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Note

Maltotetraose-forming, amylase-mediated, *p*-nitrophenyl α- and β-maltopentaoside formation in an aqueous–organic solvent system: a substrate for human amylase in serum*

Koichi Ogawa[†], Takeomi Murata, and Taichi Usui[‡]
Department of Applied Biological Chemistry, Faculty of Agriculture, Shizuoka University, Shizuoka, Shizuoka 422 (Japan)

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In recent years, malto-oligosaccharide derivatives of defined structure have been used in clinical laboratories[1–6] for the measurement of alpha-amylase activity in human serum and urine. One such chromogenic substrate[2,7] is pNP-α-G_5. However, conventional methods for obtaining pNP-α-G_5, either chemically or enzymically, give only low yields of the desired compound[8,9]. We have reported that G_4-amylase from *Pseudomonas stutzeri* forms pNP-α-G_5 from maltopentaose and pNP-α-G_1 through a transglycosylation reaction in aqueous methanol[10]. We report here an efficient enzymic synthesis of pNP-α-G_5 in improved yield by utilizing the transglycosylation of G_4-amylase in a buffer medium containing Me_2SO at high concentration. A control experiment for pNP-β-G_5 production by the enzyme was performed similarly.

The enzymic synthesis of pNP-α-G_5 was as follows. To a solution of maltopentaose (120 mg) and pNP-α-G_1 (84 mg) in 1 mL of 15mM phosphate buffer (pH 9.0) containing 50% Me_2SO was added 1.9 U of G_4-amylase and the mixture was incubated for 8 h at 40°. The reaction was then stopped by the addition of two volumes of 0.2M acetate buffer (pH 3.5) and the mixture was evaporated to a syrup under diminished pressure (0.1 Torr) at 50°. The syrup was dissolved in 2 mL of 3:1 (v/v) water–methanol and put on a column (2.2 x 95 cm) of Toyopearl HW-40S. Elution was monitored by measuring the *p*-nitrophenyl-group absorbance at 295 and 485 nm, and the carbohydrate content was determined by the phenol–sulfuric acid method. The eluate was collected in 7-mL fractions. The chromatogram showed two main peaks (F-I and F-II), for which the absorption at 295 nm coincided with that at 485 nm. The first peak (F-I, tube numbers 46–52) was concentrated and lyophilized to give 26 mg of product, $[\alpha]_D^{25}$

* Abbreviations: G_4-amylase, maltotetraose-forming amylase from *Pseudomonas stutzeri*; pNP-α- or β-G_1, *p*-nitrophenyl α- or β-D-glucopyranoside; pNP-α-G_2, *p*-nitrophenyl α-maltoside; pNP-α-G_4, *p*-nitrophenyl α-maltotetraoside; pNP-α or β-G_5, *p*-nitrophenyl α- or β-maltopentaoside; Me_2SO, dimethyl sulfoxide.

† Present address: Research Laboratory of Nihon Shokuhin Kako Co., Ltd., 30 Tajima, Fuji, Shizuoka 417, Japan.
‡ To whom correspondence should be addressed.

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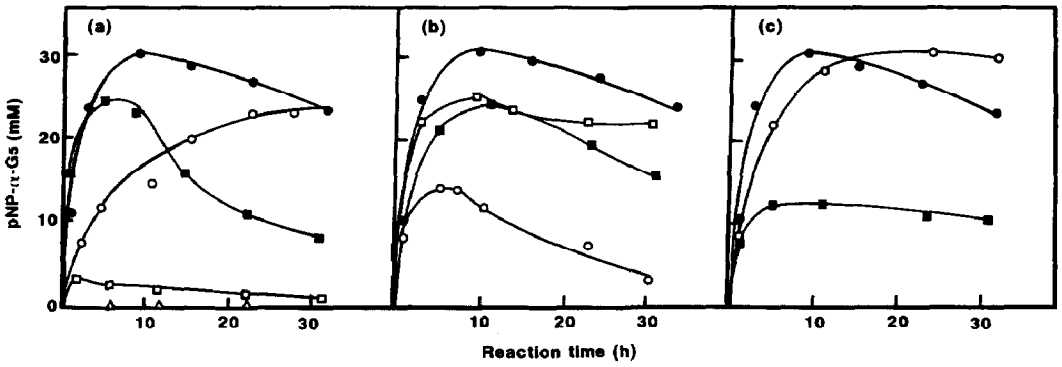

Fig. 1. Effects of Me_2SO concentrations, pH, and temperature on pNP-α-G_5 production: (*a*) The enzyme reaction was performed with maltopentaose (120 mg) and pNP-α-G_1 (84 mg) at 40° in 15mM phosphate buffer (pH 9.0) containing different Me_2SO concentrations (0%, □; 40%, ■; 50%, ●; 60%, ○; and 70%, △). G_4-amylase (1.9U) was added and samples were taken during incubation for analysis by h.p.l.c. (*b*) Substrates were dissolved in 1 mL of phosphate buffer (pH 8.0, □; pH 9.0, ●) and glycine–NaOH buffer (pH 9.0, ○; pH 10.0, ■) containing 50% Me_2SO at 40°, respectively. Other conditions were the same as those in (*a*). (*c*) Substrates were dissolved in 1 mL of phosphate buffer (pH 9.0) containing 50% Me_2SO at 30 (○), 40 (●), and 50° (■), respectively. Other conditions were the same as those in (*a*).

+210.9 (*c* 2.4, water). The yield of this product was ~24% of the amylase-catalyzed net decrease of maltopentaose. The second peak (F-II, tube numbers 90–98) contained recovered pNP-α-G_1 used as an acceptor. F-I showed only a single peak on h.p.l.c., indistinguishable from a standard sample of pNP-α-G_5. The ^{13}C- and ^1H-n.m.r. spectra could be superposed on those of authentic pNP-α-G_5 reported in our previous paper[10]. The f.a.b.-mass spectrum of F-I was also consistent with that[10] of pNP-α-G_5.

The effects of various conditions on G_4-amylase-mediate, pNP-α-G_5 formation at high substrate concentration were investigated by h.p.l.c. (Fig. 1). The efficiency of transglycosylation was greatly dependent on the concentration of Me_2SO, the pH, and the temperature. Use of a water–Me_2SO system in this reaction not only ensured a sufficient solubility of pNP-α-G_1 but also resulted in a remarkable increase in the formation of pNP-α-G_5. Thus, pNP-α-G_1 shows much higher solubility (8.4%) in a medium containing 50% Me_2SO than that (2.8%) in the presence of 50% methanol. The maximum production of pNP-α-G_5 at 50% Me_2SO was ~2-fold that at 50% methanol reported previously[10], and indicates that the yield of pNP-α-G_5 is directly influenced by the solubility of pNP-α-G_1 in an aqueous–organic solvent system. As a result, we prepared pNP-α-G_5 by taking into account the efficiency of the desired compound on the transglycosylation already mentioned.

Our method for the enzymic synthesis of pNP-α-G_5 was also employed to prepare pNP-β-G_5 by using G_4-amylase from maltopentaose and pNP-β-G_1 in an aqueous–organic solvent system. For production of pNP-β-G_5, the use of aqueous methanol ensured a sufficient solubility of pNP-β-G_1, which is more soluble (25.3%) in 50% aqueous methanol than its solubility (12.6%) in 50% Me_2SO. Thus, to a solution of maltopentaose (348 mg) and pNP-β-G_1 (253 mg) in 1 mL of 15mM phosphate buffer (pH

TABLE I

^{13}C-Chemical shifts of pNP-β-G_5 in D_2O solution

Residue	α-*Glc*p-(*1→4*)-α-*Glc*p-(*1→4*)-α-*Glc*p-(*1→4*)-α-*Glc*p-(*1→4*)-β-*Glc*p-*pNP* *V* *IV* *III* *II* *I* *P*									
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	m-*Ar*	o-*Ar*	p-*Ar*	c-*Ar*[a]
I	102.13	75.25	78.54	79.53	77.62	63.18				
II	102.45	73.98	75.65[b]	79.80	74.21	63.15				
III	102.61	73.98	76.06	80.07	74.30	63.15				
IV										
V	102.66	74.50	76.06	72.04	75.45[b]	63.24				
P							119.25	128.82	145.13	164.53

[a] Phenyl carbon attached to the phenolic oxygen. [b] Assignment may be interchanged.

7.0) containing 50% methanol was added 0.9 U of G_4-amylase and the mixture was kept for 26 h at 30°. The reaction was stopped by the addition of two volumes of 0.2M acetate buffer (pH 3.5) and the mixture was evaporated to a syrup. The syrup was dissolved in 2 mL of 3:1 (v/v) water–methanol and loaded onto a column of Toyopearl HW-40S according to the same procedure as that used in the separation of pNP-α-G_5. The first peak (F–I′, tube numbers 52–62) was concentrated and lyophilized to give 172 mg of product, $[\alpha]_D^{25}$ +91.2 (*c*, 2.5 water). The yield of this product was ~54% of the amylase-catalyzed net decrease of maltopentaose. The second peak (F-II′, tube numbers 99–109) contained recovered pNP-β-G_1 used as an acceptor. On h.p.l.c., F-I′ showed only a single peak having a retention time of a standard sample of pNP-β-G_5. The f.a.b.-m.s. spectrum showed two major ions in the high-mass region at *m/z* 950 and 972, consistent with $[M + H]^+$ and $[M + Na]^+$, respectively; ^{1}H-n.m.r. (H_2O): δ 5.21 (d, 1 H, *J* 7.7 Hz), 5.38 (d, 1 H, *J* 3.5 Hz), 7.20 (d, 2 H, *J* 8.8 Hz, *o*-Ph), and 8.19 (d, 2 H, *J* 9.2 Hz, *m*-Ph). The ^{13}C-n.m.r. data are shown in Table I.

Anal. Calc. for $C_{36}H_{55}NO_{28}\cdot 0.5H_2O$: C, 44.67; H, 5.89; N, 1.44. Found: C, 44.52; H, 5.89; N, 1.26.

The rate of pNP-β-G_5 production was also dependent on the concentration of methanol, the pH, and the temperature (Fig. 2). The maximum pNP-β-G_5 production at 50% methanol was ~1.6-fold of that in the presence of 50% Me_2SO (data not shown). Thus, an aqueous-methanol system was more suited for pNP-β-G_5 production than that of an aqueous-Me_2SO system.

In conclusion, the efficiency of G_4-amylase-catalyzed transglycosylation is greatly influenced by the solubility of the acceptor molecule (pNP-α-G_1 or pNP-β-G_1) in an aqueous–organic solvent rather than any solvent effect. The increased solubility of

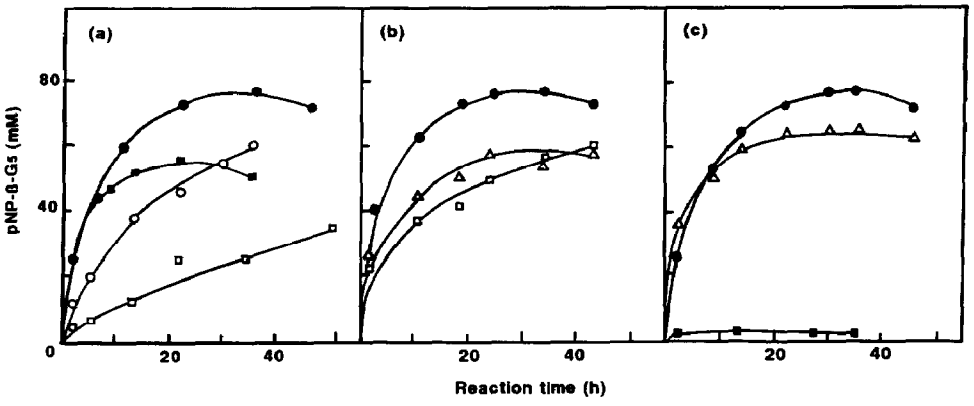

Fig. 2. Effects of methanol concentrations, pH, and temperature on pNP-β-G_5 production: (*a*) The enzyme reaction was performed with maltopentaose (348 mg) and pNP-β-G_1 (253 mg) at 30° in 15 mM phosphate buffer (pH 7.0) containing different methanol concentrations (40%, ■; 50%, ●; 60%, ○ and 70%, □). G_4-amylase (1.9U) was added and samples were taken during incubation for analysis by h.p.l.c. (*b*) Substrates were dissolved in 1 mL of phosphate buffer (pH 6.0, △; pH 7.0, ●; pH 8.0 □) containing 50% methanol at 30°, respectively. Other conditions were same as those in (*a*). (*c*) Substrates were dissolved in 1 mL of phosphate buffer (pH 9.0) containing 50% methanol at 30 (●), 40 (△), and 50° (■), respectively. Other conditions were same as those in (*a*).

pNP-α-G_1 in aqueous Me_2SO facilitated the production of pNP-α-G_5 in comparison with aqueous methanol, whereas the reverse was true for production of pNP-β-G_5. This enzymic method not only provides a useful technique for the preparations of pNP-α-G_5, but also for that of other chromogenic oligosaccharide analogues.

EXPERIMENTAL

Materials. — G_4-amylase from the culture filtrates of *P. stutzeri* NRRL B 3389 mutant[11] was prepared by precipitation with $(NH_4)_2SO_4$ followed by adsorption on starch granules[12]. A series of *p*-nitrophenyl α- or β-malto-oligosaccharides (degree of polymerization, 2–5) were purchased from Calbiochem-Behring Corp. Other reagents were guaranteed reagent grade.

General methods. — H.p.l.c. was performed with a YMC Packed Column Type A-014 (SIL) (6.0 mm x 30 cm) in a Shimadzu LC-3A liquid chromatograph equipped with a Shimadzu SPD-6A u.v. detector. Elution was effected with 1:4 (v/v) water–MeCN at a flow rate of 1.0 mL/min. The ^{13}C- and ^1H-n.m.r. spectra were recorded in D_2O using 1% oligosaccharide solution in 5-mm tubes at room temperature with a Jeol-GSX-400 spectrometer operating at 100 MHz in the pulsed Fourier-transform mode with complete proton decoupling. Chemical shifts are expressed in p.p.m. relative to sodium 4,4-dimethyl-4-silapentanesulfonate (TPS) as the internal standard. F.a.b.-m.s. measurements of oligosaccharides were recorded with a Jeol DX-303 HF mass spectrometer, operating at the full accelerating potential (3 kV) and coupled to a Jeol DA-500 mass data system. The sample (3 μL) in distilled water was added to the glycerol matrix, and 0.1N HCl (1 μL) was added. The molecular weight of the sample was estimated from the *m/z* value of the quasimolecular ion $(M + H)^+$ peak. Specific rotations were determined with a Digital Automatic Polarimeter PM-101 (Union Giken Corp., Ltd.). Elemental analyses were performed with a Perkin–Elmer 240C apparatus.

Amylase assay. — The G_4-amylase was assayed as follows. A mixture containing 10 mg of soluble starch in 0.2 mL of 50mM acetate buffer (pH 6.0) and an appropriate amount of enzyme in a total volume of 1.0 mL was incubated for 15 min at 40°. The reducing sugar released by the enzymic action was measured by the Somogyi–Nelson method[13,14]. One unit is defined as the amount of enzyme liberating one μmol of reducing sugar equivalent as glucose per minute under the assay conditions.

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