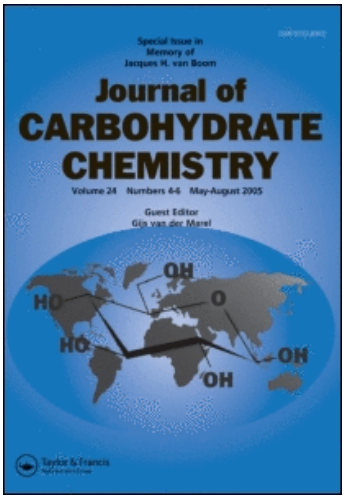

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MALTOPENTAOSE-FORMING AMYLASE-MEDIATED *p*-NITROPHENYL α-MALTOHEXAOSIDE FORMATION IN AN AQUEOUS-ORGANIC SOLVENT SYSTEM :A SUBSTRATE FOR HUMAN AMYLASE IN SERUM

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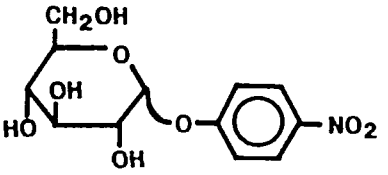
ABSTRACT

A maltopentaose-forming amylase [EC 3.2.1.1] from *Bacillus licheniformis* effectively produced *p*-nitrophenyl α-maltohexaoside (**3**) through a transglycosylation reaction from maltohexaose as a donor and *p*-nitrophenyl α-glucoside (**1**) as an acceptor in an aqueous solution containing hydrophilic organic solvents. The enzyme specifically transferred from maltohexaose to the 4-position of **1**. The yield of **3** depended on the kind of donor, concentration of solvent, pH, and temperature. By the addition of solvents such as dimethyl sulfoxide and 1-butanol, respectively, effects of acceleration on transferase activity of the enzyme and improvement of acceptor solubility were achieved, and resulted in a great increase in the formation of **3**.

INTRODUCTION

The activity of α-amylase in human serum and urine has been measured using native or modified polysaccharides in clinical laboratories for the diagnosis of pancreatic disorders.[1-4] In recent years, *p*-nitrophenyl derivatives of maltooligosaccharides having defined structure have been used as substrates for the measurement of α-amylase activity in serum.[5-10] *p*-Nitrophenyl α-maltopentaoside (**2**), *p*-nitrophenyl α-maltohexaoside (**3**) and *p*-nitrophenyl α-maltoheptaoside (**4**) are useful as such

chromogenic substrates. Normal organic chemical methods for obtaining these compounds have been developed,[11,12] but these



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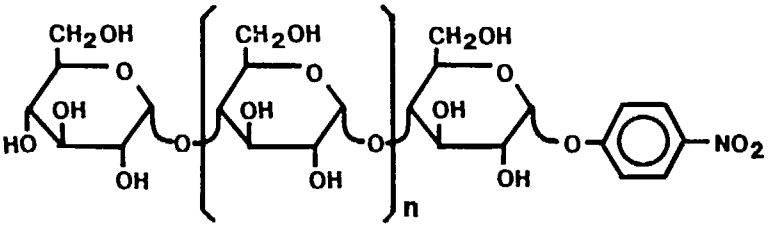

2: n=3 3: n=4 4: n=5

compounds were complicated by multiple protection and deprotection steps and by different problems in regioselectivity. Wallenfels *et al.* have enzymatically synthesized *p*-nitrophenyl α-maltooligosaccharides by cyclomaltodextrin glucanotransferase [EC 2.4.1.16],[13] although it was difficult to isolate pure-grade desired compounds from by-products such as homologous series of *p*-nitrophenyl α-maltooligosaccharides. Recently, we have reported that maltotetraose-forming amylase [EC 3.2.1.60] from *Pseudomonas stutzeri* (G_4-amylase) effectively produced 2 from maltopentaose (donor) and *p*-nitrophenyl α-glucoside (1) (acceptor) in an aqueous solution containing dimethyl sulfoxide or methanol.[14,15] Based on the same procedure, we have further prepared 4 from maltohexaose (donor) and 1 (acceptor) by transglycosylation of maltohexaose-forming amylase [EC 3.2.1.98] from *Klebsiella pneumoniae* in aqueous-methanol or -ethanol buffer systems.[16] This paper describes an efficient and simple procedure for preparation of 3 utilizing the transferase activity of maltopentaose-forming amylase [EC 3.2.1.1] from

Bacillus licheniformis (G_5-amylase), which is generally known as a representative thermostable α-amylase,[17] in a buffer containing organic solvent.

RESULTS AND DISCUSSION

The G_5-amylase-catalyzed transglycosylation in an aqueous-dimethyl sulfoxide solvent system was efficient, allowing accumulation of 3 in preparative amounts as described in the EXPERIMENTAL section. Purification of the transglycosylation product by column chromatography was straightforward, since the transglycosylation was stereospecific and no anomerisation of the acceptor glycoside occurred. Thus, the reaction was performed in one-pot synthesis of 25 mg of 3 from 171 mg of maltohexaose and 52 mg of 1. The yield of 3 was approximately 13% of the amylase-catalyzed net decrease of maltohexaose. Conditions for maximal yield of the desired compound were established by investigating the effects of various conditions on transglycosylation as shown below.

Effect of pH, Temperature and Donors on Production of *p*-Nitrophenyl α-Maltohexaoside (3) in an Aqueous solution. G_5-amylase caused considerable transglycosylation in an aqueous solution in contrast with G_4-amylase-mediated transglycosylation at high concentration of organic solvent reported previously.[14,15] The effects of various conditions on the transglycosylation in an aqueous medium were investigated by HPLC. However, the solubility of 1 was only about 1.5% in an aqueous medium at 35 °C. Figure 1 shows effects of pH, and temperature on 3 production in an aqueous solution. The maximum rate of 3 production was obtained at pH 8-9, and at 35-45 °C. Effects of donors such as a series of maltooligosaccharides (DP = 2~7) and short-chain amylose (average DP = 17) on 3 production were examined as in Fig. 2. The maximum production of 3 using maltohexaose as a donor, which is a minimum substrate for the enzyme hydrolysis, was apparently larger than that using maltoheptaose, while the enzyme showed only a little transferase activity on short-chain amylose. On the contrary, 3

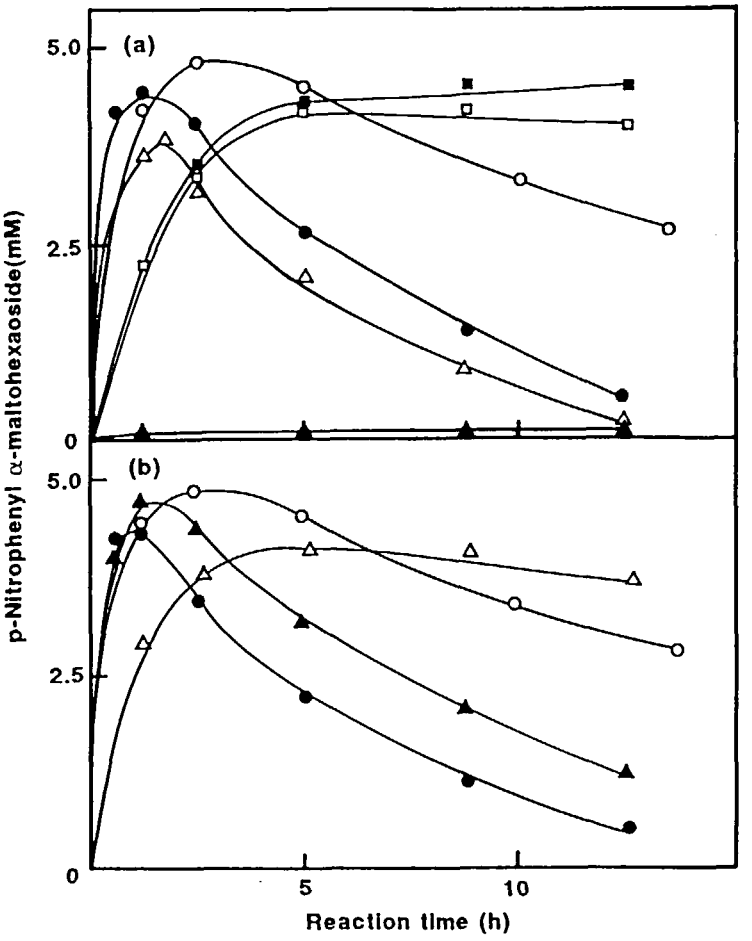

Fig. 1. Effects of pH and temperature on 3 production in an aqueous solution : (a) The enzyme reactions were performed with maltohexaose (42.8 mg) and 1 (13 mg) at 35 °C in 1 mL of 15 mM citrate buffer (pH 3.0, ▲), acetate buffer (pH 5.0, △), phosphate buffer (pH 7.0, ●), Tris-HCl buffer (pH 8.0, ○ ; 9.0, ■), and glycine-NaOH buffer (pH 10.0, □). The amylase (2.0 U) was added and samples were taken during incubation for analysis by HPLC. (b) Substrates were dissolved in 1 mL of 15 mM Tris-HCl buffer (pH 8.0) at 25 °C (△), 35 °C (○), 45 °C (▲), and 55 °C (●). Other conditions were the same as those in (a).

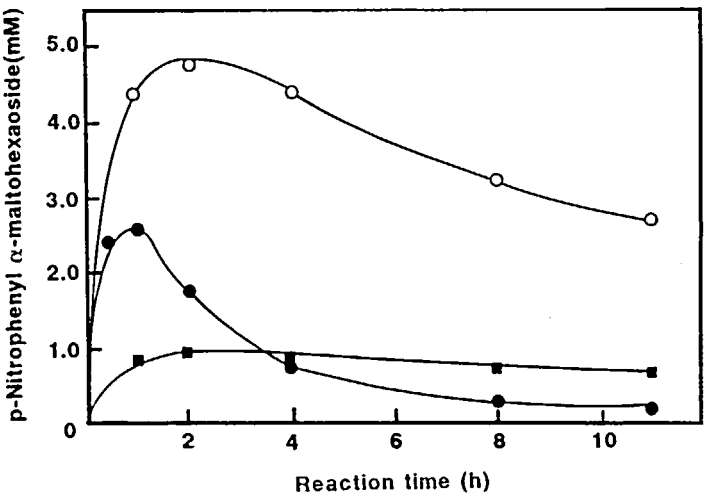

Fig. 2. Effects of donors on 3 production. The enzyme reactions were performed with various donors and 1 (molar ratio, donor : 1 = 1:1) at 35 °C in 1 mL of 15 mM Tris-HCl buffer (pH 8.0). Other conditions were the same as those in Fig. 1. Symbols were maltohexaose (○), maltoheptaose (●), and short-chain amylose (■).

production was never observed when using maltose, maltotriose, maltotetraose, and maltopentaose as donors. (Data not shown) Thus, 3 production seemed to depend on the degree of polymerization of donors.

Solvent Effects on Production of *p*-Nitrophenyl α-Maltohexaoside (3). A sufficient solubility of 1 as an acceptor was ensured in medium containing hydrophilic organic solvents such as dimethyl sulfoxide and alcohols. An ~4-fold increase in solubility is observed with addition of organic solvent. Figure 3 shows the solvent effects on 3 production at higher substrate concentration. Conditions of pH and temperature used were based on the optimum as those in Fig. 1. The maximum productions of 3 at 40% dimethyl sulfoxide, 20% 1-butanol, and 20% 2-propanol, were about 2-3 fold that in the absence of organic solvent. These results indicate that an appropriate concentration of appropriate organic solvents in this reaction result in a great increase of 3 formation. In

contrast, the acceleration of transglycosylation was little affected by addition of methanol.

Proposed Transglycosylation Mode. G_5-amylase is known to be an endo-acting α-amylase on starch and amylose, but it hydrolyzes maltooligosaccharides such as maltohexaose and maltoheptaose with a pattern resembling an exo-acting enzyme.[18] Using maltohexaose as a donor in the transglycosylation reaction system, it is probable that the amylase attacks maltohexaose as an initial substrate to give enzyme-bound maltopentaose and glucose. In the transglycosylation, enzyme-bound maltopentaose is subsequently transferred to the 4-position of 1 to form 3. On the other hand, in the hydrolysis reaction, enzyme-bound maltopentaose is attacked by a water molecule to release maltopentaose. As described above, the use of appropriate concentrations of solvents such as dimethyl sulfoxide, 1-butanol and 2-propanol, improved not only transferase activity of the enzyme but also resulted in a great increase of formation of 3. From these data, we established an efficient enzymatic preparation of 3 using maltohexaose and 1 as substrates in an aqueous-organic solvent system.

EXPERIMENTAL

Materials. Crude G_5-amylase (termamyl L-60) was purchased from Novo Industry Co., Japan and purified by the method of Saito. A series of *p*-nitrophenyl α-maltooligosaccharides (DP = 2-6) were purchased from Calbiochem-Behring Corp. Other reagents were guaranteed reagent grade.

Amylase Assay. G_5-amylase was assayed by using a substrate solution containing 0.5% reduced short-chain amylose in 0.9 mL of 0.1 M phosphate buffer (pH 7.0). After incubation of the substrates solution for 3 min at 40 °C, 0.1 mL of suitably diluted enzyme solution was added, and the amount of reducing sugar liberated by the enzyme action for 30 min was measured by the Somogyi-Nelson method.[19,20] One unit (U) was defined as the amount of the enzyme that hydrolyzes one μmol of glycosidic bonds per minute.

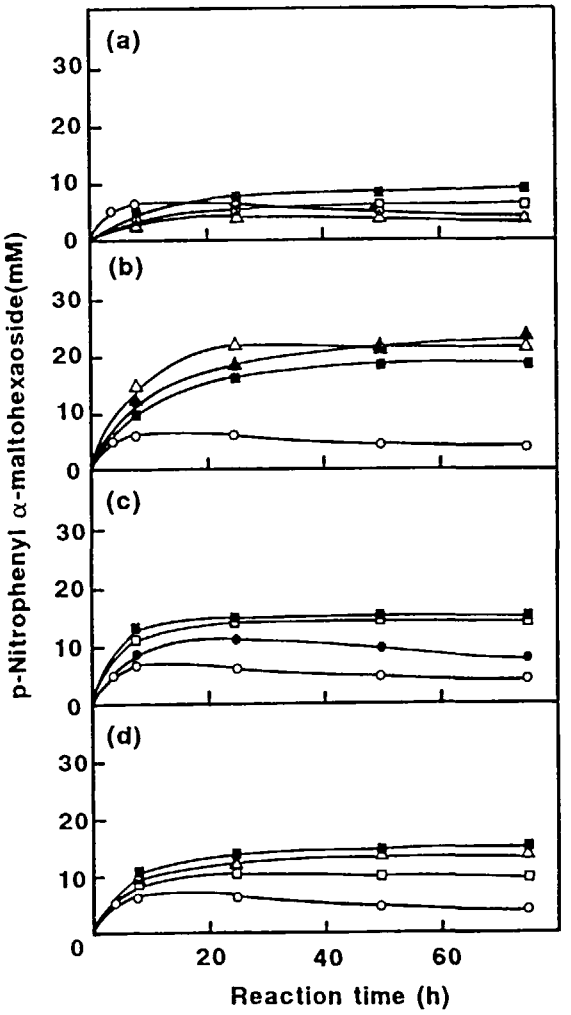

Fig. 3. Solvents effects on 3 production. The enzyme reactions were performed with maltohexaose (171 mg) and 1 (52 mg) at 35 °C in 1 mL of 15 mM of Tris-HCl buffer (pH 8.0) containing different solvent concentrations : (a) methanol (0%, ○ ; 10%, □ ; 20%, ■ ; and 30%, △), (b) DMSO (0%, ○ ; 20%, ■ ; 30%, △ ;and 40%, ▲), (c) 1-butanol (0%, ○ ; 5%, ◐ ; 10%, □ and 20%, ■), and (d) 2-propanol (0%, ○ ; 10%, □ ; 20%, ■ and 30%, △). The amylase (2.0 U) was added and samples were taken during incubation for analysis by HPLC.

Analytical Methods. HPLC were performed with a YMC packed column AQ-312 (ODS) column (10 × 150 mm) in a Hitachi L-6000 pump equipped with a Shimadzu ultraviolet detector. Elution was effected with water-methanol (9:1,v/v) at a flow rate of 1.0 mL/mim. ^{13}C and ^{1}H NMR spectra were determined with a Jeol FX-90 spectrometer operating at 22.5 MHz in the pulsed Fourier-transform mode with complete proton decoupling and 90 MHz, respectively. Chemical shifts are expressed in ppm relative to 3-(trimethylsilyl)propanesulfonate sodium salt (TPS) as an internal standard. FAB MS spectra 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 0.1 N HCl (1 μL) was added. The molecular weight of the sample was estimated from the *m/z* value of the quasi molecular-ion $[M+H]^+$ peak. Specific rotation was determined with a Digital Automatic Polarimeter PM-101 apparatus (Union Giken Corp., Ltd.). Elemental analysis was performed using a Perkin-Elmer 240C apparatus.

Preparation of *p*-Nitrophenyl α-Maltohexaoside (3). Maltohexaose (171 mg) and 1 (52 mg) were dissolved in 1 mL of 20 mM Tris-HCl buffer (pH 8.0) containing 40% dimethyl sulfoxide. To this solution, 2.0 U of G_5-amylase was added and the mixture was incubated for 75 h at 35 °C. After the reaction was stopped by the addition of two volumes of 1.0 M acetic acid for the reaction mixture, it was concentrated to a syrup under diminished pressure (0.1 Torr) at 50 °C. The concentrate was dissolved in 2 mL of water-methanol (3:1, v/v) solution and was put on a Toyopearl HW-40S column (2.2 × 90 cm). The column was eluted with water-methanol (3:1, v/v) at a flow rate of 40 mL/hr at room temperature. Elution was monitored by measuring the absorbance at 295 nm due to *p*-nitrophenyl group, and at 485 nm (carbohydrate content determined by 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 52-58) was concentrated and lyophilized to give a yield of 26 mg. The second peak (F-II, tube numbers 90-98) contained recovered 1 used as an acceptor. F-I showed only a single peak on HPLC and its retention time coincided with that of a standard sample of 3; $[\alpha]_D^{25}$+214.0 (*c* 1.0 water); ^{1}H NMR (90 MHz, D_2O) δ 5.39-5.37 (*d*, 5H, *J*=3.7 Hz, H-1',-1'',-1''',-1'''', -1'''''), 5.83 (*d*, 1H, *J*=3.0 Hz, H-1),7.20 (*d*, 2H, o-Ph), 8.19 (*d*, 2H, m-Ph); ^{13}C NMR (90 MHz, D_2O) δ 63.11 (C-6,-6',-6'', -6''',-6'''',-6'''''), 72.05 (C-4'''''), 73.35 (C-2), 74.00 (C-2',-2'',-2''',-2'''',-2'''''), 74.22 (C-5,-5',-5'',-5''', -5''''), 75.41 (C-3, C-5''''''), 75.95 (C-3',-3'',-3''', -3'''',-3'''''), 79.96 (C-4), 80.29 (C-4',-4'',-4''',-4''''), 99.36 (C-1), 102.66 (C-1', -1'',-1''',-1'''',-1'''''), 119.40 (m-Ph), 128.61 (o-Ph), 144.75 (p-Ph), 164.20 (Ph-carbon attached to the phenolic oxygen). *Anal.* Calcd for $C_{42}H_{65}O_{33}N \cdot H_2O$: C, 44.60; H, 5.93; N, 1.24. Found; C, 44.30 ; H 5.99 ; N 1.21. FAB MS spectrum of F-I gave two major ions in the high-mass region at m/z 1112 and 1134, which are consistent with the molecular ion $[M+H]^+$ and $[M+Na]^+$, respectively.

ACKOWLEDGMENTS

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