Agricultural and Biological Chemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb19

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Published online: 08 Sep 2014.

To cite this article: Koichi Ogawa, Osamu Uejima, Teruo Nakakuki, Taichi Usui & Keiji Kainuma (1990) Enzymatic Synthesis of p-Nitrophenyl α-Maltoheptaoside by Transglycosylation of Maltohexaose-forming Amylase, Agricultural and Biological Chemistry, 54:3, 581-586

To link to this article: http://dx.doi.org/10.1080/00021369.1990.10869998

Agric. Biol. Chem., **54** (3), 581–586, 1990

Enzymatic Synthesis of *p*-Nitrophenyl α-Maltoheptaoside by Transglycosylation of Maltohexaose-forming Amylase

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Received July 17, 1989

An extracellular maltohexaose-forming amylase [EC 3.2.1.98] from *Klebsiella pneumoniae* mutant is a normal hydrolytic enzyme that hydrolyzes short-chain amylose ($\overline{DP}=23$) to give about 40% maltohexaose. Transglycosylation from maltoheptaose to the 4-position of *p*-nitrophenyl α-glucoside was efficiently induced through the use of maltohexaose-forming amylase in an aqueous methanol solution. The enzyme specifically produced *p*-nitrophenyl α-maltoheptaoside (13% of the *p*-nitrophenyl α-glucoside) from maltoheptaose as a donor and *p*-nitrophenyl α-glucoside as an acceptor. The yield of *p*-nitrophenyl α-maltoheptaoside depended on the concentration of methanol solvent, the pH, and temperature. Furthermore, the use of the aqueous methanol system in the reaction not only improved the solubility of *p*-nitrophenyl α-glucoside but also greatly increased the formation of *p*-nitrophenyl α-maltoheptaoside, which is a useful substrate for assay of human amylase in serum and urine.

The measurement of α-amylase activity in serum, urine, or other body fluids has been widely used in clinical laboratories for the diagnosis of pancreatic disorders.[1-3] In recent years, maltooligosaccharides or their *p*-nitrophenyl derivatives having defined structures have been used as substrates for the measurement of α-amylase activity in serum.[4,5] pNP-α-G_7 is one such substrate.[6] However, organic synthetic methods for these compounds are cumbersome and impractical for industrial production. Wallenfels *et al.*[7] have enzymatically synthesized pNP-α-G_7 from α-cyclodextrin and pNP-α-G_1, although it was difficult to obtain pure-grade pNP-α-G_7. Recently, Usui and Murata have reported that the enzymatic synthesis of *p*-nitrophenyl α-maltopentaoside can be done by transglycosylation of maltotetraose-forming amylase from *Pseudomonase stutzeri*.[8] By the same method, we tried to prepare pNP-α-G_7 using G_6-forming amylase. The enzyme was discovered in 1971 by Kainuma *et al.*[9] as a cell-bound enzyme from *Klebsiella pneumoniae* and they reported that the enzyme had a transferase activity.[10] After that, Nakakuki *et al.*[11,12] found that an ultraviolet-induced mutant from *Klebsiella pneumoniae* produced an extracellular G_6-forming amylase. Furthermore, Monma *et al.*[13] described the formation of maltohexaose from maltotetraose by the transfer action of the extracellular en-

Abbreviations: $\overline{DP}$, average degree of polymerization; DP, degree of polymerization; pNP-α-G_1, *p*-nitrophenyl α-glucoside; G_6-forming amylase, maltohexaose forming amylase; pNP-α-G_7, *p*-nitrophenyl α-maltoheptaoside; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance spectroscopy; TPS, 3-(trimethylsilyl)propanesulfonate sodium salt; IU, one International unit (IU) is defined by the International Commission on Enzmes as the amount of enzyme that hydrolyzes one μmol of glycosidic bonds per minute under optimal conditions.

zyme.

This paper describes a useful enzymatic synthesis of pNP-α-G_7 using the transfer activity of the enzyme in an aqueous methanol system.

Materials and Methods

Materials. G_6-forming amylase from a *Klebsiella pneumoniae* mutant was kindly donated by Kirin Brewery Co., Ltd. Pure-grade maltoheptaose was prepared by Nihon Shokuhin Kako Co., Ltd. A series of *p*-nitrophenyl α-D-maltooligosaccharides (DP, 2–7) was purchased from Calbiochem-Behring Corp. Other reagents were guaranteed reagent grade.

Amylase assay. G_6-forming amylase was assayed using a substrate solution of 0.5 ml of 0.4% reduced short-chain amylose and 0.4 ml of 0.1 M phosphate buffer (pH 7.0). After incubation of the substrate solution for 3 min at 40°C, 0.1 ml of suitably diluted enzyme solution was added, and the reducing sugars released by the enzyme action for 30 min was measured by the Somogyi–Nelson method.[14,15]

Analytical methods. The saccharide composition of G_6-forming amylase reaction products under various conditions were identified by HPLC. The HPLC system consisted of a Hitachi 655A-11 pump, a Shimazu SPD-6A UV spectrophotometric detector, a Hitachi 655A-30 RI monitor, and a Hitachi D-2000 integrator. The reaction mixtures were eluted from a column (4.0 mm × 25 cm) packed with TSK-Gel NH_2-60 (Toso Co., Ltd.) at a flow rate of 1.0 ml/min with water–acetonitrile (1 : 4, v/v). The elution was monitored at 310 nm for *p*-nitrophenyl residue and by differential refractometer for the carbohydrate content. ^{13}C- and ^1H-NMR spectra were taken with a JOEL FX-90 spectrometer operating at 22.5 MHz in the pulsed fourier-transform mode with complete proton decoupling and at 90 MHz, respectively. Chemical shifts are expressed in ppm relative to TPS as an internal standard.

Results and Discussion

Enzymatic synthesis

Maltohexaose (520 mg) and pNP-α-G_1 (130 mg) were dissolved in 10 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 30% methanol. They were incubated with 2.0 IU of G_6-orming amylase at 35°C for 6 hr. After the reaction was stopped by the addition of two volumes of 0.2 M acetate buffer (pH 3.5), the reaction mixture was evaporated. The concentrated solution (3 ml) was put on a Toyopearl HW-40S column. Elution was monitored at 310 nm for *p*-nitrophenyl residue and by differential refractometer for the carbohydrate content as in Fig. 1. Peaks 1, 2, and 3 were found to contain the high-molecular weight oligosaccharides, maltoheptaose + maltohexaose, and glucose, respectively. The chromatograph of the absorbance at 310 nm shows two main peaks. Some small peaks around FI were presumed to be *p*-nitrophenyl derivatives of maltooligosaccharides. The first peak (FI) was collected and freeze-dried to give a yield of 68 mg. The second peak (FII) contained recovered pNP-α-G_1 used as an acceptor. Therefore, FI was expected to be the product of the enzymatic transglycosylation.

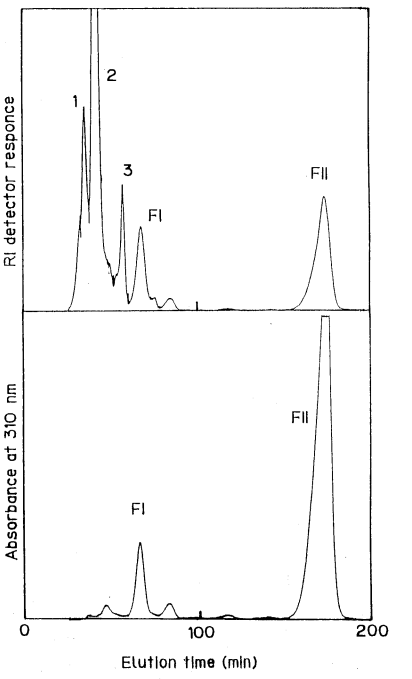

Fig. 1. Toyopearl HW-40S Chromatographic Separation of Incubation Mixture.

The reaction was done as described in *Enzymatic synthesis*. Chromatography was done on a column (2.2 × 95 cm) of Toyopearl HW-40S at room temperature. The column was eluted with water–methanol (v/v, 3 : 1) at a flow rate of 310 ml/hr. 1, high molecular weight oligosaccharides; 2, maltoheptaose and maltohexaose; 3, glucose.

Table I. CARBON-13 CHEMICAL SHIFTS[a] OF pNP-α-G_7 IN D_2O SOLUTION

Compound	Residue or group	C-1	C-2	C-3	C-4	C-5	C-6	*m*-ph	*o*-ph	*p*-ph	C-ph[b]
pNP-α-G_7	Glycoside residue	99.23	73.45	76.08	79.73	74.32	63.16				
	Internal residue	102.98	74.03	76.08	80.08	74.32	63.16				
	Terminal residue	102.98	74.32	75.49	72.08	75.64	63.16				
	p-Nitrophenyl group							119.50	128.76	144.95	164.20

[a] In ppm downfield from TPS.
[b] Phenyl carbon attached to the phenolic oxygen.

Characterization

HPLC analysis of FI showed only a single peak, at a retention time consistent with that of a standard sample of pNP-α-G_7. ^{13}C- and ^1H-NMR were used for further elucidation of the structure of this fraction. The spectral data could be superimposed on those of authentic pNP-α-G_7. The carbon resonances of the oligomer were easily assigned by comparing their spectra with the earlier data for the *p*-nitrophenyl α-maltopentaoside[8] as listed in Table I. The ^1H-NMR spectra in D_2O at 80°C showed a glycosidic signal at 5.82 (H-1) ppm and two anomeric signals at 5.39–5.37 (other H-1) involving linkages. The rate of intensities of 5.37–5.39 ppm and 5.82 ppm was 6 : 1. The coupling constant $J_{1,2}$ of each signal was near 3.4 Hz, indicating the cis configuration. This was indicated the enzymatic products contained only α-1,4-glycosidic linkages. From these data, FI was identified as pNP-α-G_7. The yield of the product was about 13% of pNP-α-G_1 as an acceptor.

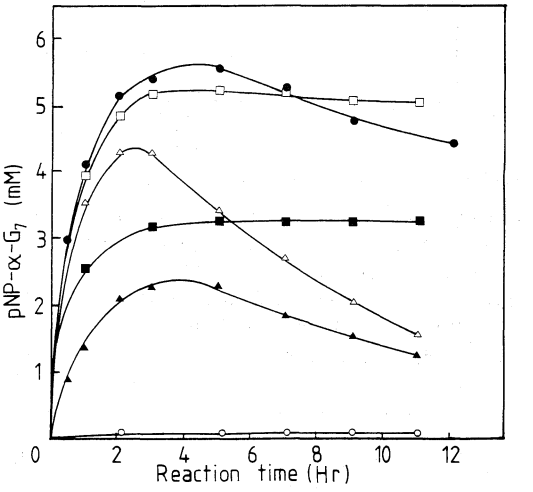

Fig. 2. Effects of Methanol Concentrations on pNP-α-G_7 Production.

Maltoheptaose (520 mg) and pNP-α-G_1 (130 mg) in 10 ml of 20 mM Tris–HCl buffer (pH 8.0) containing various methanol concentrations (0%, ▲; 20%, △; 30%, ●; 35%, □; 40%, ■ and 50%, ○) were incubated with the enzyme (2.0 IU) at 35°C, and samples were taken during the incubation for analysis by HPLC.

Effects of methanol concentration, pH, temperature, and some solvents on transglycosylation

To investigate the transglycosylation mechanism, at first the pNP-α-G_7 production in various methanol concentrations in methanol-aqueous systems was examined by HPLC. At higher methanol concentrations up to 40%, transglycosylation was efficient and the pNP-α-G_7 production was greatly dependent on methanol concentration as shown in Fig. 2. In this case, the pNP-α-G_7 production at 30% methanol was the largest and that the yield was twice as large as in the absence of methanol. At 50% methanol, the enzyme seemed to be inactivated, because both reactions of transglycosylation and hydrolysis were never observed by HPLC. In this system, to accelerate the transglycosylation, it was necessary to use a high methanol concentration to the extent that the enzyme could be active.

Figure 3 shows the effects of pH on the transglycosylation reaction. At pH 8.0, the pNP-α-G_7 production was the largest, while the enzyme was completely inactivated at pH 4.0.

The courses of pNP-α-G_7 production from initial maltoheptaose and pNP-α-G_1 substrates

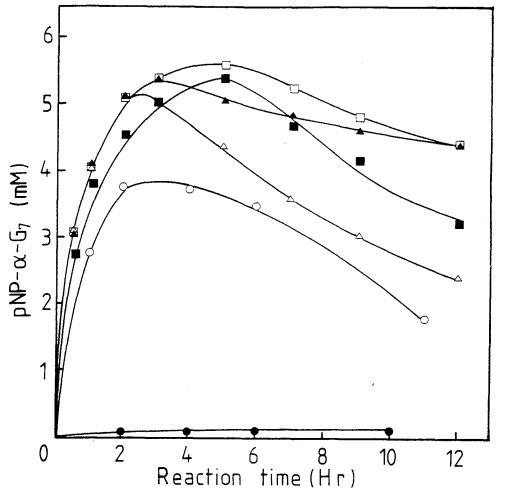

Fig. 3. Effects of pH on pNP-α-G_7 Production.

Conditions were the same as those in Fig. 2. The substrates were incubated at 35°C in acetate buffer (pH 4.0, ●), 20 mM acetate buffer (pH 5.0, ○), 20 mM phosphate buffer (pH 6.0, △), 20 mM phosphate buffer (pH 7.0, ▲), 20 mM Tris–HCl buffer (pH 8.0, □) or 20 mM Tris–HCl buffer (pH 9.0, ■) containing 30% methanol.

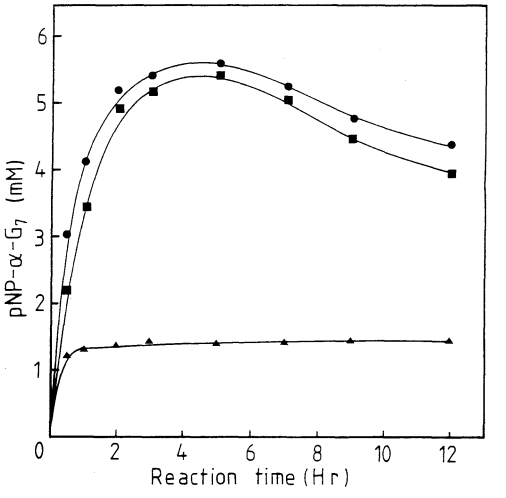

Fig. 4. Effects of Temperature on pNP-α-G_7 Production.

Conditions were the same as those in Fig. 2. The substrates were incubated at 25°C (■), 35°C (●) and 45°C (▲) in 10 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 30% methanol.

incubated at 25, 35, and 45°C were followed by HPLC. As shown in Fig. 4, the transglycosylation activity at 35°C was the largest, while that at 45°C was apparently lower. It seemed

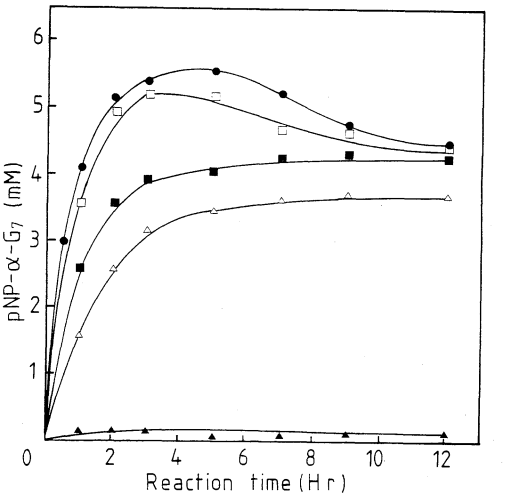

Fig. 5. Effects of Various Solventls on pNP-α-G_7 Production.

Conditions were the same as those in Fig. 2. The substrates were incubated at 35°C in 10 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 30% methanol (●), ethanol (□), 1-propanol (▲), 2-propanol (■), and dimethyl sulfoxide (△).

to be considerably inactivated at 45°C.

Figure 5 shows the effects of various hydrophilic organic solvents on the transglycosylation reaction. The transglycosylation activity was examined in various aqueous–organic solvent systems as mentioned above. When methanol and ethanol were used, the pNP-α-G_7 production was apparently larger than that of other solvents such as 2-propanol and dimethyl sulfoxide. On the contrary, the use of 1-propanol inactivated the enzyme.

Furthermore, the effects of molar ratios of maltoheptaose (donor) and pNP-α-G_1 (acceptor) on the pNP-α-G_7 production were examined in the aqueous-methanol system as shown in Fig. 6. The maximum molar ratio, which means the maximum production of pNP-α-G_7 (mM) obtained to maltoheptaose (mM) during the reaction, decreased with increasing the molar ratio of maltoheptaose to pNP-α-G_1 used, while that of pNP-α-G_7 obtained to pNP-α-G_1 increased. However, the yield of pNP-α-G_7 production as a dry solid was the largest when molar ratio of donor to acceptor was 1 : 1.

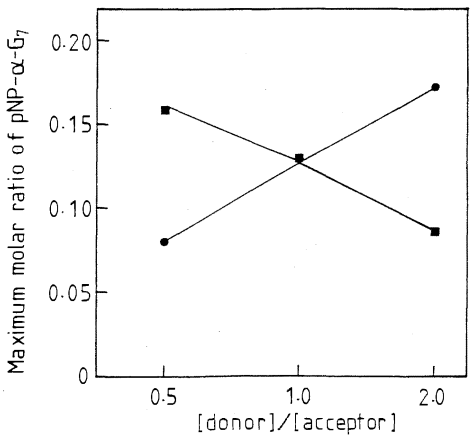

Fig. 6. Effects of Molar Ratio of Donor (Maltoheptaose) and Acceptor (pNP-α-G_1) on pNP-α-G_7 Production.

Maltoheptaose (260, 520, and 1040 mg) and pNP-α-G_1 (130 mg) were dissolved in 10 ml of 20 mM Tris–HCl buffer (pH 8.0) containing 30% methanol. Reaction conditions are described in *Enzymatic Synthesis*. ■—■, maximum molar ratio of pNP-α-G_7 obtained to maltoheptaose; ●—●, maximum molar ratio of pNP-α-G_7 obtained to pNP-α-G_1.

Proposed transglycosylation mechanism of G_6-forming amylase

As described above, the transglycosylation was achieved efficiently under moderate conditions of methanol concentration, pH, and temperature. The main transfer action mode of G_6-forming amylase on maltoheptaose and pNP-α-G_1 may be summarized as follows:

$$\text{GGGGGGG}^* + \text{E} \rightleftharpoons \text{GGGGGG}\cdot\text{E} + \text{G}^* \quad (1)$$

$$\text{GGGGGG}\cdot\text{E} + \text{pNP-}\alpha\text{-G}_1 \rightleftharpoons \text{GGGGGGG-pNP} + \text{E} \quad (2)$$

$$\text{GGGGGGG-pNP} + \text{E} + \text{H}_2\text{O} \longrightarrow \text{GGGGGG} + \text{pNP-}\alpha\text{-G}_1 + \text{E} \quad (3)$$

(G = glucose; E = G_6-forming amylase; pNP-α-G_1 = *p*-nitrophenyl α-glucoside; G* = reducing-end glucose)

At the initial reaction, maltoheptaose is attacked by the enzyme to give glucose and enzyme-bound maltohexaose, and the bound maltohexaose at the catalytic site of the enzyme is subsequently transferred to the pNP-α-G_1 to release pNP-α-G_7 (1 and 2). Also, it was presumed that the prolonged reaction reduced the production of pNP-α-G_7 by the hydrolysis reaction of the enzyme as shown in equation 3. Furthermore, the solubility of pNP-α-G_1 which was about 1% in the absence of methanol increased by about 3% by the addition of methanol. As mentioned above, the use of appropriate concentrations of methanol not only improved the solubility of pNP-α-G_1 and decreased the water content in the reaction mixture but also resulted in a great increase in the formation of pNP-α-G_7. As reported by Usui and Murata,[8] the addition of methanol in this system may be important in strengthening enzyme-enzyme interaction by changing the enzyme conformation or the solvation environment such as the increase of hydrophobicity and the decrease of dielectric constant on the surface of the enzyme. In this enzymatic reaction system, pNP-α-G_7 was produced from maltoheptaose and pNP-α-G_1 by transglycosylation with a relatively high yield. Therefore, the enzymatic reaction provides a novel and practical technique for the industrial production of pNP-α-G_7, which is a useful substrate for the assay of α-amylase.

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