, lyophilised, and their structure was confirmed by HPLC and ^1H NMR (500 MHz). NMR data are in accordance with those in the chemical synthesis and have been published in Ref [5]. Fig. 2 shows the results of HPLC analysis and product recovery after SEC. Calculating with the release of G-1-P, the productivity of enzymatic synthesis results in 70–75% yield for G_4-, G_5-, and G_6-CNP oligomers. The yields obtained with the enzyme system are superior to those obtained with the chemical synthesis.

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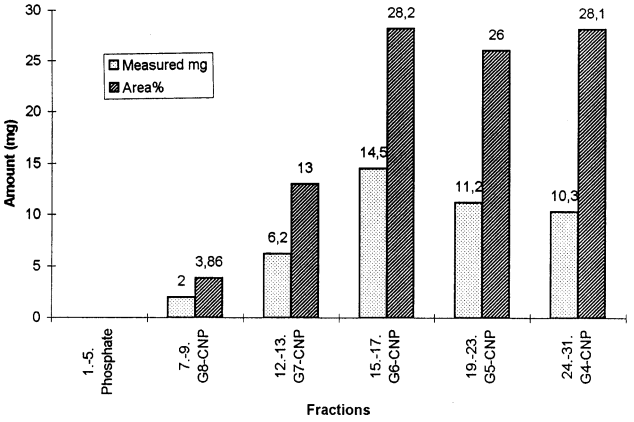

Fig. 2. HPLC and SEC separation of reaction products.

References

[1] J.F. Robyt, D. French, *J. Biol. Chem.*, 245 (1970) 3917–3918.

[2] E.H. Ajandouz, G.J. Marchis-Mouren, *Carbohydr. Res.*, 268 (1995) 267–277.

[3] L. Kandra, G. Gyémánt, E. Farkas, A. Lipták, *Carbohydr. Res.*, 298 (1997) 237–242.

[4] N. Saito, *J. Jpn. Soc. Starch Sci.*, 29 (1982) 153–160.

[5] E. Farkas, L. Jánossy, J. Harangi, L. Kandra, A. Lipták, *Carbohydr. Res.*, 303 (1997) 407–415.

[6] D.J. Graves, J.H. Wang, in P.D. Boyer (Ed.), *The Enzymes*, Vol. VIII, Academic Press, London, 1972, pp. 435–482.

[7] S.L. Haynie, G.M. Whitesides, *Appl. Biochem. Biotech.*, 23 (1990) 155–170.

[8] B. Pfannemüller, *Stärke*, 11 (1968) 341.

[9] C. Niemann, R. Nuck, B. Pfannemüller, W. Saenger, *Carbohydr. Res.*, 197 (1990) 187–196.

[10] H. Waldmann, D. Gygax, M.D. Bednarski, W.R. Shangraw, G.M. Whitesides, *Carbohydr. Res.*, 157 (1986) C4–C7.

[11] G. Ziegast, B. Pfannemüller, *Carbohydr. Res.*, 160 (1987) 185–204.

[12] M. Yoshida, N. Nakamura, K. Horikoshi, *Starch*, 49 (1997) 21–26.

[13] A. Percy, H. Ono, D. Watt, K. Hayashi, *Carbohydr. Res.*, 308 (1998) 423–429.

[14] E. Samain, C. Lancelon-Pin, F. Férigo, V. Moreau, H. Chanzy, A. Heyraud, H. Driguez, *Carbohydr. Res.*, 271 (1995) 217–226.

[15] K. Titani, A. Koide, J. Hermann, L.H. Ericsson, S. Kumar, R.D. Wade, K.A. Walsh, H. Neurath, E.H. Fischer, *Proc. Natl. Acad. Sci. USA*, 74 (1977) 4762–4777.

[16] J. Hajdu, K.R. Acharya, D.J. Stuart, P.J. McLaughlin, D. Barford, N.G.O. Oikonomakos, H. Klein, L.N. Johnson, *EMBO J.*, 6 (1987) 539–546.

[17] L.N. Johnson, E.A. Stura, M.S.P. Sansom, Y.S. Babu, *Biochem. Soc. Trans.*, 11 (1983) 142–144.

[18] D.H. Brown, C.F. Cori, *The Enzymes*, Vol. V, Academic Press, London, 1961, p. 207.

[19] V. Dombradi, *Int. J. Biochem.*, 13 (1980) 125–139.