Substrate Specificity of Human and Bovine Pancreatic α -Amylases Using End-blocked Maltooligosaccharides

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β -D-Galactosyl and β -N-acetylglucosaminyl maltooligosaccharide derivatives, which are substituted at the nonreducing-end glucosyl residue of *p*-nitrophenyl α -maltooligosaccharides (maltotriose to maltopentaose), were regioselectively synthesized using the transglycosylation reaction of β -D-galactosidase from *Bacillus circulans* and hen-egg white lysozyme, respectively. On porcine and human pancreatic α -amylases, the hydrolytic pattern and kinetic parameter (k_o/K_m) of both end-blocked maltooligosaccharides, *p*-nitrophenyl 4⁵-O- β -D-galactosyl- α -maltopentaoside (Gal⁴G5P), *p*-nitrophenyl 4⁴-O- β -D-galactosyl- α -maltotetraoside (Gal⁴G4P) and *p*-nitrophenyl 4³-O- β -D-galactosyl- α -maltotrioside (Gal⁴G3P), were very close to those of *p*-nitrophenyl α -maltohexaoside (G6P), *p*-nitrophenyl α -maltopentaoside (G5P) and *p*-nitrophenyl α -maltotetraoside (G4P), respectively, corresponding to their degrees of polymerization, while *p*-nitrophenyl 3⁵-O- β -N-acetylglucosaminyl- α -maltopentaoside (NG5P) and *p*-nitrophenyl 3⁴-O- β -N-acetylglucosaminyl- α -maltotetraoside (NG4P) behaved as G5P and G4P, respectively, as if the GlcNAc group is ignored. On the contrary, *p*-nitrophenyl 6⁵-O- β -D-galactosyl- α -maltopentaoside (Gal⁶G5P) caused a remarkable decrease in the k_o/K_m value compared with G5P, and *p*-nitrophenyl 6⁴-O- β -D-galactosyl- α -maltotetraoside (Gal⁶G4P) acted only slightly for the α -amylases. A series of maltooligosaccharides with both ends blocked were shown to be advantageous as probes for substrate recognition at the active site in porcine and human pancreatic α -amylases.

Mammalian α -amylases (1,4- α -D-glucan glucanohydrolase, EC 3.2.1.1) are present mainly in pancreatic and salivary glands. The structure and function of porcine pancreatic α -amylase (PPA) are well documented.^{1–5)} In particular, a 3-nm-long crevice is apparent in the molecule, and it can accommodate a chain of five glucose residues. This observation is consistent with the five-subsite model in which the catalytic site is located between subsites 3 and 4 (subsite corresponds to the nonreducing end).⁶⁾ Maltooligosaccharide analogs with reducing-end terminal residues other than glucose such as *p*-nitrophenyl and 2-chloro-4-nitrophenyl groups are frequently employed to discern the requirements for binding and reaction.^{7–11)} Stud-

ies with the modified substrates have been proved to be advantageous for investigation of the mechanism of enzyme action, since the modification of part of the substrate will affect the interaction with the active site of the enzyme and the mode of action of the enzyme will be altered.^{12,13)} The maltooligosaccharides with both ends blocked have been reported to be very suitable as novel substrates for the analytical use of human α -amylase in serum through a conjugated reaction involving glucoamylase and α -glucosidase.^{14–16)} From such aspect, a series of maltooligosaccharide derivatives with both ends blocked were designed as substrate analogs for mammalian α -amylases, because systematic trends in kinetic studies on the structural modification of substrates would be helpful in revealing the requirements for binding and catalytic specificity.

The present paper describes that the sugar modification at the nonreducing end residues of *p*-nitro-

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phenyl α -maltooligosaccharides (degree of polymerization 3–5) is carried out by employing the transglycosylation of lysozyme and β -D-galactosidase, and the resulting end-blocked derivatives are useful as tools for examining the interaction between the substrate and subsite structures of HPA (human pancreatic α -amylase) and PPA.

MATERIALS AND METHODS

Materials. PPA was firstly obtained in the crystalline state from porcine pancreatic juice as described by Sakano *et al.*¹⁷⁾ The crystalline preparation was applied to a column of HiLoad 16/10 Q-Sepharose equilibrated with 20 mM Tris-HCl buffer (pH 8.4) containing 1 mM CaCl_2 . Isozyme I was eluted from this column, but isozyme II was absorbed. The combined fraction of isozyme I (showing a single band on SDS-polyacrylamide electrophoresis) was dialyzed against 25 mM phosphate buffer (pH 6.9) containing 30 mM NaCl and 1 mM CaCl_2 , and concentrated using an Amicon Diaflo unit equipped with a PM-10 membrane operating at 2 kg/cm², and stored at 4°C. Isozyme I was used in the present study. Human pancreatic α -amylase (HPA) was purified from pancreatic juice according to the method of Matsuura *et al.*¹⁸⁾ with our modification.¹⁹⁾ Crude enzyme from human pancreatic juice was precipitated with ammonium sulfate added to 75%. The precipitate was dissolved in 20 mM Tris-HCl buffer containing 2 mM CaCl_2 (pH 8.8). After the solution was dialyzed with an Amicon Diaflo unit, it was applied to a column of Mono Q HR5/5 with the same buffer. After being washed with the same buffer, the column was eluted with a gradient of the buffer containing 0–0.4 M NaCl. A main peak containing α -amylase activity was eluted from the column, and the combined fraction collected. The enzyme fraction gave a single protein band on SDS-polyacrylamide electrophoresis. Commercially available β -D-galactosidase preparations (Biolacta, Daiwa Kasei Co., Ltd., Osaka, Japan) prepared from the culture filtrates of *B. circulans* were directly used for enzymatic synthesis without further purification. Gal⁴G5P and Gal⁶G5P were prepared from lactose and G5P using the transglycosylation

of β -D-galactosidase,¹⁵⁾ and NG5P was prepared from GlcNAc₂ and G5P using lysozyme¹⁴⁾ by our methods. G6P, G5P, and G4P were prepared in each case using specific amylases as previously reported.^{20–22)} All other chemicals were obtained from commercial sources.

Analytical methods. HPLC was performed with a YMC-packed column Type AQ-312 (ODS) (6×150 mm) or a TSK-Gel G-Oligo-PW (7.8×300mm) in a Hitachi liquid chromatograph equipped with a Hitachi L-4000 UV detector. Elution of the first column was effected with water/methanol (88/12, v/v) at a flow rate of 0.9 mL/min at 40°C, and elution of the second column was with distilled water at a flow rate of 0.9 mL/min at 50°C. ¹H- and ¹³C-NMR spectra were determined with a JEOL JNM-EX 270 spectrometer operating at 68MHz in the pulsed Four-transform mode with complete proton decoupling and 270 MHz, respectively. Chemical shifts are expressed in δ relative to sodium 3-(trimethylsilyl)-propionate (TPS) as an internal standard. The FAB-MS spectra was measured using a DX-303 HF mass spectrometer operating at full accelerating potential (3 kV) and coupled to a JEOL DA-500 mass data system. The samples in distilled water were added to the glycerol matrix. 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 DIP-101 apparatus (Union, Giken Corp., Ltd.).

Enzyme assays. β -D-Galactosidase and lysozyme activities were spectrophotometrically assayed with *o*-nitrophenyl β -D-galactopyranoside and ethylene glycol chitin, respectively, as previously reported.^{23,24)} α -Amylase activity was assayed as follows: a mixture containing 1 mM G5P, 120 U of α -D-glucosidase and an appropriate amount of mammalian pancreatic α -amylase in 80 mM 3,3-dimethylglutaric acid/5 M sodium hydroxide buffer (pH 6.9) containing 40 mM NaCl and 5 mM CaCl_2 in a total volume of 1 mL was incubated for 15 min at 37°C. After inactivation of the reaction by adding 3.0 mL of 0.2 M borate (pH 9.8), the liberated *p*-nitrophenol was determined spectrophotometrically at 405 nm. One unit of the enzyme

activity was defined as the amount of enzyme liberating *p*-nitrophenol 1 μ mol/min.

Cleavage frequencies and kinetic studies. Incubations with *p*-nitrophenylated oligosaccharides as substrates (0.05–10 mM) were carried out at 37 °C in 0.5 mL of 80 mM 3,3-dimethylglutaric acid containing 40 mM NaCl and 5 mM CaCl₂ (pH 6.9). Reactions were initiated by the addition of enzyme (1–100 nM). Samples (100 μ L) were taken at intervals (0, 3, 6, 9, 12 min) during the incubation, and inactivated by adding 200 μ L of 1 M acetic acid. The amount of each *p*-nitrophenylated product formed at an early stage (within 15% hydrolysis) from the initial substrates during incubation with enzyme was analyzed for HPLC. From the chromatograms of the digests of each substrate with enzyme, the amount of the products was calculated from the peak areas on the chromatograms. The amount of each product increased linearly with time in the initial stage of the reaction. On the basis of these data, the frequency of amylase-catalyzed cleavage of glycosidic linkages was determined.

On the basis of measurement of the amount of products as described above, the initial velocities (ν) were also obtained directly from the initial slopes of the time-course plots of the reaction. Six different substrate concentrations (0.05–10 mM) were used per experiment. The k_o and K_m values for synthetic maltooligosaccharide substrates were calculated from the plots of ν against the substrate concentrations using the Michaelis-Menten equation and the least squares method (Sakoda and Hiromi).²⁵⁾

Preparation of nonreducing-end modified maltooligosaccharides.

(a) *p*-Nitrophenyl 4'-O- β -D-galactosyl- α -maltotetraoside (Gal⁴G4P) and *p*-nitrophenyl 6'-O- β -D-galactosyl- α -maltotetraoside (Gal⁶G4P).

Lactose (844 mg, 616 mM) and G4P (972 mg, 305 mM) dissolved in 4 mL of 20 mM phosphate buffer (pH 7.0) were incubated with β -D-galactosidase (1.72 U) from *B. circulans* for 8 h at 40 °C. At the termination of reaction, the reaction mixture was directly loaded onto a Toyopearl HW-40S column (4.5 $\times$ 89 cm). Elution was monitored by measuring the absorbance at 300 nm (characteristic

absorption of *p*-nitrophenyl group) and 485 nm (carbohydrate content, determined by the phenol-sulfuric acid method). The chromatogram shows two main peaks, for which the absorption at 300 nm coincides with that at 485 nm. The first peak (F-1, tubes 60–67) was combined, concentrated, and lyophilized to obtain a yield of 176 mg. The second peak (F-2, tubes 73–83) contained recovered G4P used as the acceptor. F-1 was dissolved in 1 mL of water, a 1/10 vol of which was put on a YMC-packed AQ-323 ODS column (20 $\times$ 250 mm) in a HPLC system. Elution of the column was effected with water/methanol (9/1, v/v). The F-1 fraction was separated into two peaks (F-1-1 and F-1-2). The eluates corresponding to the F-1-1 and F-1-2 peaks were combined, concentrated and lyophilized. The remaining aliquots were similarly worked up as above. As a result, F-1-1 and F-1-2 afforded Gal⁴G4P (124 mg) and Gal⁶G4P (19 mg), respectively.

Compound Gal⁴G4P had $[\alpha]_D^{+175.6}$ (c 1, H₂O) and m/z 950. ¹H-NMR data (D₂O) was δ 8.22 (2 H, d, J 9.2 Hz, *m*-Ph), 7.28 (2H, d, J 9.2 Hz, *o*-Ph), 5.82 (1H, d, J 3.3 Hz, H-1), 5.42 (1H, d, J 3.3 Hz, H-1'), 5.37 (2H, d, J 3.0 Hz, H-1'' and H-1'''), and 4.45 (1H, d, J 8.1 Hz, H-1''').

Compound Gal⁶G4P had m/z 950. ¹H-NMR data (D₂O) was δ 8.23 (2H, d, J 9.2 Hz, *m*-Ph), 7.29 (2H, d, J 9.2 Hz, *o*-Ph), 5.82 (1H, d, J 3.6 Hz, H-1), 5.43 (1H, d, J 3.6 Hz, H-1'), 5.37 (1H, d, J 3.0 Hz, H-1''), 5.36 (1H, d, J 3.0 Hz, H-1'''), and 4.43 (1H, d, J 7.7 Hz, H-1''').

(b) *p*-Nitrophenyl 4'-O- β -D-galactosyl- α -maltotrioside (Gal⁴G3P) and *p*-nitrophenyl 6'-O- β -D-galactosyl- α -maltotrioside (Gal⁶G3P).

Incubation conditions were the same as those for (a). Lactose (220 mg) and G3P (201 mg) dissolved in 1 mL of the same buffer were incubated with the

β -D-galactosidase (0.43 U) for 8 h at 40 °C. The reaction mixture was applied to a Toyopearl HW-40S column (2.2 $\times$ 89 cm, 5 mL/tube). The chromatogram shows two main peaks (F-1 and F-2) at 300 and 485 nm. The F-1 fraction (tubes 73–80) was combined, concentrated, and lyophilized to afford a yield of 41 mg. Peak F-2 contained recovered G3P used as the acceptor. F-1 was further resolved by HPLC in order to obtain two separate

components (F-1-a and F-1-b). F-1-a and F-1-b gave Gal⁴G3P (29 mg) and Gal⁶G3P (3 mg), respectively.

Compound Gal⁴G3P had $[\alpha]_D +181.0$ (c1, H₂O) and m/z 788. ¹H-NMR data (D₂O) was δ 8.22 (2H, d, J 9.2 Hz, *m*-Ph), 7.27 (2H, d, J 9.2 Hz, *o*-Ph), 5.79 (1H, d, J 3.3 Hz, H-1), 5.41 (1H, d, J 3.3 Hz, H-1'), 5.37 (1H, d, J 3.0 Hz, H-1''), and 4.42 (1H, d, J 7.7 Hz, H-1''').

Compound Gal⁶G3P had $[\alpha]_D$ 167.5 (c1, H₂O) and m/z 788. ¹H-NMR data (D₂O) was δ 8.24 (2 H, d, J 7.9 Hz, *m*-Ph), 7.28 (2H, d, J 8.9 Hz, *o*-Ph), 5.80 (1H, d, J 3.0 Hz, H-1), 5.41 (1H, d, J 3.6 Hz, H-1'), 5.35 (1H, d, J 3.3 Hz, H-1''), and 4.40 (1H, d, J 7.6 Hz, H-1''').

(c) ***p*-Nitrophenyl 3⁴-O- β -N-acetylglucosaminyl- α -maltotetraoside (NG4P).** *N*, *N*'-diacetylchitobiose (GlcNAc₂, 240 mg) and G4P (390 mg) dissolved in 1 mL of 50 mM acetate buffer (pH 4.5) were incubated with hen egg white lysozyme (50 U) for 120 h at 50°C. At the termination of reaction, the precipitate formed during the reaction was removed by centrifugation and the supernatant was loaded onto a Toyopearl HW-40S column (2.2 $\times$ 89 cm, 3 mL/tube). The chromatogram shows two main peaks (F-1 and F-2) at 300 and 485 nm. The first peak (F-1, tubes 97–107) was combined, concentrated, and lyophilized to obtain a yield of 27 mg. The second peak (F-2, tubes 121–138) contained recovered G4P used as the acceptor. The F-1 fraction was further resolved by HPLC on a ODS column, as before, to obtain mainly one separate component (F-1'). The eluates corresponding to the F-1' peak were combined, concentrated and lyophilized to obtain compound NG4P (20 mg).

Compound NG4P had $[\alpha]_D +143.8$ (c1, H₂O) and m/z 991. ¹H-NMR data (D₂O) was δ 8.26 (2 H, d, J 9.2 Hz, *m*-Ph), 7.31 (2H, d, J 9.5 Hz, *o*-Ph), 5.83 (1H, d, J 3.6 Hz, H-1), 5.44 (1H, d, J 3.6 Hz, H-1'), 5.38 (1H, d, J 4.0 Hz, H-1''), 5.37 (1H, d, J 4.0 Hz, H-1'''), 4.75 (1H, d, J 8.4 Hz, H-1'''), and 2.05 (s, 3H, Ac).

(d) ***p*-Nitrophenyl 3³-O- β -N-acetylglucosaminyl- α -maltotrioside (NG3P).** Incubation conditions were the same as those for (c). GlcNAc₂ (240 mg) and G3P (360 mg) dissolved in 1 mL of the

same buffer were incubated with the lysozyme. The transfer product was separated by the same column as used in (c). The eluates show two main peaks (F-1, tubes 65–71; and F-2, tubes 85–96) at 300 and 485 nm. The F-1 fraction obtained a yield of 25 mg. The F-2 fraction contained recovered G3P used as the acceptor. F-1 was further resolved by HPLC as in (c) to obtain mainly one separate component (F-1'). The eluates corresponding to the F-1' peak were combined, concentrated and lyophilized to afford compound NG3P (19 mg).

Compound NG3P had $[\alpha]_D +153.2$ (c1, H₂O) and m/z 829. ¹H-NMR data (D₂O) was δ 8.22 (2 H, d, J 9.2 Hz, *m*-Ph), 7.27 (2H, d, J 9.2 Hz, *o*-Ph), 5.79 (1H, d, J 3.0 Hz, H-1), 5.41 (1H, d, J 3.6 Hz, H-1'), 5.35 (1H, d, J 3.6 Hz, H-1''), 4.73 (1H, d, J 8.4 Hz, H-1'''), and 2.03 (s, 3H, Ac).

RESULTS AND DISCUSSIONS

Synthesis of maltooligosaccharide analogs with both ends blocked.

In order to examine how PPA and HPA are recognized by the maltooligosaccharide analog with nonreducing end residues other than glucose, a series of heterooligosaccharides were prepared by using glycosidases to catalyze highly regioselective transfer reaction from the donor to the nonreducing end of acceptor sugars. We have already reported that the enzymatic synthesis of end-blocked maltooligosaccharide analogs Gal⁴G5P and Gal⁶G5P can be achieved by utilizing the transglycosylation activity of *B. circulans* β -D-galactosidase from lactose as a donor to G5P as the acceptor.¹⁵⁾ The synthetic concept was introduced to the preparation of the reduction of chain-length from $n = 5$ to 3–4 in Gal⁴GnP and Gal⁶GnP. Thus, when G4P was the acceptor and lactose the galactosyl donor, two galactosyl pentasaccharide isomers were obtained at a 11.4% overall yield (based on the acceptor added) and in a ratio of 9:1. These were separated by chromatography on a Toyopearl HW-40S column and purified by HPLC with a ODS column, and were shown to be the (1–4)-(Gal⁴G4P, major isomer) and (1–6)-(Gal⁶G4P, minor isomer) transfer products. This was also the case for the formation of the two isomers Gal⁴G3P

and Gal⁶G3P upon the reaction with the G3P acceptor. Gal⁴G3P and Gal⁶G3P were obtained at a 12.7% overall yield and a ratio of 9:1. In both cases, more β -(1-4)-linked product than the β -(1-6)-linked product was formed in the initial stage of reaction, but the relation between this yield was reversed in the later stage of reaction, as well as Gal⁴G5P and Gal⁶G5P production. We have also demonstrated that hen egg white lysozyme produces NG5P by transferring the GlcNAc residue from (GlcNAc)₂ to the 3-position at the nonreducing end of the glucosyl group of G5P.¹⁴⁾ In a similar reaction, 3-*O*-substituted *N*-acetylglucosaminyl-oligomers NG4P and NG3P were synthesized from (GlcNAc)₂ donor, and G4P and G3P acceptors respectively. The yields of NG4P and NG3P were

3.3 and 4.0% based on the addition of G4P and G3P, respectively. These structures were elucidated by their ¹H- and ¹³C-NMR spectra. NMR signal assignments were made using carbon-proton shift correlation spectroscopy (CH-COSY) as shown in Tables 1 and 2.

Hydrolysis of synthetic modified maltooligosaccharide analogs: reaction product and bond cleavage frequency.

The modes of action of HPA and PPA on the nonreducing-end modified maltooligosaccharides prepared as described above were examined. The amount of each product formed at an early stage (within 15% hydrolysis) from the initial substrate during incubation with enzyme was analyzed by

Table 1. ¹³C chemical shifts of both-end blocked galactosyl-maltooligosaccharides in D₂O.

Compound		C-1	C-2	C-3	C-4	C-5	C-6	<i>m</i> -Ar	<i>o</i> -Ar	<i>p</i> -Ar	<i>c</i> *-Ar
Gal ⁴ G3P	I	99.26	73.53	75.37	79.54	74.26	62.64				
	II	102.50	73.98	76.09	79.78	74.35	63.18				
	III	102.37	73.19	76.09	80.95	74.35	63.94				
	IV	102.66	73.77	76.37	71.36	78.14	63.83				
	P							119.52	128.83	145.12	164.17
Gal ⁶ G3P	I	99.17	73.51	75.47	79.42	74.30	62.91				
	II	102.69	74.14	76.05	80.05	74.47	63.34				
	III	102.41	74.30	74.54	71.93	73.94	71.27				
	IV	106.16	73.57	75.56	71.46	77.93	63.81				
	P							119.48	128.82	145.12	164.11
Gal ⁴ G4P	I	99.32	73.53	75.44	79.72	74.25	62.69				
	II	102.66	74.03	76.13	79.97	74.36	63.22				
	III	102.66	74.09	76.13	80.19	74.36	63.22				
	IV	102.47	74.17	76.13	80.02	74.29	62.95				
	V	105.71	73.81	75.44	71.40	78.19	63.88	119.54	128.86	145.05	164.23
Gal ⁶ G4P	I	99.30	73.52	75.53	79.71	74.24	62.95				
	II	102.60	74.08	76.09	80.24	74.35	63.21				
	III	102.66	74.08	76.09	80.37	74.35	63.39				
	IV	102.81	73.51	74.59	72.00	74.02	71.36				
	V	106.19	73.62	75.65	71.50	77.96	63.84	119.52	128.83	145.08	164.19
	P										

*Phenyl carbon attached to phenolic oxygen.

Gal β 1-4Glc α 1-4Glc α 1-4Glc α -pNP(Gal⁴G3P)
IV III II I P

Gal β 1-6Glc α 1-4Glc α 1-4Glc α -pNP(Gal⁶G3P)
IV III II I P

Gal β 1-4Glc α 1-4Glc α 1-4Glc α 1-4Glc α -pNP(Gal⁴G4P)
V IV III II I P

Gal β 1-6Glc α 1-4Glc α 1-4Glc α 1-4Glc α -pNP(Gal⁶G4P)
V IV III II I P

Table 2. ^{13}C chemical shifts of both-end blocked *N*-acetylglucosaminyl-maltooligosaccharides in D_2O .

Compound		C-1	C-2	C-3	C-4	C-5	C-6	CH ₃ (NHCOCH ₃)	C=O (NHCOCH ₃)	<i>m</i> -Ar	<i>o</i> -Ar	<i>p</i> -Ar	<i>c</i> *-Ar
NG3P	I	99.23	73.49	75.14	79.30	74.39	62.42						
	II	102.56	74.03	76.01	79.49	74.39	63.89						
	III	102.47	74.16	84.59	70.58	74.39	63.23						
	IV	104.52	58.54	76.42	72.61	78.58	63.42	24.99	177.62				
	P									119.48	128.80	145.08	164.15
NG4P	I	99.28	73.53	75.18	79.40	74.33	62.95						
	II	102.55	74.06	76.10	79.50	74.33	63.23						
	III	102.55	74.06	76.10	79.98	74.44	63.23						
	IV	102.61	74.22	84.64	70.63	74.44	63.23						
	V	104.52	73.81	75.44	71.40	78.19	63.88	25.03	177.65				
	P									119.52	128.83	145.12	164.17

*Phenyl carbon attached to phenolic oxygen.

GlcNAc β 1-3Glc α 1-4Glc α 1-4Glc α -pNP(NG3P)
IV III II I P

GlcNAc β 1-3Glc α 1-4Glc α 1-4Glc α 1-4Glc α -pNP(NG4P)
V IV III II I P

HPLC. The cleavage is easily localized here due to the nitrophenyl labeling of the glucosidic chain at its reducing end. From the chromatograms of the digests of each substrate with the α -amylases, the amounts of the products were calculated from their peak areas on the chromatograms. The amounts of each product increased linearly with time in the initial stage of the reaction (data not shown). Based on the product analysis, the frequency of amylase-catalyzed cleavage of glycosidic linkages in a series of maltooligosaccharide analogs with both ends blocked was determined as summarized in Figs.1 and 2. In this case, the data on GnP ($n=3-5$) were given as a control to ascertain the effect of the modification. The action patterns on different kinds of substrates of PPA and HPA were substantially similar except for Gal⁶G5P. The number of susceptible bonds varies with the length and structural difference of the substrate. For 4-*O*-Gal substituted Gal⁴G5P, the major cleavage occurs at the third and fourth glucosidic bonds from the nitrophenyl group at about the same frequency as well as G6P. For Gal⁴G4P ($n=5$), the major cleavage occurs at bond 3 (frequencies 0.97 for PPA and 0.88 for HPA) as well as G5P. Thus, the hydrolytic pattern of Gal⁴G5P and Gal⁴G4P greatly corresponds to that of G6P and G5P, respectively. One should remark that this galactosyl residue be-

haves exactly the same as the glucosyl residue. Gal⁴G3P is cut exclusively at bond 2 by the enzymes. The hydrolysis of the 6-*O*-Gal substituted substrate Gal⁶G5P for PPA is significantly different from HPA. The preferred cleavage of the former enzyme is at bond 3, whereas the latter is at bond 2. For Gal⁶G4P, one bond is cut preferentially at bond 2, although it was hydrolyzed slowly to form GP. Gal⁶G3P did not act as substrate toward the two amylases under the conditions used. For 3-*O*-GlcNAc substituted NG5P and NG4P, one bond is cut almost exclusively at bonds 3 and 2, respectively. NG3P ($n=4$) was hydrolyzed very slowly to form GP and phenol in the ratio of 0.40 and 0.60 for PPA. The same tendency was also observed for HPA.

Kinetic parameters for the hydrolysis of modified maltooligosaccharide analogs.

The kinetic parameters for the hydrolysis of the end-blocked maltooligosaccharides by PPA and HSA are determined with GnP as control samples and listed in Tables 3 and 4. The catalytic behavior of the two enzymes were very similar. The kinetic parameters for both end-blocked derivatives Gal⁴GnP and NGnP increase with chain length as well as GnP. For both enzymes, despite introduction of the Gal group at the terminal position, the

Substrate	Porcine pancreatic α -amylase
G6P	G-G-G-G-G-G-P (0.56 [*]) G-G-G-G-G-G-P (0.44)
G5P	G-G-G-G-G-P (0.94) G-G-G-G-G-P (0.06)
G4P	G-G-G-G-P (0.80) G-G-G-G-P (0.20)
G3P	G-G-G-P (0.65) G-G-G-P (0.19) G-G-G-P (0.16)
Gal ⁴ G5P	L-G-G-G-G-G-P (0.54) L-G-G-G-G-G-P (0.41) L-G-G-G-G-G-P (0.05)
Gal ⁴ G4P	L-G-G-G-G-P (0.97) L-G-G-G-G-P (0.03)
Gal ⁴ G3P	L-G-G-G-P (1.0)
Gal ⁶ G5P	L ₄ -G-G-G-G-G-P (0.61) L ₄ -G-G-G-G-G-P (0.39)
Gal ⁶ G4P	L ₄ -G-G-G-G-P (0.96) L ₄ -G-G-G-G-P (0.03) L ₄ -G-G-G-G-P (0.01)
NG5P	N-G-G-G-G-G-P (0.96) N-G-G-G-G-G-P (0.04)
NG4P	N-G-G-G-G-P (1.0)
NG3P	N-G-G-G-P (0.60) N-G-G-G-P (0.40)

Substrate	Human pancreatic α -amylase
G6P	G-G-G-G-G-G-P (0.45 [*]) G-G-G-G-G-G-P (0.43)
G5P	G-G-G-G-G-P (0.12) G-G-G-G-G-P (0.83) G-G-G-G-G-P (0.10)
G4P	G-G-G-G-G-P (0.07) G-G-G-G-P (0.66) G-G-G-G-P (0.34)
G3P	G-G-G-P (0.88) G-G-G-P (0.07) G-G-G-P (0.05)
Gal ⁴ G5P	L-G-G-G-G-G-P (0.55) L-G-G-G-G-G-P (0.45)
Gal ⁴ G4P	L-G-G-G-G-P (0.88) L-G-G-G-G-P (0.12)
Gal ⁴ G3P	L-G-G-G-P (1.0)
Gal ⁶ G5P	L ₄ -G-G-G-G-G-P (0.83) L ₄ -G-G-G-G-G-P (0.17)
Gal ⁶ G4P	L ₄ -G-G-G-G-P (0.91) L ₄ -G-G-G-G-P (0.06) L ₄ -G-G-G-G-P (0.03)
NG5P	N-G-G-G-G-G-P (0.96) N-G-G-G-G-G-P (0.04)
NG4P	N-G-G-G-G-P (1.0)
NG3P	N-G-G-G-P (0.74) N-G-G-G-P (0.26)

Fig. 1. Products of the initial stage of reaction of porcine pancreatic α -amylase on substrates.

G, glucosyl residue; L-, 4-*O*-galactosyl residue; L₄-, 6-*O*-galactosyl residue; N, *N*-acetylglucosaminyl residue; P, C₆H₅NO₂-*, Frequency; **, Arrowhead indicates the catalytic site and each box numbered in order represents a subsite.

Fig. 2. Products of the initial stage of reaction of human pancreatic α -amylase on substrates.

Abbreviations are according to the same as Fig. 1.

Table 3. Kinetic parameters of porcine pancreatic α -amylase.

Substrate	K_m (mM)	k_o (s ⁻¹)	k_o/K_m (s ⁻¹ mM ⁻¹)
G6P	0.11	710	6500
G5P	0.14	617	4400
G4P	0.41	549	1300
G3P	1.60	19	12
Gal ⁴ G5P	0.10	592	5900
Gal ⁴ G4P	0.15	518	3500
Gal ⁴ G3P	0.60	494	820
Gal ⁶ G5P	0.27	352	1300
Gal ⁶ G4P	1.60	86	54
NG5P	0.15	555	3700
NG4P	0.58	506	870
NG3P	2.30	5	2

k_o/K_m value of Gal⁴G5P is close to that of G6P rather than G5P. This is also the same tendency for the comparison of Gal⁴G4P with G5P, and Gal⁴G3P with G4P. HSA and HPA have been shown to have five binding sites for the glucose residue of the substrates.^{6,26)} Taking these earlier data and the present kinetic results into account, we reasonably concluded that Gal⁴G4P as well as G5P are predominantly bound to subsites geometrically complementary to five glucose residues, 1, 2, 3, 4 and 5 as in Fig. 3. Thus, the 4-*O*-substituted Gal moiety of Gal⁴G4P interacts with subsite 1 and mimics a glucose residue. On the contrary,

Table 4. Kinetic parameters of human pancreatic α -amylase.

Substrate	K_m (mM)	k_o (s ⁻¹)	k_o/K_m (s ⁻¹ mM ⁻¹)
G6P	0.07	605	7600
G5P	0.17	596	3500
G4P	0.41	590	1400
G3P	5.00	56	11
Gal ⁴ G5P	0.08	602	6900
Gal ⁴ G4P	0.18	591	3300
Gal ⁴ G3P	0.84	399	480
Gal ⁶ G5P	0.76	405	530
Gal ⁶ G4P	1.80	13	7
NG5P	0.15	585	3900
NG4P	0.69	501	730
NG3P	7.70	18	2

the catalytic efficiency of Gal⁶G4P was remarkable reduced for HSA and HPA in comparison with that of G5P. The k_o/K_m values of Gal⁶G4P for PPA and HPA are 1.2 and 0.2% of those of G5P, respectively. Gal⁶G4P acted only slightly as a substrate for these enzymes. These results indicate that the 6-*O*-substituted Gal moiety at the nonreducing end of the glycosyl group prohibits the action of these enzymes. The catalytic efficiency of NG5P for HPA and PPA corresponds approximately to that of G5P hydrolytic pattern mentioned above, as if the GlcNAc group is ignored. The k_o/K_m values of NG4P for PPA and HSA are 52 and 67% of those of G4P, respectively. NG3P acted slightly as

a substrate (k_o/K_m : 2 for HPA and PPA), whereas Gal⁴G3P still acts as a fairly good substrate (k_o/K_m : 480 for HPA and 820 for PPA).

These results suggest that it is very difficult for the 3-*O*-substituted GlcNAc group to interact with subsite 1. Furthermore, taking into account the results of Figs. 1 and 2, subsite 2 was, in contrast to subsite 1, strict for binding the glycosyl group at the nonreducing end since it did not interact with sugar residues other than glucose. This indicates that the steric compatibility of the terminal molecule of substrates such as Gal⁴G5P and Gal⁴G4P has, to a certain extent, flexibility for recognition at subsite 1. Qian *et al.* have shown electron density corresponding to the fully occupied subsite in the active site of PPA soaked with acarbose (a pseudotetrasaccharide α -amylase inhibitor) by X-ray analysis.²⁷⁾ This may explain the flexibility of subsite 1 for binding the galactosyl group at the nonreducing end.

In conclusion, the present results show that the 4-*O*-substituted Gal group at the nonreducing end of GalⁿGnP ($n=3-5$) does not prohibit reaction of the amylases, but gives some affinity to the subsite, as much as glucose. Thus, Gal moiety mimics a glucose residue and interacts with the subsite in a similar manner. On the contrary, the 6-*O*-substituted Gal group of Gal⁶G5P acts as a barrier for binding with enzyme. The introduction of the 3-*O*-substituted GlcNAc group at the terminal position of NG5P does not appreciably influence the en-

Fig. 3. Productive binding models of Gal⁴G4P and G5P on the subsites.
*Flexible binding position.

zyme action. Maltooligosaccharides with both ends blocked would be useful substrates for discerning the requirements for binding and reaction of amylase.

This work was supported by the Shimadzu Scientific Foundation.

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(Received September 14, 1999; Accepted December 13, 1999)

マルトオリゴ糖両末端修飾化合物を用いたヒトおよびブタ膵臓 α -アミラーゼの基質特異性

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p-ニトロフェル α -マルトオリゴ糖 (重合度 3-5) を基質に用い、グリコシダーゼの糖転移反応を利用して、非還元末端グルコシルにガラクトシル基および *N*-アセチルグルコサミニル基を位置選択的導入した一連のマルトオリゴ糖両末端修飾誘導体を合成した。ガラクトシル基を修飾した: *p*-ニトロフェル 4⁵-*O*- β -D-ガラクトシル- α -マルトペンタオシド (Gal⁴G5P), *p*-ニトロフェル 4⁴-*O*- α -D-ガラクトシル- α -マルトテトラオシド (Gal⁴G4P) そして *p*-ニトロフェル 4³-*O*- β -D-ガラクトシル- α -マルトトリオシド (Gal⁴G3P) のヒトおよびブタ膵臓 α -アミラーゼに対する水解様式と動力学パラメーターは、それぞれの糖重合度に対応して *p*-ニトロフェル α -マルトヘキサオシド (G6P), *p*-ニトロフェル α -マルトペンタオシド (G5P) そして *p*-ニトロフェル α -マルトテトラオシド (G4P) のそれらと近似した値を示し、活性部位上であたかも Gal 残基を Glc 残基 1 個分に置き換えたような結合特異性を示した。一方、*N*-アセチルグルコサミニル基修飾基質である *p*-ニトロフェル 3⁵-*N*-アセチルグルコサミニル- α -マルトペンタオシド (NG5P) と *p*-ニトロフェル 3⁴-*N*-アセチルグルコサミニル- α -マルトテトラオシド (NG4P) 速度パラメーターはそれぞれ G5P と G4P に対応しており、活性部位上であたかも末端 GlcNAc 残基を無視するかのような結合特異性を示した。このように一連の両末端修飾基質は両 α -アミラーゼの活性部位における基質認識プローブとして有用であることが実証できた。