Carbohydrate Research, 180 (1988) 137–146
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

PREPARATION OF CARBOXYMETHYL DERIVATIVES OF *p*-NITROPHENYL α-MALTOPENTAOSIDE AS SUBSTRATE OF α-AMYLASES

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(Received May 15th, 1987; accepted for publication, June 26th, 1987)

ABSTRACT

p-Nitrophenyl α-maltotetraosides and α-maltopentaosides having a carboxymethyl group at the nonreducing-end glucosyl group were prepared by the action of *Bacillus macerans* cyclomaltodextrin glucanotransferase on a mixture of monocarboxymethyl-substituted cyclomaltoheptaose and *p*-nitrophenyl α-D-glucopyranoside, followed by digestion with glucoamylase, and purification by chromatography. The modes of actions of human pancreatic and salivary α-amylases on these derivatives were studied. The maltopentaoside derivatives were suitable as substrates for assays of α-amylases coupled with glucoamylase and α-D-glucosidase.

INTRODUCTION

The assay of α-amylase activity in human serum and urine is helpful in the diagnosis of pancreatic disease. Starch and amylose have been used as substrate for such assays, based on different principles[1–3]. Maltooligosaccharides or their *p*-nitrophenyl derivatives with defined structures, such as *p*-nitrophenyl α-maltopentaoside, have been used as substrates for coupled-enzymic assays[4,5]. For example, *p*-nitrophenyl α-maltopentaoside is hydrolyzed by α-amylase to maltooligosaccharides and *p*-nitrophenyl α-D-glycosides. The coupled enzyme, α-D-glucosidase, acts on the products to release *p*-nitrophenol. These substrates are not very suitable for the coupled-enzyme method, because they are slightly hydrolyzed by α-D-glucosidase. *p*-Nitrophenyl maltooligosaccharides with a pyridylamino group at C-6 of the nonreducing D-glucosyl end-group is resistant to α-D-glucosidase and glucoamylase[6–13]. The carboxymethyl group is also a blocking group that confers resistance to the action of glucoamylase, as seen in carboxymethylamylose[14]. We report herein a simple new procedure for the preparation of *p*-nitrophenyl α-maltotetraosides and *p*-nitrophenyl α-maltopentaosides with nonreducing

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end-groups substituted with carboxymethyl groups, and the mode of action of two human α-amylases on these substrates.

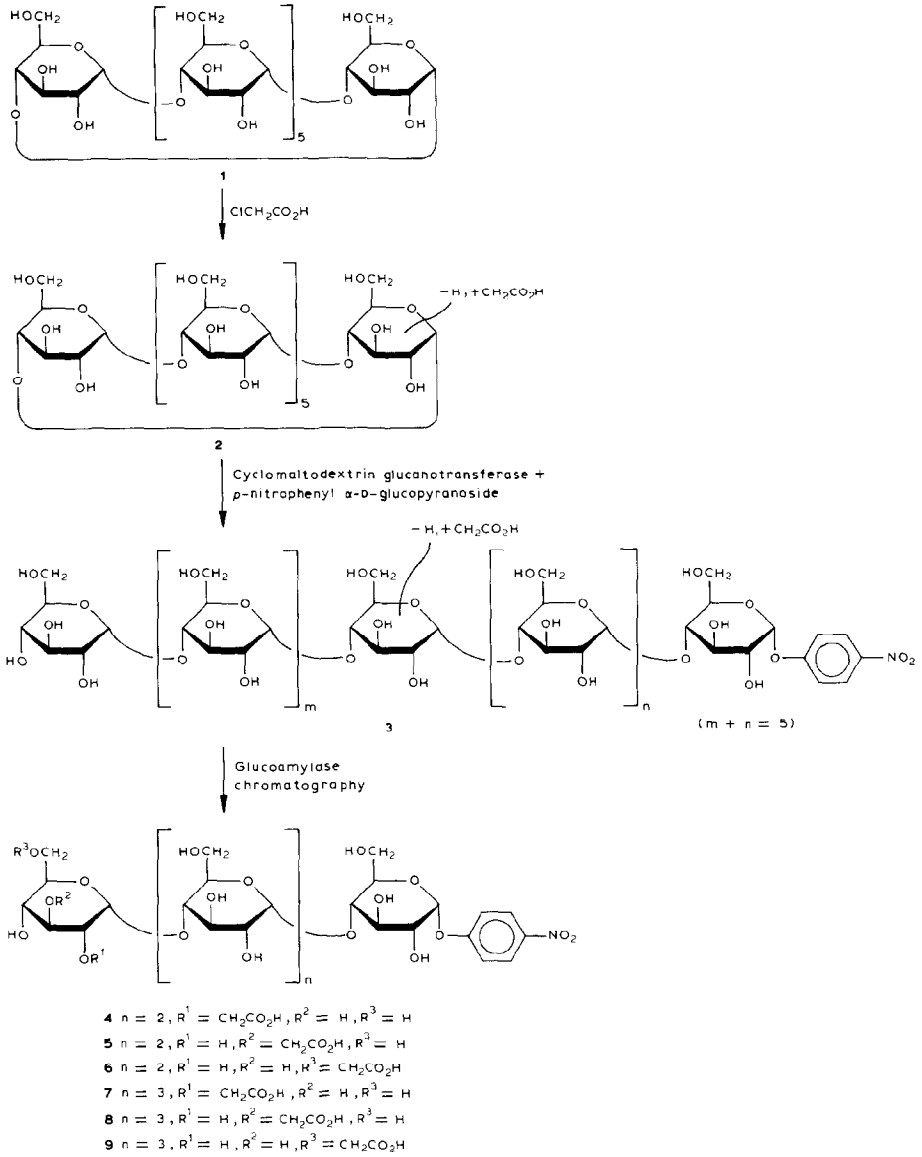
EXPERIMENTAL

Materials. — A crude preparation of cyclomaltodextrin glucanotransferase (EC 2.4.1.19; *B. macerans*, 10 450 units/L) was from Japan Maize Products Co., Ltd., Shizuoka, Japan. The activity of the enzyme was defined elsewhere[15]. Human salivary α-amylase (EC 3.2.1.1) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Human pancreatic α-amylase (EC 3.2.1.1) was purified from pancreatic juice by the method of Matsuura *et al.*[16]. The activity of human α-amylases is defined as the amount of enzyme that hydrolyzes 1 μmol of *p*-nitrophenyl *O*-(2-*O*-carboxymethyl)-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranoside (**7**) per min from a 1.0mM solution of **7** at pH 6.9 and 37°. Glucoamylase (EC 3.2.1.3; *Rhizopus delemar*, 36.5 units/mg) and α-D-glucosidase (EC 3.2.1.20; *Saccharomyces carlsbergensis*, 122 units/mg) were purchased from Toyobo Co., Ltd., Osaka, Japan. Methyl 2-*O*-carboxymethyl-α-D-glucopyranoside, methyl 3-*O*-carboxymethyl-α-D-glucopyranoside, and methyl 6-*O*-carboxymethyl-α-D-glucopyranoside were prepared by the method of Shyluk and Timell[17]. Other reagents were manufactured by Wako Pure Chemical Industries Ltd., Osaka, Japan.

G.l.c. analysis. — D-Glucose and *O*-carboxymethyl-D-glucoses in the substrates were analyzed by g.l.c. (column, 2% OV-17 on Chromosorb W; 0.4 × 200 cm) after methanolysis (1.4M HCl in methanol for 2 h at 90°), followed by trimethylsilylation with hexamethyldisilazane and chlorotrimethylsilane in pyridine[18]. The temperature was programmed from 110 to 230° at the rate of 4° increments per min.

High-pressure liquid chromatography (l.c.). — The l.c. apparatus used was a Shimadzu model LC-6A (Kyoto, Japan). For the separation of the carboxymethyl derivatives **4–9**, a column (4.6 × 150 mm) packed with Wakosil $5C_{18}$ (reversed phase; Wako) was eluted at the flow rate of 1.5 mL/min with 0.1% acetic acid containing 0.5% butanol, the pH of which was adjusted to 4.5 by the addition of 10M NH_4OH. Elution was monitored by the absorbance at 305 nm. The digest of the compounds **4–9** by α-amylases was assayed by the same procedure with the exception for the elution buffer, 0.1% acetic acid containing 12% acetonitrile.

F.a.b.-mass spectrometry. — F.a.b.–mass spectra were obtained with a Jeol HX-100 double-focusing mass spectrometer (Nihon Denshi Co., Ltd., Tokyo, Japan) fitted with a 2.33-tesla magnet and equipped with a f.a.b. ion source and a post-accelerating system. A mixture of sample solution (1–2 μL) containing the sample (10–20 μg), glycerol (0.5 μL), and M HCl (0.5 μL) was bombarded with a xenon neutral atom beam, accelerated by a 7-keV potential. A Jeol DA-5000 mass data analysis system was used as a processor, and the mol. wts. of the samples were estimated from the quasi-molecular-ion signals, $[M + H]^+$ in f.a.b. mass spectra (M, mol. wt.).



4 n = 2, R^1 = CH_2CO_2H, R^2 = H, R^3 = H
5 n = 2, R^1 = H, R^2 = CH_2CO_2H, R^3 = H
6 n = 2, R^1 = H, R^2 = H, R^3 = CH_2CO_2H
7 n = 3, R^1 = CH_2CO_2H, R^2 = H, R^3 = H
8 n = 3, R^1 = H, R^2 = CH_2CO_2H, R^3 = H
9 n = 3, R^1 = H, R^2 = H, R^3 = CH_2CO_2H

Preparation of the substrates. — To a mixture of cyclomaltoheptaose (100 g) (**1**) and NaOH (93 g) in water (370 mL) was added a 16.3% monochloroacetic acid solution (270 mL). After being stirred for 4 h at 50°, the mixture was made neutral with 6M HCl, and trichloroethylene (100 mL) was added. The mixture was stirred strongly to precipitate unmodified **1** as an inclusion compound with trichloroethylene. The precipitate (**1**, 26 g) was filtered off, and the carboxymethyl-substituted cyclomaltoheptaose (**2**) in the water layer was precipitated by adding

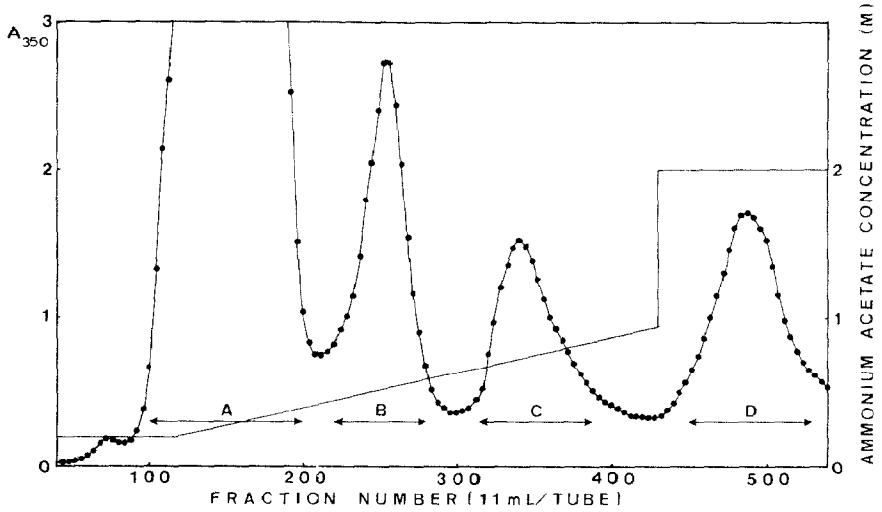


Fig. 1. Ion-exchange chromatography of the glucoamylase digests of transglycosylation products on Dowex 1-X4. The elution was done as described in the Experimental section.

acetone (2 L). The crude **2** was filtered off and dried under reduced pressure (yield, 69 g). The molar ratio of glucose, 2-, 3-, and 6-*O*-carboxymethyl-D-glucose residues in **2** was 40:6:3:1.

A mixture of **2** (12 g), *p*-nitrophenyl α-D-glucopyranoside (3.6 g), and cyclomaltodextrin glucanotransferase (3000 units) in 50mM ammonium acetate buffer (pH 6.5, 300 mL) was incubated for 3 h at 37°. After adjustment of the pH to 4.5 with acetic acid, glucoamylase (2500 units) was added, and the incubation continued for 12 h more. The solution was deposited onto a column (2.8 × 57 cm) of Dowex 1-X4 (100–200 mesh), equilibrated with 0.1M ammonium acetate. After being washed with 1.3 L of the same solution, the column was developed by a linear gradient (made by mixing 2.0 L of this solution with 2.0 L of M ammonium acetate) at the flow rate of 70 mL/h. Then, the elution of the column with 2.0M ammonium acetate (1.3 L) was monitored by the absorbance at 350 nm of the *p*-nitrophenyl groups. Under these conditions, *p*-nitrophenol remained bound to the column. D-Glucose and **2** were eluted with 0.1M ammonium acetate (monitored by t.l.c.; data not shown). The elution pattern is shown in Fig. 1. Each fraction (A–D) was lyophilized and analyzed by l.c. (Fig. 2). Fraction A was found to be *p*-nitrophenyl α-D-glucopyranoside. Compounds **4**–**9** were further purified to give a single peak on l.c. The yields of **7** and **8** mainly depended on the *p*-nitrophenyl α-D-glucopyranoside concentration. The conditions just described gave an optimum yield (0.78 g of **7** and 0.38 g of **8**). Purification of **2** was not necessary, because cyclomaltodextrin glucanotransferase did not act on di(carboxymethyl)-substituted **1**.

Compounds **4**–**9** were analyzed by g.l.c. (Fig. 3) and the data are summarized

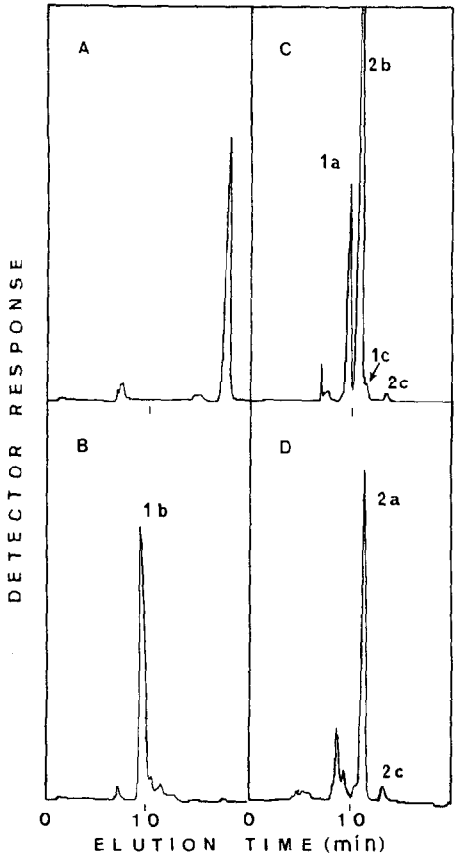


Fig. 2. L.c. analysis of the peaks separated by ion-exchange chromatography (Fig. 1). The column (4.6 × 150 mm) was eluted with ammonium acetate buffer, pH 4.5, containing 0.5% butanol at the flow rate of 1.5 mL/min. A–D; Fractions A–D of Fig. 1.

TABLE I

SUGAR COMPOSITION[a] OF COMPOUNDS **4–9**

Component	*Compound*[b]					
	4	**5**	**6**	**7**	**8**	**9**
D-Glucose	2.8	2.9	2.8	3.9	3.7	3.8
2-*O*-Carboxymethyl-D-glucose	0.9	0	0	0.9	0	0
3-*O*-Carboxymethyl-D-glucose	0	0.9	0	0	0.9	0
6-*O*-Carboxymethyl-D-glucose	0	0	1.0	0	0	0.9

[a]Molar ratio. [b]The value of the *p*-nitrophenyl group was taken as unity. The concentration of the *p*-nitrophenyl group was estimated from the absorbance at 305 nm in 0.1M acetic acid (a 100μM solution of *p*-nitrophenyl α-glycoside gave an absorbance of 1.08 with a 1-cm light path).

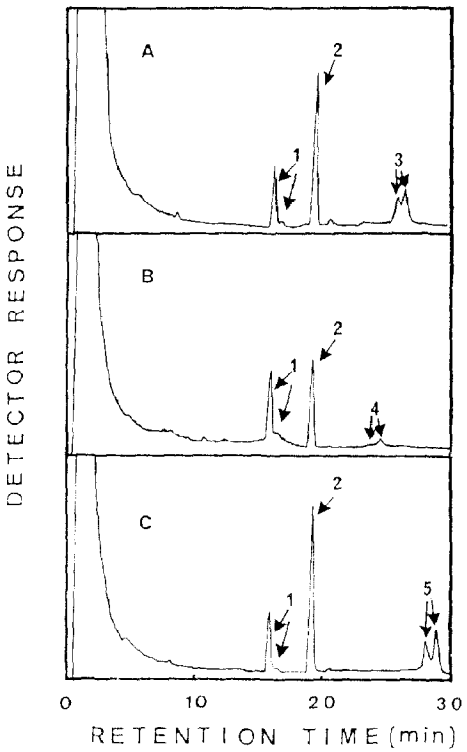


Fig. 3. Component analysis of compounds **7**, **8**, and **9** by g.l.c. The compounds were methanolyzed and trimethylsilylated before g.l.c., as described in the Experimental section: (A) **7**, (B) **8**, and (C) **9**. Peaks: 1, D-mannose (standard); 2, D-glucose; 3, methyl 2-*O*-carboxymethyl-D-glucoside; 4, methyl 3-*O*-carboxymethyl-D-glucoside; and 5, methyl 6-*O*-carboxymethyl-D-glucoside.

in Table I. They indicated that **4**, **5**, and **6** were derivatives of *p*-nitrophenyl α-maltotetraoside, and that **7**, **8**, and **9** were derivatives of *p*-nitrophenyl α-maltopentaoside. The nonreducing D-glucosyl end-groups of **4**–**9** were substituted with carboxymethyl groups, because **4**–**9** had been obtained after glucoamylase digestion. The mol. wts. of **4** and **7**, as analyzed by f.a.b.–m.s., were consistant with their structure.

Anal. Compound **4**, calc. for $C_{32}H_{47}NO_{23}$: 845.2; found $(M + H)^+$: 846.2. Compound **7**, calc. for $C_{38}H_{57}NO_{30}$: 1007.3; found $(M + H)^+$: 1008.2.

RESULTS AND DISCUSSION

Action of human α-amylases on **4**, **5**, **7**, **8**, p-*nitrophenyl α-maltotetraoside, and* p-*nitrophenyl α-maltopentaoside.* — Glucoamylase and α-D-glucosidase cannot hydrolyze **4**–**9**, because of the carboxymethyl group at the nonreducing D-glucosyl end-group. This shielding is needed for a substrate to be suitable for α-amylase

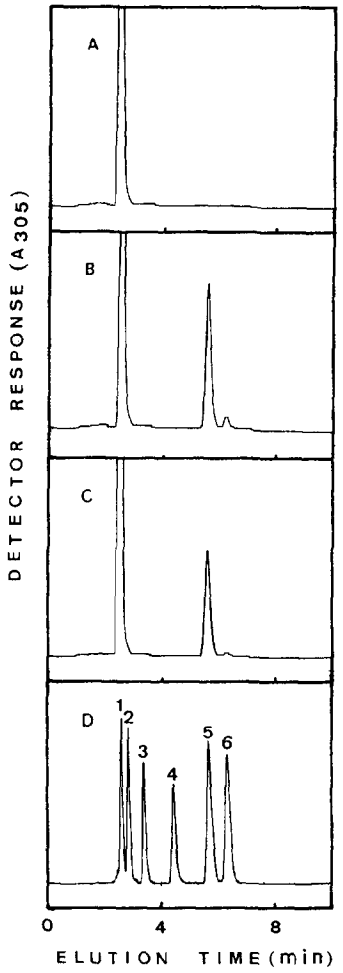


Fig. 4. Analysis of the α-amylases digests of **7** by l.c. Elution of the column (4.6 × 150 mm) with 0.1% acetic acid solution containing 12% acetonitrile at the flow rate of 1.5 mL/min was monitored by measurement of A_{305} (*p*-nitrophenyl group): (A) **7**, (B) pancreatic α-amylase digest of **7**, (C) salivary α-amylase digest of **7**, and (D) standard mixture. Peaks: 1, **7**; 2, *p*-nitrophenyl α-maltopentaoside; 3, *p*-nitrophenyl α-maltotetraoside; 4, *p*-nitrophenyl α-maltotrioside; 5, *p*-nitrophenyl α-maltoside; and 6, *p*-nitrophenyl α-D-glucoside.

assays coupled with these enzymes. The mode of action of human pancreatic and salivary α-amylases on **4, 5, 7**, and **8** was examined, but not that on **6** and **9** because of the low amounts available. A mixture of 1.1mM substrate solution in 50mM 4-morpholinoethane sulfonic acid (MES)–NaOH buffer, pH 6.9, containing 2mM $CaCl_2$ and 20mM NaCl (100 μL) and of α-amylase solution (10 μL; 30 units/L) was incubated for 10 min at 37°. The reaction was stopped by the addition of 100mM acetic acid (500 μL), and the sample (100 μL) was analyzed by l.c. The chromatograms of α-amylase digests of **7** are shown in Fig. 4 as an example. The amounts

TABLE II

RATIOS OF PRODUCTS AT THE INITIAL STAGE OF THE ACTION OF TWO HUMAN α-AMYLASES ON SUBSTRATE OLIGOSACCHARIDES

Substrates	*Ratio of products with salivary α-amylase*[a]			*Ratio of products with pancreatic α-amylase*[a]		
	G-G-G-P:	*G-G-P:*	*G-P*	*G-G-G-P:*	*G-G-P:*	*G-P*
p-Nitrophenyl α-maltotetraoside	0	:0.74	:0.26	0	:0.55	:0.45
4	0	:0.02	:0.98	0	:0.01	:0.99
5	0	:0.33	:0.67	0	:0.23	:0.77
p-Nitrophenyl α-maltopentaoside	0.12	:0.80	:0.08	0.12	:0.72	:0.16
7	0	:0.93	:0.07	0	:0.91	:0.09
8	0	:0.79	:0.21	0	:0.68	:0.32

[a]Abbreviations: G-G-G-P, *p*-nitrophenyl α-maltotrioside; G-G-P, *p*-nitrophenyl α-maltoside; and G-P, *p*-nitrophenyl α-D-glucopyranoside.

TABLE III

RELATIVE RATES OF HYDROLYSIS OF SUBSTRATES BY HUMAN α-AMYLASES

Substrates	*Salivary α-amylase*	*Pancreatic α-amylase*
p-Nitrophenyl α-maltotetraoside	0.75	0.70
4	0.31	0.40
5	0.15	0.11
p-Nitrophenyl α-maltopentaoside	1.00[a]	1.00[a]
7	0.66	0.66
8	0.62	0.55

[a]Value taken as unity.

of the products were calculated from their peak areas on chromatograms. Under the conditions used, these carboxymethyl derivatives were eluted at the same position.

p-Nitrophenol was eluted from this column with 0.1% acetic acid containing 35% of acetonitrile, but *p*-nitrophenol was not detected in any digest (data not shown). Table II gives the ratios of the products that were formed in the early stages of hydrolysis. Analysis of the hydrolyzates of six substrates by the two enzymes showed the binding modes of the substrates to their active sites. The binding modes of **4, 5, 7**, and **8** were influenced by the position of the carboxymethyl group at the nonreducing D-glucosyl end-group. Table III shows the rate of hydrolysis of the six substrates. The rate of hydrolysis of the O-2-substituted substrates was higher than that of the O-3-substituted substrates.

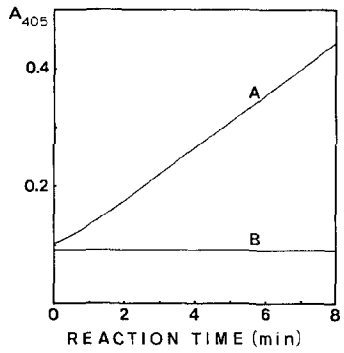


Fig. 5. Course of the digestion of **7** with α-amylase of human serum. The digestion of **7** and the measurement of the *p*-nitrophenol liberated is described in the text: (A) serum α-amylase (500 units/L), and (B) reagent blank.

Assay of pancreatic and salivary α-amylases in human serum. — Substrate **7** is mainly hydrolyzed into *p*-nitrophenyl α-maltoside and *O*-(2-*O*-carboxymethyl-α-D-glucopyranosyl)-(1→4)-*O*-α-D-glucopyranosyl-(1→4)-α-D-glucopyranose by the two enzymes. Thus, the modified compound is suitable as a substrate for the assay of the enzymes in serum, although the rates of hydrolysis of **7** by the enzymes are slower than those of *p*-nitrophenyl α-maltopentaoside. The activity of α-amylases was assayed by spectrophotometric determination at 405 nm of the *p*-nitrophenol released by coupling with glucoamylase and α-D-glucosidase.

To a solution of mM **7** and the coupled enzymes (20 units/mL of glucoamylase and 10 units/mL of α-glucosidase) in 50mM MES–NaOH buffer, pH 6.9, containing 2mM $CaCl_2$ and 20mM NaCl (3 mL), was added the serum from a patient with

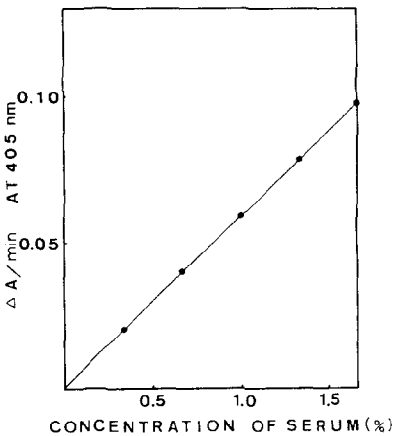


Fig. 6. Relationship between the concentration of serum (α-amylase) and the initial rate of the enzymic reaction. Increase of absorbance at 405 nm with time was measured as shown in Fig. 5.

hyperamylasemia (50 μL). The mixture was incubated at 37° and the reaction monitored by the increase of absorbance at 405 nm. The rate of the α-amylase reaction became linear after about 1-min lag period under the conditions chosen (Fig. 5). The relationship between the concentration of α-amylases and the release of *p*-nitrophenol was linear (Fig. 6). These results suggest that the new substrates, especially **7**, may be used as substrates for clinical assays of α-amylase activity in serum.

ACKNOWLEDGMENTS

The authors thank Professor Yasutsugu Shimonishi and Dr. Toshifumi Takao, Institute for Protein Research, Osaka University, for their kind help in recording the f.a.b.–mass spectra, and Mr. Satoru Okajima, Wako Pure Chemical Industries, Ltd., for his technical assistance in the preparation of the monocarboxymethyl-substituted cyclomaltoheptaose.

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